Mathematical Models for T cell Activation through Kinetic Proofreading

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Abstract

T cells play a crucial role in the adaptive immune system, protecting the body from diseases. An intact antigen discrimination mechanism of the T cell receptors is essential for survival. T cells must be able to discriminate between few pathogenic antigens and abundant self antigens. Failure of this mechanism can lead to immune deficiency or autoimmunity. A simple mathematical model that captures this ability of antigen discrimination is given by the kinetic proofreading model, which includes successive phosphorylation steps of the complexes built by the T cell receptor and the antigen presented. We show that extending this model by enzyme-substrate reactions which explicitly model the kinase Lck involved, fundamentally alters the characteristics of the basic kinetic proofreading model. Whereas the basic model is known to have a unique steady state, we show that including Lck in the system enables it to support multiple steady states. More precisely, there exist kinetic parameters and initial conditions for which the system exhibits more than one positive steady state. This capacity to support multiple steady states persists when incorporating ZAP-70, a molecule recruited upon antigen binding to the receptor. The existence of multiple steady states provides scope for interpretation regarding the association of these to possible "on" and "off" states of T cell signaling or to potential healthy and ill states of the immune system. Furthermore, we find that the trajectories of the systems do not approach the boundary and establish that at least two phosphorylation steps are required for multistationarity to occur. Moreover, we investigate the minimal kinetic proofreading system with Lck and a single phosphorylation step and identify conditions under which the unique positive steady state is globally asymptotically stable. These findings provide detailed insights into the complex mechanisms of T cell activation and immune response.

Contents

Introduction

This work is concerned with the qualitative analysis of a deterministic mathematical model consisting of ordinary differential equations that address the early phase of T cell activation. T cells carry receptors on their surface which can detect peptide fragments that are presented by specific molecules, referred to as major histocompatibility complexes (MHCs), on the surface of other cells. The process of T cell activation is highly complex and not all interactions involved have been discovered yet. Therefore, it is crucial to gain comprehensive information about the underlying basic mechanisms contributing significantly to this intricate process. Extensive research about T cell activation in the last decades has revealed several features of the activation mechanism. A decisive factor for the strength of the immune response initiated is the affinity of the antigen to the T cell receptor. The affinity describes the binding strength of the complex formed by antigen-presenting MHC molecule (pMHC) and T cell receptor (TCR). T cell reaction to antigens is highly sensitive, specific and fast. T cells have to detect foreign antigens even when they are only present in a very low concentration and discriminate between these and highly abundant self antigens that should not trigger an immune response, and this task has to be performed as fast as possible. Experimental measurement of T cell activation involves assessing the concentration of downstream molecules. These molecules accumulate after the binding of the antigen to the T cell receptor initiates the intracellular signaling pathways to stimulate an immune response. Various studies have reported different relationships between the affinity of the pMHC-TCR complex and the T cell activation signal initiated. The affinity range of the ligands used, the induced pMHC dose and the general setting, if it is an *in vitro* or *in vivo* study, are determining factors which lead to different observations in the degree of T cell activation [71, 15]. The phenomenon that antigens with a higher affinity to the T cell receptor initiate a stronger response than antigens with a lower affinity, even if the latter are present in a very high concentration, is in T cell modeling referred to as antigen discrimination. This antigen discrimination is a crucial feature of T cell activation and has been observed for physiological affinities [103]. For supraphysiological affinities, that means engineered high-affinity T cell receptors, an optimal affinity has been observed [53, 61, 103, 71, 15], though it was reported to be lost with higher ligand doses [36, 49]. Similarly, optimal concentrations for high affinity ligands have been reported, whereas low affinity ligands do not show such an optimum but follow a sigmoidal dose-response curve [3, 71, 13, 61]. T cell activation with physiological affinities of the pMHC-TCR complex shows further features in addition to antigen discrimination. Research has revealed antagonism [69, 68, 19, 98] and digital signaling of T cells [2, 17, 56, 45]. Antagonists are antigens that have a similar structure as the agonist that is supposed to trigger an immune response. Antagonists themselves do not trigger an immune response, but when present together with the actual agonist antigen, they reduce the response induced by the agonist. This phenomenon is called antagonism. Digital signaling refers to the observation that some enzymes and molecules of the downstream signaling seem to exist in only two modes, “on” and “off”, or are present in either high concentrations or not at all [62]. Hence, the signaling of a single T cell is assumed to be of digital nature, either initiating an activation signal or not.

Various models have been proposed to reveal the mechanisms behind these observations. None of them captures all of the features of T cell activation mentioned [62, 103]. A simple mechanism that models the key feature antigen discrimination, which is essential for an intact immune response, is given by the kinetic proofreading model proposed by McKeithan in 1995 [70]. The model postulates that the pMHC-TCR complex has to be phosphorylated several times before it is able to initiate an intracellular activation signal. Thus, peptides with a higher affinity to the T cell receptor are more likely to induce an activation signal than peptides with lower affinity, which dissociate before

reaching the fully phosphorylated status. The kinetic proofreading model has been incorporated in more complex models that aim to capture other features of T cell activation in addition to antigen discrimination. In 1996 Rabinowitz et al. [82] proposed a first model including negative feedback to explain antagonism. Whereas the dissociation of the pMHC molecule from the T cell receptor results in McKeithan's kinetic proofreading model in the immediate loss of the phosphate residues of the T cell receptor, in Rabinowitz' model the T cell receptor stays phosphorylated for some time which allows another ligand to bind to the receptor. Furthermore, the singly phosphorylated complex in the model leads to a negative signal and the doubly phosphorylated complex sends the positive activation signal. Thus, any ligand can send both negative and positive signals. Antagonists only bind long enough to the receptor to initiate a negative signal but do not reach the second phosphorylation step which initiates an immune response. How exactly an incomplete phosphorylation of the T cell receptor could suppress the initiation of the activation signal was not known yet to this date. Further research on T cell activation has identified the phosphatase Shp-1 to be involved in the T cell activation process and act as a negative regulator [81, 97]. In addition to the negative feedback by the phosphatase Shp-1, positive feedback by the kinase ERK has been observed [97]. The inclusion of both the negative and the positive feedback in the kinetic proofreading model has first been examined with a stochastic model by Chan et al. [11]. The authors associated the negative feedback to improved discrimination and the positive feedback to improved sensitivity of the T cell receptor. A deterministic model including kinetic proofreading with negative and positive regulation as a core model was given by Altan-Bonnet and Germain [2]. Their simulations are in accordance with the experimental observations of antigen discrimination, antagonism and the digital nature of the T cell activation. Hence, the model captures the features of T cell activation observed for physiological affinities [103]. The observations for supraphysiological affinities are not explained by this model. Another stochastic model was given by Lipniacki et al. [66] for which the authors identified negative and positive feedback as the source of bistability. The mechanism of the negative feedback has been examined more closely in a deterministic model by François et al. [33]. The authors extended McKeithan's basic kinetic proofreading model by the phosphatase Shp-1 which is modeled to be activated by the singly phosphorylated pMHC-TCR complex. Since antagonists can bind long enough to be singly phosphorylated, they can activate the phosphatase Shp-1 and in this way diminish the activation signal. Hence, the inclusion of this negative feedback captures the antagonism phenomenon. This negative feedback also gives rise to an optimal concentration. Lever et al. [62] focused on the observations for supraphysiological ligand affinities and proposed a model that adds a non-signaling state to the kinetic proofreading model. As a consequence, the model exhibits an optimal affinity. However, such an optimal affinity has been observed experimentally only for supraphysiological and not for physiological affinities. Furthermore, the model does not capture the reported loss of the optimum under high ligand concentrations. For this reason, the authors proposed an additional model where the T cell receptor is able to sustain the intracellular activation signal initiated for some time after the ligand has dissociated. This sustained signaling model produces an optimal affinity which is lost for high ligand concentrations. In a further work, Lever et al. [61] addressed the observation of an optimal concentration for high affinity ligands. In this work, the authors proposed an incoherent feedforward loop where inhibitory and stimulating intracellular signals are initiated simultaneously. Combined with the limited signal mechanism, this created a model that reflects the observations of an optimal ligand concentration for high affinity ligands and a sigmoidal response curve for low affinity ligands. Thus, the model agrees with the features observed for supraphysiological affinities. On the other hand, this model does not address the digital signaling and antagonism, features in the scope of physiological affinities, that some of the other models can reproduce.

None of the existing models captures all physiological and supraphysiological features of T cell activation so far. Most of the models for the T cell activation, in particular the more elaborate ones, have been investigated using computer simulations. Some deterministic models have been analyzed qualitatively by determining the characteristics of the system of ordinary differential equations associated to the respective model. Such analyses have been done for McKeithan's

kinetic proofreading model and the negative feedback proposed in the model of François et al. [95, 85]. Other models, such as the model by Altan-Bonnet and Germain, are too elaborate for a rigorous analysis of the qualitative behavior of the trajectories of the associated system of differential equations. However, an analysis of core components of such an intricate model can help to reveal the key characteristics of the mechanisms involved. Minimal models are crucial to identify fundamental principles. We extend McKeithan's basic kinetic proofreading model by enzyme-substrate reactions which explicitly model the kinase Lck which is responsible for the phosphorylation of the complexes. Additionally, we include the molecule ZAP-70 which can bind to the receptor upon its phosphorylation and is involved in initiating the intracellular activation signal. This model corresponds to the core model of Altan-Bonnet and Germain. We analyze the corresponding ordinary differential equations resulting from the assumption of mass action kinetics. We show that this system differs in its key characteristics from McKeithan's kinetic proofreading system. Precisely, it is able to admit multiple steady states. Thus, negative or positive feedback loops are not necessary elements to induce multistationarity in the context of T cell activation. This underlines the importance of minimal models to unravel complex phenomena. This work shall contribute to a deeper understanding of the mechanisms involved in T cell activation.

The first chapter of this work gives an introduction to basic immunobiology and the role that T cells play in the immune system, as well as the structure of the T cell receptor on which the mathematical model is based. The chapter is addressed to readers unfamiliar with immunobiology. Readers familiar with or not interested in the biological background of the mathematical model may skip this chapter. In the second chapter, we give a more detailed overview about the existing deterministic models for T cell activation that incorporate kinetic proofreading as core mechanism. In particular, we address the qualitative mathematical analyses on the basis of the associated systems of ordinary differential equations, resulting from the assumption of mass action kinetics for the reactions involved. Following this review of the existing models and their known characteristics, we introduce the mathematical models we want to investigate, which result from the extension of the kinetic proofreading model by explicitly modeling the kinase Lck and the molecule ZAP-70, respectively. The next chapter then addresses the capability for multistationarity that these systems possess provided the kinetic proofreading mechanism encompasses at least two phosphorylation steps. We prove this ability by applying an algorithm, the Advanced Deficiency Algorithm, a tool which belongs to the Chemical Reaction Network Theory. Thus, chapter three starts with an introduction to Chemical Reaction Network Theory and the Advanced Deficiency Algorithm. After these have been introduced, we apply the algorithm to the kinetic proofreading system with Lck and ZAP-70, respectively, to prove that both systems are able to support multiple positive steady states. Chapter four completes the question of multistationarity by showing that both systems are uniformly persistent, which implies that none of the trajectories approaches the boundary. In particular, there are no steady states on the boundary. Some results in this chapter are formulated for general mass action kinetic systems and are not specific for the kinetic proofreading systems examined. The last chapter addresses the question of what changes if the number of phosphorylation steps of the kinetic proofreading system with Lck is reduced to a single phosphorylation step. This reduction of the kinetic proofreading mechanism to a minimum has consequences for the dynamics of the system. A single phosphorylation step is not sufficient to create the capacity for multistationarity. We deduce conditions under which the single steady state is globally asymptotically stable by applying a generalized Bendixson criterion. The matrices and matrix measure for this criterion are introduced in the first section of the chapter, followed by the application to the kinetic proofreading system with a single phosphorylation step. The analysis of the models in this work, resulting from the gradual extension of the most basic kinetic proofreading model by two additional molecules involved in the T cell activation process, reveals characteristics, namely multistationarity, that have not yet been attributed to the pure kinetic proofreading mechanism but rather to the extension by negative or positive feedback regulation.

1 Biological Background

This chapter serves as an introduction to the biological setting of the mathematical model examined in this work, a kinetic proofreading model that describes the early activation of T cells. The first section gives a general introduction to the immune system, and the second section addresses the structure of the T cell receptor. Readers familiar with or not interested in immunobiology may skip this chapter and proceed directly to the introduction of the mathematical model in Chapter 2. The acronyms introduced in this chapter are commonly used in immunobiology and are listed in the glossary.

1.1 Basic Immunobiology

We are continually exposed to a wide variety of infectious microorganisms such as viruses, bacteria, fungi and parasites. Nevertheless, we only become ill rarely. Responsible for defending our body against these pathogens is the immune system [109]. The immune system has a multi-layered organization increasing in complexity and specificity. The first layer is provided by physical barriers such as the skin and the mucosa that aim to prevent pathogens from gaining access to underlying tissue. The next layer is the non-specific chemical barrier that consists of antimicrobial compounds and factors of the humoral immune system, soluble factors found in the body fluids. If a pathogen breaches the physical and chemical barriers, then the immune system utilizes its immune cells, the third layer of immune defense, beginning with a rapid but non-specific response provided by the innate immune system. If the innate immune response is overcome by an infectious pathogen, then the adaptive immune system comes into play, the last immune defense line [109]. In contrast to the innate immune response, responses by the adaptive immune system take days rather than hours to develop [74]. The adaptive immune response can be divided into two components: humoral and cellular immune response.

There are a multitude of cells and soluble factors that can be considered part of the immune system [109]. Most cells of the immune system arise from the bone marrow, where many of them develop and mature [74]. Key roles in the immune response are played by the white blood cells, also called leukocytes (from the Greek leuko “white” and cyte “cell”), originating from multipotent stem cells in the bone marrow, more precisely from hematopoietic stem cells that give rise to blood cells. Blood cells are categorized in the lymphoid or the myeloid lineage. The lymphoid lineage includes T and B cells and natural killer cells, also referred to as lymphocytes. The letter T in T cells arises from the fact that, unlike most other immune cells, they mature in the thymus. B cells, that mature in humans in the bone marrow, got their name as they were first discovered in the bursa fabricii of birds [80]. Phagocytes (from the Greek phagein “to eat” and kutos “hollow vessel”) belong to the myeloid lineage and include various granulocytes, macrophages and dendritic cells. Some but not all phagocytes live up to their name and perform phagocytosis, a process where particles are absorbed into the cell. Lymphocytes and phagocytes represent the two major families of white blood cells. Each type of white blood cell has specialized functions, allowing them to be categorized into the innate and adaptive immune systems. Additionally, the cells classified under the adaptive immune system can be further associated with the humoral and cellular immunity [87, 109].

Suppose a virus enters the body overcoming the physical and chemical barriers. This virus carries molecules on its surface. These are called antigens and on the basis of these antigens the cells of the immune system identify the invading pathogen via receptors on their surface. The cells of the innate

immune system express a number of receptors that allow them to recognize different pathogens. However, this number of innate recognition receptors is limited. They recognize simple molecules and regular patterns of molecular structure known as pathogen-associated molecular patterns that are part of many microorganisms but not of the host body's own cells [74]. These receptors can bind to the virus antigens and therefore detect it. Phagocytes and natural killer cells can be assigned to the innate immune system though they also play a role in the adaptive immune response. Whereas the phagocytes detect pathogens or dead cells and cellular debris, the natural killer cells detect infected cells. Phagocytosis is the main mechanism of the innate immune system [80]. Responsible for the phagocytosis are three cell types. Neutrophil granulocytes ingest and destroy pathogens but do not present antigens. The task of the macrophages is mainly to destroy pathogens. They act as general scavenger cells in the body by removing dead cells and cellular debris. Furthermore, they ingest and digest pathogens and can present antigens to T cells, more precisely to helper T cells. Dendritic cells are specialized in antigen presentation to helper T cells to stimulate the adaptive immune response. They form the bridge between innate and adaptive immune responses [74]. Cells that present antigens on their surface are also referred to as APCs, antigen-presenting cells. Thus, macrophages, dendritic cells and infected cells can be APCs. To display antigens, they possess specific proteins on the cell surface, the major histocompatibility complex (MHC) proteins [87].

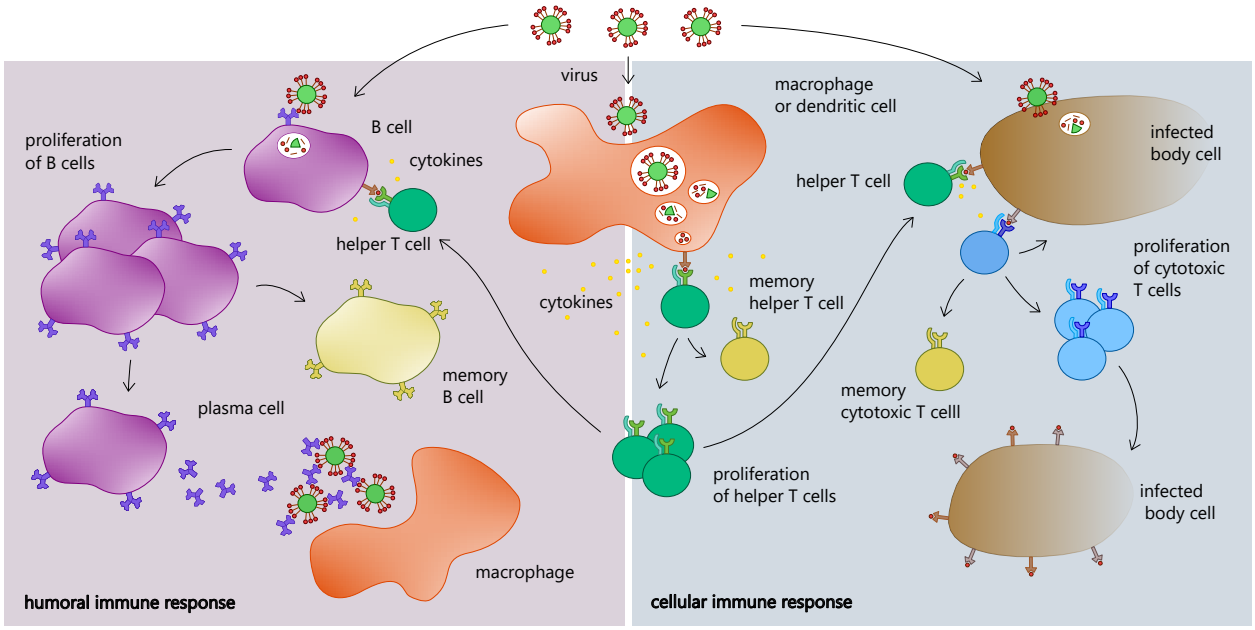
T and B cells are crucial components of the adaptive immune system [87]. Both of them carry receptor molecules on their surface. In contrast to the cells of the innate immune system, every T or B cell expresses just one kind of receptor molecule that recognizes only one specific antigen. Thus, the adaptive immune system is highly specific. The vast diversity of specific receptors is generated primarily by DNA changes as chromosomal rearrangements and other mutations that occur just after the T and B cells are formed in the bone marrow. Hence, the adaptive immune system is "predeveloped" [87]. T and B cells that have not yet encountered their specific antigen are known as naive lymphocytes. Activation and proliferation of these cells occur when their receptors bind to a specific antigen and simultaneously receive a co-stimulation signal transmitted by cytokines. That means the cell divides to form a clone of cells that all recognize and react to the same antigen. This proliferation process is called clonal selection, as the antigen "selects" a particular T or B cell for proliferation [87]. The activated cells that have differentiated further into fully functional lymphocytes are known as effector lymphocytes [74]. If an effector lymphocyte meets its antigen, no co-stimulation is necessary. There are different types of effector cells. When a T cell encounters an antigen that its receptor can bind for the first time, it proliferates and differentiates into one of several distinct functional types of effector T cells: cytotoxic T cells, helper T cells, regulatory T cells and memory T cells. Cytotoxic T cells kill other cells that are infected with viruses or other intracellular pathogens bearing the antigen. Helper T cells provide signals, often in form of specific cytokines, to activate the functions of other cells, especially B cells and cytotoxic T cells. Regulatory T cells suppress the activity of other lymphocytes and help to limit the possible damage of immune responses. The memory cells are responsible for the long-lasting immunity that can follow exposure to disease or vaccination. Memory cells will readily differentiate into effector cells on a second exposure to their specific antigen. Upon activation, a B cell proliferates and differentiates into plasma cells and memory B cells. Plasma cells produce antibodies, a class of proteins also known as immunoglobulins [74]. Cells that further differentiate into memory cells divide at low rate, perpetuating the clone. Memory B and T cells can survive in the body for decades. They can rapidly start dividing to produce more effector and memory cells if they detect their antigen during a new infection [87]. Plasma cells, effector B cells that make antibodies, are the workhorses of the humoral immune response and cytotoxic T cells those of the cellular immune response [87]. Activated helper T cells stimulate both cell types by releasing cytokines.

The adaptive immune system's humoral response is mediated by antibodies that are found in the fluid component of blood and in extracellular fluids [74]. Antibody molecules are composed of two distinct regions. One is a constant region, which takes one of only four or five distinguishable

forms. The variable region, by contrast, can be composed of a vast number of different amino acid sequences that allow antibodies to recognize an equally vast variety of antigens [74]. The B cell receptors are specific antibodies anchored in the plasma membrane of the B cell surface. The B cell gets activated when such an antibody on the cell surface recognizes and binds to its specific antigen, for example viral antigen. The B cell can detect free viruses and directly bind to them through the antibodies that function as receptors on the cell surface. When an antigen binds to the antibody on the B cell surface, the B cell ingests the complex formed by the antigen and the antibody and degrades the antigen using enzymes. The degraded fragments of the pathogenic antigen are then presented on the cell surface via MHC proteins, specifically by class II MHC proteins. This class of MHC proteins is recognized by the receptor of helper T cells. When a helper T cells binds to the antigen fragment presented by the B cell, it releases cytokines. These cytokines are signaling proteins that stimulate the proliferation and differentiation of the B cell into antibody-secreting plasma cells or into memory B cells. Consequently, the plasma cells produce and release antibodies which bind to the antigens on the virus's surface. This process neutralizes the pathogenic antigens, enabling their absorption and digestion by phagocytic cells (see left part of Figure 1.1).

Whereas antibodies can recognize nearly any type of chemical structure, T cell receptors formed by integral membrane proteins on the surface usually recognize protein antigens and do so very differently from antibodies [74, 87]. Antigen receptors, such as an individual antibody, recognize only a small portion of the antigen's molecular structure, called an epitope. Large antigens, such as proteins, can contain multiple epitopes. Consequently, they may bind to different antibodies, with each antibody recognizing a specific epitope on the antigen's surface. Antibodies generally recognize epitopes on the surface of the antigen, whereas T cell receptors recognize epitopes that are buried within antigens. To allow this, first the antigens must be degraded, and the epitope delivered to and presented by an MHC protein on the surface of other cells [74]. There are two main types of MHC molecules, called MHC class I and MHC class II, that have slightly different structure. MHC class I molecules are expressed on most cells of the body. Besides presenting the epitope of an antigen on the cell surface, the MHC molecules also serve as important self-identifying labels [87]. MHC class II proteins are expressed by cells whose antigen presentation is directed to helper T cells. Dendritic cells, macrophages, B cells and cytotoxic T cells carry both classes of MHC molecules on their surfaces as all of them interact with helper T cells. Due to the two different types of MHC proteins that are presenting the relevant antigen fragments, helper T cells and cytotoxic T cells are able to differentiate between healthy body cells that present the antigen to stimulate the T cell proliferation and infected cells that have to be killed. The T cells express either a cell-surface protein called CD8 or another called CD4. They are involved in the antigen recognition by recognizing different regions on MHC molecules and are therefore known as co-receptors. CD8 recognizes a region of the MHC class I protein, while CD4 recognizes a region of the MHC class II protein. Thus, the two co-receptors functionally distinguish T cells. Cytotoxic T cells carry CD8 proteins and so recognize antigen-presenting MHC class I molecules, which are expressed on most cells of the body [74]. The antigens presented can be fragments of virus proteins in virus-infected cells or abnormal proteins made by cancer cells as a result of somatic mutations [87]. MHC class I molecules bearing viral peptides are recognized by CD8-bearing cytotoxic T cells, which then kill the infected cell. Helper T cells carry the co-receptor CD4. CD4 T cells recognize antigen presented by MHC class II proteins, which are expressed by cells that carry out phagocytosis and present the antigens to helper T cells to activate the adaptive immune response as well as by B cells and cytotoxic T cells, which are stimulated by the cytokines released by helper T cells.

The cellular immune response is carried out by cytotoxic T cells (see right part of Figure 1.1). They recognize and destroy virus-infected body cells, cancer cells or transplants. Cytotoxic T cells primarily circulate in the body as resting cells and have to be activated by a suitable antigen. First, the virus-infected cell or tumor cell degrades the antigens via enzymes into fragments. These relevant fragments, the epitopes, that the T cell receptor can recognize when bound to an MHC



Suppose a pathogen, for example a virus, has overcome the physical and chemical barriers. A macrophage or a dendritic cell can detect and digest the virus. Thereupon it presents the antigen of the virus or more precisely its epitopes on the surface via a class II MHC molecule acting now as an APC. The antigen fragment is now presented in a way that a helper T cell receptor can recognize it. The binding of the T cell receptor to the class II MHC molecule carrying the antigen fragment together with the cytokines, signal molecules released by the phagocyte, activates the naive helper T cell. The thus activated helper T cell releases cytokines which stimulate its own proliferation. Most cells differentiate into effector T helper cells that, upon antigen recognition, release cytokines which in turn stimulate naive B cells and naive cytotoxic T cells to proliferate. A few of the T cells develop not into effector cells but into memory cells. Playing a role in the activation of B cells and cytotoxic T cells alike, the helper T cells initiate both the humoral and the cellular immune response.

(a) The humoral immune response is driven by B cells. When a B cell receptor, which is an antibody anchored in the membrane of a B cell, encounters an antigen on the surface of a virus to which it is able to bind, it absorbs the virus, decomposes it and presents the antigens, or its epitopes, respectively, via a class II MHC molecule on the surface. That means the naive B cell itself is acting as an APC for helper T cells. The T cell receptor of a helper T cell can bind to the class II MHC molecule presenting the antigen fragment on the surface. Again the helper T cell releases cytokines this time directly next to the naive B cell and and thus activates it. The B cell proliferates, and most of the cells mature into plasma cells that produce antibodies, whereas a few develop into memory cells. The antibodies produced and released by the plasma cells bind to the antigen on the virus surface, building up an antigen-antibody complex that is absorbed and digested, for example by macrophages.

(b) The cellular immune response is carried out by cytotoxic T cells. Once a body cell is infected by a virus, it decomposes the virus and transports and presents the antigen fragments on the surface via a class I MHC molecule, acting as an APC. This process is the same whether the diseased cell was affected by a virus or is for example a cancer cell. The antigen fragments presented on the class I MHC molecule are recognized by the receptor of a cytotoxic T cell which then binds to the infected body cell. This partly activates naive cytotoxic T cells. Naive cytotoxic T cells only get fully activated and trigger the apoptosis, the programmed cell death, of the diseased body cell if cytokines released by helper T cells diffuse to the receptors of the cytotoxic T cells.

Figure 1.1: Scheme of the humoral and cellular immune response that form the adaptive immune response, the last defense layer of the immune system.

protein, are presented on the surface by the class I MHC proteins of the body cell. When a cytotoxic T cells recognizes and binds to such an MHC protein presenting the specific antigen, the cytotoxic T cell gets partly activated. To get fully activated, the cytotoxic T cell must detect specific cytokines, signal molecules released by an activated helper T cell. Only when these cytokines diffuse to a cytotoxic T cell bound to an infected body cell that presents the right antigen or, more precisely, epitope of this antigen on its class I MHC protein on the cell surface, the cytotoxic T cell gets fully activated, meaning it divides about ten times and differentiates to fully activated cytotoxic T cells. Another contact with the infected cell or cancer cell via the binding of the class I MHC protein engaged with an antigen and the T cell receptor ensures that the right cell is to be attacked and the bound activated cytotoxic T cells initiate apoptosis, a programmed cell death, of the diseased cell. A small part of the activated cytotoxic T cells differentiates further into memory cells. Activation of naive cytotoxic T cells without the help of T helper cells leads to an early cytotoxic T cell response but induces no or a defective repertoire of memory cytotoxic T cells that do not provide protection in case of a second exposure [75]. Helper T cells can bind to the same cell presenting the pathogenic antigen via a class II MHC molecule, while the cytotoxic T cell is bound to the class I MHC molecules, and release cytokines. The close proximity ensures that the cytokines reach the cytotoxic T cell and thus activate it fully. Some bacteria and parasites and all viruses replicate inside cells, where they cannot be detected by antibodies, which access only the blood and extracellular space. The destruction of these intracellular invaders is the function of the cytotoxic T cells, which are responsible for the cell-mediated immune response of the adaptive immunity [74].

1.2 T cell Receptor Complex

The ability of T cells and B cells to recognize and respond to their specific antigen is central to adaptive immunity. Each T cell bears several thousand antigen receptors on its surface, each receptor consisting of two different polypeptide chains, termed the T cell receptor α (TCRα) and β (TCRβ) chains (see Figure 1.2). These variable chains have exquisite specificity for antigen, allowing each T cell to detect the presence of one type of pathogen [74]. After the receptor has detected and bound such a pathogen, the information that antigen receptor engagement has occurred needs to be transduced into the intracellular compartment of the T cell. Thus, the fully functional antigen receptor complex must include proteins that can transduce a signal across the plasma membrane [74].

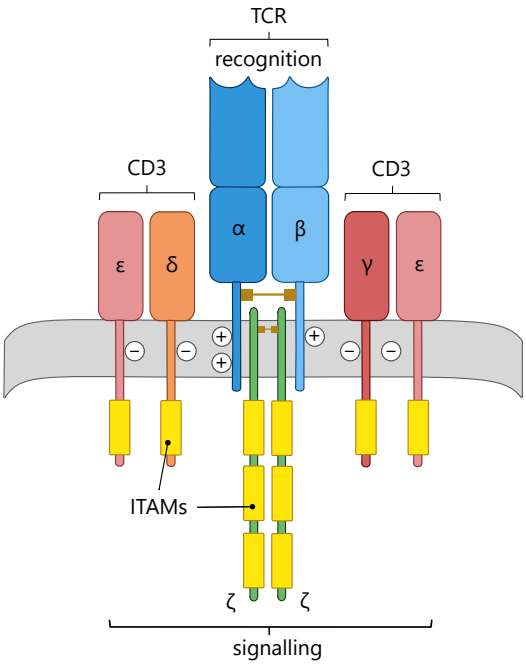


Figure 1.2: The T cell receptor complex consists of variable antigen-recognition proteins and invariant signaling proteins. The variable TCRα and TCRβ chain belong to the antigen-binding proteins of the receptor complex while the ζ chains and the ε, γ and δ chains of the CD3 protein carry out the signaling function of the receptor. The complex gains its stability through charge interactions in the transmembrane region. Marked yellow are the ITAMs, the immunoreceptor tyrosine-based activation motifs on the variable chains that get phosphorylated when the TCR binds its antigen and so transduce the signal of the bound ligand across the plasma membrane. [74]

Besides the α and β chains, the T cell receptor complex (TCR) also includes two CD3 proteins, each with an extracellular domain linked to an invariant intracellular chain, and two ζ chains predominantly located within the intracellular compartment (see Figure 1.2). The CD3 proteins are necessary for the T cell receptor to be expressed on the cell surface. The ζ chains as well as the invariant chains of the CD3 proteins carry out the signaling function of the receptor. It is thought that each of the T cell receptor chains interacts with one of the CD3 molecules and one ζ chain. These interactions are mediated by charge interactions between the transmembrane domains. The TCRα and TCRβ chains carry positive charges in the transmembrane regions whereas the CD3 protein and the ζ chains are provided with negative charges in their transmembrane domains. These interactions stabilize the α and β dimer during its production and transport to the plasma membrane [74]. The variable chains of the T cell receptor, the α and β chains, are responsible for binding the antigen whereas the associated invariant chains, the chains of the CD3 proteins and the ζ chains, carry out the signaling function of the receptor. The invariant chains contain regions lying on the intracellular domain which can be phosphorylated. These regions are called immunoreceptor tyrosine-based activation motifs (ITAMs) as tyrosine phosphorylation of these regions initiates the signaling from the T cell receptor. The CD3 chains each contain one ITAM and each ζ chain contains three ITAMs, giving the T cell receptor complex a total of ten ITAMs. Each ITAM contains two tyrosine residues that become phosphorylated by specific kinases when the receptor binds its ligand, that is, its antigen [74].

There are several suggestions for the mechanism of T cell receptor triggering that involve aggregation, conformational change and segregation. Some mechanisms turned out to be insufficient for T cell receptor triggering on their own, but nevertheless they may still be valid and contribute to the complete signaling process. For example, it was observed that T cell receptor triggering can occur in the complete absence of co-receptors. Accordingly, it might still be the case that co-receptors bind to the same peptide MHC complex as the T cell receptor and in this way bring a co-receptor associated kinase in close proximity to the CD3 and T cell receptor chains that thereupon phosphorylates the ITAMs, but this mechanism alone does not suffice to explain T cell receptor triggering. One suggestion for the mechanism is the pseudodimer model that involves two T cell receptors. One binds to an antigen-presenting MHC molecule, the other one to another MHC molecule carrying a self peptide. The co-receptor associated with the T cell receptor that bound to the self molecule binds to the MHC molecule presenting the antigen fragment and in this way forms a pseudodimer [104]. This model would explain how it is possible that a very low density of antigens on the surface of the antigen-presenting cell can induce signaling. Evidence for this is clearest in the case of helper T cells, being associated with the co-receptor CD4, whereas there are conflicting data for cytotoxic T cells, being associated with the co-receptor CD8 [104]. The natural state of T cell receptors on the cell surface is still not clear, whether they exist as monomers or are present in aggregates or clusters. The appearance of such aggregations of T cell receptors, called microclusters, at least partly, has been observed, but it may still be the case that the building of these clusters does not initially trigger the signaling but results from it. Though it has been demonstrated that ITAM phosphorylation is not required for microcluster formation that does not rule out a role for other signaling pathways [104]. Another mechanism which has been suggested is the segregation or redistribution of the T cell complex with respect to other cell membrane associate proteins. The binding of the T cell receptor to the antigen-presenting MHC molecule traps the T cell receptor complex in close-contact zones thereby segregating it from the inhibitory tyrosine phosphatases, leading to stable phosphorylations of the ITAMs [104]. The segregation in turn might enhance aggregation and the forming of microclusters. Another mechanism that most likely plays an additional part in T cell receptor triggering are conformational changes in the T cell receptor complex that predispose it to dimerization and aggregation [104]. That again would give an explanation for T cell triggering at very low densities of antigen-presenting MHC molecules. Several models for this mechanism exist, but as the structure of the intact T cell receptor complex is not fully known yet, none of these can explain in molecular detail how the binding of the T cell receptor

to the antigen-carrying MHC molecule can lead to changes in the CD3 cytoplasmic domains [104]. In resting T cells there is a delicate balance between tyrosine phosphorylation of the T cell receptor complex, that means phosphorylation of the ITAMs on the ζ chains of the T cell receptor and the different chains of the CD3 protein, and dephosphorylation, where the dephosphorylation dominates. In principle, T cell receptor triggering can be induced by any mechanism that tilts this balance in favor of phosphorylation [104].

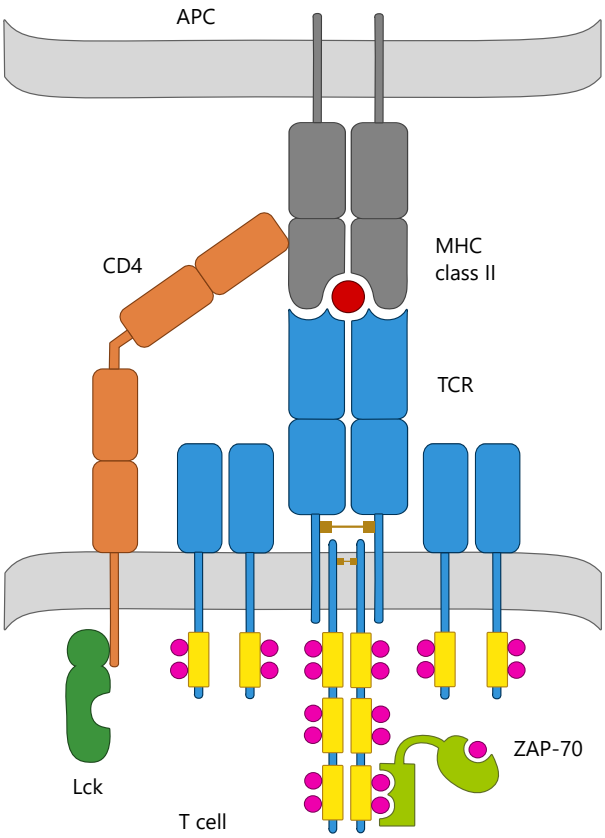


Figure 1.3: Engagement of co-receptor enhances phosphorylation of the ITAMs. After the T cell receptor has bound to an MHC molecule presenting its specific antigen, co-receptors can also bind to the same MHC molecule. In the panel, the principle is pictured with a CD4 protein found in helper T cells that binds to a class II MHC molecule. Signaling within microclusters may differ from this arrangement [74]. The co-receptor associated kinase Lck phosphorylates both tyrosine residues in the ITAMs on the chains of the T cell receptor complex. After an ITAM has been fully phosphorylated, the tyrosine kinase ZAP-70 binds to the ITAM enabling ZAP-70 to be phosphorylated and activated by Lck. The activated ZAP-70 then phosphorylates other intracellular signaling molecules. [74]

Though the exact T cell receptor triggering mechanism is not known yet, it is known that the first intracellular signal that is generated after the T cell has detected its specific antigen is the phosphorylation of both tyrosines residues in the ITAMs of the T cell receptor complex. This signal is initiated with the help of the CD4 or CD8 co-receptors (see Figure 1.3). These are in the intracellular domain associated with a kinase, the tyrosine kinase Lck. The extracellular domain of the co-receptor binds to the antigen-carrying MHC molecule, CD4 to class II MHC and CD8 to class I MHC molecules, respectively. The engagement of the extracellular domain of the co-receptor with the MHC molecule brings the co-receptor and particularly with it the associated kinase Lck in close proximity to the T cell receptor, resulting in more efficient phosphorylation of the ITAMs of the T cell receptor complex by Lck. The kinase Lck, associated to the co-receptors, is thought to be the kinase primarily responsible for the phosphorylation of the ITAMs of the T cell receptor complex [74]. Lck is important for T cell receptor signaling, during the selection of developing T cells in the thymus as well as in naive and effector T cells, but is less important for the activation or maintenance of memory cytotoxic T cells. After both tyrosine residues within an ITAM are phosphorylated by the kinase Lck, another tyrosine kinase, ZAP-70, the ζ-chain-associated protein kinase, can bind to it. The kinase ZAP-70 carries the activation signal onward. Once bound, ZAP-70 is phosphorylated by Lck at three tyrosine residues. Together these phosphorylations activate ZAP-70 by disrupting the autoinhibited form of inactive ZAP-70 allowing the ZAP-70 kinase domain to adopt the active conformation. The active ZAP-70 then phosphorylates further proteins that again initiate downstream signaling molecules [74].

For an effective immune response, T and B cells must be able to respond to their specific antigen even at extremely low levels. This is especially important for T cells, as an antigen-presenting cell will display many different peptides on its surface from both self and foreign proteins, and the number of peptide MHC complexes specific for a particular T cell receptor is likely to be very low. Some estimates suggest that a naive helper T cell can become activated by fewer than 50 antigenic peptide MHC complexes displayed by an antigen-presenting cell and effector cytotoxic T cells may be even more sensitive. These estimates based on *in vitro* studies may not be precise for cells *in vivo*, but it is clear that the antigen receptors on T cells confer remarkable sensitivity to antigen [74]. The ability of the T cell receptor to differentiate between its specific pathogen and self peptides is called antigen discrimination. Key characteristics of the T cell activation are thus the exquisite specificity of the antigen discrimination, the high sensitivity of activation and the speed of the biochemical response that occurs within a few seconds after T cell activation [29].

2 Modeling T cell Activation

An elementary model that comes to mind when thinking about modeling T cell activation is the assumption that the activation of T cells is proportional to the number of antigen presented via an MHC molecule that bound to a T cell receptor. Though this model captures the observation that ligands with higher affinity require fewer molecules to trigger T cell activation than ligands with lower affinity, it fails to explain the phenomenon of antigen discrimination as even a ligand with low affinity would eventually trigger the T cell receptor after its concentration was raised sufficiently [103]. This contradicts the T cell receptor's key characteristic of antigen discrimination. Thus, this model is insufficient to explain T cell activation. Therefore, we focus on models that incorporate the principle of the kinetic proofreading. Of course there are numerous approaches to construct a model for T cell activation. Here we consider exclusively deterministic models that strictly describe the early phase of T cell activation, that is, the first minutes after the antigen encounters the receptor. The kinetic proofreading concept models the binding of an antigen presented by an MHC molecule to a T cell receptor triggering an intracellular signal that finally results in proliferation and the release of cytokines, signal molecules that mobilize the adaptive immune system (see Chapter 1). Following mass action kinetics, the deterministic models examined, models that incorporate kinetic proofreading, can be described as systems of ordinary differential equations and their characteristics can be detected by analyzing the qualitative behavior of these systems. The basic assumption underlying these models is that population effects are negligible and the activation of a T cell results from the recognition of a foreign antigen by its own receptor and not from the interaction with other immune cells or cytokines [85]. As our objective is to keep the model simple to gain insights into the dynamics of the molecular mechanisms triggering T cell activation and resulting in the mobilization of the adaptive immune system, we focus on the dynamics of the antigen discrimination process of an individual T cell.

2.1 Kinetic Proofreading

To account for the T cell receptor's ability for antigen discrimination, the ability to discriminate between foreign and self antigens with only moderately lower affinity, McKeithan suggested in 1995 the kinetic proofreading model [70]. The model is based on a model that was formulated for protein synthesis and DNA polymerization, showing how the specificity of the substrate binding of an enzyme can be improved through introducing intermediate stages in the formation of the product [70, 76]. The term kinetic proofreading was introduced in the context of a similar model to address mechanisms where at least two independent substrate recognition steps are coupled to increase the specificity of the binding event [41, 8]. In McKeithan's model the complex of T cell receptor and antigen-carrying MHC molecule undergoes a number of successive modifications before transmitting activation signals to the intracellular domain (see Figure 2.1). These modification steps can be identified with the phosphorylation of the ITAMs of the T cell receptor complex. Starting with the binding of a T cell receptor to its specific antigen presented by an MHC molecule on the surface of an antigen-presenting cell, the complex is phosphorylated several times. Depending on the antigen's affinity to the receptor or the lifetime of the complex formed by the antigen-carrying MHC molecule and the T cell receptor, the complex undergoes a varying number of phosphorylation steps before dissociating. The kinetic proofreading model now assumes that the activation signal for the T cell to start the adaptive immune defense is given by the complex that completed all phosphorylation steps whereas the partly phosphorylated complexes are not able to send the same strong intracellular activation signal. In this way, the lifetime of the complex of antigen-carrying MHC molecule and T

cell receptor determines how many phosphorylation steps are carried out. This implies that ligands with slightly lower affinity, MHC molecules presenting antigens that are structurally similar but not the exact match for the T cell receptor, do not reach the last phosphorylation step as the lifetime of the complex is not long enough but the complex decays and thus does not trigger the strong activation signal within the T cell. Therefore, McKeithan's kinetic proofreading model is able to explain T cell antigen discrimination.

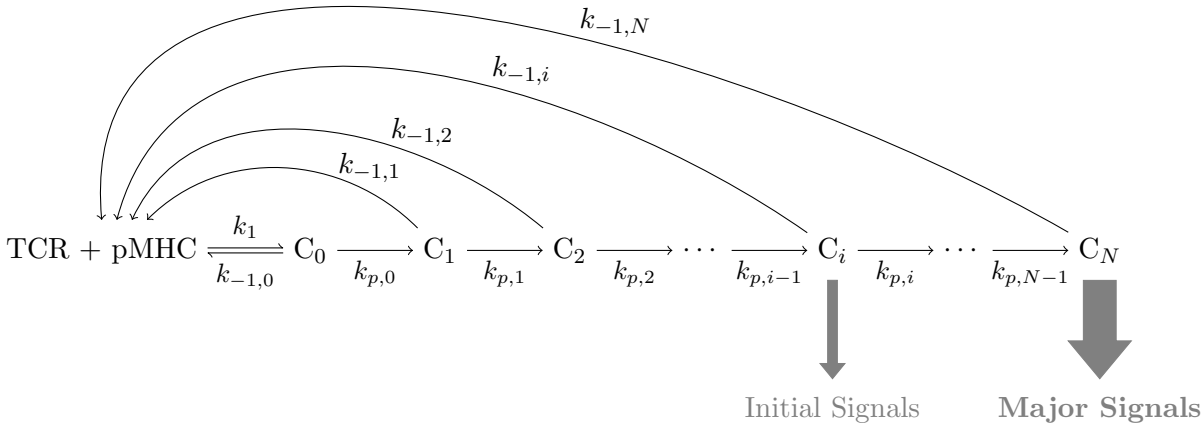


Figure 2.1: Kinetic proofreading model. T cell receptor complex (TCR) and peptide-carrying MHC molecule (pMHC) form a complex C_0 that undergoes a series of phosphorylations, either reaching the state of maximal phosphorylation sending an activation signal, or getting only partly phosphorylated, potentially sending weak activation signals, and decomposing back into T cell receptor complex and peptide-carrying MHC molecule. McKeithan assumed equal reaction constants for the phosphorylation steps to keep the model simple, i.e. $k_{p,i} \equiv k_p$ for all $0 \leq i \leq N-1$ and $k_{-1,i} \equiv k_{-1}$ for all $0 \leq i \leq N$ [70].

Concretely, the kinetic proofreading model follows mass action kinetics and involves as variables the concentration of T cell receptors, R, the concentration of MHC molecules carrying antigen, M, the concentration of complexes formed by the binding of a T cell receptor to an MHC molecule, C_0, and the concentration of phosphorylated complexes C_i where $1 \leq i \leq N$ for some $N \in \mathbb{N}$, with N being the maximal number of phosphorylation steps. In the following, we will refer to the concentration of some molecule in italic notation. That is, for example C_i denotes the molecule which we find in the representation of the reactions and C_i denotes its concentration which occurs as variable in the differential equations associated to the reactions. The assumption of mass action kinetics is that the rate with which a chemical reaction takes place is directly proportional to the product of the concentration of its reactants. McKeithan kept the model simple by assuming the same association and dissociation rates for the phosphorylated complexes (cf. Figure 2.1). The T cell receptor and the antigen-carrying MHC molecule associate with the rate k_1 and dissociate with the rate k_{-1}. The phosphorylation of the complexes $\mathrm{C}_0, \ldots, \mathrm{C}_{N-1}$ occurs at a rate of k_p, while the dissociation of the complexes, leading to the separation of the T cell receptor (TCR) and the antigen respectively peptide-carrying MHC molecule (pMHC), takes place with the same rate k_{-1} as the dissociation of the unphosphorylated complex C_0. In particular, the partly phosphorylated complexes are either phosphorylated once more or they decompose, but the phosphorylation steps are not reversible.

Sontag [95] analyzed McKeithan's kinetic proofreading model without the simplification that $k_{p,i} \equiv k_p$ for all $0 \leq i \leq N-1$ and $k_{-1,i} \equiv k_{-1}$ for all $0 \leq i \leq N$ (cf. Figure 2.1). Sontag proved that the system of differential equations resulting from this model with mass action kinetics has a unique steady state that is globally asymptotically stable for every set of positive rate constants and total concentration of T cell receptors and peptide-carrying MHC molecules by applying the Deficiency Zero Theorem of Chemical Reaction Network Theory (see Chapter 3). Specifically, the system of ordinary differential equations that models McKeithan's kinetic proofreading reads as

follows:

$$\begin{aligned}
\dot{C}_0 &= k_1 \left(R_{\text{tot}} - \sum_{i=0}^{N} C_i \right) \left(M_{\text{tot}} - \sum_{i=0}^{N} C_i \right) - (k_{-1,0} + k_{p,0})\, C_0 \\
\dot{C}_i &= k_{p,i-1} C_{i-1} - (k_{-1,i} + k_{p,i})\, C_i \qquad \text{for } 1 \leq i \leq N-1 \\
\dot{C}_N &= k_{p,N-1} - k_{-1,N} C_N
\end{aligned}$$

where $N \in \mathbb{N}$ corresponds to the number of phosphorylation steps and R_{tot} and M_{tot} represent the total concentration of TCR and pMHC, that is, the sum of free TCR, R, and complex bound TCR and the sum of free pMHC, M, and complex bound pMHC, respectively. Due to this two conserved quantities,

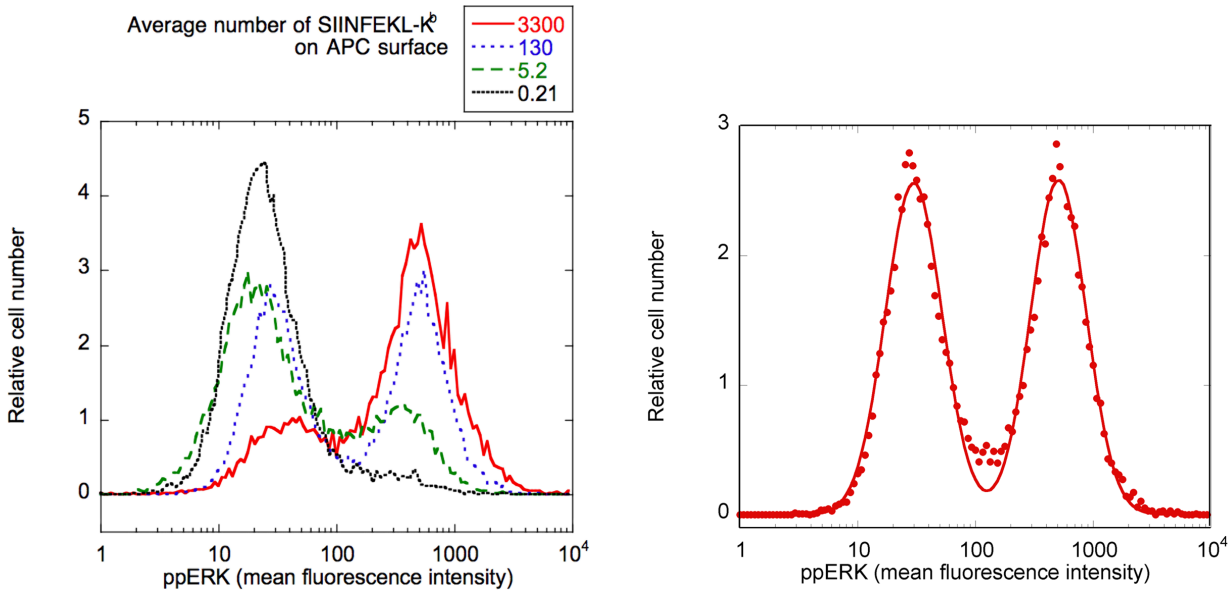
$$R_{\text{tot}} = R + \sum_{i=0}^{N} C_i \quad \text{and} \quad M_{\text{tot}} = M + \sum_{i=0}^{N} C_i,$$

the system of differential equations characterizing the kinetic proofreading model reduces to the above time derivatives of the complexes.

2.2 Kinetic Proofreading with Competing Positive and Negative Feedback Loops

Altan-Bonnet and Germain [2] observed in 2005 a digital response in the downstream signal initiated by the activated T cell. The intracellular signal pathway carrying forward the signal of the T cell receptor includes the MAPK cascade, the mitogen-activated protein kinase cascade, a series of protein kinases that become phosphorylated and activated upon cellular stimulation by ligands and lead to new gene expression by phosphorylating key transcription factors. In T and B cells the last MAPK in this series is ERK, extracellular signal-related kinase [74]. Altan-Bonnet and Germain measured the ERK phosphorylation using APCs, antigen-presenting cells, with calibrated numbers of ligand to activate T cells. They found that after three minutes of contact with APCs, the pattern of staining, that is, the pattern of fluorescence of doubly phosphorylated ERK (ppERK), is strictly bimodal (see Figure 2.2), that means the ERK response of T cells is essentially digital [2]. The bimodal distribution of ppERK response shows that there are two peaks depending on the average number of agonist pMHC on the surface of the APC (cf. Figure 2.2(a)). Figure 2.2(b) shows the digital response of ppERK to a medium number of pMHC activating the T cell receptors and a fit of the bimodal distribution as a sum of two discrete log-normal distributions. Due to this Altan-Bonnet and Germain assumed a switch-like ppERK response of the individual cell [2]. The most T cells showing ppERK activity are found at two different mean fluorescence activities of ppERK, one peak at a low intensity, indicating a weak activation signal transmitted from the T cell receptor, the other peak at a high intensity, indicating a strong transmitted activation signal.

To account for this digital ppERK response, Altan-Bonnet and Germain suggested a model including negative and positive feedback. Their model is based on kinetic proofreading but includes further pathways of phosphorylation and dephosphorylation of regulatory proteins. The core model of kinetic proofreading comprises the successive double phosphorylation of three ITAMs through the kinase Lck as well as the co-receptor CD8 carried by cytotoxic T cells and the kinase ZAP-70 that is able to bind to a doubly phosphorylated ITAM (see Figure 2.3). Unlike the kinase Lck that accounts for the phosphorylation steps, the dephosphorylation of the pMHC-TCR complexes is not mediated by an enzyme in the model. It is rather assumed that an active phosphatase is highly abundant and the dissociation of the complexes permits the rapid dephosphorylation by this phosphatase [2]. The model incorporates three ITAMs and, similar to McKeithan's model, assumes the successive phosphorylation of their sites. These three ITAMs in Altan-Bonnet and



(a) Relative number of T cells by mean fluorescence intensity of ppERK. The different colored graphs belong to different average numbers of agonist pMHC ligands per APC. SIINFEKL-K^b is a monoclonal antibody, i.e. an antibody that only binds to one specific epitope, here to an epitope of the agonist pMHC. That way the number of agonist pMHC on the surface of the APC can be measured.

(b) Sum of two log-normal distributions as fit of the distribution of ppERK among naive T cells activated with an average of 130 SIINFEKL-K^b ligands on the surface of each APC (corresponding to the blue colored data in the left panel).

Figure 2.2: Digital nature of ERK activation in naive T cells. The panels show the distribution of ERK phosphorylation among individual naive T cells after 3 min of activation at different levels of presentation oft the agonist pMHC. [2]

Germain's model correspond to the three ITAMs of a ζ chain (cf. Figure 1.2). The sequential phosphorylation process in the model is based on studies that identified a specific order in the phosphorylation of ζ chains [54, 77, 44]. Altan-Bonnet and Germain assume that immediately after an ITAM is phosphorylated at both its phosphorylation sites, a ZAP-70 molecule binds to it. After passing through all three ITAM phosphorylation steps, carrying now three ZAP-70 molecules, the ZAP-70 molecules of the complex are phosphorylated by Lck and that way enabled to phosphorylate other signaling molecules. We refer to these complexes that passed all ITAM phosphorylation steps and carry at least one activated ZAP-70 molecule as the fully phosphorylated complexes. The fully phosphorylated complexes transmit the activation signal by activating further signal pathways, including the MAPK cascade. Furthermore, every complex carrying at least one ZAP-70 molecule can induce a negative feedback by activating the phosphatase Shp-1. Shp-1 has to be phosphorylated before it can be enzymatically active. The network schema (Figure S6 in the SI of [2]) illustrates the phosphorylation of Shp-1 by the complexes carrying a ZAP-70 molecule but not binding a Lck molecule. Once it is phosphorylated, the Shp-1 molecule can bind to a T cell receptor complex not involving Lck, which means, again, it can bind to the complexes that are not intermediates of an enzyme-substrate reaction. The phosphorylated and complex-bound Shp-1 is activated by Lck binding to the complex, phosphorylating the complex and activating Shp-1. Binding of phosphorylated Shp-1 to fully phosphorylated complexes results in the direct activation of Shp-1 without an additional Lck that has to bind to the complex. The phosphorylated Shp-1 dissociates from the complex and acts as an active phosphatase dephosphorylating the ITAMs which corresponds to a negative feedback in the kinetic proofreading system. This negative feedback is opposed to a positive feedback that sets in with a time delay initiated by the MAPK cascade which is triggered by the fully phosphorylated complexes. More precisely, the fully phosphorylated complexes, the complexes whose ITAMs have undergone all phosphorylation steps and are carrying

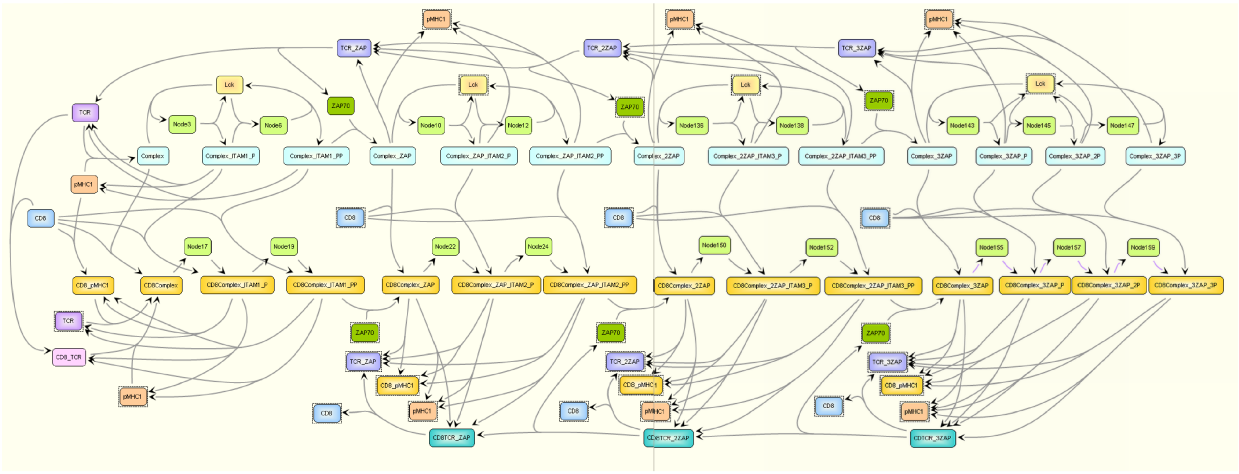


Figure 2.3: Main network of T cell receptor activation. The phosphorylation steps of the pMHC-TCR complexes are mediated by the kinase Lck. The phosphorylation by Lck is modeled as a enzyme-substrate reaction, that is, the binding of the substrate to the enzyme Lck is reversible but the transfer of a phosphate residue to it is not. The graphic indicates only the binding reaction which has a higher reaction rate than the unbinding of the substrate from the enzyme. The upper part of the panel thus corresponds to McKeithan's kinetic proofreading model including the kinases Lck and ZAP-70. The protein ZAP-70 binds to an ITAM right after both its phosphorylation sites are phosphorylated by Lck. Only then the next ITAM is phosphorylated. The lower part of the panel extends the model by the inclusion of the co-receptor CD8. [2]

at least one active ZAP-70 molecule, phosphorylate an adapter protein. The adapter protein then initiates the MAPK cascade, which in turn activates the kinase ERK. The doubly phosphorylated kinase ERK, ppERK, acts as a positive feedback by serine phosphorylation of Lck in the complexes, a biochemical modification that prevents the kinase from binding to the phosphatase Shp-1 and thus protecting the ITAMs from dephosphorylation [2]. The activated kinase ppERK binds to complexes not involving a Lck molecule and results in a kinetic proofreading scheme corresponding to the absence of Shp-1.

To compare experiments and computer simulations, Altan-Bonnet and Germain implemented a set of ordinary differential equations using the software JDesigner and Matlab. Altogether, the model of Altan-Bonnet and Germain is a very elaborate model including lots of variables and reactions. Whereas the positive feedback through ppERK is of digital nature due to its activation via the MAPK cascase, the negative feedback mediated by the phosphatase Shp-1 is analog [2]. It has been observed in experiments that the presence of antagonists, which are ligands similar to the agonist intended to bind to the T cell receptor and trigger a response but are not able to initiate a T cell response on their own, can nevertheless reduce the activation signal initiated by the agonist. This phenomenon is referred to as antagonism [103]. Further research showed that the negative feedback through the phosphatase Shp-1 is involved in this suppression by antagonists [97, 2]. Altan-Bonnet and Germain made the counter-intuitive observation that at low concentrations, strong antagonists diminish the T cell activation more than weak antagonists. That is, strong antagonists result in an increased negative feedback until a concentration is reached that overcomes the threshold to activate the T cell and induce an activation signal themselves.

2.3 Kinetic Proofreading with Negative Feedback

In 2013, François, Voisinne, Siggia, Altan-Bonnet and Vergassola [33] proposed a model based on kinetic proofreading including only the negative feedback caused by Shp-1 activation that can

largely be solved analytically. As in McKeithan's kinetic proofreading model the T cell receptor complex, denoted by TCR and its concentration by R, and a peptide-carrying MHC molecule, L_1, form a complex C_0 with a binding rate κ. In the previous kinetic proofreading models the peptide-carrying MHC molecule was denoted by pMHC and its concentration by M. The notation is changed here because the inclusion of antagonism in the model introduces two different peptides or ligands, respectively. The complex C_0 decomposes again into the two components TCR and L_1, with a rate ν_1, or is phosphorylated with a rate ϕ. It follows a series of phosphorylations with the constant phosphorylation rate ϕ until the complex reaches the fully phosphorylated state C_N (see upper part of Figure 2.4). The fully phosphorylated complex C_N transmits the activation signal for the T cell to induce the immune response. Both fully and partially phosphorylated complexes C_j, $1 \leq j \leq N$, can decompose in the same way as the unphosphorylated complex C_0 with rate ν into the two components T cell receptor and ligand carrying MHC molecule. Additionally, François et al. introduced a dephosphorylation rate which was not present in McKeithan's model (cf. Figure 2.1). The phosphorylated complexes C_j, $1 \leq j \leq N$, can lose a phosphate residue, resulting in the complex C_{j-1}. Thus, these complexes can undergo phosphorylation, dephosphorylation, or decomposition. The dephosphorylation is modeled with a default rate b. It is assumed that these phosphorylations are catalyzed by a constitutive phosphatase that is available in a sufficiently high concentration such that it does not have to be included in the model. The same applies to the kinase Lck which is also not explicitly modeled just as in McKeithan's basic kinetic proofreading model and in contrast to the more detailed and complicated model of Altan-Bonnet and Germain. In addition to the default dephosphorylation rate, François et al. included the enzyme Shp-1 in the model that, once activated, contributes to the dephosphorylation of the phosphorylated complexes. Initially the phosphatase Shp-1, denoted by S, exists in its inactive form but gets activated by the singly phosphorylated complex C_1. The Shp-1 now contributes with a rate γ to the dephosphorylation of the phosphorylated complexes. This leads to a negative feedback. As more antigen-carrying MHC molecules bind to a T cell receptor and get phosphorylated by Lck, more Shp-1 phosphatases are activated. This, in turn, increases the dephosphorylation rate. As a consequence, the number of complexes passing through all phosphorylation steps is reduced, resulting in a diminished concentration of fully phosphorylated complexes C_N and, therefore, a decrease in the activation signal.

Furthermore, François et al. extended their model by including antagonism. For simplicity, François et al. assumed the same kinetic binding, phosphorylation and dephosphorylation rates for the antagonist as for the agonist. The core difference between agonist and antagonist is the binding time of the antigen presented by an MHC molecule to the T cell receptor complex, following the "lifetime dogma". The agonist has a longer binding time to the T cell receptor complex. Even though the antagonist can bind to the latter as well, the engagement is not as stable, leading to an earlier decomposition of antigen-carrying MHC molecule and T cell receptor. This is represented in the model through the higher dissociation rate ν_2 of the agonist. Therefore, more agonists that form a complex with the T cell receptor reach the final state of phosphorylation than antagonists whose complexes are more likely to decompose early on. In the model, the combination of fully phosphorylated agonist complexes and fully phosphorylated antagonist complexes initiate the activation signal for the T cell (see Figure 2.4). Antagonism in T cell response denotes the experimental observation that antagonistic ligands are incapable of activating T cells by themselves but can reduce the response elicited by agonistic ligands when both of them are present [103]. François et al. show that in their model the ligand competition for the receptors is negligible for the effect of antagonism but identify the phosphatase Shp-1 as the source of it [33]. Both simply phosphorylated complexes, the one formed by the agonist, C_1, and the one formed by the antagonist, D_1, activate Shp-1 and activated Shp-1 dephosphorylates both types of complexes equally. Suppose now a mixture of few agonists and a large number of antagonists is given. As the lifetime of the complexes including the antagonist is much shorter than that of the agonist, the former rarely reach the fully phosphorylated state, whereas the latter become fully phosphorylated and trigger a response. Both phosphorylation pathways are linked through Shp-1. Therefore, a high concentration

of antagonistic ligands does not trigger a cell response itself but reduces the activation signal by activating the phosphatase Shp-1, which in turn diminishes the amount of fully phosphorylated complexes involving the agonistic ligand. and, with it, the activation signal. Furthermore, the negative feedback by Shp-1 results in the fact that there is a range of Shp-1 concentration for which there is an optimal concentration of MHC molecules carrying agonistic ligands that triggers a strong response, before the response is reduced under higher MHC concentration due to the activation of Shp-1.

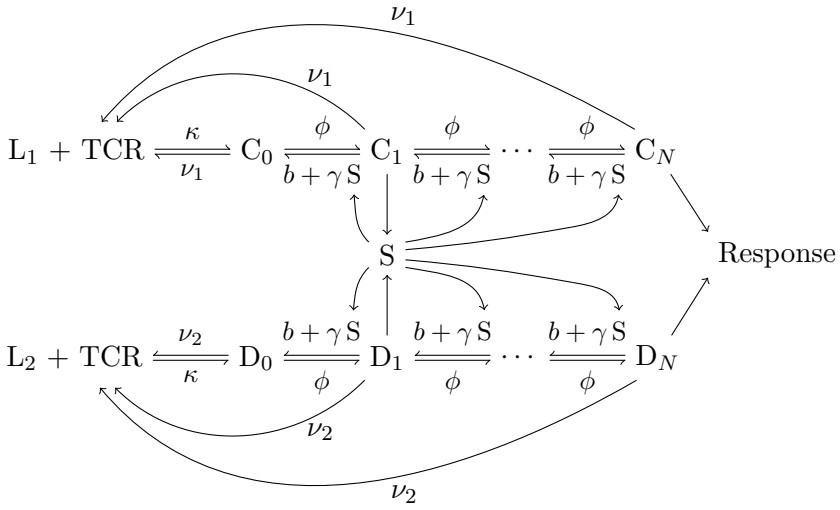


Figure 2.4: Model of François et al. including agonist, antagonist and negative feedback through activated Shp-1. L_1 represents the agonist carrying MHC molecule and L_2 that carrying the antagonist. TCR represents the T cell receptor, while C_i and D_i represent the ligand receptor complexes after i phosphorylation steps that take place with a phosphorylation rate ϕ. The two ligands, agonist (L_1) and antagonist (L_2), are modeled with the same binding rate κ but different dissociation rates ν_1 and ν_2, respectively. The abbreviation S represents the active Shp-1. The singly phosphorylated complexes activate inactive Shp-1 and the activated Shp-1, S, contributes with a rate γ to the dephosphorylation rate b. In this way, the activation of Shp-1 through the singly phosphorylated complexes leads to a negative feedback as the dephosphorylation rate of the complexes rises, and therefore fewer complexes reach the fully phosphorylated states C_N and D_N that induce the activation signal.

Following mass action kinetics, the above modeling results can be summed up in a system of ordinary differential equations where the concentration of complexes involving antagonistic antigen is represented by D_i, $0 \leq i \leq N$, and the concentration of the complexes with agonistic ligands by C_i as introduced above. There are three conserved quantities, the total concentration of Shp-1, S_{tot}, that is the sum of the concentrations of active Shp-1, S, and inactive Shp-1, the total concentration of T cell receptors, R_{tot}, which is the sum of free receptors, R, and receptors that are bound in complexes, C_i or D_i, and the total concentration of ligands, $L_{1,\text{tot}}$ and $L_{2,\text{tot}}$, the sum of free ligands L_1 and L_2, that is, antigen presented by an MHC molecule that did not yet engage with a T cell receptor, and ligands bound to a T cell receptor complex, C_i and D_i, respectively. With α being the binding rate of inactive Shp-1 to the singly phosphorylated complexes and β being the rate with which the active phosphatase turns back into its inactive state, this model results in the following system of ordinary differential equations [33]:

$$\dot{S} = \alpha\,(C_1 + D_1)\,(S_{\text{tot}} - S) - \beta S$$

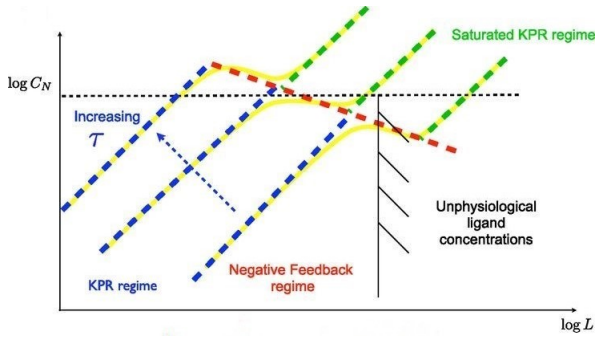
$$\dot{C}_0 = \kappa \left(L_{1,\text{tot}} - \sum_{i=0}^{N} C_i \right) \left(R_{\text{tot}} - \sum_{i=0}^{N} (C_i + D_i) \right) + (b + \gamma S) C_1 - (\phi + \nu_1) C_0$$

$$
\begin{aligned}
\dot{C}_i &= \phi C_{i-1} + (b+\gamma S)C_{i+1} - (\phi + b + \gamma S + \nu_1)C_i, \qquad 1 \le i \le N-1 \\
\dot{C}_N &= \phi C_{N-1} - (b + \gamma S + \nu_1)C_N \\
\dot{D}_0 &= \kappa \left(L_{2,\mathrm{tot}} - \sum_{i=0}^{N} D_i \right) \left(R_{\mathrm{tot}} - \sum_{i=0}^{N} (C_i + D_i) \right) + (b+\gamma S)D_1 - (\phi + \nu_2)D_0 \\
\dot{D}_i &= \phi D_{i-1} + (b+\gamma S)D_{i+1} - (\phi + b + \gamma S + \nu_2)D_i, \qquad 1 \le i \le N-1 \\
\dot{D}_N &= \phi D_{N-1} - (b + \gamma S + \nu_2)D_N.
\end{aligned}
\tag{2.1}
$$

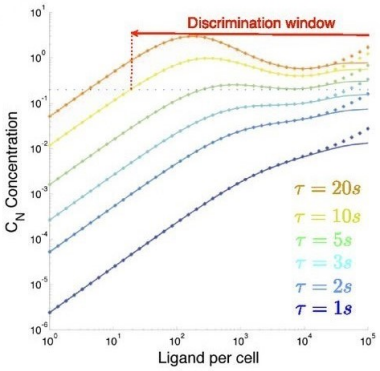
First, François et al. examined the case where there is no antagonist present, that is, the case where $L_2 = 0$. Under the assumption that $\sum_{i=0}^{N} C_i << R$, which corresponds to the situation that the concentration of ligands is rather small compared to the concentration of the T cell receptors, they obtained a steady state approximation for the concentration of C_N and therefore for the signal strength to activate an immune response. François et al. further investigated two limiting cases. The first, called the kinetic proofreading regime, corresponds to the presence of very few ligands such that the phosphatase Shp-1 is not turned on, and its concentration can be assumed to be zero, $S = 0$. The second corresponds to an intermediate concentration of Shp-1. That is, there is a high concentration of ligands that are sufficient to activate a significant amount of Shp-1 without saturating it [33]. This intermediate non-saturating regime represents a region where the negative feedback through the activated phosphatase Shp-1 dominates. That is, the concentration of fully phosphorylated complexes, C_N, decreases with the increasing concentration of ligands, L_1. Consequently, the kinetic proofreading system with negative feedback does not show a monotone dependence of the signal strength on the agonist concentration, in contrast to the pure kinetic proofreading system. François et al. derived a steady state approximation for the concentration of fully phosphorylated complexes in the intermediate regime. The comparison of their analytical derivation and the numerical integration of these steady state concentrations, along with the schematic diagram of the three regimes examined, can be seen in Figure 2.5.

Including antagonism, that is, a second ligand, $L_2 \neq 0$, under the same assumption that the number of both ligands is rather small compared to the concentration of T cell receptors, leads to more complicated terms for the approximation of the steady state concentrations of the fully phosphorylated agonist, C_N, and antagonist, D_N. François et al. compared their analytical approximations to a numerical integration of the equations for this case as well, and found that both showed the same qualitative course of the response function. This response function, the concentration $C_N + D_N$ in dependence of the present agonist concentration, shows exactly the counter-intuitive observation that stronger antagonists can initiate a stronger feedback and therefore reduce the T cell activation signal (see Figure 2.6).

Rendall and Sontag [85] rigorously analyzed the mathematical model of François et al. in 2017. First, they proved that starting with positive concentrations, none of the quantities can approach zero, and used this to show that for any fixed choice of parameters there exists at least one steady state and its concentrations are strictly positive. This leads to the question of multistationarity, which they examined next. Rendall and Sontag were able to prove that the agonist-only case, corresponding to the above system of ordinary differential equations (2.1) where the quantities L_2 and D_i, $1 \le i \le N$, representing the antagonist and its complexes, are set to zero, exhibits multistationarity for particular parameters when there are three phosphorylation steps, $N = 3$. For $N = 2$ and $N = 1$ multistationarity is not possible, but in the latter case the system is able to exhibit damped oscillations as proved by Rendall and Sontag [85]. The question of stability of the steady state or states of this system is not easily determined, and to gain some information about the stability, the authors considered limiting cases of the agonist-only system. Setting the binding rate of inactive Shp-1 to the singly phosphorylated complexes and the concentration of active Shp-1 to zero, $\alpha = 0$ and $S = 0$, results in a system without negative feedback. This system differs from the generalized version of McKeithan's kinetic proofreading system analyzed by Sontag



(a) Schematic diagram of the different regimes and respective limiting cases. The blue slope represents the region where only few ligands are present, and their concentration is not high enough to activate the phosphatase Shp-1 so that the curve in this region follows that of kinetic proofreading without negative feedback. The red slope represents the region where some Shp-1 enzymes are turned on but not yet all of them, the intermediate regime. The negative feedback results in a decreasing C_N-concentration. A further increase in the ligand concentration results in more binding events between antigen presented on an MHC molecule and T cell receptors, leading to a higher concentration of singly phosphorylated complexes, which in turn activate more Shp-1 until all phosphatase Shp-1 present is turned on and contributes to the increased dephosphorylation rate, resulting in a dynamic that corresponds to simple kinetic proofreading with the maximum dephosphorylation rate $b + \gamma S_{\text{tot}}$, where S_{tot} represents the total concentration of Shp-1. This regime is indicated by the green slope. The three blue and green slopes correspond to three different dissociation times and the solid yellow lines indicate interpolations for these dissociation times [33].



(b) Intermediate regime. Numerical integration for the steady-state concentration of fully phosphorylated complexes, C_N, per cell in dependence on ligands presented per cell for different lifetimes τ, that is, for different dissociation rates $\nu = \tau^{-1}$. The crosses indicate the analytical derivation for the limit case, assuming no receptor saturation of the phosphatase Shp-1. The dotted line indicates the threshold on C_N for activation of response [33]. A strong ligand (orange curve) already triggers a response at very low concentrations whereas a less strong ligand (green curve) needs to be present at a much higher concentration to activate the T cell and a weak ligand (blue curve) has to exist in very high concentrations to trigger the T cell or even may not be able to do so. For the stronger ligands we see a region where the C_N-concentration decreases with increasing concentration of L_1 due to the negative feedback caused by activated Shp-1 which also damps the increase of the curve of weaker ligands. This leads to a wider discrimination window compared to simple kinetic proofreading without a negative feedback, as weak ligands only trigger a signal at higher concentrations, and therefore an improved discrimination between self and non-self antigens.

Figure 2.5: Intracellular activation signal for T cells in form of the concentration of fully phosphorylated complexes, C_N, for $N = 5$, depending on the antigen concentration, the ligands $L = L_1$ for the agonist-only case ($L_2 = D_i = 0$ for all $0 \leq i \leq N$). Log-log plot. For parameters see [33].

(cf. Figure 2.1) only in the dephosphorylation rate b, which does not exist in the latter one. The limiting case of the agonist-only system possesses, just as McKeithan's system, a unique steady state that is globally asymptotically stable and hyperbolic [85]. By gradually reducing the assumptions made for the limiting case, information about a stable steady state can be deduced for the full system. Setting only $\alpha = 0$ results in a system with constantly decreasing Shp-1 concentration. It can be concluded from the first limiting case that this system possesses a hyperbolic sink on the boundary. That is, with $S = 0$ and all other variables positive, the full agonist system where α is not zero but sufficiently small exhibits a hyperbolic sink as well. Furthermore, Rendall and Sontag proved this hyperbolic sink to be the unique steady state and to be globally asymptotically stable by showing that for small α a solution can have no ω-limit points other than the steady state. This property can also be deduced for the full system including the antagonist in a similar manner [85].

The further mathematical analysis of the system by Rendall and Sontag addresses the observation by François et al. that the agonist-only case shows a non-monotone dependency of the concentration of fully phosphorylated complexes, C_N, on the antigen concentration, L_1, in other words, the observation of a signal strength that does not necessarily increase with a higher ligand concentration. Rendall and Sontag analyzed the dependency of C_N not only on the present ligand concentration

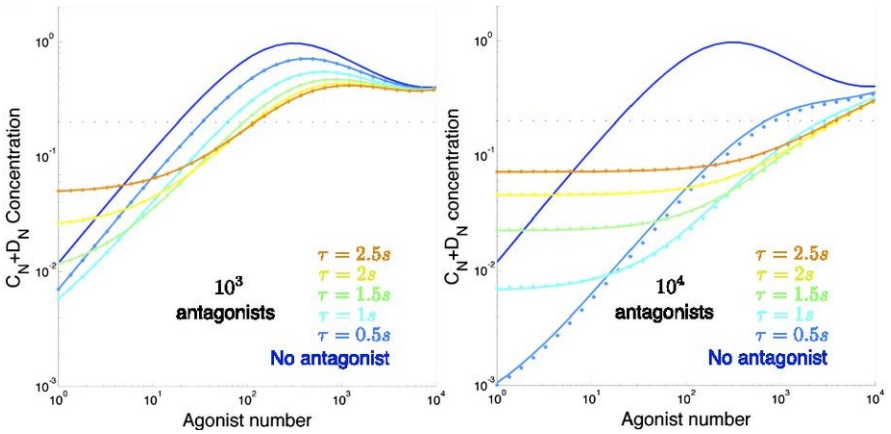


Figure 2.6: Antagonism. Comparing the left and right panels, featuring lower and higher antagonist concentrations, reveals that the relative strength of antagonists cannot be ranked simply by their binding time without reference to their concentrations [33]. We can consider the orange curve one of a strong antagonist. At both antagonist concentrations, that is, in both panels, we observe that compared to the no-antagonist case the presence of relatively strong antagonists increases the $C_N + D_N$ concentration at low agonist levels and decreases it as soon as the agonist concentration is raised. At very low agonist levels a strong antagonist can significantly contribute to inducing an activation signal as the agonists are too few in number to induce the same immune response. With increasing agonist level, however, the number of agonists is sufficient to trigger a strong immune response. Now the strong antagonist primarily contributes to the activation of Shp-1, which increases the negative feedback. A weak antagonist, as represented by the light blue curve, is less likely to reach the fully phosphorylated state than a strong antagonist. However, their binding time can still be long enough to get singly phosphorylated and activate the phosphatase Shp-1 and diminish the activation signal. At high agonist levels, the strong antagonist can diminish the activation signal more than a weak antagonist due to the increased portion of activated Shp-1. [33]

but also on the dissociation rate $\nu_1 = \tau^{-1}$, the inverse of the binding time of the ligand to the receptor. Concretely, they defined a response function $\log C_N^* = F(\log L_1, \nu_1)$ where log quantities are used for convenience and C_N^* denotes a steady state concentration of the fully phosphorylated complex. In case that there is more than one steady state, this function should be thought of as a multi-valued function [85]. The authors proved that if the number of phosphorylation steps is higher than two, that is, for all $N > 2$, there exists a range for L_1 in which the response function is decreasing. Within this range of L_1, the response function can be an increasing function of ν_1 [85]. This counter-intuitive dependency of ν_1 means that it is possible that the concentration of fully phosphorylated complexes can increase with an increasing dissociation rate, that is, a shorter binding time. This was proved for the regime where the C_N concentration decreases due to the negative feedback of activated Shp-1. A diminished binding time could result in a reduced C_1 concentration, which, in turn, would lead to a diminished activation of Shp-1 phosphatases, potentially decreasing the impact of the negative feedback or even overcoming it and resulting in an increased C_N concentration. That means that the kinetic proofreading system with negative feedback does not only fulfill the experimental observations of an optimal ligand concentration (cf. Figure 2.5 and Figure 2.6), but for the agonist-only case also that of an optimal affinity of the antigen-presenting MHC molecule to the T cell receptor [61, 53, 103]. Rendall and Sontag provide an example for an explicit set of parameters under which the response function, with fixed ligand concentration L_1, as a function of the dissociation rate ν_1 exhibits a maximum. This indicates an optimal dissociation rate or lifetime or affinity, respectively [85]. Including the antagonist, Rendall and Sontag were able to show for the full system, in an analogous way to the agonist-only case, that there exist parameters and a regime for the antagonist L_2 where the signal initiated, corresponding

in the model to the sum of the fully phosphorylated complexes, $C_N + D_N$, decreases with L_2 when the agonist concentration L_1 is held fixed. This implies that the increase of a self antigen concentration can lead to a reduced T cell activation signal to a foreign antigen [85].

2.4 Kinetic Proofreading with Lck (and ZAP-70)

Whereas there exists a comprehensive mathematical analysis for the kinetic proofreading system with negative feedback (2.1), as discussed above, the elaborate model of Altan-Bonnet and Germain (cf. Section 2.2) poses analytical challenges in examining its associated differential equations. However, there are no difficulties to formulate part of Altan-Bonnet and Germain's main network of T cell activation (cf. Figure 2.3) in a system of differential equations. The main network of their model comprises a kinetic proofreading core expanded by the inclusion of the kinases Lck and ZAP-70, the phosphorylation of the latter and the co-receptor CD8. The model we want to analyze corresponds effectively to Altan-Bonnet and Germain's kinetic proofreading core model without including the co-receptor CD8 (cf. upper part of Figure 2.3). That is, we want to investigate the implications of including the kinases into McKeithan's classic kinetic proofreading model. First, we incorporate the kinase Lck in McKeithan's kinetic proofreading model (cf. Figure 2.1), which is responsible for the phosphorylation of the T cell receptor complexes, specifically its ITAMs (cf. Section 1.2). We aim to investigate characteristics of the kinetic proofreading system with explicit modeling of the kinase Lck. Altan-Bonnet and Germain defined a number of six phosphorylation steps in their model, representing the double phosphorylation of three ITAMs. The T cell receptor complex consists of the T cell receptor, which includes two ζ chains, each with three ITAMs, and two CD3 proteins, each formed by two chains with one ITAM (cf. Figure 1.2). This configuration totals ten ITAMs, and as each ITAM offers two phosphorylation sites, it potentially allows for up to twenty phosphorylation steps. Therefore, we will analyze the model as McKeithan for an arbitrary number of phosphorylation steps N. Next, we want to include not only the kinase Lck but ZAP-70 as well. The kinase ZAP-70 can bind to an ITAM with both of its tyrosine residues phosphorylated. Once bound to a phosphorylated ITAM, ZAP-70 itself can be phosphorylated, leading to its activation. Activated ZAP-70 is able to phosphorylate other intracellular signaling molecules, forwarding the T cell activation signal (cf. Figure 1.3). Since Altan-Bonnet and Germain assumed a number of three ITAMs in their model, the phosphorylation of these is followed by a number of three phosphorylation steps for the three ZAP-70 molecules that have to be activated. When we include the kinase ZAP-70 in the kinetic proofreading model with Lck and an arbitrary number of N phosphorylation steps, N should be an even number as each ITAM possesses two tyrosine residues that must be phosphorylated before ZAP-70 can bind to it. Consequently, the model contains $N/2$ additional phosphorylation steps for the ZAP-70 kinases.

Including the kinase Lck in the kinetic proofreading model, we follow the kinetics of an enzyme reaction. The conversion of a substrate, here a complex formed by the antigen-presenting MHC molecule and the T cell receptor, into a product, in this case the phosphorylated complex, is catalysed by an enzyme, the kinase Lck. Whereas the binding of the substrate to the enzyme is still reversible, the catalysis that follows is not (see Figure 2.7). The product of these catalyses, the phosphorylated complexes, can decompose again into the T cell receptor and the antigen-carrying MHC molecule just as in the classic kinetic proofreading model (cf. Figure 2.1). With regard to the Chemical Reaction Network Theory that we will apply in the next chapter to get information about the possibility of multistationarity of the system, we display the model in a second graph. In this representation, every reaction is represented by exactly one arrow. That is, there are no branching arrows and all substrates and all products of a reaction are mapped together (see Figure 2.8). To describe the model, we use the same abbreviations for the molecules as in Figure 2.8, that is, R and M for the T cell receptor and the pMHC molecule, C_0 for the complex formed by them, C_i, $1 \leq i \leq N$, for the phosphorylated complexes, B_i for the enzyme-substrate complexes and L for Lck. Again, we use the abbreviations in italics to denote the variables for the concentrations of the

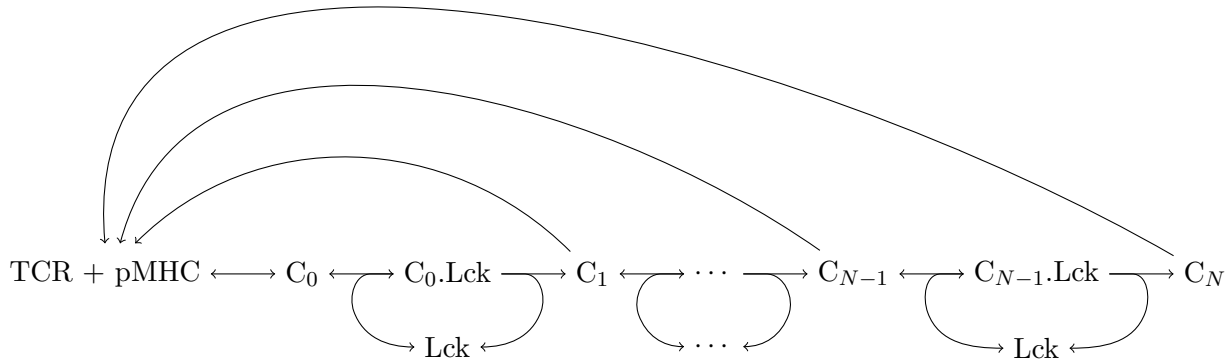


Figure 2.7: Kinetic proofreading with Lck. T cell receptor (TCR) and peptide-carrying MHC molecule (pMHC) form a complex (C_0) to which the kinase Lck can bind, forming the enzyme-substrate complex (C_0.Lck). This enzyme-substrate complex can either decompose again into enzyme and substrate or undergo phosphorylation. While this phosphorylation is irreversible, similar to McKeithan's kinetic proofreading system, the phosphorylated complex C_1 may decompose into T cell receptor and antigen-carrying MHC molecule, losing its phosphate residue. With the help of the kinase Lck, the complex is successively phosphorylated. It can either reach the maximum number of phosphorylations, N, inducing an intracellular activation signal, or decompose at any intermediate state of the phosphorylation steps. The amount of fully phosphorylated complexes, C_N, or its concentration, respectively, serves as a measure for the strength of the T cell activation signal.

respective molecules. By following mass action kinetics, we derive the system of ordinary differential equations associated to the model:

$$
\begin{aligned}
\dot{R} &= -k_1 RM + k_2 C_0 + \sum_{i=1}^{N} k_{6+4(i-1)} C_i = -k_1 RM + k_2 C_0 + \sum_{i=0}^{N-1} k_{6+4i} C_{i+1} = \dot{M} \\
\dot{C}_0 &= k_1 RM - k_2 C_0 - k_3 C_0 L + k_4 B_0 \\
\dot{C}_i &= k_{5+4(i-1)} B_{i-1} - k_{6+4(i-1)} C_i - k_{3+4i} C_i L + k_{4+4i} B_i, \qquad 1 \le i \le N-1 \\
\dot{C}_N &= k_{5+4(N-1)} B_{N-1} - k_{6+4(N-1)} C_N \\
\dot{B}_i &= k_{3+4i} C_i L - k_{4+4i} B_i - k_{5+4i} B_i, \qquad 0 \le i \le N-1 \\
&= k_{3+4i} C_i L - (k_{4+4i} + k_{5+4i}) B_i \\
\dot{L} &= -\sum_{i=0}^{N-1} k_{3+4i} C_i L + \sum_{i=0}^{N-1} k_{4+4i} B_i + \sum_{i=0}^{N-1} k_{5+4i} B_i = -\sum_{i=0}^{N-1} k_{3+4i} C_i L + \sum_{i=0}^{N-1} (k_{4+4i} + k_{5+4i}) B_i.
\end{aligned}
\tag{2.2}
$$

Resulting from mass action kinetics, the right-hand side of the differential equations (2.2) consists of polynomials and is therefore continuously differentiable and consequently locally Lipschitz. This ensures the existence and uniqueness of a solution for every choice of initial concentrations. In our model, there is neither input nor output of molecules, leading to the preservation of all molecules as part of the complexes. This results in three conserved quantities: the total number of T cell receptors, antigen-presenting MHC molecules and enzymes Lck. The total concentration of T cell receptors, R_{tot}, consists of the amounts of free T cell receptors, R, of T cell receptors bound to an MHC molecule in different stages of phosphorylation, C_i, and of the latter complexes bound

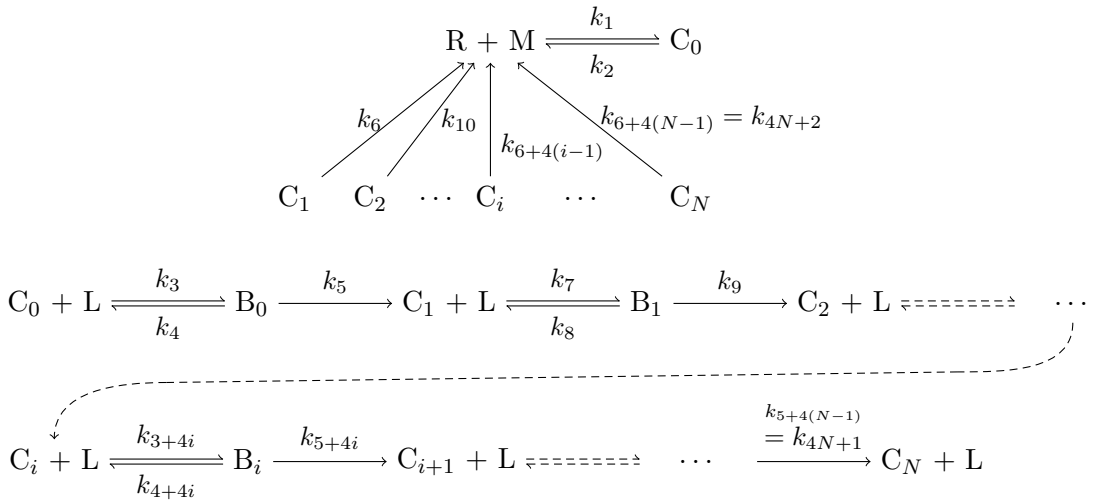


Figure 2.8: Kinetic proofreading with Lck, mapped with reaction constants k_i. The T cell receptor is abbreviated with R, the antigen-carrying MHC molecule with M and the kinase Lck with L. The complexes B_i stand for the enzyme-substrate complexes C_i.Lck.

to the kinase Lck in an enzyme-substrate complex, B_i. Similarly, we derive the total quantity of antigen-presenting MHC molecules, M_{tot}. The total concentration of enzymes Lck involved, L_{tot}, consists of the amount of free Lck, L, and those enzymes that are involved in an enzyme-substrate complex, B_i. We assume all these conserved quantities to be positive:

$$\begin{aligned} R_{\text{tot}} &= R + \sum_{i=0}^{N} C_i + \sum_{i=0}^{N-1} B_i > 0, \\ M_{\text{tot}} &= M + \sum_{i=0}^{N} C_i + \sum_{i=0}^{N-1} B_i > 0, \\ L_{\text{tot}} &= L + \sum_{i=0}^{N-1} B_i > 0. \end{aligned} \tag{2.3}$$

As the mathematical model strictly follows mass action kinetics, each quantity decays in dependence on its own present concentration. Consequently, in every differential equation of the system (2.2), all the terms that contribute negatively to the rate of change include the quantity they describe as a factor. Systems of this type, that is, systems that follow mass action kinetics and where every variable contributes to a conserved quantity, have the property that starting with positive or nonnegative variables, the solutions of the differential systems stay positive or nonnegative, respectively [84]. A concise proof of this is given in Chapter 5. Hence, it is ensured from the mathematical point of view that starting with nonnegative concentrations, none of the concentrations can reach negative values which would be biologically implausible. Consequently, our analysis will focus solely on the region where all quantities are nonnegative. This region is bounded as all concentrations are nonnegative and each is part of a conserved quantity:

$$0 \le R \le R_{\text{tot}}, \qquad 0 \le M \le M_{\text{tot}}, \qquad 0 \le L \le L_{\text{tot}},$$

$$0 \le \sum_{i=0}^{N-1} B_i \le \min\{L_{\text{tot}}, R_{\text{tot}}, M_{\text{tot}}\} =: B_{\max} \quad \Rightarrow \quad 0 \le B_i \le B_{\max} \quad \text{for all } 0 \le i \le N-1,$$

$$0 \le \sum_{i=0}^{N} C_i \le \min\{R_{\text{tot}}, M_{\text{tot}}\} =: C_{\max} \quad \Rightarrow \quad 0 \le C_i \le C_{\max} \quad \text{for all } 0 \le i \le N.$$

Next, we want to expand the kinetic proofreading model with Lck (cf. Figure 2.8) by the tyrosine kinase ZAP-70 that binds to doubly phosphorylated ITAMs which in turn enables ZAP-70 to be phosphorylated by Lck (cf. Figure 1.3 and Section 1.2). Once activated, ZAP-70 phosphorylates other intracellular signaling molecules, initiating the immune response. Including ZAP-70, we follow the modeling approach of Altan-Bonnet and Germain (cf. upper part of Figure 2.3). For the expansion with this further enzyme, a couple of additional assumptions have to be made. First, we assume that if an ITAM is phosphorylated at one of its tyrosine residues, the phosphorylation of its second tyrosine residue is favored over the phosphorylation of a yet unphosphorylated ITAM. That is, we assume that once an ITAM is singly phosphorylated, the next catalysis by Lck is the phosphorylation of the second tyrosine residue of the same ITAM. The next assumption is that the affinity between doubly phosphorylated ITAMs and the kinase ZAP-70 is so high that ZAP-70 binds immediately to the doubly phosphorylated ITAM. The binding is modeled as reversible reaction, though the unbinding rate is expected to be very low. Furthermore, we suppose that the phosphorylation of the bound ZAP-70 molecules only occurs after all ITAMs of the T cell receptor complex have been successfully phosphorylated. The decomposition of the complexes involving ZAP-70 is modeled via the dissociation of the antigen from the receptor. That is, the ZAP-70 molecule initially stays bound to the T cell receptor while the antigen-carrying MHC molecule dissociates from the complex. Thereafter, the ZAP-70 molecules dissociate one by one from the ITAMs of the T cell receptor complex. Due to the high affinity of ZAP-70 to phosphorylated ITAMs, we assume that ZAP-70 does not immediately dissociate after the antigen-carrying MHC molecule leaves the complex. Therefore, we model the dissociation of the pMHC molecule from the T cell receptor complex where all phosphorylated ITAMs are bound to ZAP-70 molecules as reversible. This contrasts with the dissociation from complexes where ITAMs are singly or doubly phosphorylated but not bound to a ZAP-70 molecule. The dissociation of these complexes and of the complexes with phosphorylated ZAP-70 is modeled as irreversible according to the kinetic proofreading mechanism. Dephosphorylation is assumed to occur very quickly, rendering rebinding impossible after dissociation. The exception is for complexes where all phosphorylated ITAMs are protected from dephosphorylation by being bound to ZAP-70 molecules. However, it is expected that the rebinding rate in these cases is low. The model these assumptions result in is displayed in Figure 2.9. Again we consider a number of N phosphorylation steps whereby this time this number has to be even, $N = 2n$, as ZAP-70 can only bind to doubly phosphorylated ITAMs.

For the mathematical analysis and the formulation of the differential equations, we introduce variables for the complexes just as in the kinetic proofreading system with Lck. We keep the abbreviations M for the concentration of antigen-presenting MHC molecules which are not yet bound to a T cell receptor and L for the free enzymes Lck. New notations are those involving ZAP-70. We denote the free kinases ZAP-70 with Z and introduce an index j for the number of ZAP-70 molecules that are bound to a complex. The abbreviations denoting a complex carry therefore two indices, the lower one indicating the number of phosphorylated residues, the upper one indicating the number of bound ZAP-70 molecules. The complexes formed by T cell receptor, MHC molecule and ZAP-70 molecules are represented by the abbreviations C_i^j, the complexes not carrying a ZAP-70 molecule are represented by the index $j = 0$. After a Lck molecule binds to these complexes, we use the abbreviation B_i^j. As ZAP-70 itself can be phosphorylated, we need to introduce two new variables, D_i representing the fully phosphorylated complex where all ITAMs are occupied by a ZAP-70 molecule and a number i of these ZAP-70 molecules is phosphorylated itself and E_i representing these complexes involving a Lck molecule. The notation introduced above,

$$\begin{aligned} \mathrm{L} &= \mathrm{Lck}, & \mathrm{C}_i^j &= C_i.j\mathrm{ZAP}, \\ \mathrm{Z} &= \mathrm{ZAP\text{-}70}, & \mathrm{B}_i^j &= C_i.j\mathrm{ZAP.Lck}, \\ \mathrm{M} &= \mathrm{pMHC}, & \mathrm{D}_i &= C_{2n}.n\mathrm{ZAP}_i, \\ \mathrm{R}^j &= \mathrm{TCR}.j\mathrm{ZAP}, & \mathrm{E}_i &= C_{2n}.n\mathrm{ZAP}_i.\mathrm{Lck}, \end{aligned} \tag{2.4}$$

Figure 2.9: Kinetic proofreading with Lck and ZAP-70, mapped with reaction constants k_i, and an even number of phosphorylation steps $N = 2n$.

results in the following variables for the molecule concentrations for the kinetic proofreading model with Lck and ZAP-70:

$$
\begin{array}{llllll}
L, & Z, \quad M, & & & & \\
R^i, & 0 \le i \le n, & & & & \\
C^i_{2i}, & 0 \le i \le n, & C^i_{2i+1}, & 0 \le i \le n-1, & C^i_{2(i+1)}, & 0 \le i \le n-1, \\
B^i_{2i}, & 0 \le i \le n-1, & B^i_{2i+1}, & 0 \le i \le n-1, & & (\text{“}B^n_{2n}\text{”} = E_0) \\
D_i, & 1 \le i \le n, & & (\text{“}D_0\text{”} = C^n_{2n}) & & \\
E_i, & 0 \le i \le n-1. & & & &
\end{array}
$$

Here we kept the notation that the italic notation of a molecule denotes its concentration. Altogether we get a number of $5 + 8n = 5 + 4N$ species where $N = 2n$ is an even number indicating the maximal number of phosphorylation sites on the ITAMs. Using the new notation we can rewrite Figure 2.9 in favor of a clearer representation, see Figure 2.10, and formulate the differential equations corresponding to the system. To keep the formulation of the differential equations as simple as possible, we use the notation D_0 for the complex C^n_{2n} and the notation B^n_{2n} for E_0. With the reaction constants, k_i, established in Figure 2.9 and 2.10 and mass action kinetics, this results in the following system of differential equations:

$$
\begin{aligned}
\dot{M} &= -k_1 R^0 M + k_2 C^0_0 + k_6 C^0_1 + k_{10} C^0_2 \\
&\quad + \sum_{i=1}^{n-1} \left(k_{13+12(i-1)} C^i_{2i} + k_{6+12i} C^i_{2i+1} + k_{10+12i} C^i_{2(i+1)} \right) \\
&\quad - \sum_{i=1}^{n-1} k_{14+12(i-1)} R^i M + k_{13+12(n-1)} C^n_{2n} - k_{14+12(n-1)} R^n M + \sum_{i=1}^{n} D_i \\
&= k_2 C^0_0 + \sum_{i=1}^{n} k_{13+12(i-1)} C^i_{2i} + \sum_{i=0}^{n-1} \left(k_{6+12i} C^i_{2i+1} + k_{10+12i} C^i_{2(i+1)} \right) + \sum_{i=1}^{n} D_i \\
&\quad - k_1 R^0 M - \sum_{i=1}^{n} k_{14+12(i-1)} R^i M \\
\dot{R}^0 &= -k_1 R^0 M + k_2 C^0_0 + k_6 C^0_1 + k_{10} C^0_2 + k_{16n+2+1} R^1 \\
\dot{C}^0_0 &= k_1 R^0 M - k_2 C^0_0 - k_3 C^0_0 L + k_4 B^0_0 \\
\dot{C}^i_{2i} &= k_{4+12i} B^i_{2i} + k_{11+12(i-1)} C^{i-1}_{2i} Z + k_{14+12(i-1)} R^i M && 1 \le i \le n-1 \\
&\quad - k_{3+12i} C^i_{2i} L - \left(k_{12+12(i-1)} + k_{13+12(i-1)} \right) C^i_{2i} \\
\dot{C}^i_{2i+1} &= k_{5+12i} B^i_{2i} - k_{7+12i} C^i_{2i+1} L + k_{8+12i} B^i_{2i+1} - k_{6+12i} C^i_{2i+1} && 0 \le i \le n-1 \\
\dot{C}^i_{2(i+1)} &= k_{9+12i} B^i_{2i+1} - k_{11+12i} C^i_{2(i+1)} Z + k_{12+12i} C^{i+1}_{2(i+1)} - k_{10+12i} C^i_{2(i+1)} && 0 \le i \le n-1 \\
\dot{C}^n_{2n} &= k_{11+12(n-1)} C^{n-1}_{2n} Z - k_{12+12(n-1)} C^n_{2n} - k_{13+12(n-1)} C^n_{2n} + k_{14+12(n-1)} R^n M \\
&\quad - k_{3+12n} C^n_{2n} L + k_{4+12n} E_0 \\
&= k_{4+12n} E_0 + k_{11+12(n-1)} C^{n-1}_{2n} Z + k_{14+12(n-1)} R^n M \\
&\quad - k_{3+12n} C^n_{2n} L - \left(k_{12+12(n-1)} + k_{13+12(n-1)} \right) C^n_{2n} \\
\dot{B}^i_{2i} &= k_{3+12i} C^i_{2i} L - \left(k_{4+12i} + k_{5+12i} \right) B^i_{2i} && 0 \le i \le n-1 \\
\dot{B}^i_{2i+1} &= k_{7+12i} C^i_{2i+1} L - \left(k_{8+12i} + k_{9+12i} \right) B^i_{2i+1} && 0 \le i \le n-1 \\
\dot{D}_i &= k_{5+12n+4(i-1)} E_{i-1} + k_{4+12n+4i} E_i - k_{3+12n+4i} D_i L - k_{6+12n+4(i-1)} D_i && 1 \le i \le n-1
\end{aligned}
$$

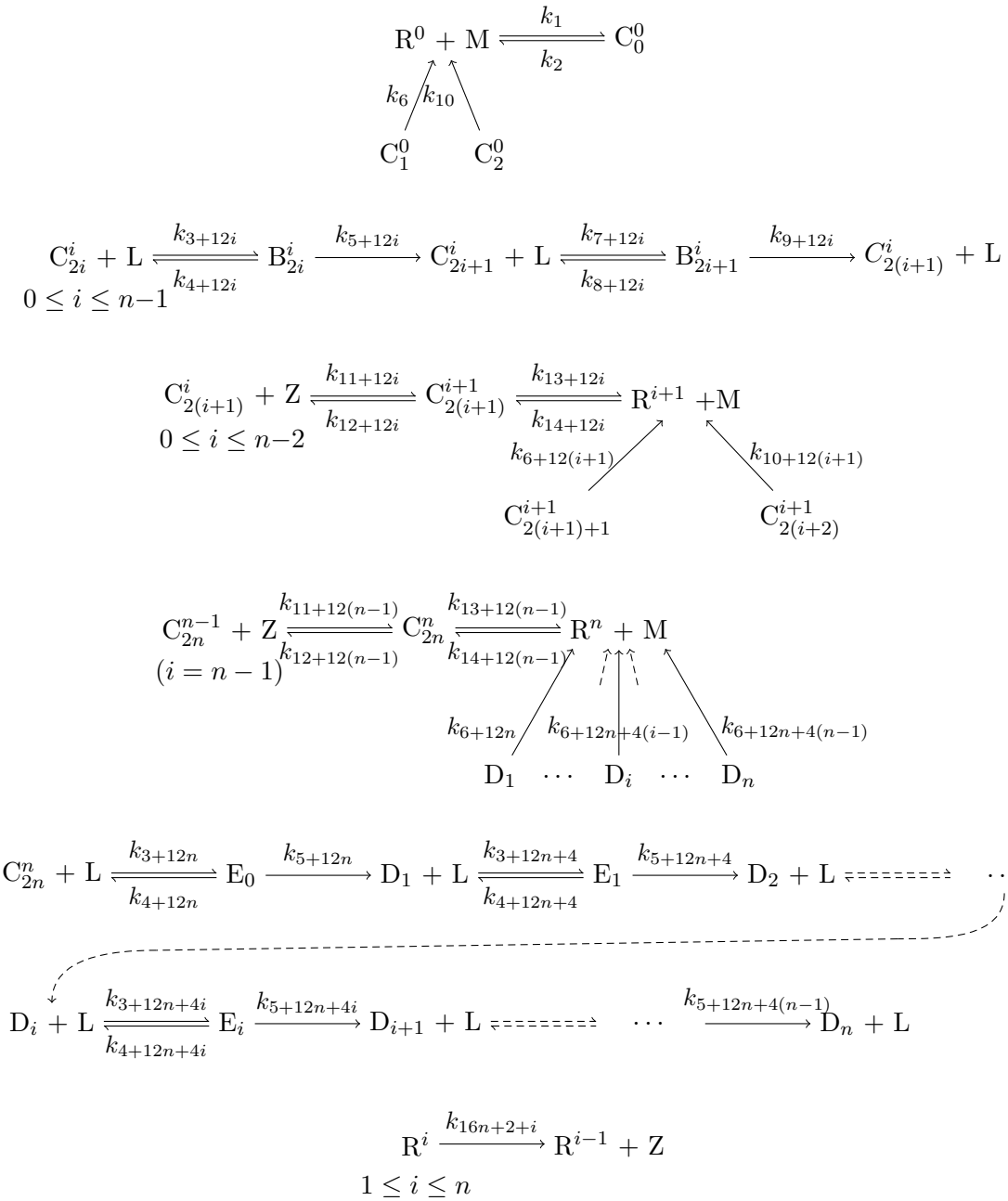


Figure 2.10: Kinetic proofreading with Lck and ZAP-70, mapped with reaction constants, k_i, and an even number of phosphorylation steps $N = 2n$. For identification of the variables see (2.4).

$$
\begin{aligned}
\dot{D}_n &= k_{5+12n+4(n-1)}E_{n-1} - k_{6+12n+4(n-1)}D_n \\
\dot{E}_0 &= k_{3+12n}C^n_{2n}L - \left(k_{4n+12n} + k_{5+12n}\right)E_0 \\
&= k_{3+12n}D_0L - \left(k_{4n+12n} + k_{5+12n}\right)E_0 \\
\dot{E}_i &= k_{3+12n+4i}D_iL - \left(k_{4+12n+4i} + k_{5+12n+4i}\right)E_i && 1 \le i \le n-1 \\
\dot{R}^i &= k_{13+12(i-1)}C^i_{2i} - k_{14+12(i-1)}R^iM + k_{6+12i}C^i_{2i+1} + k_{10+12i}C^i_{2(i+1)} && 1 \le i \le n-1 \\
&\quad + k_{16n+2+i+1}R^{i+1} - k_{16n+2+i}R^i \\
\dot{R}^n &= k_{13+12(n-1)}C^n_{2n} - k_{14+12(n-1)}R^nM + \sum_{i=1}^{n} k_{6+12n+4(i-1)}D_i - k_{16n+2+n}R^n \\
\dot{Z} &= -\sum_{i=0}^{n-1} k_{11+12i}C^i_{2(i+1)}Z + \sum_{i=0}^{n-1} k_{12+12i}C^{i+1}_{2(i+1)} + \sum_{i=1}^{n} k_{16n+2+i}R^i \\
\dot{L} &= -\sum_{i=0}^{n-1} k_{3+12i}C^i_{2i}L + \sum_{i=0}^{n-1}\left(k_{4+12i} + k_{5+12i}\right)B^i_{2i} - \sum_{i=0}^{n-1} k_{7+12i}C^i_{2i+1}L \\
&\quad + \sum_{i=0}^{n-1}\left(k_{8+12i} + k_{9+12i}\right)B^i_{2i+1} - \sum_{i=0}^{n-1} k_{3+12n+4i}D_iL + \sum_{i=0}^{n-1}\left(k_{4+12n+4i} + k_{5+12n+4i}\right)E_i \\
&= \sum_{i=0}^{n-1}\Big(\left(k_{4+12i} + k_{5+12i}\right)B^i_{2i} + \left(k_{8+12i} + k_{9+12i}\right)B^i_{2i+1} + \left(k_{4+12n+4i} + k_{5+12n+4i}\right)E_i\Big) \\
&\quad - \sum_{i=0}^{n} k_{3+12i}C^i_{2i}L - \sum_{i=0}^{n-1} k_{7+12i}C^i_{2i+1}L - \sum_{i=1}^{n-1} k_{3+12n+4i}D_iL.
\end{aligned}
\tag{2.5}
$$

The abbreviation $D_0 = C^n_{2n}$ was used in the equations for $\dot{E}_0$ and $\dot{L}$. The conservation laws give rise to four conserved quantities, the amount of total T cell receptors, free and occupied, R_{tot}, the total amount of antigen-presenting MHC molecules, free and bound to a T cell receptor, M_{tot}, the total concentration of the kinase Lck, free and bound to a complex, L_{tot}, and the total amount of ZAP-70 kinases, free and bound to an ITAM in a T cell receptor complex, Z_{tot}:

$$
\begin{aligned}
R_{\text{tot}} &= \sum_{i=0}^{n} R^i + \underbrace{\sum_{i=0}^{n} C^i_{2i} + \sum_{i=0}^{n-1}\Big(C^i_{2i+1} + C^i_{2(i+1)} + B^i_{2i} + B^i_{2i+1}\Big) + \sum_{i=1}^{n} D_i + \sum_{i=0}^{n-1} E_i}_{=:S} \\
M_{\text{tot}} &= M + S \\
L_{\text{tot}} &= L + \sum_{i=0}^{n-1}\Big(B^i_{2i} + B^i_{2i+1} + E_i\Big) \\
Z_{\text{tot}} &= Z + \sum_{i=1}^{n} i\Big(R^i + C^i_{2i}\Big) + \sum_{i=1}^{n-1} i\Big(C^i_{2i+1} + C^i_{2(i+1)} + B^i_{2i} + B^i_{2i+1}\Big) + \sum_{i=1}^{n} nD_i + \sum_{i=0}^{n-1} nE_i.
\end{aligned}
\tag{2.6}
$$

The arguments for local existence and uniqueness of a solution of the above differential system for every choice of initial concentrations as well as for the property that, starting with nonnegative variables, the solution always stays in the region where all quantities are nonnegative, are the same as in the system only including Lck. In an analogous manner to the system only including Lck, we can state a bounded region in which the nonnegative solutions of the above system lie:

$$
\begin{aligned}
&0 \le M \le M_{\text{tot}}, \quad 0 \le L \le L_{\text{tot}}, \quad 0 \le Z \le Z_{\text{tot}} \\
&0 \le R^i \le R_{\text{tot}} && 0 \le i \le n \\
&0 \le B^0_i \le \min\{R_{\text{tot}}, M_{\text{tot}}, L_{\text{tot}}\} =: B_{\max,0} \quad (\ge B_{\max}) && 0 \le i \le 1
\end{aligned}
$$

$$
\begin{aligned}
&0 \le B_i^j \le \min\{R_{\mathrm{tot}}, M_{\mathrm{tot}}, L_{\mathrm{tot}}, Z_{\mathrm{tot}}\} =: B_{\max} && 1 \le j \le n-1, \quad 2j \le i \le 2j+1\\
&0 \le E_i \le \min\{R_{\mathrm{tot}}, M_{\mathrm{tot}}, L_{\mathrm{tot}}, Z_{\mathrm{tot}}\} = B_{\max} && 0 \le i \le n-1\\
&0 \le C_i^0 \le \min\{R_{\mathrm{tot}}, M_{\mathrm{tot}}\} =: C_{\max,0} \quad (\ge C_{\max}) && 0 \le i \le 2\\
&0 \le C_i^j \le \min\{R_{\mathrm{tot}}, M_{\mathrm{tot}}, Z_{\mathrm{tot}}\} =: C_{\max} && 1 \le j \le n, \qquad 2j \le i \le 2(j+1)\\
&0 \le D_i \le \min\{R_{\mathrm{tot}}, M_{\mathrm{tot}}, Z_{\mathrm{tot}}\} = C_{\max} && 1 \le i \le n.
\end{aligned}
$$

The mathematical proof for the invariance of this compact subset of the nonnegative orthant will be given in Chapter 5. In the following sections we want to analyze the systems just introduced, the kinetic proofreading system including Lck (2.2) and the kinetic proofreading system including Lck and ZAP-70 (2.5).

3 Multistationarity

As the name suggests, the Chemical Reaction Network Theory (CRNT) is a theory dealing with systems based on chemical reactions, whose dynamics are modeled as ordinary differential equations. The variables are the concentrations of the substances involved in the reactions. The underlying assumption here is that the substances are present at approximately homogeneous concentrations. That is, there are enough molecules of each substance to talk about concentrations in the first place, and therefore no necessity for stochastic modeling, and there are hardly any spatial differences in the concentrations. The CRNT formalizes these kinds of systems of ordinary differential equations and provides tools to answer questions like the existence, uniqueness and stability of steady states. It was originated in the 1960s by Aris [4] who was one of the first to systematically formulate mathematical approaches for the analysis of chemical reaction networks. In the 1970s Jackson, Horn and Feinberg [42, 24] picked up this idea to develop a mathematical theory for chemical reaction networks and laid the foundation for what is today known as Chemical Reaction Network Theory.

3.1 Chemical Reaction Network Theory

In this section we introduce the basics of CRNT necessary for the analysis of our systems of kinetic proofreading with Lck and ZAP-70, respectively. As main sources for this serve lecture notes of Feinberg [26] and of Rendall [84], a work of Gunawardena [38] as well as a recently published book on CRNT written by Feinberg [25] and his previously published papers [28, 27].

By the term *chemical reaction network* we understand a finite set of *species* $\mathcal{S}$ together with a finite set of *complexes* $\mathcal{C}$, that correspond to the reactants and products of the chemical reactions involved, and a finite set of these *reactions* $\mathcal{R}$. Every reaction can be assigned a nonnegative real-valued *rate function*. We denote the set of rate functions by $\mathcal{K}$. These rate functions dictate the kinetics of the system, determining the rate at which each reaction occurs. In the literature, the inclusion or exclusion of the set of rate functions varies in the definition of a chemical reaction network. Since some statements in CRNT apply to networks with arbitrary kinetics, we define a chemical reaction network as the triple $(\mathcal{S}, \mathcal{C}, \mathcal{R})$ to which we can assign a set of rate functions $\mathcal{K}$. The set of species encompasses all molecules involved in the network. The set of complexes therefore consists of different combinations of species, precisely linear combinations. A species can participate in a complex with multiple molecules, the number of molecules involved in the complex is known as the *stoichiometric coefficient*. These chemical reaction networks can be represented graphically by associating each reaction with an edge of a directed graph where the nodes correspond to the complexes. For example, Figures 2.8 and 2.9 for the kinetic proofreading systems including the kinases Lck and ZAP-70, represent chemical reaction networks. To introduce the terminology used in CRNT, we consider a simpler example reducing the kinetic proofreading model with Lck to a single phosphorylation step (see Figure 3.1). To examine the dynamics of this system, we study the time evolution of the concentrations of the species involved, that is, of the molecules involved. The molar concentration of the individual species $s \in \mathcal{S}$ at time t is denoted $c_s(t)$ and the mixture composition at time t is denoted $c(t)$, representing the molar concentrations of the different species [28]. The rate functions then depend on the concentration $c(t)$ of the species. We assume *mass action kinetics* for the kinetic proofreading systems we are looking at. That is, we assume that the likelihood of an encounter between two different molecules is proportional to the product of the concentrations of these two species [28]. The reactions rates are therefore assumed to be proportional to the concentrations of the reactants to the power of their stoichiometric coefficients. For the above

$$\begin{array}{ccccc} \mathrm{TCR} + \mathrm{pMHC} & \underset{k_2}{\overset{k_1}{\rightleftharpoons}} & \mathrm{C_0} & & \\ \uparrow{\scriptstyle k_6} & & & & \\ \mathrm{C_1} & & & & \\ \mathrm{C_0} + \mathrm{Lck} & \underset{k_4}{\overset{k_3}{\rightleftharpoons}} & \mathrm{C_0.Lck} & \xrightarrow{k_5} & \mathrm{C_1} + \mathrm{Lck} \end{array}$$

Figure 3.1: Kinetic proofreading with Lck and one phosphorylation step.

example, the chemical reaction network $(\mathcal{S}, \mathcal{C}, \mathcal{R})$ consists of the following species, complexes and reactions:

$$\begin{aligned} \mathcal{S} &= \{\mathrm{TCR}, \mathrm{pMHC}, \mathrm{C_0}, \mathrm{Lck}, \mathrm{C_0.Lck}, \mathrm{C_1}\}, \\ \mathcal{C} &= \{\mathrm{TCR} + \mathrm{pMHC}, \mathrm{C_0}, \mathrm{C_0} + \mathrm{Lck}, \mathrm{C_0.Lck}, \mathrm{C_1} + \mathrm{Lck}, \mathrm{C_1}\}, \\ \mathcal{R} &= \{\mathrm{TCR} + \mathrm{pMHC} \to \mathrm{C_0}, \mathrm{C_0} \to \mathrm{TCR} + \mathrm{pMHC}, \mathrm{C_0} + \mathrm{Lck} \to \mathrm{C_0.Lck}, \mathrm{C_0.Lck} \to \mathrm{C_0} + \mathrm{Lck}, \\ &\qquad \mathrm{C_0} + \mathrm{Lck} \to \mathrm{C_1} + \mathrm{Lck}, \mathrm{C_1} \to \mathrm{TCR} + \mathrm{pMHC}\}. \end{aligned}$$

and the rate functions in $\mathcal{K}$ are given by the assumption of mass action kinetics and the proportionality constants, $k_1, \ldots, k_6$, called *rate constants*. Denoting the reaction constants with k_i, we tacitly numbered the reactions to get a simpler setting. To be able to analyze the system, we will do the same with the other sets and impose an artificial ordering on each set such that we can talk about the i-th species or the i-th complex [25]. If $\mathcal{I}$ is a finite set of objects, for example species $\mathcal{S}$, complexes $\mathcal{C}$, or reactions $\mathcal{R}$, then we denote the vector space of real-valued functions on $\mathcal{I}$ by $\mathbb{R}^{\mathcal{I}}$. That is, a vector in $\mathbb{R}^{\mathcal{I}}$ associates a real number with each element $i \in \mathcal{I}$ [25]. For example, the set of species $\mathcal{S}$ is associated with the vector-space $\mathbb{R}^{\mathcal{S}}$. This vector space $\mathbb{R}^{\mathcal{S}}$ has a natural basis given by the characteristic functions of the elements of $\mathcal{S}$. The characteristic function of the species with index i is denoted by ω_i. In this way, we can identify the vector space $\mathbb{R}^{\mathcal{S}}$ with $\mathbb{R}^m$ where m is the number of species [84]. As the set $\mathcal{S}$ carries no algebraic structure, we will use the index i as an abbreviation for the vector ω_i [25]. We can therefore express the complexes as vectors in $\mathbb{R}^{\mathcal{S}}$,meaning that the i-th species is represented in the i-th column of the vector. In our example, the expression $\mathrm{TCR} + \mathrm{pMHC}$ is a surrogate for $\omega_{\mathrm{TCR}} + \omega_{\mathrm{pMHC}} \in \mathbb{R}^{\mathcal{S}}$, and we can express the complex $\mathrm{TCR} + \mathrm{pMHC}$ as vector $y = (1, 1, 0, 0, 0, 0)$ in $\mathbb{R}^{\mathcal{S}}$, or $\mathbb{R}^6$ as $\mathcal{S}$ contains six species, by following the ordering given by the order of the species listed in the set $\mathcal{S}$. The stoichiometric coefficients that indicate the number of molecules of the species involved in a reaction are thus given by the entries y_i. As the concentration of all species is nonnegative, the entries of the vectors representing the complexes are nonnegative as well. Denoting by $\mathbb{R}_+$ the positive real numbers and by $\overline{\mathbb{R}}_+$ the nonnegative real numbers, all complexes lie in $\overline{\mathbb{R}}_+^{\mathcal{S}}$. An alternative notation for the set of complexes $\mathcal{C}$ in our example is therefore given by

$$\mathcal{C} = \left\{ \begin{pmatrix} 1 \\ 1 \\ 0 \\ 0 \\ 0 \\ 0 \end{pmatrix}, \begin{pmatrix} 0 \\ 0 \\ 1 \\ 0 \\ 0 \\ 0 \end{pmatrix}, \begin{pmatrix} 0 \\ 0 \\ 1 \\ 1 \\ 0 \\ 0 \end{pmatrix}, \begin{pmatrix} 0 \\ 0 \\ 0 \\ 0 \\ 1 \\ 0 \end{pmatrix}, \begin{pmatrix} 0 \\ 0 \\ 0 \\ 1 \\ 0 \\ 1 \end{pmatrix}, \begin{pmatrix} 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 1 \end{pmatrix} \right\} \subset \overline{\mathbb{R}}_+^{\mathcal{S}} \cong \overline{\mathbb{R}}_+^6.$$

As mentioned before, the stoichiometric coefficients of the species that are involved in a specific reaction and therefore part of a complex all equal one in our example. For the species not involved in a complex, we can identify zero as the stoichiometric coefficient. Thus, we can read the stoichiometric coefficients from the entries y_i of the above formulation of $\mathcal{C}$ via vectors $y \in \overline{\mathbb{R}}_+^{\mathcal{S}}$. With the help of the natural bases it is possible to identify linear mappings between spaces of functions on finite sets

with matrices [84]. The dynamics of a chemical reaction network takes place in the species space $\mathbb{R}^{\mathcal{S}}$, while the network itself is a relation on the set of complexes $\mathcal{C}$ [38]. The set of reactions comprises the networks' reactions in the form $y \to y'$ with $y, y' \in \mathcal{C}$, that is, the reaction of a complex y that contains the reactants to a complex y' that consists of all products of this reaction. Thus, we can identify such a reaction $y \to y'$ as a pair (y, y') in $\mathcal{R} \subset \mathcal{C} \times \mathcal{C} \subset \overline{\mathbb{R}}_+^{\mathcal{S}} \times \overline{\mathbb{R}}_+^{\mathcal{S}}$. In terms of the CRNT we give the following formal definition.

Definition 1. [38, 25, 84] A *chemical reaction network* is a triple $(\mathcal{S}, \mathcal{C}, \mathcal{R})$ where $\mathcal{S}$ is a finite set of species, $\mathcal{C}$ is a finite set of multisets of species, called complexes, and $\mathcal{R}$ is a relation on $\mathcal{C}$, denoted $y \to y'$ for $y, y' \in \mathcal{C}$, which represents the reaction converting y to y'. The definition of the sets is such that every species $s \in \mathcal{S}$ is involved in a complex $y \in \mathcal{C}$ and every complex is a reactant or product of a reaction $y \to y' \in \mathcal{R}$ and $\mathcal{R}$ does not include any trivial reaction $y \to y$.

Definition 2. [25, 6] Every reaction of a chemical reaction network $(\mathcal{S}, \mathcal{C}, \mathcal{R})$ can be assigned a *rate function* $\kappa_{y\to y'} : \overline{\mathbb{R}}_+^{\mathcal{S}} \to \overline{\mathbb{R}}_+$ such that $\kappa_{y\to y'}(c)$ depends precisely on the concentrations c_s of the species occurring in the reactant complex y of the reaction $y \to y' \in \mathcal{R}$ and $\kappa_{y\to y'}(c) = 0$ if and only if $c_s = 0$ for some species occurring in the reactant complex. A *kinetics* for a reaction network is an assignment where every reaction $y \to y' \in \mathcal{R}$ is assigned such a continuously differentiable rate function $\kappa_{y\to y'}$. The set of assigned rate functions is denoted by $\mathcal{K}$.

For models where some complex is added or withdrawn from the system, which would graphically correspond to a reaction "$\to y$" or "$y \to$" where the reactant or the product is not included in the model, a zero complex is added to the set of complexes, namely "$0 \to y$" or "$y \to 0$".

The time evolution for a chemical reaction network is given by the system of ordinary differential equations consisting of the time evolution of the concentration of the different species $i \in \mathcal{S}$

$$\frac{dc_i}{dt} = \sum_{y\to y' \in \mathcal{R}} \kappa_{y\to y'}(c)(y'_i - y_i),$$

or, referring to the imposed ordering, it is given by

$$\frac{dc}{dt} = \sum_{y\to y' \in \mathcal{R}} \kappa_{y\to y'}(c)(y' - y),$$

respectively. As the kinetic proofreading systems with Lck (2.2) and ZAP-70 (2.5) that we are looking at follow mass action kinetics, that is, the reaction rate is proportional to the power of concentration of a substance which is equal to the number of molecules of this substance going into the reaction [84], we can simplify this formulation for the time evolution. For systems following mass action kinetics, the reaction rate for the reaction $y \to y'$ is given by

$$\kappa_{y\to y'}(c) = k_{y\to y'} \prod_{i\in\mathcal{S}} c_i^{y_i} = k_{y\to y'} c^y \quad \text{with} \quad c^y := \prod_{i\in\mathcal{S}} c_i^{y_i},$$

where $k_{y\to y'}$ is a positive constant, the rate constant, for the reaction $y \to y' \in \mathcal{R}$ and the y_i's denote the stoichiometric coefficients.

Following mass action kinetics, the reaction rates depending on the present concentrations of the species $c(t)$ are given by

$$\begin{aligned}
&\kappa_{\mathrm{TCR+pMHC}\to\mathrm{C_0}}(c) = k_1 c_{\mathrm{TCR}} c_{\mathrm{pMHC}}, && \kappa_{\mathrm{C_0}\to\mathrm{TCR+pMHC}}(c) = k_2 c_{\mathrm{C_0}},\\
&\kappa_{\mathrm{C_0+Lck}\to\mathrm{C_0.Lck}}(c) = k_3 c_{\mathrm{C_0}} c_{\mathrm{Lck}}, && \kappa_{\mathrm{C_0.Lck}\to\mathrm{C_0+Lck}}(c) = k_4 c_{\mathrm{C_0.Lck}},\\
&\kappa_{\mathrm{C_0+Lck}\to\mathrm{C_1+Lck}}(c) = k_5 c_{C_0} c_{\mathrm{Lck}}, && \kappa_{\mathrm{C_1}\to\mathrm{TCR+pMHC}}(c) = k_6 c_{\mathrm{C_1}}.
\end{aligned}$$

The resulting system of differential equations corresponds to system (2.2) for $N = 2$ with the notation $c = (c_{\mathrm{TCR}}, c_{\mathrm{pMHC}}, c_{\mathrm{C_0}}, c_{\mathrm{Lck}}, c_{\mathrm{C_0.Lck}}, c_{\mathrm{C_1}}, c_{\mathrm{C_1.Lck}}, c_{\mathrm{C_2}}) = (R, M, C_0, L, B_0, C_1, B_1, C_2)$. Only considering systems following mass action kinetics, the same information that is given with the functions in $\mathcal{K}$ is contained in the rate constants. With the condition that a chemical reaction network follows mass action kinetics, we can replace the set $\mathcal{K}$ of functions by the *set of rate constants* denoted by $\mathcal{K}_m$, here $\mathcal{K}_m = \{k_1, \dots k_6\}$. The stoichiometric coefficients all equal one in this example as every reaction only involves a single molecule of a species.

We call the vectors $y' - y$ for $y \to y' \in \mathcal{R}$ the *reaction vectors* and the subspace spanned by these vectors the *stoichiometric subspace*.

Definition 3. [38] The stoichiometric subspace, S, of a chemical reaction network is the vector subspace of $\mathbb{R}^{\mathcal{S}}$ defined by $S = \operatorname{span}\{y' - y \,|\, y \to y' \in \mathcal{R}\}$.

Following the ordering for the species and the reactions, we construct a matrix N whose columns correspond to the reaction vectors. This matrix is called *stoichiometric matrix*. The dimension of the stoichiometric matrix therefore is $m \times r$ where m is the number of species and r is the number of reactions. The entry with indices (i, j) is the net production of the species i in the reaction j [84]. In our example, the stoichiometric matrix is given by

$$N = \begin{pmatrix} -1 & 1 & 0 & 0 & 0 & 1 \\ -1 & 1 & 0 & 0 & 0 & 1 \\ 1 & -1 & -1 & 1 & 0 & 0 \\ 0 & 0 & -1 & 1 & 1 & 0 \\ 0 & 0 & 1 & -1 & -1 & 0 \\ 0 & 0 & 0 & 0 & 1 & -1 \end{pmatrix}.$$

Definition 4. [28] The *rank of a chemical reaction network* $(\mathcal{S}, \mathcal{C}, \mathcal{R})$, denoted s, is defined as the rank of its set of reaction vectors $\left\{y' - y \in \mathbb{R}^{\mathcal{S}} \,|\, y \to y' \in \mathcal{R}\right\}$.

The rank of a chemical reaction network therefore corresponds to the rank of the stoichiometric matrix, $s = \operatorname{rank}(N)$. In our example of the kinetic proofreading model with Lck and a single phosphorylation step, the stoichiometric matrix given above satisfies $s = \operatorname{rank}(N) = 3$.

Definition 5. [25] Let $(\mathcal{S}, \mathcal{C}, \mathcal{R})$ be a reaction network and let $S \subset \mathbb{R}^{\mathcal{S}}$ be its stoichiometric subspace. Two vectors $c, c' \in \overline{\mathbb{R}}^{\mathcal{S}}_+$ are *stoichiometrically compatible* if $c' - c$ lies in S.

Definition 6. [27, 22] Let $(\mathcal{S}, \mathcal{C}, \mathcal{R})$ be a reaction network and let $S \subset \mathbb{R}^{\mathcal{S}}$ be its stoichiometric subspace. An element $v \in \mathbb{R}^{\mathcal{S}}$ is *sign compatible with the stoichiometric subspace* if there is an element $\sigma \in S$ such that $\operatorname{sign}(v_s) = \operatorname{sign}(\sigma_s)$ for all $s \in \mathcal{S}$. Thus, the vector $v \in \mathbb{R}^{\mathcal{S}}$ is sign compatible with the stoichiometric subspace if there exists a vector $\sigma \in S$ and a set of positive numbers, $\{p_s\}$, such that $v_s = p_s \sigma_s$ for all $s \in \mathcal{S}$.

Stoichiometric compatibility is an equivalence relation that induces a partition of $\overline{\mathbb{R}}^{\mathcal{S}}_+$ into equivalence classes, called the stoichiometric compatibility classes for the network.

Definition 7. [25] The *stoichiometric compatibility class* containing $c \in \overline{\mathbb{R}}^{\mathcal{S}}_+$ is the set $(c + S) \cap \overline{\mathbb{R}}^{\mathcal{S}}_+$. The *positive stoichiometric compatibility class* containing $c \in \mathbb{R}^{\mathcal{S}}_+$ is the set $(c + S) \cap \mathbb{R}^{\mathcal{S}}_+$.

A chemical reaction network is said to *support multiple steady states* if there exists a set of reaction rates such that the corresponding system of differential equations admits two distinct steady states in the same stoichiometric compatibility class [22, 51]. Thus, a mass action kinetic system supports

multiple steady states if there exists a positive rate constant for every reaction such that the system admits two distinct steady states. Another partition, but this time a partition of $\mathcal{C}$, is induced by the reaction paths.

Definition 8. [38] The complex $y \in \mathcal{C}$ is *directly linked* to the complex $y' \in \mathcal{C}$, denoted $y \leftrightarrow y'$, if either $y \to y' \in \mathcal{R}$ or $y' \to y \in \mathcal{R}$. If $y, y' \in \mathcal{C}$, then y is said to be *linked* to y', denoted $y \sim y'$, if either $y = y'$ or there are $y_1, \dots y_n \in \mathcal{C}$ such that $y = y_1 \leftrightarrow y_2 \leftrightarrow \dots \leftrightarrow y_n = y'$. The equivalence classes of complexes under $\sim$ are termed *linkage classes*. We reserve the symbol l to indicate the number of linkage classes in a network.

Definition 9. [38] If $y, y' \in \mathcal{C}$, then y *ultimately reacts* to y', denoted $y \Rightarrow y'$, if either $y = y'$ or there exist $y_1, \dots, y_n \in \mathcal{C}$ such that $y = y_1 \to y_2 \cdots \to y_n = y'$. The complex y is *strongly linked* to y', denoted $y \approx y'$, if both $y \Rightarrow y'$ and $y' \Rightarrow y$ hold. The equivalence classes under $\approx$ are called *strong linkage classes*.

Definition 10. [28] A *terminal strong linkage class* is a strong linkage class containing no complex that reacts to a complex in a different strong linkage class. In other words, if the complex $y \in \mathcal{C}$ lies within a terminal strong linkage class and $y \to y' \in \mathcal{R}$, then y' lies in the same strong linkage class. A terminal strong linkage class is *nontrivial* if it contains more than one complex.

Definition 11. [25] A chemical reaction network $(\mathcal{S}, \mathcal{C}, \mathcal{R})$ is *reversible* if the reacts to relation $\to$ is symmetric, that is, if $y' \to y$ is a member of $\mathcal{R}$ whenever $y \to y'$ is a member of $\mathcal{R}$. A reaction network is *weakly reversible* if its ultimately reacts to relation $\Rightarrow$ is symmetric. That is, if $y' \Rightarrow y$ whenever $y \Rightarrow y'$.

To put it in other words, two complexes are directly linked if the network includes a reaction from one of the two complexes to the other. Two complexes are called linked to each other if they are connected through undirected reaction paths. The direction of the paths comes into play with the definition of strong linkage classes. If a complex ultimately reacts to another complex, then there exists a directed path from the former to the latter one. That is, in a strong linkage class, every complex can react to any other complex in this linkage class via a chain of reactions. That is exactly the idea of the definition of a weakly reversible network, which contains no final complexes that cannot react to any other product. Thus, if every linkage class is a strong linkage class, the network is weakly reversible and the other way around. A strong linkage class that is terminal comprises only complexes that do not react to a complex of a different strong linkage class. Hence, we can identify a weakly reversible network as well as a network where every strong linkage class is a terminal strong linkage class. Then every complex within the terminal strong linkage classes can react to any of the other complexes in the same terminal strong linkage class via a chain of reactions. Thus, a weakly reversible network is given when every strong linkage class is a terminal strong linkage class or when every linkage class is a strong linkage class, respectively. That is, weakly reversible networks are characterized by the fact that, for them, linkage classes, strong linkage classes and terminal strong linkage classes coincide [25].

To clarify these definitions, we look again at our example of the kinetic proofreading system with Lck and one phosphorylation step. The complex C_0 is linked to the complexes TCR + pMHC and C_1 (see Figure 3.2). To the complex TCR + pMHC it is even directly linked, not so to the complex C_1. As the complexes C_0, TCR + pMHC and C_1 are all linked, they belong to the same linkage class. C_0 ultimately reacts to the complex TCR + pMHC but not to the complex C_1. The complex TCR + pMHC reacts as well ultimately to the complex C_0 but not to C_1, whereas the latter ultimately reacts to the former. The three complexes are therefore in the same linkage class but not in a common strong linkage class. One strong linkage class which is also a nontrivial terminal strong linkage class is formed by the two complexes TCR + pMHC and C_0, whereas the complex C_1

forms its own strong linkage class which is not terminal as C_1 reacts to complexes of another strong linkage class. The second linkage class of the system is formed by the reactions including the kinase Lck. Here the complexes $C_0 + Lck$ and $C_0.Lck$ ultimately react to the complex $C_1 + Lck$, whereas $C_1 + Lck$ itself does not react to any other complex. Therefore, we have again two different strong linkage classes in this linkage class, formed by $C_0 + Lck$ and $C_0.Lck$ and by the single complex $C_1 + Lck$, respectively. The latter is a terminal strong linkage class. Hence, our system comprises two linkage classes, $l = 2$, and four strong linkage classes of which two are terminal strong linkage classes. To be weakly reversible, every strong linkage class would have to be a terminal strong linkage class. As this is not the case in our example, the system is not weakly reversible.

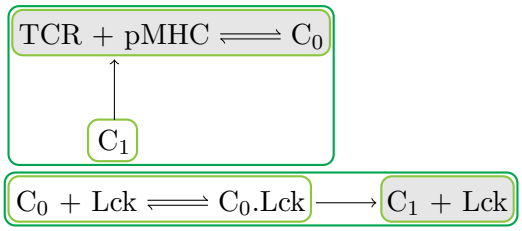


Figure 3.2: Linkage classes of the kinetic proofreading system with Lck and one phosphorylation step. The linkage classes are encircled green, the strong linkage classes are encircled lime green and the terminal strong linkage classes are shaded gray.

Another characteristic that is assigned to chemical reaction networks is the *deficiency*, a nonnegative number that arises from considerations about an upper bound to the rank of the reaction network. First, we think about a network with only one linkage class. That is, all complexes are linked by a sequence of reactions. The network cannot exceed the rank of a star-like arrangement of complexes [25]. The rank of a reaction network is defined as the number of linearly independent reaction vectors. A reversible reaction leads to two linearly dependent reaction vectors which only differ in sign, so every reaction, reversible or irreversible, can at most add one reaction vector to the set of linearly independent reaction vectors. Suppose now there are two different reaction pathways that link the complex y to the complex y', then the reaction vector $y' - y$ is the result of two different sums of reaction vectors. Therefore, some of the reaction vectors involved are linearly dependent. Thus, to get the maximal number of linearly independent reaction vectors, we look at a network where there is just a single path for every complex y to another complex y'. Such a network is given by a star-like arrangement where there is one central complex and all other complexes react to or are product of this complex. Alternatively, we could look at a chain of reactions starting with one complex that reacts to the next and so on, until the final complex is reached. Such a network with a total of n complexes produces $n - 1$ linearly independent reaction vectors. Suppose now we are looking at a reaction network with l linkage classes. Then there are no reactions between the linkage classes, that is, the maximal number of linearly independent reaction vectors is given by $(n - 1) - (l - 1) = n - l$. Thus, for every reaction network an upper bound for the rank s of the reaction network is given by the difference between the amount of complexes n and the number of linkage classes l:

$$n - l \geq s.$$

This implies $n - l - s \geq 0$ with equality holding precisely when the reaction vectors for the network are as linearly independent as the partition of complexes into linkage classes will allow [25]. This measure of the linear independence of the reaction vectors is defined as the deficiency of a network, denoted δ.

Definition 12. [25] The *deficiency*, δ, of a reaction network with n complexes, l linkage classes and rank s is defined by $\delta := n - l - s$.

In our example, the deficiency of the kinetic proofreading network with Lck and one phosphorylation step is given by $\delta = n - l - s = 6 - 2 - 3 = 1$. Similarly, we can look at the different linkage classes individually, identifying their rank and deficiency. We denote by s_i the *rank of a linkage class* and by δ_i the *deficiency of a linkage class*, $1 \leq i \leq l$. Denoting by n_i the number of complexes in a linkage class, the deficiency of a linkage class is given by $\delta_i = n_i - 1 - s_i$, $1 \leq i \leq l$. In general, the sum of the rank of linkage classes is not the same as the rank of the whole network. Since the rank of a linkage class is defined by the number of linearly independent reaction vectors involved in the linkage classes' reactions, the reaction vector of a linkage class could still be a linear combination of the reactions vectors of the other linkage classes. Therefore, the sum of the ranks of the different linkage classes can exceed the rank of the entire network. However, it is clearly not possible for the sum of the ranks of the linkage classes to be smaller than the rank of the whole network. Thus, for every network the following inequality holds:

$$\sum_{i=1}^{l} s_i \geq s,$$

with equality holding precisely when the stoichiometric subspace of the whole network, S, is the direct sum of the stoichiometric subspaces of the linkage classes [25]. This implies the inequality

$$\sum_{i=1}^{l} \delta_i \leq \delta$$

for the sum of the linkage classes' deficiencies, with equality holding precisely when the equality in the rank inequality is given. If a network has deficiency zero, all its linkage classes also have deficiency zero. Taking the deficiency as a measure of the linear independence of the reaction vectors of the network, we expect networks with deficiency zero to be somewhat simpler to handle than networks with a higher deficiency. The research by Horn, Jackson, and Feinberg in CRNT provides insights into the existence of steady states and periodic solutions for general networks with deficiency zero, especially those following mass action kinetics.

Theorem 1. (Deficiency Zero Theorem) [26, 28] *Let $(\mathcal{S}, \mathcal{C}, \mathcal{R})$ be a chemical reaction network of deficiency zero. Then the following statements hold for the underlying system of differential equations:*

(i) If the network is not weakly reversible, then, for arbitrary kinetics $\mathcal{K}$, there exists neither a positive steady state nor a periodic orbit in $\mathbb{R}^{\mathcal{S}}_{+}$.

(ii) If the network is weakly reversible and follows mass action kinetics, then, for any set of rate constants $\mathcal{K}_m$, there exists precisely one steady state in each positive stoichiometric compatibility class and this steady state is asymptotically stable and there cannot exist a nontrivial periodic orbit in $\mathbb{R}^{\mathcal{S}}_{+}$.

A positive stoichiometric compatibility class is the partition on $\mathbb{R}^{\mathcal{S}}_{+}$ induced by the equivalence relation of stoichiometric compatibility. Thus, the Deficiency Zero theorem tells us that in the case of a weakly reversible network following mass action kinetics and having deficiency zero, there is a unique positive steady state that is asymptotically stable for every choice of positive initial concentrations. The choice of initial concentrations for the species determines the stoichiometric compatibility class within which we are operating. Asymptotic stability of the steady state is to be understood relative to its stoichiometric compatibility class [28, 38]. If the system does not have any steady states on the boundary $\partial\overline{\mathbb{R}}^{\mathcal{S}}_{+}$, then the unique steady state in each positive stoichiometric compatibility class is even globally asymptotically stable [91, 95]. For networks following mass action kinetics, it is even possible to make a statement about the existence of a unique steady state when their reaction networks are not maximally independent, that is, not as linearly independent

as the linkage classes would allow, in other words, when the network's deficiency is not zero. A network has deficiency zero if and only if the deficiency of every linkage class is also zero and the sum of all these linkage classes equals the deficiency of the whole network. The Deficiency One theorem applies to networks that fulfill the condition that the sum of the linkage class deficiencies equals the deficiency of the entire network. In these networks, the deficiencies of the linkage classes can be either zero or one.

Theorem 2. (Deficiency One Theorem) [28, 51] *Let $(\mathcal{S}, \mathcal{C}, \mathcal{R})$ be a chemical reaction network following mass action kinetics. Let δ be its deficiency and l its number of linkage classes whose respective deficiency is denoted by δ_i. Suppose the following conditions are satisfied:*

(i) $\delta_i \leq 1, \quad i = 1, \dots l,$

(ii) $\delta = \sum_{i=1}^{l} \delta_i,$

(iii) *Each linkage class contains just one terminal strong linkage class.*

Then, for any positive rate constants, the corresponding system of differential equations can admit no more than one steady state in each positive stoichiometric compatibility class. If the system admits a positive steady state for some rate constants, then each positive stoichiometric compatibility class contains precisely one steady state. If the network is weakly reversible, then the system admits a steady state for every choice of rate constants.

Returning to our example, the kinetic proofreading system with Lck and one phosphorylation step, we summarize which characteristics we already identified: the system follows mass action kinetics and the network is not weakly reversible, it has two linkage classes which each contain exactly one terminal strong linkage class (cf. Figure 3.2) and the network's deficiency is one, $\delta = 1$. Thus, we cannot apply the Deficiency Zero theorem to learn about the existence of a unique positive steady state, but we can check the conditions for the Deficiency One theorem of which we already found (iii) to be satisfied. To determine the deficiencies of the linkage classes, we identify which reaction vector belongs to which of the two linkage classes. In the beginning, we numbered the reactions in order of their occurrence after the binding of the antigen-presenting MHC molecule to the T cell receptor (see Figure 3.1). Therefore, the first, second and sixth column of the stoichiometric matrix N are reaction vectors belonging to the first linkage class, and the columns three, four and five represent the reaction vectors of the second linkage class. Consequently, the corresponding sets of reaction vectors are given by

$$\left\{ \begin{pmatrix} -1 \\ -1 \\ 1 \\ 0 \\ 0 \\ 0 \end{pmatrix}, \begin{pmatrix} 1 \\ 1 \\ -1 \\ 0 \\ 0 \\ 0 \end{pmatrix}, \begin{pmatrix} 1 \\ 1 \\ 0 \\ 0 \\ 0 \\ -1 \end{pmatrix} \right\} \quad \text{and} \quad \left\{ \begin{pmatrix} 0 \\ 0 \\ -1 \\ -1 \\ 1 \\ 0 \end{pmatrix}, \begin{pmatrix} 0 \\ 0 \\ 1 \\ 1 \\ -1 \\ 0 \end{pmatrix}, \begin{pmatrix} 0 \\ 0 \\ 0 \\ 1 \\ -1 \\ 1 \end{pmatrix} \right\}.$$

We observe that within each set, there exists one pair of linearly dependent reaction vectors. Consequently, the respective dimension of the subspace generated by the reaction vectors is two, $s_1 = s_2 = 2$. Each of the linkage classes contains three complexes, $n_1 = n_2 = 3$. That gives us the linkage classes' deficiencies

$$\delta_2 = \delta_1 = n_1 - 1 - s_1 = 3 - 1 - 2 = 0.$$

We see that both linkage classes have deficiency zero and therefore satisfy condition (i) but not condition (ii) of the Deficiency One theorem. The deficiencies of the linkages classes do not add up to the deficiency of the whole network, $\delta_1 + \delta_2 = 0 + 0 = 0 \neq 1 = \delta$. The name of the Deficiency

One theorem is based on the deficiencies of the linkage classes, which are not permitted to exceed one. However, the deficiency of the whole system may be higher than one. As we have just seen, the Deficiency One theorem can even fail for networks with deficiency one. To investigate the possibility of multistationarity for networks following mass action kinetics with deficiency one where all linkage classes have deficiency zero, Feinberg [23] developed an algorithm translating the question of multistationarity into questions of linear equalities and inequalities. To be able to apply this algorithm, we have to introduce a few more terms about chemical reaction networks. Suppose the chemical reaction network $(\mathcal{S}, \mathcal{C}, \mathcal{R})$ has a positive steady state $c^* \in \mathbb{R}^{\mathcal{S}}_+$, then

$$\sum_{y \to y' \in \mathcal{R}} \kappa_{y \to y'}(c^*)(y' - y) = 0 \tag{3.1}$$

where $\kappa_{y \to y'} : \overline{\mathbb{R}}^{\mathcal{S}}_+ \to \overline{\mathbb{R}}_+$ denote again the rate functions assigning a positive rate $\kappa_{y \to y'}(c^*)$ to each reaction in $\mathcal{R}$.

Definition 13. [27] The reaction vectors of the network are *positively dependent* if a set of positive numbers $\left\{\alpha_{y \to y'}\right\}_{y \to y' \in \mathcal{R}}$ exists such that

$$\sum_{y \to y' \in \mathcal{R}} \alpha_{y \to y'}(y' - y) = 0.$$

Thus, any network admitting a positive steady state $c^* \in \mathbb{R}^{\mathcal{S}}_+$ has positively dependent reaction vectors setting $\alpha_{y \to y'} = \kappa_{y \to y'}(c^*)$. Conversely, assume we have a network with positively dependent reaction vectors that follows mass action kinetics. Then for any choice of a concentration vector $c \in \mathbb{R}^{\mathcal{S}}_+$, there is an assignment of rate constants $k \in \mathbb{R}^{\mathcal{R}}_+$, that is, a positive constant $k_{y \to y'}$ for every reaction $y \to y' \in \mathcal{R}$, such that the network following mass action kinetics admits c as a steady state [27].

Definition 14. [27, 25] Two directly linked complexes, $y \leftrightarrow y'$, of a reaction network are called a *cut pair* if the linkage class containing y and y' admits a partition into two sets $\mathcal{W}$ and $\mathcal{W}'$ with $y \in \mathcal{W}$ and $y' \in \mathcal{W}'$ such that the only link between $\mathcal{W}$ and $\mathcal{W}'$ is $y \leftrightarrow y'$.

In our example of the kinetic proofreading with Lck and one phosphorylation step (cf. Figure 3.2) all directly linked complexes are cut pairs. We call a complex *terminal* if it lies in a terminal strong linkage class.

Definition 15. [27] A chemical reaction network $(\mathcal{S}, \mathcal{C}, \mathcal{R})$ is called *regular* if it satisfies the following three conditions:

(R1) The reaction vectors of the network are positively dependent.

(R2) Each linkage class in the network contains just one terminal strong linkage class.

(R3) Each pair of directly linked terminal complexes is a cut pair.

The second condition (R2) that each linkage class contains just one terminal strong linkage class is also a condition in the Deficiency One theorem (Theorem 2, condition (iii)). To see why we require this network property, we look at the following simple network [27]:

$$2\mathrm{A} \xleftarrow{k_1} \mathrm{A} + \mathrm{B} \xrightarrow{k_2} 2\mathrm{B}$$

The system consists of only one linkage class and this linkage class contains two terminal strong linkage classes, {2A} and {2B}. Following mass action kinetics the differential equations for the system are given by

$$\frac{dc_\mathrm{A}}{dt} = 2k_1 c_\mathrm{A} c_\mathrm{B} - k_1 c_\mathrm{A} c_\mathrm{B} - k_2 c_\mathrm{A} c_\mathrm{B} = (k_1 - k_2) c_\mathrm{A} c_\mathrm{B}$$
$$\frac{dc_\mathrm{B}}{dt} = 2k_2 c_\mathrm{A} c_\mathrm{B} - k_2 c_\mathrm{A} c_\mathrm{B} - k_1 c_\mathrm{A} c_\mathrm{B} = (k_2 - k_1) c_\mathrm{A} c_\mathrm{B}.$$

For $k_1 \neq k_2$, no positive steady state exists. In contrast, when $k_1 = k_2$, every choice of c_A and c_B corresponds to a steady state, resulting in an infinite number of steady states within each positive stoichiometric compatibility class [27].

As an example, we check if the network for kinetic proofreading with Lck and one phosphorylation step is regular. As we did not argue so far if the system possesses a positive steady state (a consequence of Brouwer's fixed point theorem as we will discuss in Chapter 4), we simply check if there exist positive numbers $\alpha_{y\to y'}$ such that the reaction vectors are positively dependent by definition, that is, such that the following equation is satisfied:

$$\alpha_{\mathrm{TCR+pMHC}\to\mathrm{C_0}} \begin{pmatrix} -1 \\ -1 \\ 1 \\ 0 \\ 0 \\ 0 \end{pmatrix} + \alpha_{\mathrm{C_0}\to\mathrm{TCR+pMHC}} \begin{pmatrix} 1 \\ 1 \\ -1 \\ 0 \\ 0 \\ 0 \end{pmatrix} + \alpha_{\mathrm{C_0+Lck}\to\mathrm{C_0.Lck}} \begin{pmatrix} 0 \\ 0 \\ -1 \\ -1 \\ 1 \\ 0 \end{pmatrix}$$
$$+ \alpha_{\mathrm{C_0.Lck}\to\mathrm{C_0+Lck}} \begin{pmatrix} 0 \\ 0 \\ 1 \\ 1 \\ -1 \\ 0 \end{pmatrix} + \alpha_{\mathrm{C_0.Lck}\to\mathrm{C_1+Lck}} \begin{pmatrix} 0 \\ 0 \\ 0 \\ 1 \\ -1 \\ 1 \end{pmatrix} + \alpha_{\mathrm{C_1}\to\mathrm{TCR+pMHC}} \begin{pmatrix} 1 \\ 1 \\ 0 \\ 0 \\ 0 \\ -1 \end{pmatrix} = \begin{pmatrix} 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \end{pmatrix}.$$

By setting

$$\alpha_{\mathrm{TCR+pMHC}\to\mathrm{C_0}} := 2, \qquad \alpha_{\mathrm{C_0}\to\mathrm{TCR+pMHC}} := 1, \qquad \alpha_{\mathrm{C_0+Lck}\to\mathrm{C_0.Lck}} := 2,$$
$$\alpha_{\mathrm{C_0.Lck}\to\mathrm{C_0+Lck}} := 1, \qquad \alpha_{\mathrm{C_0.Lck}\to\mathrm{C_1+Lck}} := 1, \qquad \alpha_{\mathrm{C_1}\to\mathrm{TCR+pMHC}} := 1,$$

all sums add up to zero:

$$-\alpha_{\mathrm{TCR+pMHC}\to\mathrm{C_0}} + \alpha_{\mathrm{C_0}\to\mathrm{TCR+pMHC}} + \alpha_{\mathrm{C_1}\to\mathrm{TCR+pMHC}} = -2 + 1 + 1 = 0$$
$$\alpha_{\mathrm{TCR+pMHC}\to\mathrm{C_0}} - \alpha_{\mathrm{C_0}\to\mathrm{TCR+pMHC}} - \alpha_{\mathrm{C_0+Lck}\to\mathrm{C_0.Lck}} + \alpha_{\mathrm{C_0.Lck}\to\mathrm{C_0+Lck}} = 2 - 1 - 2 + 1 = 0$$
$$-\alpha_{\mathrm{C_0+Lck}\to\mathrm{C_0.Lck}} + \alpha_{\mathrm{C_0.Lck}\to\mathrm{C_0+Lck}} + \alpha_{\mathrm{C_0.Lck}\to\mathrm{C_1+Lck}} = -2 + 1 + 1 = 0$$
$$\alpha_{\mathrm{C_0+Lck}\to\mathrm{C_0.Lck}} - \alpha_{\mathrm{C_0.Lck}\to\mathrm{C_0+Lck}} - \alpha_{\mathrm{C_0.Lck}\to\mathrm{C_0+Lck}} = 2 - 1 - 1 = 0$$
$$\alpha_{\mathrm{C_0.Lck}\to\mathrm{C_0+Lck}} - \alpha_{\mathrm{C_1}\to\mathrm{TCR+pMHC}} = 1 - 1 = 0.$$

Thus, the equations hold and the reaction vectors are therefore positively dependent. Furthermore, each linkage class contains just one terminal strong linkage class (cf. Figure 3.2), and as all directly linked complexes are cut pairs, this is in particular true for the terminal complexes. Therefore, all three regularity conditions are satisfied.

Definition 16. [27] A set $\mathcal{A}$ of complexes is *absorptive* if $\mathcal{A}$ is not empty and if there is no reaction from a complex in $\mathcal{A}$ to a complex not in $\mathcal{A}$. That is, $\mathcal{A} \subset \mathcal{C}$ is absorptive if $\mathcal{A} \neq \emptyset$ and $y \in \mathcal{A}$ and $y \to y' \in \mathcal{R}$ imply that $y' \in \mathcal{A}$.

Terminal strong linkage classes and linkage classes are therefore absorptive, whereas strong linkage classes are generally not absorptive.

Definition 17. [27] A *confluence vector* g for a reaction network $(\mathcal{S}, \mathcal{C}, \mathcal{R})$ is an element in $\mathbb{R}^{\mathcal{C}}$ such that

(i) $\displaystyle\sum_{y\in\mathcal{C}} g_y y = 0,$

(ii) For each linkage class $\mathcal{L}$: $\displaystyle\sum_{y\in\mathcal{L}} g_y = 0,$

(iii) For each absorptive set $\mathcal{A} \subset \mathcal{C}$ that is not the union of linkage classes: $\displaystyle\sum_{y\in\mathcal{A}} g_y > 0.$

Suppose that $c^* \in \mathbb{R}^{\mathcal{S}}_+$ is a steady state for a system following mass action kinetics, for $y \in \mathcal{C}$ set

$$g_y(c^*) := \sum_{\substack{y'\to y\in\mathcal{R}\\ (y \text{ fixed})}} k_{y'\to y} c^{*y'} - \sum_{\substack{y\to y'\in\mathcal{R}\\ (y \text{ fixed})}} k_{y\to y'} c^{*y}.$$

As we are looking at a steady state, we know that equation (3.1) holds. That is, the concentration of each species does not change over time. In other words, for every species the same amount is consumed as a reactant in reactions as it is produced as a product in other reactions. This holds for the species but certainly not for the complexes. What we just defined with $g_y(c^*)$ for every $y \in \mathcal{C}$, can be interpreted as the net current into complex y from all other complexes and satisfies the conditions for a confluence vector[27].

For regular deficiency one networks, the existence of nonzero confluence vectors is ensured [27]. The reaction vectors of a regular network are positively dependent. Let $\{\alpha_{y\to y'}\}_{y\to y'\in\mathcal{R}}$ be the respective set of positive numbers from Definition 13, then a confluence vector is given by setting

$$g_y := \sum_{\substack{y'\to y\in\mathcal{R}\\ (y \text{ fixed})}} \alpha_{y'\to y} - \sum_{\substack{y\to y'\in\mathcal{R}\\ (y \text{ fixed})}} \alpha_{y\to y'}.$$

For a regular deficiency one network following mass action kinetics, it is possible to determine the capacity to support multiple steady states within a stoichiometric compatibility class. The *Deficiency One Algorithm* developed by Feinberg [23] rephrases the question of the existence of two positive steady states within the same stoichiometric compatibility class into the question of the existence of a vector $\mu \in \mathbb{R}^{\mathcal{S}}$ satisfying certain linear equalities and inequalities. Suppose c^* and c^{**} are two distinct positive steady states in a mass action kinetic system. Then the reaction rates are given by $\kappa_{y\to y'}(c) = k_{y\to y'} c^y$ for a $k_{y\to y'} \in \mathbb{R}_+$ for every reaction $y \to y' \in \mathcal{R}$, and the equations

$$\sum_{y\to y'\in\mathcal{R}} k_{y\to y'} c^{*y}(y - y') = 0 \quad \text{and} \quad \sum_{y\to y'\in\mathcal{R}} k_{y\to y'} c^{**y}(y - y') = 0$$

hold. This is equivalent to the existence of reactions constants $k_{y\to y'}$ such that

$$\sum_{y\to y'\in\mathcal{R}} k_{y\to y'}(y - y') = 0 \quad \text{and} \quad \sum_{y\to y'\in\mathcal{R}} k_{y\to y'} e^{y\cdot\mu}(y - y') = 0$$

where

$$\mu_i := \ln\left(\frac{c_i^{**}}{c_i^*}\right). \tag{3.2}$$

This vector $\mu \in \mathbb{R}^{\mathcal{S}}$ is nonzero exactly when the steady states c^* and c^{**} are distinct, that is, the entry for at least one species is distinct. Given a pair of stoichiometrically compatible steady state compositions, c^* and c^{**}, then the vector $c^* - c^{**}$ lies in the stoichiometric subspace and the components $(c^* - c^{**})_i$ coincide in sign with μ_i. Thus, the vector $c^* - c^{**}$ and the vector μ are sign compatible, and μ is sign compatible with the stoichiometric subspace [22]. If there exists a nonzero vector μ that is sign compatible with the stoichiometric subspace satisfying the equalities and inequalities created by the algorithm, this vector is called a *signature* for the reaction network. If there exists any signature, then the network has the capacity to support multiple positive steady states. If no signature exists, then the network cannot support multiple positive steady states, no matter what positive values the rate constants take. In other words, if a signature is found, then there exists a set of positive rate constants such that the corresponding system of differential equations admits two distinct positive steady states in the same stoichiometric compatibility class, and if no signature exists, then, for any rate constants, the corresponding system does not admit more than one positive steady state.

The equalities and inequalities produced in the Deficiency One Algorithm are based on a partition of the complexes into three sets, called *shelves*. If the shelves chosen for the complexes lead to a system of equalities and inequalities for which there exists a nonzero solution $\mu \in \mathbb{R}^{\mathcal{S}}$ that is sign compatible with the stoichiometric subspace of the system, that is, a signature μ, then the reaction network is able to support multiple positive steady states within a stoichiometric compatibility class. For simplicity, we will in the following refer to the system of equalities and inequalities also as inequality system. If the inequality system produced does not have a signature, then we choose a different partitioning into shelves for the complexes and check if the new inequality system has a signature. If no partition results in an inequality system with a signature, then the network cannot support multiple positive steady states within a stoichiometric compatibility class.

Deficiency One Algorithm

Suppose we are looking at a regular chemical reaction network $(\mathcal{S}, \mathcal{C}, \mathcal{R})$ following mass action kinetics that has deficiency one and two or more linkage classes, each of deficiency zero, then the Deficiency One Algorithm is given by the following steps [23, 22]:

Step 1: Find a Confluence Vector.

> For regular deficiency one networks, the existence of nonzero confluence vectors is ensured, and all confluence vectors are colinear to each other. If the network is not weakly reversible, all confluence vectors point in the same direction. If it is weakly reversible, the confluence vectors can point in opposite directions.

Thus, for a regular deficiency one network, an answer to the question of capacity for multistationarity is ensured. In the case of a network that is not weakly reversible, there is just one confluence vector to be found to perform the algorithm. For our kinetic proofreading example, the absorptive sets correspond precisely to the linkage classes and the terminal strong linkage classes (cf. Figure 3.2). For $g \in \mathbb{R}^{\mathcal{C}}$ to be a confluence vector for our example, the following conditions have to be satisfied:

(i) $g_{\mathrm{TCR+pMHC}}(\omega_{\mathrm{TCR}} + \omega_{\mathrm{pMHC}}) + g_{\mathrm{C_0}}\omega_{\mathrm{C_0}} + g_{\mathrm{C_0+Lck}}(\omega_{\mathrm{C_0}} + \omega_{\mathrm{Lck}}) + g_{\mathrm{C_0.Lck}}\omega_{\mathrm{C_0.Lck}}$
$+ g_{\mathrm{C_1+Lck}}(\omega_{\mathrm{C_1}} + \omega_{\mathrm{Lck}}) + g_{\mathrm{C_1}}\omega_{\mathrm{C_1}} = 0$

(ii) $g_{\mathrm{TCR+pMHC}} + g_{\mathrm{C_0}} + g_{\mathrm{C_1}} = 0 \quad \text{and} \quad g_{\mathrm{C_0+Lck}} + g_{\mathrm{C_0.Lck}} + g_{\mathrm{C_1+Lck}} = 0$

(iii) $g_{\mathrm{TCR+pMHC}} + g_{\mathrm{C_0}} > 0 \quad \text{and} \quad g_{\mathrm{C_1+Lck}} > 0.$

Thus, we have to find a solution $g \in \mathbb{R}^{\mathcal{C}}$ for the following equalities and inequalities:

$$\begin{aligned} g_{\text{TCR+pMHC}} &= 0 \\ g_{\text{C}_0} + g_{\text{C}_0+\text{Lck}} &= 0 \\ g_{\text{C}_0+\text{Lck}} + g_{\text{C}_1+\text{Lck}} &= 0 \\ g_{\text{C}_0.\text{Lck}} &= 0 \\ g_{\text{C}_1+\text{Lck}} + g_{\text{C}_1} &= 0 \end{aligned} \qquad \begin{aligned} g_{\text{TCR+pMHC}} + g_{\text{C}_0} + g_{\text{C}_1} &= 0 \\ g_{\text{C}_0+\text{Lck}} + g_{\text{C}_0.\text{Lck}} + g_{\text{C}_1+\text{Lck}} &= 0 \end{aligned} \qquad \begin{aligned} g_{\text{TCR+pMHC}} + g_{\text{C}_0} &> 0 \\ g_{\text{C}_1+\text{Lck}} &> 0. \end{aligned}$$

A solution and therefore a confluence vector is given for example by

$$g_{\text{TCR+pMHC}} = 0, \quad g_{\text{C}_0} = 1, \quad g_{\text{C}_0+\text{Lck}} = -1, \quad g_{\text{C}_0.\text{Lck}} = 0, \quad g_{\text{C}_1+\text{Lck}} = 1 \quad g_{\text{C}_1} = -1.$$

Step 2: Choose a Partitioning into Shelves.

The reactive complexes are partitioned into three sets, called *shelves*, the upper shelf $\mathcal{U}$, the middle shelf $\mathcal{M}$ and the lower shelf $\mathcal{L}$. All complexes that are not in a trivial terminal strong linkage class have to be partitioned according to the following rules:

(i) All complexes that are not in a terminal strong linkage class must be placed in the middle shelf.

(ii) All complexes that are in the same terminal strong linkage class must be placed in the same shelf.

(iii) If there are no trivial terminal strong linkage classes, then neither the upper shelf nor the lower shelf can be empty.

(iv) If there is exactly one trivial terminal strong linkage class, then the upper shelf and the lower shelf cannot both be empty.

In our example, there is exactly one trivial terminal strong linkage class, containing the complex $\text{C}_1 + \text{Lck}$. All other complexes have to be placed in one of the shelves according to the rules above. The complexes that are not in a terminal strong linkage class are the complexes C_1, $\text{C}_0 + \text{Lck}$ and $\text{C}_0.\text{Lck}$. Thus, these three complexes must be placed in the middle shelf. The complexes $\text{TCR} + \text{pMHC}$ and C_0 lie within the same terminal strong linkage class and must therefore be placed in the same shelf. As the network possesses exactly one trivial terminal strong linkage class, upper and lower shelf cannot both be empty, so we have to place the two complexes in either one of them. Choosing the upper shelf for the latter, we obtain the following partition:

$$\begin{aligned} \mathcal{U} &= \{\text{TCR} + \text{pMHC}, \text{C}_0\} \\ \mathcal{M} &= \{\text{C}_0 + \text{Lck}, \text{C}_0.\text{Lck}, \text{C}_1\} \\ \mathcal{L} &= \emptyset. \end{aligned}$$

Step 3: Add Shelving Inequalities.

We say a complex y lies in a higher shelf than a complex y', if y lies in the upper shelf and y lies in the middle or lower shelf, or if y lies in the middle shelf and y' lies in the lower shelf. If y is in a higher shelf than y', then the inequality

$$y \cdot \mu > y' \cdot \mu$$

is added to the linear system of equalities and inequalities in μ.

The shelving inequalities for our example arising for the partition chosen in Step 2 are thus:

$$\begin{aligned} &\mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}} > \mu_{\mathrm{C}_0} + \mu_{\mathrm{Lck}} && \mu_{\mathrm{C}_0} > \mu_{\mathrm{C}_0} + \mu_{\mathrm{Lck}} \\ &\mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}} > \mu_{\mathrm{C}_0.\mathrm{Lck}} && \mu_{\mathrm{C}_0} > \mu_{\mathrm{C}_0.\mathrm{Lck}} \\ &\mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}} > \mu_{\mathrm{C}_1} && \mu_{\mathrm{C}_0} > \mu_{\mathrm{C}_1}. \end{aligned}$$

Step 4: Add Middle Shelf Equalities.

If y and y' are in the middle shelf, then the equality

$$y \cdot \mu = y' \cdot \mu$$

is added to the system.

Hence, we add the equality

$$\mu_{\mathrm{C}_0} + \mu_{\mathrm{Lck}} = \mu_{\mathrm{C}_0.\mathrm{Lck}} = \mu_{\mathrm{C}_1}$$

to the linear system so far consisting of the above inequalities.

Step 5: Add Upper and Lower Shelf Inequalities and Equalities.

Let $\{y, y'\}$ be a cut pair and $\mathcal{W}(y)$ and $\mathcal{W}(y')$, with $y \in \mathcal{W}(y)$ and $y' \in \mathcal{W}(y')$, the partition of the linkage class containing y and y' such that the only reactions between $\mathcal{W}(y)$ and $W(y')$ are the reactions between y and y'. We define

$$\left[g, y \leftrightarrow y', y\right] := \sum_{\tilde{y} \in \mathcal{W}(y)} g_{\tilde{y}},$$

that is, the sum of the confluence vectors resulting from the partition of the linkage class by a cut pair.

(i) If $\{y, y'\}$ is a cut pair in the upper shelf, then according to the sign of $[g, y \leftrightarrow y', y]$ the following inequality or equality is added to the system:

$$\begin{aligned} y \cdot \mu > y' \cdot \mu \quad &\text{if } [g, y \leftrightarrow y', y] > 0, \\ y \cdot \mu = y' \cdot \mu \quad &\text{if } [g, y \leftrightarrow y', y] = 0, \\ y \cdot \mu < y' \cdot \mu \quad &\text{if } [g, y \leftrightarrow y', y] < 0. \end{aligned}$$

(ii) If $\{y, y'\}$ is a cut pair in the lower shelf, then according to the sign of $[g, y \leftrightarrow y', y]$ the following inequality or equality is added to the system:

$$\begin{aligned} y \cdot \mu < y' \cdot \mu \quad &\text{if } [g, y \leftrightarrow y', y] > 0, \\ y \cdot \mu = y' \cdot \mu \quad &\text{if } [g, y \leftrightarrow y', y] = 0, \\ y \cdot \mu > y' \cdot \mu \quad &\text{if } [g, y \leftrightarrow y', y] < 0. \end{aligned}$$

We note that from the definition of a confluence vector (Definition 17 (ii)), it follows that $[g, y \leftrightarrow y', y'] = -[g, y \leftrightarrow y', y]$. Thus, defining the sign of $[g, y \leftrightarrow y', y]$ leads to the same inequalities as defining the sign of $[g, y' \leftrightarrow y, y']$. Given a cut pair $\{y, y'\}$, we can arbitrarily choose one of the complexes to generate the equalities and inequalities in this step.

For our example, there is exactly one cut pair $\{\mathrm{TCR}+\mathrm{pMHC}, \mathrm{C}_0\}$ in the upper shelf and the lower shelf is empty. Thus, we have only one inequality that has to be added to the system. With the confluence vector chosen in Step 1, we get

$$\begin{aligned}[g, \mathrm{TCR}+\mathrm{pMHC} \leftrightarrow \mathrm{C}_0, C_0] &= g_{\mathrm{C}_0} = 1 \\ &= -(0-1) = -\left(g_{\mathrm{TCR+pMHC}} + g_{\mathrm{C}_1}\right) = -\left[g, \mathrm{TCR}+\mathrm{pMHC} \leftrightarrow \mathrm{C}_0, \mathrm{TCR}+\mathrm{pMHC}\right].\end{aligned}$$

Thus, according to the sign of $[g, \mathrm{TCR}+\mathrm{pMHC} \leftrightarrow \mathrm{C}_0, \mathrm{C}_0] = 1 > 0$ we add the following inequality to the linear system:

$$\mu_{\mathrm{C}_0} > \mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}}.$$

Step 6: Check for Solutions.

> We now have a complete linear system in μ that has to be examined for a solution. That is, no more inequalities or equalities have to be added. If a nonzero solution vector $\mu \in \mathbb{R}^{\mathcal{S}}$ sign compatible with the stoichiometric subspace is found, then there exist reaction rates such that two distinct positive steady states occur. If such a solution is not found, the next step of the algorithm has to be performed.

Summarizing the added equalities and inequalities, our system is given by:

$$\mu_{\mathrm{C}_0} > \mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}} > \mu_{\mathrm{C}_0} + \mu_{\mathrm{Lck}} = \mu_{\mathrm{C}_0.\mathrm{Lck}} = \mu_{\mathrm{C}_1}.$$

The question is if there exists a solution for $\mu \in \mathbb{R}^{\mathcal{S}}$ that is sign compatible with the stoichiometric subspace (Definition 6). The stoichiometric subspace, $S = \mathrm{span}\{y' - y : y \to y' \in \mathcal{R}\} \subset \mathbb{R}^{\mathcal{S}}$, is spanned by the reaction vectors, thus by the linearly independent columns of the stoichiometric matrix N:

$$S = \mathrm{span}\left\{\begin{pmatrix}-1\\-1\\1\\0\\0\\0\end{pmatrix}, \begin{pmatrix}0\\0\\-1\\-1\\1\\0\end{pmatrix}, \begin{pmatrix}0\\0\\0\\1\\-1\\1\end{pmatrix}\right\},$$

where we imposed the ordering $(\mathrm{TCR}, \mathrm{pMHC}, \mathrm{C}_0, \mathrm{Lck}, \mathrm{C}_0.\mathrm{Lck}, \mathrm{C}_1)$. Thus, any vector $\sigma \in S$ is a linear combination of the above vectors and to check if there exists a nonzero solution vector $\mu \in \mathbb{R}^{\mathcal{S}}$ sign compatible with the stoichiometric subspace, we are looking for scalars $a_1, a_2, a_3 \in \mathbb{R}$ such that

$$\begin{aligned} p_{\mathrm{TCR}}(-a_1) &= \mu_{\mathrm{TCR}} \\ p_{\mathrm{pMHC}}(-a_1) &= \mu_{\mathrm{TCR}} \\ p_{\mathrm{C}_0}(a_1 - a_2) &= \mu_{\mathrm{C}_0} \\ p_{\mathrm{Lck}}(-a_2 + a_3) &= \mu_{\mathrm{Lck}} \\ p_{\mathrm{C}_0.\mathrm{Lck}}(a_2 - a_3) &= \mu_{\mathrm{C}_0.\mathrm{Lck}} \\ p_{\mathrm{C}_1} a_3 &= \mu_{\mathrm{C}_1} \end{aligned}$$

where $p_s \in \mathbb{R}^+$, $s \in \mathcal{S}$. From $\mu_{\mathrm{C}_0} > \mu_{\mathrm{C}_0} + \mu_{\mathrm{Lck}}$ we know that $\mathrm{sign}\,(\mu_{\mathrm{Lck}}) < 0$. From $p_{\mathrm{Lck}}(-a_2+a_3) = \mu_{\mathrm{Lck}}$ and $p_{\mathrm{C}_0.\mathrm{Lck}}(a_2 - a_3) = \mu_{\mathrm{C}_0.\mathrm{Lck}}$ we get $\mathrm{sign}\,(\mu_{\mathrm{C}_0.\mathrm{Lck}}) = -\mathrm{sign}\,(\mu_{\mathrm{Lck}}) > 0$. Thus, $\mathrm{sign}\,(\mu_{\mathrm{C}_0}) =$

$\operatorname{sign}(\mu_{\mathrm{TCR}}) = \operatorname{sign}(\mu_{\mathrm{pMHC}}) = \operatorname{sign}(\mu_{\mathrm{C}_1}) > 0$. This implies

$$\left.\begin{aligned} -a_1 &> 0 \\ a_1 - a_2 &> 0 \\ a_2 - a_3 &> 0 \\ a_3 &> 0 \end{aligned}\right\} \quad \Rightarrow \quad 0 > a_1 > a_2 > a_3 > 0,$$

a contradiction. Thus, the produced linear system in μ possesses no solution that is sign compatible with the stoichiometric subspace. The confluence vector and complex partitioning chosen do not provide a signature for the network.

Step 7: Repeat Steps 2 through 6.

> If no signature exists for the linear system of equalities and inequalities produced, another partition might create a system that possesses a solution vector sign compatible with the stoichiometric subspace. However, not all possible partitions need to be checked. Exchanging the complexes in the upper and lower shelf leads to the inversion of the inequalities of the original partitioning. Thus, there exists a solution for the inverted system exactly when there is a solution for the original system.

In our case, the only other partitioning is such an inversion.

Step 8: Repeat Steps 1 through 7.

> If the given reaction network is weakly reversible, there are two possible directions for a confluence vector. Then the algorithm has to be performed for a confluence vector pointing in the opposite direction which produces reversed inequalities in Step 5.

As the network we are investigating is not weakly reversible, this step does not need to be performed. Thus, we have shown that there exists no signature for the kinetic proofreading system with a single phosphorylation step. That is, the network is not able to support multiple positive steady states within the same stoichiometric compatibility class. Thus, the kinetic proofreading system with one phosphorylation step possesses at most one positive steady state. In fact, it possesses exactly one steady state as we will argue in Chapter 4.

In the case of a regular deficiency one network with mass action kinetics and linkage classes that have deficiency zero, the Deficiency One Algorithm provides an answer to whether the network can support multiple positive steady states within a stoichiometric compatibility class. That is, if there exist rate constants such that the system exhibits at least two different steady states. We have shown that the kinetic proofreading system with one phosphorylation step cannot support multiple positive steady states, no matter which rate constants we choose. How does this characteristic change when we admit one more phosphorylation step to the system?

$$\begin{array}{ccccc} \mathrm{TCR} + \mathrm{pMHC} & \underset{k_2}{\overset{k_1}{\rightleftharpoons}} & \mathrm{C}_0 & & \\ \quad {\scriptstyle k_6}\nearrow \quad \nwarrow{\scriptstyle k_{10}} & & & & \\ \mathrm{C}_1 \qquad\quad \mathrm{C}_2 & & & & \end{array}$$

$$\mathrm{C}_0 + \mathrm{Lck} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{C}_0.\mathrm{Lck} \xrightarrow{k_5} \mathrm{C}_1 + \mathrm{Lck} \underset{k_8}{\overset{k_7}{\rightleftharpoons}} \mathrm{C}_1.\mathrm{Lck} \xrightarrow{k_9} \mathrm{C}_2 + \mathrm{Lck}$$

Figure 3.3: Kinetic proofreading with Lck and two phosphorylation steps.

The network for the kinetic proofreading system with two phosphorylation steps (see Figure 3.3) consists of eight species, ten complexes, $n = 10$, and ten reactions. Just as for the network with one phosphorylation step there are two linkage classes, $l = 2$. The corresponding stoichiometric matrix with the imposed ordering of the species $\{\mathrm{TCR}, \mathrm{pMHC}, \mathrm{C}_0, \mathrm{Lck}, \mathrm{C}_0.\mathrm{Lck}, \mathrm{C}_1, \mathrm{C}_1.\mathrm{Lck}, \mathrm{C}_2\}$ is given by

$$N = \begin{pmatrix} -1 & 1 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 1 \\ -1 & 1 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 1 \\ 1 & -1 & -1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & -1 & 1 & 1 & 0 & -1 & 1 & 1 & 0 \\ 0 & 0 & 1 & -1 & -1 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 & -1 & -1 & 1 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 1 & -1 & -1 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & -1 \end{pmatrix}.$$

With $s = \mathrm{rank}(N) = 5$, we get a deficiency of

$$\delta = n - l - s = 10 - 2 - 5 = 3 \neq 1.$$

Thus, adding one phosphorylation step is enough to get a network with a deficiency higher than one so that we cannot apply the Deficiency One Algorithm any more to answer the question of the existence of multiple positive steady states within the same stoichiometric compatibility class. For networks with a deficiency higher than one, Ellison [22] developed the *Advanced Deficiency Algorithm*.

3.2 Advanced Deficiency Algorithm

The Advanced Deficiency Algorithm can be applied to mass action kinetic networks with a deficiency higher than zero to determine if the system is able to support multiple positive steady states within a stoichiometric compatibility class. For networks satisfying certain linearity conditions, this question can be answered conclusively. The section is based on the PhD thesis of Ellison [22], who developed the Advanced Deficiency Algorithm. A second source is the PhD thesis of Ji [51], which summarizes the algorithm and further develops it, focusing on networks that do not satisfy the conditions for the Advanced Deficiency Algorithm. The Advanced Deficiency Algorithm answers the question of multistationarity by reformulating it into systems of inequalities and equalities that need to be solved. These systems are formulated in terms of the vector $\mu \in \mathbb{R}^{\mathcal{S}}$, which was introduced in the Deficiency One Algorithm, and additional parameters that we will introduce throughout the algorithm. Before being able to apply the Advanced Deficiency Algorithm, the introduction of a few more terms and definitions is necessary. We begin where we left off in the last section, with the information stored in the stoichiometric matrix.

To determine the rank of the stoichiometric matrix N, we determined the number of linearly independent reaction vectors. Any reversible reaction gives rise to two linearly dependent reaction vectors. Thus, we can reduce the dimension of the matrix that has to be examined by choosing one reaction for every reversible reaction.

Definition 18. [22, 51] An *orientation* $\mathcal{O}$ is defined as a subset of the set of all reactions in a reaction network $(\mathcal{S}, \mathcal{C}, \mathcal{R})$ such that for every reaction $y \to y' \in \mathcal{R}$ either $y \to y' \in \mathcal{O}$ or $y' \to y \in \mathcal{O}$, but not both. We define the map $L_{\mathcal{O}} : \mathbb{R}^{\mathcal{O}} \to \mathbb{R}^{\mathcal{S}}$ via

$$L_{\mathcal{O}}(\alpha) := \sum_{y \to y' \in \mathcal{O}} \alpha_{y \to y'} \left(y' - y\right).$$

With $\overline{r}$ denoting the number of reactions in the orientation $\mathcal{O}$ and m denoting the number of species,

$L_{\mathcal{O}}$ maps from $\mathbb{R}^{\overline{r}}$ to $\mathbb{R}^m$. If $y \to y'$ is the i-th reaction in the orientation, then $\alpha_{y\to y'}$ is the i-th component of α.

We can represent the linear map $L_{\mathcal{O}}$ by a matrix. Precisely, this is equivalent to the stoichiometric matrix restricted to the reactions in $\mathcal{O}$, denoted $N|_{\mathcal{O}}$. In this representation, every reversible reaction is represented only by one column.

Definition 19. [22, 51] Let $\mathcal{O}$ be an orientation and let $d := \dim(\ker(L_{\mathcal{O}}))$ and $v^1, \dots v^d$ be a basis for $\ker(L_{\mathcal{O}})$. With s denoting the dimension of the stoichiometric subspace and $\overline{r}$ denoting the number of reactions in orientation, the equation $d = \overline{r} - s$ holds. We define a *w-vector*, $w_{y\to y'} = \left(w_{y\to y'_1}, \dots, w_{y\to y'_d}\right) \in \mathbb{R}^d$ where $y \to y' \in \mathcal{R}$ as follows:

(i) If $y \to y' \in \mathcal{O}$, we set $w_{y\to y'_i} = v^i_{y\to y'}$.

That is, $w_{y\to y'} = \sum_{i=1}^{d} \left(v^i_{y\to y'} e^i\right)$ where e^i is the i-th vector of the basis of $\mathbb{R}^d$.

(ii) If $y \to y' \in \mathcal{R}\backslash\mathcal{O}$, then $y' \to y \in \mathcal{O}$, and we define $w_{y\to y'} = w_{y'\to y}$.

For every reaction, the dimension of the kernel of $L_{\mathcal{O}}$ will always be equal to or greater than the deficiency, with equality holding if and only if every pair of directly linked complexes in the reaction network is a cut pair [22]. The w-vectors are used to define the colinearity classes of the reaction network [22].

Definition 20. [22, 51] Two reactions $y \to y'$ and $\tilde{y} \to \tilde{y}'$ are in the same *colinearity class*, if there exists a nonzero number $\alpha \in \mathbb{R}\backslash\{0\}$ such that

$$w_{y\to y'} = \alpha w_{\tilde{y}\to\tilde{y}'}.$$

The *zero colinearity class* is defined as the colinearity class containing the reactions whose w-vectors are equal to the zero vector. A colinearity class is called a *reversible colinearity class* if every reaction in the colinearity class is reversible. If any reaction in the colinearity class is irreversible, then the colinearity class is called an *irreversible colinearity class*. An empty colinearity class is considered reversible by definition. For a reaction network, suppose there exist a zero colinearity class and k nonzero colinearity classes. We denote them as $CC_0, CC_1, \dots, CC_k$, where CC_0 is the zero colinearity class.

Thus, both directions of a reversible reaction are always assigned to the same colinearity class. Irrespective of the chosen orientation $\mathcal{O}$ and the basis of the kernel of $L_{\mathcal{O}}$, the colinearity classes identified remain consistent. Any reactions being part of the same colinearity class for one choice will be in the same colinearity class for any other choice. In particular, the zero colinearity class will always contain the same reactions [22]. The colinearity classes can be used to partition the network into subnetworks. Each subnetwork comprises exactly the reactions of a single colinearity class. Within these subnetworks, we can now define linkage classes in an analogous way to the linkage classes of the whole network, called colinkage sets.

Definition 21. [22] A linkage class of the subnetwork given by a colinearity class is called a *colinkage set* for the reaction network. A *strong colinkage set* and a *terminal strong colinkage set* are defined in an analogous way.

For every colinearity class a representative w-vector can be chosen, referred to as colinearity class vector [51]. This representative vector is chosen in a way that it is related to the w-vectors of the

reactions in the colinearity class and helps to examine the relationships between different colinearity classes [22].

Definition 22. [51, 22] We define a *colinearity class vector* w_i by choosing a representative from all w-vectors within a colinearity class in the following way:

(i) For an irreversible colinearity class CC_i, we can pick any positive multiple of $w_{y\to y'}$ for some irreversible reaction $y \to y' \in CC_i$.

(ii) For a reversible colinearity class CC_i, we can pick any nonzero multiple of $w_{y\to y'}$ for some $y \to y' \in CC_i$.

The colinearity class vector for a zero colinearity class is thus the zero vector. To describe how nonzero colinearity classes are related to each other, we define sets of colinearity classes.

Definition 23. [22, 51] A *coplanar set* $\mathcal{T}$ is defined as a set of nonzero colinearity classes with the following properties:

(i) The set $\mathcal{T}$ has three or more colinearity classes in it.

(ii) The colinearity class vectors for all colinearity classes in $\mathcal{T}$ lie in the same two-dimensional linear subspace, and all such colinearity classes whose colinearity class vectors lie in the two-dimensional linear subspace are in $\mathcal{T}$.

In other words, each colinearity class vector in $\mathcal{T}$ can be written as linear combination of any other two colinearity class vectors in $\mathcal{T}$. Coplanar sets do not partition the colinearity classes. A colinearity class may belong to more than one coplanar set or to no coplanar set at all. A reaction network may not even have coplanar sets [22]. Similar to the definition of the linkage classes for complexes, we can define an equivalence relation on the nonzero colinearity classes [22].

Definition 24. [22, 51] Two nonzero colinearity classes CC_i and CC_j are *directly linked*, if there exists a coplanar set $\mathcal{T}$ containing both colinearity classes, this is denoted by $CC_i \sim CC_j$. Two nonzero colinearity classes CC_i and CC_j are *connected* if at least one of the following conditions is satisfied:

(i) CC_i and CC_j are the same colinearity class.

(ii) $CC_i \sim CC_j$.

(iii) There exist colinearity classes $CC_1, \ldots, CC_t$ such that $CC_i \sim CC_1 \sim \cdots \sim CC_t \sim CC_j$.

Two colinearity classes are in the same *connected class* if and only if they are connected.

Connected classes partition the nonzero colinearity classes. Every colinearity class belongs to exactly one connected class. The relationship between nonzero colinearity classes, coplanar sets and connected classes can be expressed graphically [22]. We can construct a *connecting graph* where all the nodes are coplanar sets and nonzero colinearity classes and the edges connect the colinearity classes to the coplanar set they belong to [22, 51].

Now we have defined all the terms necessary to proceed with the execution of the Advanced Deficiency Algorithm [22, 51].

Advanced Deficiency Algorithm

The Advanced Deficiency Algorithm can be applied to any chemical reaction network $(\mathcal{S}, \mathcal{C}, \mathcal{R})$ following mass action kinetics with a deficiency higher than zero [22].

Step 1: Choose an Initial Orientation.

By the definition of an orientation $\mathcal{O}$, every irreversible reaction is an element of $\mathcal{O}$, and for each reversible reaction, one of the reactions has to be chosen. Which direction of a reversible reaction is chosen is arbitrary and will not affect the results of the algorithm.

As an example, we will perform the Advanced Deficiency Algorithm for the kinetic proofreading system with Lck and two phosphorylation steps (cf. Figure 3.3). We arbitrarily choose the orientation of complex bindings for the reversible reactions:

$$\mathcal{O} = \Big\{ \mathrm{TCR} + \mathrm{pMHC} \xrightarrow{k_1} \mathrm{C}_0, \mathrm{C}_0 + \mathrm{Lck} \xrightarrow{k_3} \mathrm{C}_0.\mathrm{Lck}, \mathrm{C}_0.\mathrm{Lck} \xrightarrow{k_5} \mathrm{C}_1 + \mathrm{Lck}, \mathrm{C}_1 \xrightarrow{k_6} \mathrm{TCR} + \mathrm{pMHC},$$
$$\mathrm{C}_1 + \mathrm{Lck} \xrightarrow{k_7} \mathrm{C}_1.\mathrm{Lck}, \mathrm{C}_1.\mathrm{Lck} \xrightarrow{k_9} \mathrm{C}_2 + \mathrm{Lck}, \mathrm{C}_2 \xrightarrow{k_{10}} \mathrm{TCR} + \mathrm{pMHC} \Big\}.$$

Step 2: Find the Colinearity Classes.

Having chosen an orientation, we can now find w-vectors and with them colinearity classes by definition (Definition 19 and 20). At this point there are already two necessary conditions for a network to support multiple steady states:

(i) The zero colinearity class of a reaction network has to be reversible.

(ii) There is no colinearity class that contains two irreversible reactions, $y \to y'$ and $\tilde{y} \to \tilde{y}'$, such that $w_{y \to y'} = \alpha w_{\tilde{y} \to \tilde{y}'}$ for some $\alpha < 0$.

Thus, if the zero colinearity class of a reaction network is irreversible or there are two irreversible reactions $y \to y'$ and $\tilde{y} \to \tilde{y}'$ in the same colinearity class such that $w_{y \to y'} = \alpha w_{\tilde{y} \to \tilde{y}'}$ for some $\alpha < 0$, then the reaction network cannot support multiple steady states, no matter what positive values the constants take. In fact, if the zero colinearity class is irreversible, the network cannot support any positive state at all [22]. In these cases, the question of multistationarity is answered for the network, and we exit the algorithm.

We keep the ordering of the species and reactions imposed from the kinetic proofreading system with only one phosphorylation step and add the species C_1.Lck and C_2 and the reaction numbers seven to ten which are identified by the numbering of the rate constants (cf. Figure 3.3). This leads to the following representation for the linear map $L(\alpha) = N|_{\mathcal{O}}\,\alpha$:

$$N|_{\mathcal{O}} = \begin{array}{l} \text{species} \\ \mathrm{TCR} \\ \mathrm{pMHC} \\ \mathrm{C}_0 \\ \mathrm{Lck} \\ \mathrm{C}_0.\mathrm{Lck} \\ \mathrm{C}_1 \\ \mathrm{C}_1.\mathrm{Lck} \\ \mathrm{C}_2 \end{array} \begin{array}{c} \begin{array}{ccccccc} \text{reactions} & & & & & & \\ 1 & 3 & 5 & 6 & 7 & 9 & 10 \end{array} \\ \begin{pmatrix} -1 & 0 & 0 & 1 & 0 & 0 & 1 \\ -1 & 0 & 0 & 1 & 0 & 0 & 1 \\ 1 & -1 & 0 & 0 & 0 & 0 & 0 \\ 0 & -1 & 1 & 0 & -1 & 1 & 0 \\ 0 & 1 & -1 & 0 & 0 & 0 & 0 \\ 0 & 0 & 1 & -1 & -1 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 & -1 & 0 \\ 0 & 0 & 0 & 0 & 0 & 1 & -1 \end{pmatrix} \end{array}.$$

First, we determine the dimension of the kernel of $L_{\mathcal{O}}$ and find a basis for it. We see that $\operatorname{rank}(N|_{\mathcal{O}}) = 5$ as the first row is identical to the second, the fourth row is obtained by adding the fifth and seventh rows and then multiplying by -1, and the eighth row equals the multiplication by -1 of the sum of the first, third, fifth, sixth and seventh row. As $\dim(L_{\mathcal{O}}) = \dim(\ker(L_{\mathcal{O}})) + \dim(\operatorname{im}(L_{\mathcal{O}})) = \dim(\ker(L_{\mathcal{O}})) + \dim((\operatorname{rank} N|_{\mathcal{O}}))$ we get $d = \dim(\ker(L_{\mathcal{O}})) = 2$. Now we choose a basis $v^1, v^2 \in \mathbb{R}^{\mathcal{O}}$ for $\ker(L_{\mathcal{O}})$ setting

$$\begin{array}{l} \\ \mathrm{TCR} + \mathrm{pMHC} \xrightarrow{1} \mathrm{C}_0 \\ \mathrm{C}_0 + \mathrm{Lck} \xrightarrow{3} \mathrm{C}_0.\mathrm{Lck} \\ \mathrm{C}_0.\mathrm{Lck} \xrightarrow{5} \mathrm{C}_1 + \mathrm{Lck} \\ \mathrm{C}_1 \xrightarrow{6} \mathrm{TCR} + \mathrm{pMHC} \\ \mathrm{C}_1 + \mathrm{Lck} \xrightarrow{7} \mathrm{C}_1.\mathrm{Lck} \\ \mathrm{C}_1.\mathrm{Lck} \xrightarrow{9} \mathrm{C}_2 + \mathrm{Lck} \\ \mathrm{C}_2 \xrightarrow{10} \mathrm{TCR} + \mathrm{pMHC} \end{array} \begin{array}{c} \begin{array}{cc} v^1 & v^2 \end{array} \\ \begin{pmatrix} 1 & 0 \\ 1 & 0 \\ 1 & 0 \\ 1 & -1 \\ 0 & 1 \\ 0 & 1 \\ 0 & 1 \end{pmatrix} \end{array} =: \begin{pmatrix} v^1 & v^2 \end{pmatrix}.$$

This provides the following w-vectors:

$$w_{\mathrm{TCR+pMHC}\to\mathrm{C}_0} = w_{\mathrm{C}_0\to\mathrm{TCR+pMHC}} = w_{\mathrm{C}_0+\mathrm{Lck}\to\mathrm{C}_0.\mathrm{Lck}} = w_{\mathrm{C}_0.\mathrm{Lck}\to\mathrm{C}_0+\mathrm{Lck}} = w_{\mathrm{C}_0.\mathrm{Lck}\to\mathrm{C}_1+\mathrm{Lck}} = \begin{pmatrix} 1 \\ 0 \end{pmatrix}$$

$$w_{\mathrm{C}_1\to\mathrm{TCR+pMHC}} = \begin{pmatrix} 1 \\ -1 \end{pmatrix}$$

$$w_{\mathrm{C}_1+\mathrm{Lck}\to\mathrm{C}_1.\mathrm{Lck}} = w_{\mathrm{C}_1.\mathrm{Lck}\to\mathrm{C}_1+\mathrm{Lck}} = w_{\mathrm{C}_1.\mathrm{Lck}\to\mathrm{C}_2+\mathrm{Lck}} = w_{\mathrm{C}_2\to\mathrm{TCR+pMHC}} = \begin{pmatrix} 0 \\ 1 \end{pmatrix}.$$

As none of the w-vectors are colinear to each other, the colinearity classes are given exactly by the reactions having the same w-vector:

$$\begin{aligned} CC_1 &= \{\mathrm{TCR} + \mathrm{pMHC} \to \mathrm{C}_0, \mathrm{C}_0 \to \mathrm{TCR} + \mathrm{pMHC}, \mathrm{C}_0 + \mathrm{Lck} \to \mathrm{C}_0.\mathrm{Lck}, \mathrm{C}_0.\mathrm{Lck} \to \mathrm{C}_0 + \mathrm{Lck}, \\ &\qquad \mathrm{C}_0.\mathrm{Lck} \to \mathrm{C}_1 + \mathrm{Lck}\}, \\ &= \{\mathrm{C}_0 \rightleftharpoons \mathrm{TCR} + \mathrm{pMHC}, \mathrm{C}_0 + \mathrm{Lck} \rightleftharpoons \mathrm{C}_0.\mathrm{Lck}, \mathrm{C}_0.\mathrm{Lck} \to \mathrm{C}_1 + \mathrm{Lck}\} \\ CC_2 &= \{\mathrm{C}_1 \to \mathrm{TCR} + \mathrm{pMHC}\}, \\ CC_3 &= \{\mathrm{C}_1 + \mathrm{Lck} \to \mathrm{C}_1.\mathrm{Lck}, \mathrm{C}_1.\mathrm{Lck} \to \mathrm{C}_1 + \mathrm{Lck}, \mathrm{C}_1.\mathrm{Lck} \to \mathrm{C}_2 + \mathrm{Lck}, \mathrm{C}_2 \to \mathrm{TCR} + \mathrm{pMHC}\} \\ &= \{\mathrm{C}_1 + \mathrm{Lck} \rightleftharpoons \mathrm{C}_1.\mathrm{Lck}, \mathrm{C}_1.\mathrm{Lck} \to \mathrm{C}_2 + \mathrm{Lck}, \mathrm{C}_2 \to \mathrm{TCR} + \mathrm{pMHC}\}. \end{aligned}$$

For the same reason, no colinearity class contains two irreversible reactions with $w_{y\to y'} = \alpha w_{\tilde{y}\to\tilde{y}'}$ for some $\alpha < 0$. Furthermore, there is no zero colinearity class. Thus, both necessary conditions to continue with the algorithm in search for multiple steady states are fulfilled.

Step 3: Find the Colinkage Sets.

Find the linkage classes in the subnetworks given by the colinearity classes.

We display the subnetworks given by the colinearity classes to identify the linkage classes within the subnetworks, the colinkage sets. Terminal strong colinkage sets are shaded gray.

Colinearity Class	Colinkage Sets
CC_1	$\mathrm{TCR} + \mathrm{pMHC} \underset{2}{\overset{1}{\rightleftharpoons}} \mathrm{C}_0$
	$\mathrm{C}_0 + \mathrm{Lck} \underset{4}{\overset{3}{\rightleftharpoons}} \mathrm{C}_0.\mathrm{Lck} \xrightarrow{5} \mathrm{C}_1 + \mathrm{Lck}$
CC_2	$\mathrm{C}_1 \xrightarrow{6} \mathrm{TCR} + \mathrm{pMHC}$
CC_3	$\mathrm{C}_1 + \mathrm{Lck} \underset{8}{\overset{7}{\rightleftharpoons}} \mathrm{C}_1.\mathrm{Lck} \xrightarrow{9} \mathrm{C}_2 + \mathrm{Lck}$
	$\mathrm{C}_2 \xrightarrow{10} \mathrm{TCR} + \mathrm{pMHC}$

Step 4: Choose Colinearity Class Vectors.

> Choose a colinearity class vector for each colinearity class according to the rules defining a colinearity class vector (Definition 22).

In our example, all three colinearity classes are irreversible. Thus, we can pick in each case any positive multiple of a w-vector belonging to an irreversible reaction of the particular colinearity class. As we found out in Step 2 for the kinetic proofreading system with two phosphorylation steps, all reactions within the same colinearity class possess the same w-vector. Thus, colinearity class vectors for the colinearity classes CC_1, CC_2 and CC_3 are given by

$$w_1 = \begin{pmatrix} 1 \\ 0 \end{pmatrix}, \quad w_2 = \begin{pmatrix} 1 \\ -1 \end{pmatrix} \quad \text{and} \quad w_3 = \begin{pmatrix} 0 \\ 1 \end{pmatrix},$$

and any other choice for colinearity class vectors would be positive multiples of these.

Step 5: Realign the Orientation.

> Realign the orientation $\mathcal{O}$ such that, under the realigned orientation, all w-vectors are positive multiples of their respective colinearity class vector.
>
> This step applies only to reversible reactions, since the colinearity class vectors are defined as a positive multiple of the w-vector for all irreversible reactions. Thus, if we encounter a w-vector, $w_{y \to y'}$, of a reversible reaction $y \to y'$ that is the negative of its associated colinearity class vector, we return to Step 1 and replace this reaction in the orientation with the reaction $y' \to y$. This results in the negative of the respective column in the stoichiometric matrix for the reactions in the orientation, $N|_{\mathcal{O}}$. Consequently, we can choose an adjusted basis for $\ker(L_{\mathcal{O}})$ by changing the sign of the entries in the row corresponding to the reaction. This modification yields a w-vector that is now a positive multiple of its respective colinearity class vector. We note that the case that there are two irreversible reactions within the same colinearity class, one being a positive the other a negative multiple of the colinearity class vector, is excluded as this violates the second necessary condition in Step 2.

For our example, there is no need to realign the orientation since all reactions in the same colinearity class have the same w-vector. Thus, the colinearity class vectors coincide with the w-vectors within the colinearity class.

Step 6: Find Coplanar Sets and Connected Classes.

> Find the colinear sets given by the colinearity class vectors (Definition 23) and identify the resulting connected classes (Definition 24).

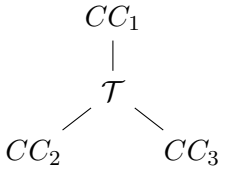
The three colinearity class vectors for the kinetic proofreading system with Lck and two phosphorylation steps, $w_1 = (1, 0)^T$, $w_2 = (1, -1)^T$ and $w_3 = (0, 1)^T$, lie within the same two-dimensional linear subspace:

$$w_2 + w_3 = w_1.$$

Thus, both conditions for a coplanar set are fulfilled, and the network possesses exactly one coplanar set containing all three colinearity classes:

$$\mathcal{T} = \{CC_1, CC_2, CC_3\}.$$

The connecting graph representing the connected classes is given by:



Step 7: Determine Linearity.

> If the inequality systems generated are entirely linear in μ and the other parameters, then the linear systems generated by the algorithm are the complete inequality and equality systems for the reaction network, and any of these systems which has a solution with a nonzero μ sign compatible with the stoichiometric subspace will be a signature of the network. In other words, if linearity conditions are met, the algorithm determines whether the network supports multiple steady states within a stoichiometric compatibility class or not. A preliminary determination of the linearity of any signatures of the reaction network is made by the following two linearity conditions:
>
> (i) *Independence Linearity Condition:*
>
> The number of coplanar sets plus the number of connected classes add up to the dimension d of the kernel of $L_{\mathcal{O}}$.
>
> (ii) *Triplet Linearity Condition:*
>
> No coplanar set contains more than three colinearity classes. That is, it contains exactly three colinearity classes.
>
> If the Independence Linearity Condition is violated, the reaction network may still have linear signatures. By introducing *pseudo-colinearity classes* to the network, the number of coplanar sets can be reduced so that the new coplanar sets and connected classes fulfill the Independence Linearity Condition. If the reaction network violates the Triplet Colinearity Condition or the introduction of pseudo-colinearity classes is not successful, most of the inequality systems produced will be incomplete, and some nonlinear equalities have to be added to obtain a complete system of inequalities and equalities [22]. As the kinetic proofreading systems we are investigating fulfill both linearity conditions, we will not execute the idea of the pseudo-colinearity classes. See Ellison [22] for details or Ji [51] for the further development of the Advanced Deficiency Algorithm to the *Higher Deficiency Algorithm* that does not need the help of pseudo-colinearity classes to determine linearity.

The kinetic proofreading with Lck and two phosphorylation steps has exactly one coplanar set and one connected class. Thus, the sum of both numbers equals the dimension of the kernel of $L_{\mathcal{O}}$ which is two. Therefore, the Independence Linearity Condition is fulfilled. The one coplanar set contains exactly three colinearity classes. Hence, the Triplet Linearity Condition holds as well. Consequently, the system of inequalities and equalities generated to answer the question of multistationarity is linear and complete. That is, we are able to answer the question of capacity for multistationarity. If we find a signature for the system produced, there exist a compatibility class and kinetic parameters such that the network exhibits multiple positive steady states. Conversely, if there exists no signature for the system, then the network is not able to admit more than one positive steady state within the same compatibility class regardless of the reaction rates.

Step 8: Choose Signs for the Colinearity Classes.

Each colinearity class is assigned a sign (positive, negative or zero) following the rules below:

(i) The zero colinearity class is assigned a zero sign.

(ii) An irreversible colinearity class is assigned a positive sign.

(iii) A nonzero reversible colinearity class can be assigned a positive, negative or zero sign as long as the following conditions are satisfied:

a) If more than one colinearity class in a coplanar set is assigned a zero sign, then every colinearity class in the coplanar set is assigned a zero sign.

Let CC_i, CC_j and CC_k be three colinearity classes from the same coplanar set.

b) If each of these three colinearity classes has a nonzero sign, then there do not exist c_i, c_j and c_k agreeing in sign with their respective colinearity classes such that $c_i w_i + c_j w_j + c_k w_k = 0$.

c) If only one of the three colinearity classes, say CC_i, has a zero sign, then there do not exist c_j and c_k agreeing in sign with their respective colinearity classes such that $c_j w_j + c_k w_k$ is a multiple of w_i.

If no choice of signs can satisfy these conditions, then the network cannot support multiple steady states. In fact, the network cannot support any positive steady state [22]. In this case, the rest of the algorithm is skipped.

The network given by the kinetic proofreading system with Lck and two phosphorylation steps possesses only irreversible colinearity classes. Thus, every colinearity class is assigned a positive sign.

Step 9: Choose Shelving for Reactions.

The reactions are partitioned into sets called *shelves* according to certain rules. In contrast to the Deficiency One Algorithm where the complexes are put into shelves, now the reactions are put into shelves and there are *upper* ($\mathcal{U}$), *middle* ($\mathcal{M}$) and *lower shelves* ($\mathcal{L}$) for every colinearity class with nonzero sign. Given a colinearity class with nonzero sign, the reactions in this colinearity class are partitioned according to the following conditions:

(i) A reaction whose reactant complex lies in a non-terminal strong colinkage set (relative to the subnetwork of the colinearity class) is placed on the middle shelf of the colinearity class.

(ii) An irreversible reaction is placed on the middle shelf of the colinearity class.

(iii) A reversible reaction whose reactant complex lies in a terminal strong colinkage set (relative to the subnetwork of the colinearity class) can be placed on the upper, middle or lower shelf of the colinearity class as long as reactions in the same colinkage set (relative to the subnetwork of the colinearity class) are placed on the same shelf of the colinearity class.

We identified the colinkage sets for the kinetic proofreading system with Lck and two phosphorylation steps in Step 3. The first colinearity class, CC_1, contains a reversible reaction that corresponds to a terminal strong colinkage set. Thus, this reaction, $\mathrm{TCR} + \mathrm{pMHC} \rightleftharpoons \mathrm{C}_0$, may be placed on an arbitrary shelf. As there are no other reactions in the colinkage set, no other reactions have to be placed on the same shelf. The reactant complexes of the other three reactions of the first colinearity class, $\mathrm{C}_0 + \mathrm{Lck} \rightleftharpoons \mathrm{C}_0.\mathrm{Lck}$ and $\mathrm{C}_0.\mathrm{Lck} \to \mathrm{C}_1 + \mathrm{Lck}$, lie in a non-terminal strong colinkage set. Thus, these reactions are placed on the middle shelf. Therefore, we can produce two different partitionings here, either putting the first two reactions on the upper or the lower shelf, or assigning them to the middle shelf as well. Placing the reactions on the upper or the lower shelf produces an inverted system, so we just have to check for solutions for one of the partitionings. We choose the upper shelf. The second colinearity class contains only a single reaction, which is irreversible and therefore has to be placed in the middle shelf of the second colinearity class. The third colinearity class consists of reversible reactions whose reactant complexes do not lie in a terminal strong colinkage set and of irreversible reactions. Thus, all reactions are placed on the middle shelf. That is, we get two possible shelvings for the first colinearity class and exactly one for the second and the third colinearity class:

Colinearity Class		Shelves
CC_1		$\mathcal{U}_1 = \emptyset$
		$\mathcal{M}_1 = \{\mathrm{TCR} + \mathrm{pMHC} \rightleftharpoons \mathrm{C}_0, \mathrm{C}_0 + \mathrm{Lck} \rightleftharpoons \mathrm{C}_0.\mathrm{Lck}, \mathrm{C}_0.\mathrm{Lck} \to \mathrm{C}_1 + \mathrm{Lck}\}$
		$\mathcal{L}_1 = \emptyset$
		$\mathcal{U}_1 = \{\mathrm{TCR} + \mathrm{pMHC} \rightleftharpoons \mathrm{C}_0\}$
	or	$\mathcal{M}_1 = \{\mathrm{C}_0 + \mathrm{Lck} \rightleftharpoons \mathrm{C}_0.\mathrm{Lck}, \mathrm{C}_0.\mathrm{Lck} \to \mathrm{C}_1 + \mathrm{Lck}\}$
		$\mathcal{L}_1 = \emptyset$
CC_2		$\mathcal{U}_2 = \emptyset$
		$\mathcal{M}_2 = \{\mathrm{C}_1 \to \mathrm{TCR} + \mathrm{pMHC}\}$
		$\mathcal{L}_2 = \emptyset$
CC_3		$\mathcal{U}_3 = \emptyset$
		$\mathcal{M}_3 = \{\mathrm{C}_1 + \mathrm{Lck} \rightleftharpoons \mathrm{C}_1.\mathrm{Lck}, \mathrm{C}_1.\mathrm{Lck} \to \mathrm{C}_2 + \mathrm{Lck}, \mathrm{C}_2 \to \mathrm{TCR} + \mathrm{pMHC}\}$
		$\mathcal{L}_3 = \emptyset$

In this step, we select a shelving by randomly choosing one for CC_1. We opt for the first option, placing all reactions on the middle shelf.

Step 10: Add Shelving Inequalities.

According to the shelving, the first equalities and inequalities for the inequality system that shall answer the question of multistationarity are constructed. Therefore, the variables M_i are introduced where M_i represents the "value" of the middle shelf of the i-th colinearity class with nonzero sign.

If the reaction $y \to y'$ is placed on the upper shelf of CC_i, then $y \cdot \mu > M_i$ is added to the inequality system. If the reaction $y \to y'$ lies on the middle shelf of CC_i, then $y \cdot \mu = M_i$ is added. If the reaction $y \to y'$ is placed on the lower shelf of CC_i, then $y \cdot \mu < M_i$ is added to the system.

For our example with the first option for the shelving of the first colinearity class where all reactions are placed on the middle shelf, we get the following equalities:

$$\begin{aligned} \mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}} = \mu_{\mathrm{C}_0} = \mu_{\mathrm{C}_0} + \mu_{\mathrm{Lck}} = \mu_{\mathrm{C}_0.\mathrm{Lck}} &= M_1 \\ \mu_{\mathrm{C}_1} &= M_2 \\ \mu_{\mathrm{C}_1} + \mu_{\mathrm{Lck}} = \mu_{\mathrm{C}_1.\mathrm{Lck}} = \mu_{\mathrm{C}_2} &= M_3. \end{aligned}$$

We note that the first equation gives us $\mu_{\mathrm{Lck}} = 0$, which leads to the equality $M_2 = M_3$.

Step 11: Add Upper and Lower Shelf Inequalities.

For the reactions in the upper and lower shelves, inequalities are added to the inequality system. To produce the inequalities for the reactions in the upper and lower shelves, only reactions in the orientation $\mathcal{O}$ are considered. Suppose that the colinearity class CC_i has a positive sign. If the reaction $y \to y' \in \mathcal{O}$ is on the upper shelf of CC_i, then $y' \cdot \mu > y \cdot \mu$ is added to the inequality system. If it is on the lower shelf of CC_i, then $y \cdot \mu > y' \cdot \mu$ is added to the inequality system. Suppose that the colinearity class CC_i has a negative sign. If the reaction $y \to y' \in \mathcal{O}$ is on the upper shelf of CC_i, then $y' \cdot \mu < y \cdot \mu$ is added to the inequality system. If it is on the lower shelf of CC_i, then $y \cdot \mu < y' \cdot \mu$ is added to the inequality system.

For the shelving we chose in the last step, all upper and lower shelves are empty. Thus, there are no inequalities to be added to the equality system constructed in the last step.

Step 12: Add Equalities for Colinearity Classes with Zero Sign.

For all colinearity classes with zero sign, equalities are added to the inequality system, since reactions in the zero colinearity classes are not partitioned into shelves and are therefore not yet incorporated in the system.

Suppose that the colinearity class CC_i has a zero sign. For each $y \to y' \in CC_i$ the equality $y \cdot \mu = y' \cdot \mu$ is added to the system.

In our example, all colinearity classes have nonzero signs, thus no equalities have to be added to the system in this step.

Step 13: Add M Inequalities and Equalities.

To complete the system, inequalities and equalities to relate the M_i's of the colinearity classes in the same coplanar set to each other, have to be added. For each coplanar set whose colinearity classes all have nonzero sign, there are three possible choices for which inequalities are added to the system. For coplanar sets that contain colinearity classes of zero sign, equalities are added to the system and there is just one possible choice of equalities.

Let CC_i, CC_j and CC_k be three colinearity classes in the same coplanar set.

(i) If all colinearity classes in the coplanar set have zero signs, then no inequalities or equalities are added.

(ii) If only one of the colinearity classes, say CC_i, has a zero sign, then $M_j = M_k$ is added to the inequality system. In other words, if among all colinearity classes in the same coplanar set, only one colinearity class has a zero sign, then all the M_i's corresponding to the rest of the colinearity classes in this coplanar set are equal.

(iii) If all three colinearity classes have nonzero sign, then there is a choice which inequalities or equalities are added to the system. Given c_i, c_j and c_k agreeing in sign with their respective colinearity classes such that $c_k w_k = c_i w_i + c_j w_j$, then either $M_i > M_k > M_j$ or $M_i = M_k = M_j$ or $M_i < M_k < M_j$ is added to the system.

For every triplet of nonzero colinearity classes in the same coplanar set, it is always possible to label the colinearity such that the equation $c_k w_k = c_i w_i + c_j w_j$ in (iii) is fulfilled for numbers c_i, c_j and c_k agreeing in sign with their respective colinearity class. This is due to rule (iii) b) in Step 8. The labeling of $c_k w_k$ is unique. If $c_k w_k$ can be written as the sum of $c_i w_i$ and $c_j w_j$, neither one of the latter vectors can be written as the sum of the other two. Therefore, case (iii) produces exactly three possible inequalities to be added to the system.

If a coplanar set contains more than three colinearity classes all having nonzero sign, then the inequalities and equalities that have to be added to the system can be found by taking three colinearity classes at a time. In the end, there are only three possible consistent choices, that is, choices that do not produce two contradictory inequalities, for example $M_i > M_j$ and $M_i < M_j$. There exists an enumeration of all colinearity classes within the coplanar set, say $CC_{n_1}, CC_{n_2}, \ldots, CC_{n_k}$, such that either $M_{n_1} > M_{n_2} > \ldots > M_{n_k}$ or $M_{n_1} = M_{n_2} = \ldots = M_{n_k}$ or $M_{n_1} < M_{n_2} < \ldots < M_{n_k}$ are the only possible consistent choices that might emerge [22, 51].

For the kinetic proofreading system with Lck and two phosphorylation steps, we know from Step 6 that all three colinearity classes lie within the same coplanar set and $w_2 + w_3 = w_1$. In Step 8 we assigned each colinearity class a positive sign. Thus, we are in case (iii) with $c_1 = c_2 = c_3 = 1$ or some positive multiple, respectively. That is, we can either add $M_3 > M_1 > M_2$ or $M_3 = M_1 = M_2$ or $M_3 < M_1 < M_2$ to the system. As we noted in Step 10, the equality $M_2 = M_3$ holds. Therefore, $M_1 = M_2 = M_3$ is the only choice that does not result in an immediate contradiction. Thus, we add the latter equation to our system, which is now complete.

Step 14: Check for Solutions to the Inequality System.

The equalities and inequalities constructed through the Steps 10 to 13 form the complete linear part of the inequality system that answers the question of multistationarity. In Step 7 is determined whether the inequality system is completely linear or not by looking at the Independence Linearity Condition and the Triplet Linearity Condition. If both linearity conditions are satisfied, the system is completely linear and no further inequalities or equalities have to be added to determine whether the network is able to support multiple steady states. In this case, we check if the inequality system produced has a solution with a nonzero μ that is sign compatible with the stoichiometric subspace S. In other words, we examine if there exist a set of M_i's and a nonzero $\mu \in \mathbb{R}^{\mathcal{S}}$ that is sign compatible with S, such that the equalities and inequalities in the system are satisfied. Such a solution is called a *signature*.

Given a reaction network following mass action kinetics, if we find a signature, then the network is able to support multiple positive steady states within a stoichiometric compatibility class, and we exit the algorithm. Otherwise, we continue with the algorithm making other choices in the steps with multiple options. If every choice was checked, and we did not find a solution for the system belonging to a mass action kinetics network, then the reaction network does not have the capacity for multiple steady states.

If the system fails to satisfy the linearity conditions in Step 7, then the system of equalities and inequalities produced in the Steps 10 to 13 is not complete yet. A solution for this incomplete system missing further nonlinear equalities is called a *pre-signature* for the reaction network. If there is no pre-signature for the system, then it cannot have a signature either, and there is no need to consider additional nonlinear equalities. In this case, the network does not have the capacity to admit multiple steady states. If a pre-signature is found, there are additional nonlinear equalities that can be added to the system, which may lead to the conclusion that the system supports multistationarity. The method is not completely decisive, however, and it is not possible to get the result that no multiple steady states are possible yet. Either the capacity for multistationarity is found or the method is inconclusive. See the works of Ellison [22] and Ji [51] for the additional nonlinear equalities that can be added to a pre-signature.

The kinetic proofreading system with Lck and two phosphorylation steps we are examining follows mass action kinetics and satisfies both linearity conditions as checked in Step 7. Thus, the system of equalities and inequalities is complete and any solution for the system is a signature. In other words, if we find numbers for M_1, M_2 and M_3 and a nonzero $\mu \in \mathbb{R}^{\mathcal{S}}$ that is sign compatible with the stoichiometric subspace, the kinetic proofreading systems supports multiple positive steady states. The complete system of equalities and inequalities we generated through the algorithm corresponds to the equations from Step 10 and the M equalities from Step 13, as there were no further inequalities to be added in the Steps 11 and 12 for this example:

$$\begin{aligned} \mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}} = \mu_{\mathrm{C}_0} = \mu_{\mathrm{C}_0} + \mu_{\mathrm{Lck}} = \mu_{\mathrm{C}_0.\mathrm{Lck}} &= M_1 \\ \mu_{\mathrm{C}_1} &= M_2 \\ \mu_{\mathrm{C}_1} + \mu_{\mathrm{Lck}} = \mu_{\mathrm{C}_1.\mathrm{Lck}} = \mu_{\mathrm{C}_2} &= M_3 \\ M_1 = M_2 = M_3 & \end{aligned}$$

which corresponds to

$$\mu_{\mathrm{Lck}} = 0 \quad \text{and} \quad \mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}} = \mu_{\mathrm{C}_0} = \mu_{\mathrm{C}_0.\mathrm{Lck}} = \mu_{\mathrm{C}_1} = \mu_{\mathrm{C}_1.\mathrm{Lck}} = \mu_{\mathrm{C}_2} = M_1 = M_2 = M_3.$$

To find a $\mu \in \mathbb{R}^{\mathcal{S}}$ that is sign compatible with the stoichiometric subspace, we have to find a vector $\sigma \in S$ and a set of positive numbers $\{p_s\}$ such that $\mu_s = p_s\sigma_s$ for every species $s \in \mathcal{S}$. The stoichiometric subspace S is given by

$$S = \operatorname{im}(L_{\mathcal{O}}) = \left\{ \begin{pmatrix} -1 \\ -1 \\ 1 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \end{pmatrix}, \begin{pmatrix} 0 \\ 0 \\ -1 \\ -1 \\ 1 \\ 0 \\ 0 \\ 0 \end{pmatrix}, \begin{pmatrix} 0 \\ 0 \\ 0 \\ 1 \\ -1 \\ 1 \\ 0 \\ 0 \end{pmatrix}, \begin{pmatrix} 0 \\ 0 \\ 0 \\ -1 \\ 0 \\ -1 \\ 1 \\ 0 \end{pmatrix}, \begin{pmatrix} 0 \\ 0 \\ 0 \\ 1 \\ 0 \\ 0 \\ -1 \\ 1 \end{pmatrix}, \right\}. \tag{3.3}$$

Thus, the question is, does there exist a set of numbers $\{a_i \in \mathbb{R}, 1 \leq i \leq 5\}$ and a set of positive numbers $\{p_s \in \mathbb{R}_+, s \in \mathcal{S}\}$ such that

$$\begin{aligned} p_{\mathrm{TCR}}(-a_1) &= \mu_{\mathrm{TCR}} \\ p_{\mathrm{pMHC}}(-a_1) &= \mu_{\mathrm{pMHC}} \\ p_{\mathrm{C_0}}(a_1 - a_2) &= \mu_{\mathrm{C_0}} \\ p_{\mathrm{Lck}}(-a_2 + a_3 - a_4 + a_5) &= \mu_{\mathrm{Lck}} \\ p_{\mathrm{C_0.Lck}}(a_2 - a_3) &= \mu_{\mathrm{C_0.Lck}} \\ p_{\mathrm{C_1}}(a_3 - a_4) &= \mu_{\mathrm{C_1}} \\ p_{\mathrm{C_1.Lck}}(a_4 - a_5) &= \mu_{\mathrm{C_1.Lck}} \\ p_{\mathrm{C_2}}(a_5) &= \mu_{\mathrm{C_2}} \end{aligned} \tag{3.4}$$

holds, and the equality system for μ is satisfied. We note that μ_{TCR} and μ_{pMHC} agree in sign. Thus, for the equality system in $\mu \in \mathbb{R}^{\mathcal{S}}$ to be fulfilled, the following equation has to hold:

$$\mathrm{sign}(-a_1) = \mathrm{sign}(a_1 - a_2) = \mathrm{sign}(a_2 - a_3) = \mathrm{sign}(a_3 - a_4) = \mathrm{sign}(a_4 - a_5) = \mathrm{sign}(a_5).$$

These signs have to be either positive or negative but cannot be zero as we are looking for a nonzero solution $\mu \in \mathbb{R}^{\mathcal{S}}$ and already identified μ_{Lck} to be zero. Suppose $\mathrm{sign}(a_5) > 0$. This implies that

$$a_1 > a_2 > a_3 > a_4 > a_5 > 0,$$

a contradiction to $\mathrm{sign}(-a_1) = \mathrm{sign}(a_5) > 0$. Supposing a_5 to be negative leads to a contradiction in an analogous way. Thus, the equality system does not have a solution that is sign compatible with the stoichiometric subspace. This means we have to proceed with the algorithm and make other choices throughout the algorithm leading to another inequality system.

Step 15: Repeat Steps 13 to 14.

> If for the inequality system produced no (pre-)signature was found, Step 13 and 14 must be repeated for every other possible choice of inequalities and equalities in Step 13. There are three choices in Step 13 for each coplanar set whose colinearity classes all have nonzero sign.

For our example, we already noticed in Step 13 that only one of the three possible choices for the coplanar set containing colinearity classes of nonzero sign can produce a system that might have a signature, whereas the other two choices lead to a contradiction. Thus, we do not repeat this step here.

Step 16: Repeat Steps 9 to 15.

> If no signature has been found yet, Steps 9 through 15 have to be repeated for all other possible partitionings of the reactions into shelves in Step 9. Similar to the Deficiency One Algorithm, the number of shelving choices that have to be checked can be reduced by skipping all the choices which are inversions. That is, skipping all shelvings that result from the switching of all upper and lower shelves from an already examined shelving, as the inversion of an inequality system will have a solution if and only if the original inequality system has a solution.

Thus, for our example there is only one more shelving option that has to be checked, the second choice for the first colinearity class in Step 9 where the upper shelf, $\mathcal{U}_1$, is nonempty. Adding the

shelving inequalities from Step 10 results in the following inequality system:

$$\begin{aligned}
\mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}} &> M_1 \\
\mu_{\mathrm{C}_0} &> M_1 \\
\mu_{\mathrm{C}_0} + \mu_{\mathrm{Lck}} = \mu_{\mathrm{C}_0.\mathrm{Lck}} &= M_1 \\
\mu_{\mathrm{C}_1} &= M_2 \\
\mu_{\mathrm{C}_1} + \mu_{\mathrm{Lck}} = \mu_{\mathrm{C}_1.\mathrm{Lck}} = \mu_{\mathrm{C}_2} &= M_3.
\end{aligned}$$

Since the first colinearity class now holds a nonempty upper shelf, we have to add an upper inequality to the system in Step 11. As the reaction $\mathrm{TCR} + \mathrm{pMHC} \to \mathrm{C}_0$ lies in the chosen orientation $\mathcal{O}$ and in the upper shelf of the first colinearity class, $\mathcal{U}_1$, the inequality

$$\mu_{\mathrm{C}_0} > \mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}}$$

has to be added to the system. Step 12 is skipped again since there are no colinearity classes with zero sign. In Step 13, adding M inequalities and equalities, the same three options $M_3 > M_1 > M_2$, $M_3 = M_1 = M_2$ or $M_3 < M_1 < M_2$ have to be added, and the resulting system checked for a solution in Step 14. Choosing the equality $M_3 = M_1 = M_2$ to start with, the following system has to be investigated for a solution:

$$\begin{array}{ccccc}
M_3 & = & M_1 & = & M_2 \\
\| & & \| & & \| \\
\mu_{\mathrm{C}_2} & & \mu_{\mathrm{C}_0.\mathrm{Lck}} & & \mu_{\mathrm{C}_1} \\
\| & & \| & & \\
\mu_{\mathrm{C}_1.\mathrm{Lck}} & & \mu_{\mathrm{C}_0} + \mu_{\mathrm{Lck}} & & \\
\| & & \wedge & & \\
\mu_{\mathrm{C}_1} + \mu_{\mathrm{Lck}} & & \mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}} & & \\
 & & \wedge & & \\
 & & \mu_{\mathrm{C}_0} & &
\end{array}$$

Now the question is again, does there exist a set of numbers $\{a_i \in \mathbb{R}, 1 \leq i \leq 5\}$ and a set of positive numbers $\{p_s \in \mathbb{R}_+, s \in \mathcal{S}\}$ such that the equalities (3.4) and the inequality system for μ are met. From $\mu_{\mathrm{C}_1} + \mu_{\mathrm{Lck}} = M_3 = M_1 = M_2 = \mu_{\mathrm{C}_1}$ it follows again that $\mu_{\mathrm{Lck}} = 0$ and

$$\operatorname{sign}(a_1 - a_2) = \operatorname{sign}(a_2 - a_3) = \operatorname{sign}(a_3 - a_4) = \operatorname{sign}(a_4 - a_5) = \operatorname{sign}(a_5).$$

Suppose the M_i's are positive, then a_5 is also positive and it follows again

$$a_1 > a_2 > a_3 > a_4 > a_5 > 0 \quad \text{and} \quad a_1 < 0,$$

a contradiction. The analogous contradiction is produced supposing the M_i's are negative. As we are looking for a nonzero solution for $\mu \in \mathbb{R}^{\mathcal{S}}$, the M_i's cannot be zero. Thus, this particular choice did not lead to a solution either. We continue with the algorithm by choosing one of the remaining inequalities for the M_i's. Picking the inequality $M_3 < M_1 < M_2$ we now get the following system:

$$\begin{array}{ccccc} M_3 & < & M_1 & < & M_2 \\ \| & & \| & & \| \\ \mu_{\mathrm{C}_2} & & \mu_{\mathrm{C}_0.\mathrm{Lck}} & & \mu_{\mathrm{C}_1} \\ \| & & \| & & \\ \mu_{\mathrm{C}_1.\mathrm{Lck}} & & \mu_{\mathrm{C}_0} + \mu_{\mathrm{Lck}} & & \\ \| & & \wedge & & \\ \mu_{\mathrm{C}_1} + \mu_{\mathrm{Lck}} & & \mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}} & & \\ & & \wedge & & \\ & & \mu_{\mathrm{C}_0} & & \end{array}$$

This time we get from $\mu_{\mathrm{C}_1} + \mu_{\mathrm{Lck}} = M_3 < M_1 < M_2 = \mu_{\mathrm{C}_1}$ that μ_{Lck} has to be negative. Thus

$$-a_2 + a_3 - a_4 + a_5 = -(a_2 - a_3) - (a_4 - a_5) < 0,$$

that is, $\mu_{\mathrm{C}_0.\mathrm{Lck}}$ or $\mu_{\mathrm{C}_1.\mathrm{Lck}}$ has to be positive. As the inequality $\mu_{\mathrm{C}_0.\mathrm{Lck}} > \mu_{\mathrm{C}_1.\mathrm{Lck}}$ holds, $\mu_{\mathrm{C}_0.\mathrm{Lck}}$ has to be positive. Consequently, M_1 and M_2 and μ_{C_0}, $\mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}}$ and μ_{C_1} have to be positive as well. Therefore, we get

$$\begin{aligned} &\operatorname{sign}(M_1) = \operatorname{sign}(M_2) = \operatorname{sign}(-a_1) = \operatorname{sign}(a_1 - a_2) = \operatorname{sign}(a_2 - a_3) = \operatorname{sign}(a_3 - a_4) > 0 \quad \text{and} \\ &\operatorname{sign}(M_3) = \operatorname{sign}(a_4 - a_5) = \operatorname{sign}(a_5). \end{aligned}$$

Choosing $M_3 > 0$ leads to the same contradiction as above:

$$0 > a_1 > a_2 > a_3 > a_4 > a_5 > 0.$$

Thus, we choose M_3 to be negative and get the following inequality:

$$0 > a_1 > a_2 > a_3 > a_4 < a_5 < 0.$$

There is no contradiction here. We set $M_3 := -1$, $M_1 := 1$ and $M_2 := 2$ and $a_i := -i$ for $1 \leq i \leq 4$ and $a_5 := -\frac{7}{2}$, then

$$\begin{aligned} p_{\mathrm{TCR}}(-a_1) = p_{\mathrm{TCR}}(+1) &= \mu_{\mathrm{TCR}} \\ p_{\mathrm{pMHC}}(-a_1) = p_{\mathrm{pMHC}}(+1) &= \mu_{\mathrm{pMHC}} \\ p_{\mathrm{C}_0}(a_1 - a_2) = p_{\mathrm{C}_0}(+1) &= \mu_{\mathrm{C}_0} \\ p_{\mathrm{Lck}}(-a_2 + a_3 - a_4 + a_5) = p_{\mathrm{Lck}}\left(-\frac{1}{2}\right) &= \mu_{\mathrm{Lck}} \\ p_{\mathrm{C}_0.\mathrm{Lck}}(a_2 - a_3) = p_{\mathrm{C}_0.\mathrm{Lck}}(+1) &= \mu_{\mathrm{C}_0.\mathrm{Lck}} \\ p_{\mathrm{C}_1}(a_3 - a_4) = p_{\mathrm{C}_1}(+1) &= \mu_{\mathrm{C}_1} \\ p_{\mathrm{C}_1.\mathrm{Lck}}(a_4 - a_5) = p_{\mathrm{C}_1.\mathrm{Lck}}\left(-\frac{1}{2}\right) &= \mu_{\mathrm{C}_1.\mathrm{Lck}} \\ p_{\mathrm{C}_2}(a_5) = p_{\mathrm{C}_2}\left(-\frac{7}{2}\right) &= \mu_{\mathrm{C}_2}. \end{aligned}$$

We choose the positive numbers p_s for $s \in \mathcal{S}$ such that there is no contradiction to the values chosen for the M_i's. We set

$$p_{\mathrm{TCR}} = p_{\mathrm{pMHC}} = 1, \quad p_{\mathrm{C}_0} = 4, \quad p_{\mathrm{Lck}} = 6, \quad p_{\mathrm{C}_0.\mathrm{Lck}} = 1, \quad p_{\mathrm{C}_1} = 2, \quad p_{\mathrm{C}_1.\mathrm{Lck}} = 2, \quad p_{\mathrm{C}_2} = \frac{2}{7}.$$

These values give us a solution for the system:

$$\begin{array}{ccccc} M_3 & & M_1 & & M_2 \\ =-1 & < & =+1 & < & =+2 \\ \| & & \| & & \| \\ \mu_{C_2} & & \mu_{C_0.Lck} & & \mu_{C_1} \\ =\frac{2}{7}\left(-\frac{7}{2}\right) & & =1(+1) & & =2(+1) \\ \| & & \| & & \\ \mu_{C_1.Lck} & & \mu_{C_0}+\mu_{Lck} & & \\ =2\left(-\frac{1}{2}\right) & & =4(+1)+6\left(-\frac{1}{2}\right) & & \\ \| & & \wedge & & \\ \mu_{C_1}+\mu_{Lck} & & \mu_{TCR}+\mu_{pMHC} & & \\ =2(+1)+6\left(-\frac{1}{2}\right) & & =1(+1)+1(+1) & & \\ & & \wedge & & \\ & & \mu_{C_0} & & \\ & & =4(+1) & & \end{array}$$

As the kinetic proofreading system with Lck and two phosphorylation steps fulfills the linearity conditions, we have not only found a pre-signature but a signature, the vector

$$\mu = \begin{pmatrix} 1 \\ 1 \\ 4 \\ -3 \\ 1 \\ 2 \\ -1 \\ -1 \end{pmatrix} \in \mathbb{R}^{\mathcal{S}} \tag{3.5}$$

that is sign compatible with the stoichiometric subspace. Thus, the kinetic proofreading system with Lck and two phosphorylation steps supports multistationarity. That is, there exist positive reaction rates such that the system admits two distinct positive steady states.

Step 17: Repeat Steps 8 to 16.

> Finally, if no pre-signature has been found so far, all other possible choices for the signs of the colinearity classes in Step 8 have to be checked. That is, for all these choices, the Steps 8 to 16 are repeated until a pre-signature is found. If the reaction network follows mass action kinetics, and after checking all possible sign choices no pre-signature is found, then the reaction network cannot support multiple steady states, no matter what positive values the rate constants take. If a pre-signature is found and the generated inequality system is not linear in μ, then a signature may exist, but it is not guaranteed. If the system follows mass action kinetics and fulfills the three linearity conditions, then every pre-signature is also a signature and the Advanced Deficiency Algorithm attests the ability of the network to support multiple positive steady states within the same stoichiometric compatibility class.

As we have already succeeded in finding a signature, we can skip the last step of the algorithm for our example. Applying the Advanced Deficiency Algorithm, we have shown that the kinetic proofreading system with Lck and two phosphorylation steps possesses positive rate constants such that the system exhibits two distinct positive steady states. The next question that arises is whether this property of multistationarity persists as we increase the number of phosphorylation steps in the kinetic proofreading mechanism. Before we will deal with this question in the next section, we

address the question of how to find specific kinetic rates under which the network displays multiple steady states once a signature has been found.

The signature obtained through the Advanced Deficiency Algorithm not only gives the information that multiple positive steady states exist but also provides some reaction rates under which the network exhibits more than one positive steady state. Moreover, it serves as a tool to assess whether the values of two different steady states can belong to a given network. The signature is given by the vector $\mu \in \mathbb{R}^{\mathcal{S}}$ that solves the inequality system produced by the algorithm. This vector, defined in equation (3.2), is constructed using the components of the logarithm of the ratio between the components of two distinct positive steady states c^* and $c^{**} \in \mathbb{R}^{\mathcal{S}}$,

$$\mu_i = \ln\left(\frac{c_i^*}{c_i^{**}}\right)$$

where $i \in \mathcal{S}$. Thus, not every ratio of stationary points is possible. Suppose c^* and c^{**} are two concentration vectors corresponding to different positive steady states. It can be determined whether they can be steady states of a certain network by examining if the vector μ is a signature of the network. For a network that follows mass action kinetics and fulfills the linearity conditions, the network supports multiple positive steady states exactly when there exists a signature for the respective inequality system produced throughout the Advanced Deficiency Algorithm. Thus, every pair of steady states has a signature associated with it. Hence, if the vector μ derived from two steady states is not a signature for the system, those steady states cannot belong to the considered network. Conversely, given a signature μ, we can deduce a pair of positive steady states in the same stoichiometric compatibility class by setting

$$c_i^* = \frac{\sigma_i e^{\mu_i}}{e^{\mu_i} - 1} \quad \text{and} \quad c_i^{**} = \frac{\sigma_i}{e^{\mu_i} - 1} \tag{3.6}$$

for a nonzero vector $\sigma \in S$ that is sign compatible with μ. As $e^{\mu_i} - 1$ and μ_i agree in sign, choosing σ sign compatible with μ, that is $\operatorname{sign}(\sigma_i) = \operatorname{sign}(\mu_i)$, produces two positive steady states. Furthermore, $c^* - c^{**}$ equals $\sigma \in S$. Thus, both steady states are stoichiometrically compatible [22]. It is possible to determine the reaction rates $\kappa_{y\to y'}$. Once we have determined the reaction rates, we get the reaction constants for mass action kinetic systems directly from the relation

$$\kappa_{y\to y'} = k_{y\to y'} e^{**y} \quad \text{where} \quad c^{**y} = \prod_{i=1}^{m} \left(c_i^{**y_i}\right).$$

Suppose now we found a signature μ for a given network and identified two positive steady states c^* and c^{**} via the equations (3.6) given above. We assign every reaction $y \to y'$ a value $c_{y\to y'}$ depending on its colinearity class vector. If the reaction $y \to y'$ belongs to the colinearity class CC_i, then by definition of the colinearity class vectors there exists a positive number $c_{y\to y'}$ such that

$$w_{y\to y'} = c_{y\to y'} w_i$$

holds. The reaction rates under which the network exhibits the above determined steady states can be found in terms of these values $c_{y\to y'}$ and certain parameters l_i that we will introduce after having given the relations for the reaction rates. In case the reaction $y \to y'$ is reversible with $y \to y'$ lying in $\mathcal{O}$ and $\mu \cdot y$ does not equal $\mu \cdot y'$, then the reaction rates for both directions can be calculated with the following equations [22]:

$$\kappa_{y\to y'} = \frac{e^{M_i} - e^{y'\cdot\mu}}{e^{y\cdot\mu} - e^{y'\cdot\mu}} c_{y\to y'} l_i \quad \text{and} \quad \kappa_{y'\to y} = \frac{e^{M_i} - e^{y\cdot\mu}}{e^{y\cdot\mu} - e^{y'\cdot\mu}} c_{y\to y'} l_i$$

where the index i indicates again the colinearity class to which the reaction belongs, $y \to y' \in CC_i$, and l_i is a parameter assigned to CC_i whose sign agrees with the sign of the colinearity class. We

will introduce these parameters below. In case that $\mu \cdot y$ equals $\mu \cdot y'$, both reaction rates can be set equal to any positive numbers that satisfy the equation:

$$\kappa_{y \to y'} - \kappa_{y' - y} = c_{y \to y'} l_i.$$

In case that the reaction $y \to y'$ is irreversible, a reaction rate is given by the equation:

$$\kappa_{y \to y'} = c_{y \to y'} l_i.$$

Every colinearity class CC_i is assigned a number l_i. Within every connected class there is a single l_i whose value is arbitrary but agreeing in sign with the sign of the colinearity class, this l_i is called a *base* l, the other l_i's assigned to the colinearity classes in the connected class will be solved in terms of this base l. An l_i is considered known if it can be expressed solely in terms of one of the base l's (and the M_i's). Base l's are known by definition [22]. The l_i's related to the colinearity classes with zero sign are set equal to zero. As base l can be chosen any l_i belonging to a nonzero colinearity class. For simplicity, we assume that the colinearity class CC_1 is chosen, thus l_1 is the base l. This constant l_1 can be chosen as an arbitrary parameter whose sign agrees with the sign of its colinearity class CC_1. To determine the other l_i's in terms of the base l, first a coplanar set satisfying certain conditions is chosen. In the coplanar set there has to be at least one l_i with nonzero sign that is known and at least one l_i that is not known. Furthermore, if the coplanar set contains a colinearity class with zero sign, then for colinearity classes CC_i and CC_j in the coplanar set, the equality $M_i = M_j$ is not in the signature. We distinguish the cases where the coplanar set chosen contains a colinearity class with zero sign and where all colinearity classes in the coplanar set chosen have nonzero signs.

We now assume that we are in the first case and there is a colinearity class CC_0 with zero sign. Within the same coplanar set there is a colinearity class CC_i with nonzero sign and known l_i and a colinearity class CC_j with unknown l_j. Since the three colinearity classes are in the same coplanar set, it is possible to find constants c_i, c_j and c_0 such that the equation

$$c_i w_i + c_j w_j + c_0 w_0 = 0$$

holds. Note that the signs of the c's will not agree in sign with the respective colinearity classes (cf. Step 8 in the Advanced Deficiency Algorithm). The unknown l_j can now be solved in terms of the known l_i by the equation

$$l_j = -\frac{c_i}{c_j} l_i.$$

Similarly, we can determine any other unknown l_k belonging to further colinearity classes CC_k in the same coplanar set in terms of the now known l_i and l_j. The equation

$$c_i' w_i + c_j' w_j + c_k' w_k = 0$$

is solved for some $c_i', c_j', c_k' \in \mathbb{R}$ and then l_k can be expressed by the equation

$$l_k = -\frac{c_i'}{c_k'} l_i.$$

Suppose now all colinearity classes in the coplanar set chosen have nonzero signs. Here we distinguish between a primary and a secondary colinearity class. The primary colinearity class CC_p is chosen as any colinearity class in the coplanar set whose l_p is known. If the l of another colinearity class is known as well, then the secondary colinearity class CC_s must be chosen such that l_s is known. If no other l of the colinearity classes in the coplanar set is known, any other colinearity class in the coplanar set can be designated the secondary colinearity class CC_s. Given the primary and

secondary classes, CC_p and CC_s, and another colinearity class CC_j from the same coplanar set whose l_j is unknown, numbers c_p, c_s and c_j can be found again such that the equation

$$c_p w_p + c_s w_s + c_j w_j = 0$$

holds. Using these c_i's, the values of l_s and l_j can be determined in terms of l_p and the M_i's by the equations [22]:

$$l_s = -\frac{c_p\left(e^{M_p} - e^{M_j}\right)}{c_s\left(e^{M_s} - e^{M_j}\right)} l_p \quad \text{and} \quad l_j = -\frac{c_p\left(e^{M_p} - e^{M_s}\right)}{c_j\left(e^{M_j} - e^{M_s}\right)} l_p.$$

To show in an example how to examine concrete steady states and reaction rates for a mass action kinetic network supporting multistationarity, we look once more at the kinetic proofreading system with Lck and two phosphorylation steps. Above, we have found in equation (3.5) the signature $\mu = (1,1,4,-3,1,2,-1,-1)^T \in \mathbb{R}^{\mathcal{S}}$ for the network and set $M_1 = 1$, $M_2 = 2$ and $M_3 = -1$. Two steady states are given by the equations for c_i^* and c_i^{**} (3.6) for any nonzero $\sigma \in S$ (3.3) that agrees in sign with μ. That means, we choose $\tilde{a}_1, \ldots, \tilde{a}_5 \in \mathbb{R}$ such that $0 > \tilde{a}_1 > \tilde{a}_2 > \tilde{a}_3 > \tilde{a}_4 < \tilde{a}_5 < 0$ and $-\tilde{a}_2 + \tilde{a}_3 - \tilde{a}_4 + \tilde{a}_5 < 0$ (cf. Step 16), and set

$$\sigma = \begin{pmatrix} -\tilde{a}_1 \\ -\tilde{a}_1 \\ \tilde{a}_1 - \tilde{a}_2 \\ -\tilde{a}_2 + \tilde{a}_3 - \tilde{a}_4 + \tilde{a}_5 \\ \tilde{a}_2 - \tilde{a}_3 \\ \tilde{a}_3 - \tilde{a}_4 \\ \tilde{a}_4 - \tilde{a}_5 \\ \tilde{a}_5 \end{pmatrix}.$$

From Step 2 and Step 4 we know that the w-vectors, $w_{y\to y'}$, coincide with their respective colinearity class vector w_i. Thus, we set $c_{y\to y'} = 1$ for all reactions. The network possesses only one coplanar set that contains exactly three colinearity classes, which have all nonzero sign. We choose l_1 to be the base l. This l_1 is now considered to be known, whereas l_2 and l_3 are unknown and have to be determined in terms of l_1. All colinearity classes have nonzero sign, therefore we assign CC_1 to be the primary colinearity class and arbitrarily choose CC_2 to be the secondary colinearity class. Next, we determine the c_i's for these three colinearity classes such that

$$c_p w_p + c_s w_s + c_3 w_3 = c_1 w_1 + c_2 w_2 + c_3 w_3 = c_1 \begin{pmatrix} 1 \\ 0 \end{pmatrix} + c_2 \begin{pmatrix} 1 \\ -1 \end{pmatrix} + c_3 \begin{pmatrix} 0 \\ 1 \end{pmatrix} = \begin{pmatrix} c_1 + c_2 \\ -c_2 + c_3 \end{pmatrix} = \begin{pmatrix} 0 \\ 0 \end{pmatrix}$$

holds. We set

$$c_p = c_1 = 1, \quad \text{and} \quad c_2 = c_s = -1 = c_3.$$

Thereby we get the following expressions for l_2 and l_3 depending on the base l, l_1:

$$l_2 = l_s = -\frac{c_p\left(e^{M_p} - e^{M_3}\right)}{c_s\left(e^{M_s} - e^{M_3}\right)} l_p = -\frac{c_1\left(e^{M_1} - e^{M_3}\right)}{c_2\left(e^{M_2} - e^{M_3}\right)} l_1 = -\frac{1}{-1}\frac{\left(e^{M_1} - e^{M_3}\right)}{\left(e^{M_2} - e^{M_3}\right)} l_1 = \frac{e^{M_1} - e^{M_3}}{e^{M_2} - e^{M_3}} l_1$$

$$l_3 = -\frac{c_p\left(e^{M_p} - e^{M_s}\right)}{c_3\left(e^{M_3} - e^{M_s}\right)} l_p = -\frac{c_1\left(e^{M_1} - e^{M_2}\right)}{c_3\left(e^{M_3} - e^{M_2}\right)} l_1 = -\frac{1}{-1}\frac{\left(e^{M_1} - e^{M_2}\right)}{\left(e^{M_3} - e^{M_2}\right)} l_1 = \frac{e^{M_1} - e^{M_2}}{e^{M_3} - e^{M_2}} l_1$$

for some $l_1 > 0$. Thus, with a given signature μ and the related values for the M_i's, specific reaction rates, $\kappa_{y\to y'}$, under which the network exhibits multiple steady states are given by

$$\kappa_{\mathrm{TCR+pMHC}\to \mathrm{C}_0} = \frac{e^{M_1} - e^{\mu_{\mathrm{C}_0}}}{e^{\mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}}} - e^{\mu_{\mathrm{C}_0}}}\, l_1$$

$$\kappa_{C_0 \to TCR+pMHC} = \frac{e^{M_1} - e^{\mu_{TCR}+\mu_{pMHC}}}{e^{\mu_{TCR}+\mu_{pMHC}} - e^{\mu_{C_0}}} l_1$$
$$\kappa_{C_0+Lck \to C_0.Lck} - \kappa_{C_0.Lck \to C_0+Lck} = l_1$$
$$\kappa_{C_0.Lck \to C_1+Lck} = l_1$$
$$\kappa_{C_1 \to TCR+pMHC} = l_2 = \frac{e^{M_1} - e^{M_3}}{e^{M_2} - e^{M_3}} l_1$$
$$\kappa_{C_1+Lck \to C_1.Lck} - \kappa_{C_1.Lck \to C_1+Lck} = l_3 = \frac{e^{M_1} - e^{M_2}}{e^{M_3} - e^{M_2}} l_1$$
$$\kappa_{C_1.Lck \to C_2+Lck} = l_3 = \frac{e^{M_1} - e^{M_2}}{e^{M_3} - e^{M_2}} l_1$$
$$\kappa_{C_2 \to TCR+pMHC} = l_3 = \frac{e^{M_1} - e^{M_2}}{e^{M_3} - e^{M_2}} l_1.$$

The reaction constants $k_{y \to y'}$ depend on the steady states. To calculate two steady states we use the equations given above in (3.6) for some $\sigma \in S$ sign compatible with μ, which can be constructed as shown above depending on five arbitrary numbers $\tilde{a}_1, \ldots, \tilde{a}_5$. Thus, there are a lot of parameters that can be chosen arbitrarily. Although it is possible to determine specific steady states and reaction rates or reaction constants once a signature is found, we are not able to determine intervals in which the values for the reaction rates must lie for the system to support multiple positive steady states. We can only provide exemplary values but cannot specify any constraints or conditions on the reaction rates. Especially, since generally there is not just a single signature for a reaction network, we selected one specific through the algorithm. Therefore, we limit the application of the Advanced Deficiency Algorithm in the subsequent sections to addressing whether a given network is generally capable of supporting multiple positive steady states without providing specific exemplary steady states.

Several toolboxes are available for analyzing chemical reaction networks, where a specific network can be entered, and the program applies Chemical Reaction Network Theory to it. One of these tools is the *Chemical Reaction Network Toolbox* [21] developed by Ellison, Ji, Knight and Feinberg, and can be downloaded from Feinberg's website. The toolbox is a closed-source software. Its application is relatively straightforward. The species and reactions, together with the information if they are reversible or not, are entered, then the program is run. The Basic Report generated by the toolbox provides the basic features of the network, that is, if the network is reversible or not, the linkage classes, the terminal and non-terminal strong linkage classes, the rank of the network, its deficiency and the deficiency of the linkage classes. If the deficiency is zero, then the report tells the consequences of the Deficiency Zero theorem. If the deficiency is one, the Deficiency One Report can be generated to obtain results from the Deficiency One theorem. For deficiencies greater than one, users can run the Higher Deficiency Report, which assesses whether the network, when using mass action kinetics, can support multiple positive steady states that are stoichiometrically compatible. However, while the report can confirm the presence of multiple steady states, it does not provide the detailed steps of the Advanced Deficiency Algorithm. If the network supports multiple steady states, the toolbox displays two exemplary positive steady states and their respective stability. For example, for the kinetic proofreading system with Lck and two phosphorylation steps, the toolbox confirms that the system exhibits multistationarity and calculates two positive steady states that are both asymptotically stable. The stability analysis applies only to the two steady states picked and does not give any general information about the stability of various steady states within the same stoichiometric compatibility class. For networks that fall outside the scope of the Deficiency Algorithms or yield inconclusive results, the toolbox offers additional tests to check specific properties that may hint at the potential for multiple steady states, including those not following mass action kinetics. However, the toolbox can only address questions regarding the support of multistationarity for a specific number of phosphorylation steps and cannot provide answers for the kinetic proofreading systems with an arbitrary number of phosphorylation steps.

Furthermore, the networks have to be relatively small to be analyzed by the toolbox. When the system gets too large or complex, the toolbox reaches its computational limits and provides the result that the tests were inconclusive. For instance, for the kinetic proofreading system with ZAP-70 and four phosphorylation steps, the result is inconclusive.

Another toolbox is *ERNEST* [96], developed in 2009 by Soranzo and Altafini. The *Reaction Network Equilibria Study Toolbox* is a Matlab package. At the time they published their toolbox, the Chemical Reaction Network Toolbox only included the Deficiency Theory. Among the deficiency theorems and the Deficiency One Algorithm, Soranzo and Altafini implemented a couple of criteria outside the Deficiency Theory that were later also included in the Chemical Reaction Network Toolbox. Applying the Matlab functions of the ERNEST toolbox to the example of the kinetic proofreading system with ZAP-70 and four phosphorylation steps gives the result that the system has the capacity for multiple steady states. The application of the toolbox is again limited to the query of multistationarity for a specific number of phosphorylation steps and cannot give a general answer for an arbitrary number of phosphorylation steps.

There are a few more toolboxes that address the potential for multiple steady states of chemical reaction networks. They comprise different criteria that mainly focus on precluding the existence of multiple steady states within the same stoichiometric compatibility class and do not include a Deficiency Algorithm. *CoNtRol* [5] is such a tool. It is an open-source web-based application by Marginean, Pantea, Banaji, Johnston and Donnell. The user only has to enter the reactions, that is, reactants and products and the information if the reaction is reversible or not. The program performs several tests, including matrix-tests related to the existence of steady states, multistationarity, and persistence, a property that will be discussed in Chapter 4. Additionally, the program calculates the second additive compound matrix, which we will introduce in Chapter 5. The application also provides a graphical output that displays the reaction network. In the case of the kinetic proofreading system with ZAP-70 and four phosphorylation steps, the results of the matrix-tests are not available, possibly due to a test timeout or misconfiguration. The python software tool *GratTeLPy* [105], Graph Theoretic Analysis of Linear Stability, developed by Walther and Hartley also takes a graph-theoretical approach to check a condition that precludes multistationarity. Another tool, in form of a maple script, was given by Feliu and Wiuf [30]. The tool is suited for moderately sized networks and focuses on determinant-based criteria that have to be fulfilled for a network to be able to support multiple steady states. Both tools are open-source and were published in 2013. However, since we want to show multistationarity and not disprove it, the examination of necessary criteria is not purposeful for us. Furthermore, the models are relatively large, which pushes the tools to their limits, and we aim at proving multistationarity for an arbitrary number of phosphorylation steps. To achieve this, we have to resort to the manual application of the Advanced Deficiency Algorithm. In the next two sections, we apply the Algorithm to the kinetic proofreading model with Lck and ZAP-70, respectively.

3.3 Multistationarity of the Kinetic Proofreading System with Lck

In this section, we apply the Advanced Deficiency Algorithm to the kinetic proofreading system with Lck and an arbitrary number of more than two phosphorylation steps to show that raising the number of phosphorylation steps does not change the ability of the system to support multiple steady states within the same stoichiometric compatibility class. The kinetic proofreading system with Lck and an arbitrary number of phosphorylation steps $N > 2$ or $N \geq 2$, respectively, is displayed in Figure 3.4.

Looking at the reaction network, we see that for one phosphorylation step the kinetic proofreading system consists of six reactions (cf. Figure 3.1), and for every additional phosphorylation step the system is expanded by four reactions (cf. Figure 3.1 and Figure 3.4). Denoting the number of

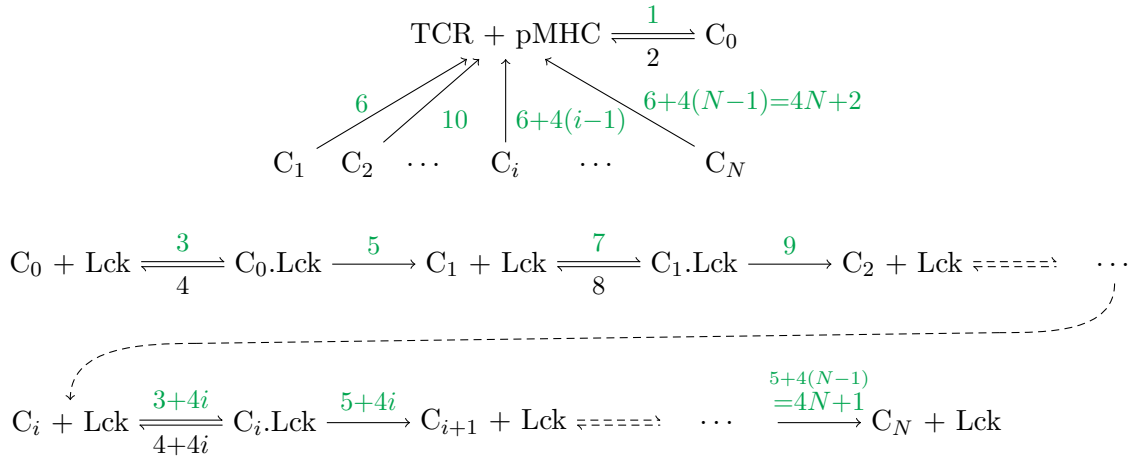


Figure 3.4: Kinetic proofreading with Lck and $N \geq 2$ phosphorylation steps. The numbering above the reaction arrows indicates an order for the reactions. The i-th reaction is assigned the reaction rate k_i. The reactions that we choose in the first step of the Advanced Deficiency Algorithm to be in orientation are colored.

reactions by r we get

$$r = 6 + 4(N-1) = 4N + 2.$$

From the four added reactions for every phosphorylation step, one pair forms a reversible reaction. Thus, the initially chosen orientation $\mathcal{O}$ consists of a number of

$$\overline{r} = 4 + 3(N-1) = 3N + 1$$

reactions. The number of species, m, and the number of complexes, $\overline{n}$, are given by

$$\begin{aligned} m &= 6 + 2(N-1) = 2N + 4, \\ \overline{n} &= 6 + 3(N-1) = 3N + 3. \end{aligned}$$

Therefore, the stoichiometric matrix, whose rows carry information about the species and whose columns represent the reactions, is a $(2N+4) \times (4N+2)$ matrix. The map $L_{\mathcal{O}}$ can be represented by the $(2N+4) \times (3N+1)$ matrix resulting from the restriction of the stoichiometric matrix to the reactions in orientation, $N|_{\mathcal{O}}$. Consequently, the following equation holds:

$$3N + 1 = \dim\left(\ker\left(L_{\mathcal{O}}\right)\right) + \dim\left(\operatorname{im}\left(L_{\mathcal{O}}\right)\right) = \dim\left(\ker\left(N|_{\mathcal{O}}\right)\right) + \operatorname{rank}\left(N|_{\mathcal{O}}\right).$$

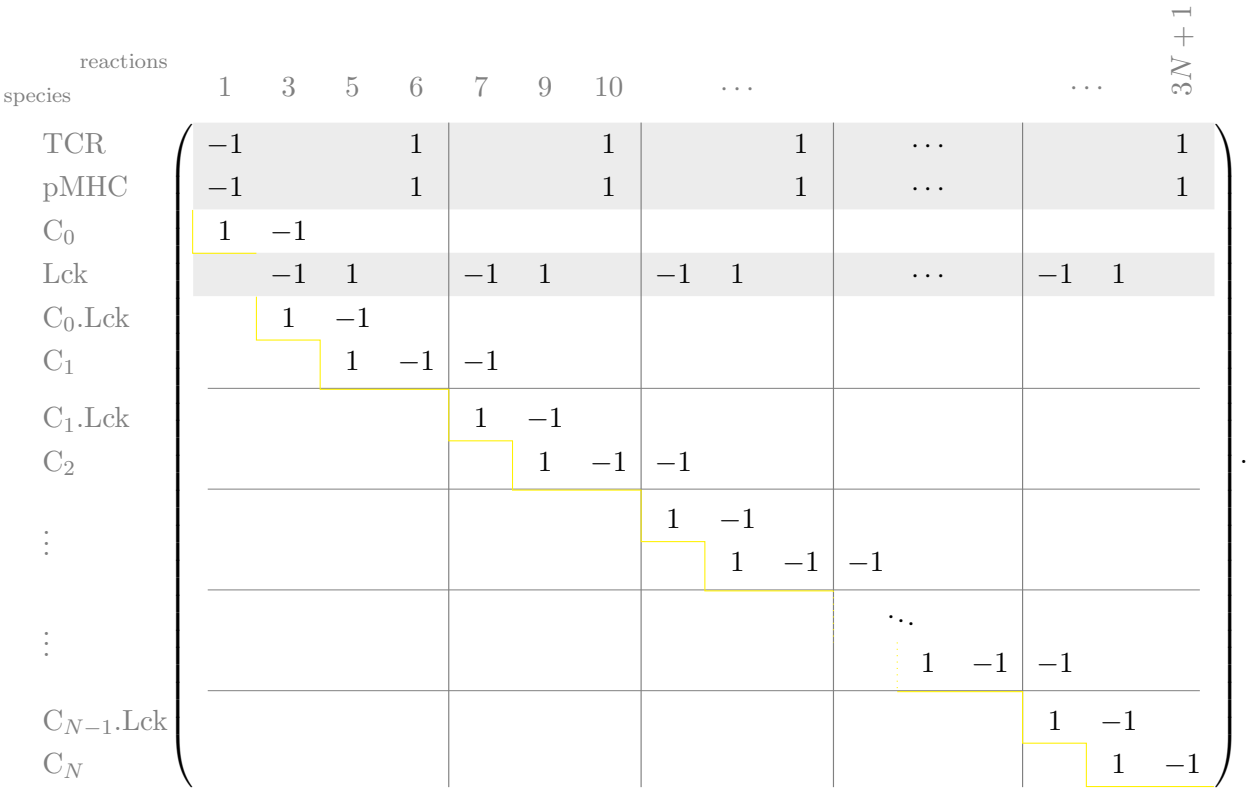
For the species we impose an ordering corresponding to the production of the species in the reaction network, that is, the ordering TCR, pMHC, C_0, Lck, C_0.Lck, C_1, C_1.Lck, C_2, …, C_{N-1}, C_{N-1}.Lck, C_N. Having gotten an overview of the network and its dimensions, we now apply the Advanced Deficiency Algorithm to the kinetic proofreading system with Lck and N phosphorylation steps, where $N > 2$.

Step 1: Choose an Initial Orientation.

We choose as orientation the reactions which are colored green in Figure 3.4. That is, for the reversible reactions, we choose the direction where two species bind to form a new complex.

Step 2: Find the Colinearity Classes.

Having chosen in Step 1 of the Advanced Deficiency Algorithm the initial orientation $\mathcal{O}$ as indicated in green in Figure 3.4, the stoichiometric matrix restricted to the reactions in orientation, $N|_{\mathcal{O}}$, is given by



Next, we determine the rank of the matrix $N|_{\mathcal{O}}$. We observe that the entries for TCR and pMHC, that is, the first and the second row, are identical. Furthermore, adding the rows corresponding to the species C_i.Lck for all $0 \leq i \leq N-1$ to the row corresponding to Lck eliminates the latter, further reducing the rank of the matrix by one. Adding the sum of the rows of all complexes C_i, $0 \leq i \leq N$, and C_i.Lck, $0 \leq i \leq N-1$, to the row of pMHC eliminates it. By eliminating the rows corresponding to TCR, pMHC, and Lck, the matrix is transformed into row-echelon form, thus

$$\operatorname{rank}\left(N|_{\mathcal{O}}\right) = m - 3 = (2N+4) - 3 = 2N+1.$$

Hence, the dimension of the kernel of $N|_{\mathcal{O}}$, which is equal to the kernel of the map $L_{\mathcal{O}}$, is given by

$$\dim\left(\ker\left(N|_{\mathcal{O}}\right)\right) = (3N+1) - (2N+1) = N.$$

Consequently, determining a basis for the kernel of $N|_{\mathcal{O}}$ yields the kernel of the mapping $L_{\mathcal{O}}$:

$$\ker\left(L_{\mathcal{O}}\right) = \operatorname{span}\left\{ \begin{pmatrix} 1\\1\\1\\1\\ \\ \\ \\ \\ \end{pmatrix}, \underbrace{\begin{pmatrix} \\ \\ -1\\1\\1\\1\\ \\ \\ \end{pmatrix}, \ldots, \begin{pmatrix} \\ \\ \\ -1\\1\\1\\1\\ \\ \end{pmatrix}, \begin{pmatrix} \\ \\ \\ \\ -1\\1\\1\\1\\ \end{pmatrix}}_{N-1 \text{ vectors}} \right\} \in \mathbb{R}^{\mathcal{O}}.$$

This provides the following w-vectors:

$$\begin{aligned}
&w_{\mathrm{TCR+pMHC}\rightleftharpoons\mathrm{C}_0} = w_{\mathrm{C}_0+\mathrm{Lck}\rightleftharpoons\mathrm{C}_0.\mathrm{Lck}} = w_{\mathrm{C}_0.\mathrm{Lck}\to\mathrm{C}_1+\mathrm{Lck}} = (1,0,\ldots,0)^T\\
&w_{\mathrm{C}_i+\mathrm{Lck}\rightleftharpoons\mathrm{C}_i.\mathrm{Lck}} = w_{\mathrm{C}_i.\mathrm{Lck}\to\mathrm{C}_{i+1}+\mathrm{Lck}} = (0,\ldots,\underset{\substack{\uparrow\\(i+1)\text{-th entry}}}{1},\ldots 0)^T \quad \text{for } 1\le i\le N-2\\
&\quad \big(\text{i.e. the } N-2 \text{ vectors: } (0,1,\ldots,0)^T,\ldots,(0,\ldots,1,0)^T\big)\\
&w_{\mathrm{C}_i\to\mathrm{TCR+pMHC}} = (0,\ldots,\underset{i}{\overset{\uparrow}{1}},\underset{i+1}{\overset{\uparrow}{-1}},\ldots,0)^T \quad \text{for } 1\le i\le N-1\\
&\quad \big(\text{i.e. the } N-1 \text{ vectors: } (1,-1,\ldots,0)^T,\ldots,(0,\ldots,1,-1)^T\big)\\
&w_{\mathrm{C}_{N-1}+\mathrm{Lck}\rightleftharpoons\mathrm{C}_{N-1}.\mathrm{Lck}} = w_{\mathrm{C}_{N-1}.\mathrm{Lck}\to\mathrm{C}_N+\mathrm{Lck}} = w_{\mathrm{C}_N\to\mathrm{TCR+pMHC}} = (0,\ldots,0,1)^T.
\end{aligned}$$

Thus, we get a number of $2N-1$ distinct w-vectors. None of these w-vectors are colinear to each other and there is no zero w-vector. Consequently, the colinearity classes are again given by the reactions having the same w-vector and there is no zero colinearity class:

$$\begin{aligned}
CC_1 &= \{\mathrm{TCR}+\mathrm{pMHC}\rightleftharpoons\mathrm{C}_0, \mathrm{C}_0+\mathrm{Lck}\rightleftharpoons\mathrm{C}_0.\mathrm{Lck}, \mathrm{C}_0.\mathrm{Lck}\to\mathrm{C}_1+\mathrm{Lck}\}\\
CC_{2i} &= \{\mathrm{C}_i\to\mathrm{TCR}+\mathrm{pMHC}\} \quad \text{for } 1\le i\le N-1\\
CC_{2i+1} &= \{\mathrm{C}_i+\mathrm{Lck}\rightleftharpoons\mathrm{C}_i.\mathrm{Lck}, \mathrm{C}_i.\mathrm{Lck}\to\mathrm{C}_{i+1}+\mathrm{Lck}\} \quad \text{for } 1\le i\le N-2\\
CC_{2N-1} &= \{\mathrm{C}_{N-1}+\mathrm{Lck}\rightleftharpoons\mathrm{C}_{N-1}.\mathrm{Lck}, \mathrm{C}_{N-1}.\mathrm{Lck}\to\mathrm{C}_N+\mathrm{Lck}, \mathrm{C}_N\to\mathrm{TCR}+\mathrm{pMHC}\}.
\end{aligned}$$

Step 3: Find the Colinkage Sets.

Colinearity Class	Colinkage Sets
CC_1	$\mathrm{TCR}+\mathrm{pMHC} \underset{2}{\overset{1}{\rightleftharpoons}} \mathrm{C}_0$
	$\mathrm{C}_0+\mathrm{Lck} \underset{4}{\overset{3}{\rightleftharpoons}} \mathrm{C}_0.\mathrm{Lck} \xrightarrow{5} \mathrm{C}_1+\mathrm{Lck}$

CC_{2i} $1 \leq i \leq N-1$	$\mathrm{C}_i \xrightarrow{4i+2} \mathrm{TCR} + \mathrm{pMHC}$
CC_{2i+1} $1 \leq i \leq N-2$	$\mathrm{C}_i + \mathrm{Lck} \underset{4i}{\overset{4i-1}{\rightleftharpoons}} \mathrm{C}_i.\mathrm{Lck} \xrightarrow{4i+1} \mathrm{C}_{i+1} + \mathrm{Lck}$
CC_{2N-1}	$\mathrm{C}_{N-1} + \mathrm{Lck} \underset{4N-1}{\overset{4N}{\rightleftharpoons}} \mathrm{C}_{N-1}.\mathrm{Lck} \xrightarrow{4N+1} \mathrm{C}_N + \mathrm{Lck}$ $\mathrm{C}_N \xrightarrow{4N+2} \mathrm{TCR} + \mathrm{pMHC}$

The terminal strong colinkage sets are shaded gray. Again the reactions $\mathrm{TCR} + \mathrm{pMHC} \rightleftharpoons \mathrm{C}_0$ form the only nontrivial terminal strong colinkage set.

Step 4: Choose Colinearity Class Vectors.

Since all colinearity classes are irreversible, we can pick for every colinearity class any positive multiple of a w-vector associated to an irreversible reaction within the same colinearity class. We simply keep the w-vectors. Thus, the colinearity class vectors are given by

$$w_{2i-1} = (0, \ldots, \underset{\uparrow}{1}_{i}, \ldots, 0)^T \quad \text{for } 1 \leq i \leq N \quad \rightarrow \quad \text{colinearity class vector for } CC_{2i-1}$$

$$w_{2i} = (0, \ldots, \underset{\uparrow}{1}_{i}, \underset{\uparrow}{-1}_{i+1}, \ldots, 0)^T \quad \text{for } 1 \leq i \leq N-1 \quad \rightarrow \quad \text{colinearity class vector for } CC_{2i}.$$

Step 5: Realign the Orientation.

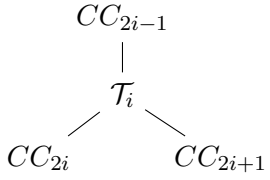
The chosen w-vectors coincide with the colinearity class vectors. Therefore, a realigning of the orientation is not necessary.

Step 6: Find Coplanar Sets and Connected Classes.

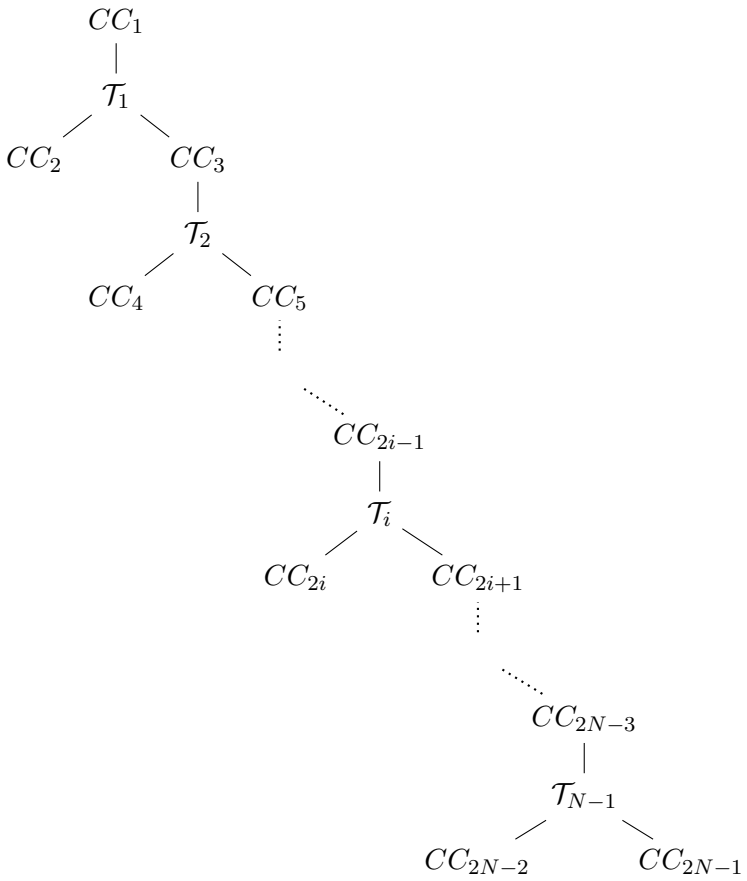
We find that exactly three colinearity class vectors each lie in the same two-dimensional subspace as

$$w_{2i} + w_{2i+1} = w_{2i-1}$$

holds for all $1 \leq i \leq N-1$. Thus, we get a number of $(N-1)$ coplanar sets, $\mathcal{T}_1, \ldots, \mathcal{T}_{N-1}$:



We observe that all colinearity classes with odd index belong to two coplanar sets, whereas the colinearity classes with even index only lie in one coplanar set. This results in a single connected class as the coplanar sets are linked via the colinkage classes with odd index. Thus, the connecting graph is given by:



Step 7: Determine Linearity.

We check whether the equalities and inequalities produced are linear in $\mu \in \mathbb{R}^{\mathcal{S}}$ and thereby form a complete system for the network by checking the two linearity conditions. The Independence Linearity Condition is fulfilled if the number of coplanar sets plus the number of connected classes is equal to the dimension of the kernel of the mapping $L_{\mathcal{O}}$. In our network we have $N-1$ coplanar sets and one connected class. Thus, the sum is equal to N, which corresponds to the dimension of the kernel of $L_{\mathcal{O}}$. Consequently, the Independence Linearity Condition is satisfied. Each coplanar set contains exactly three colinearity classes. Therefore, the Triplet Linearity Condition holds as well. With both linearity conditions being satisfied, the system of equalities and inequalities produced is complete, and we will get a definite answer whether the network is able to support multiple steady states within the same stoichiometric compatibility class or not.

Step 8: Choose Signs for Colinearity Classes.

Since all colinearity classes are irreversible, each of them is assigned a positive sign.

Step 9: Choose Shelving for Reactions.

All irreversible reactions are put on the middle shelf, as are reactions whose reactant complex lies in a non-terminal strong colinkage set. Only reversible reactions whose reactant complex lies in a terminal strong colinkage set can be placed on an arbitrary shelf as long as all reactions belonging to the same colinkage set are put on the same shelf. We already noticed in Step 3 that the reaction $\mathrm{TCR} + \mathrm{pMHC} \rightleftharpoons \mathrm{C}_0$ forms the only nontrivial terminal strong colinkage set. Thus, it is the sole reversible reaction whose reactant complex lies in a terminal strong colinkage set. Consequently, it can be placed on an arbitrary shelf. Since no other reactions share the same

colinkage set, its shelving choice does not affect other reactions. Therefore, the only colinkage class with multiple shelving possibilities is CC_1. With the shelving options for $N = 2$ in mind, we now choose for $N > 2$ the same shelving option that was successful in the former case, putting the reaction $\mathrm{TCR} + \mathrm{pMHC} \rightleftharpoons \mathrm{C}_0$ in the upper shelf of the first colinearity class. Hence, the shelving we are examining in the following steps is represented by:

Colinearity Class	Shelves
CC_1	$\mathcal{U}_1 = \{\mathrm{TCR} + \mathrm{pMHC} \rightleftharpoons \mathrm{C}_0\}$
	$\mathcal{M}_1 = \{\mathrm{C}_0 + \mathrm{Lck} \rightleftharpoons \mathrm{C}_0.\mathrm{Lck} \to \mathrm{C}_1 + \mathrm{Lck}\}$
	$\mathcal{L}_1 = \emptyset$
CC_{2i}	$\mathcal{U}_{2i} = \emptyset = \mathcal{L}_{2i}$
$1 \leq i \leq N-1$	$\mathcal{M}_{2i} = \{\mathrm{C}_i \to \mathrm{TCR} + \mathrm{pMHC}\}$
CC_{2i+1}	$\mathcal{U}_{2i+1} = \emptyset = \mathcal{L}_{2i+1}$
$1 \leq i \leq N-2$	$\mathcal{M}_{2i+1} = \{\mathrm{C}_i + \mathrm{Lck} \rightleftharpoons \mathrm{C}_i.\mathrm{Lck} \to \mathrm{C}_{i+1} + \mathrm{Lck}\}$
CC_{2N-1}	$\mathcal{U}_{2N-1} = \emptyset = \mathcal{L}_{2N-1}$
	$\mathcal{M}_{2N-1} = \{\mathrm{C}_{N-1} + \mathrm{Lck} \rightleftharpoons \mathrm{C}_{N-1}.\mathrm{Lck} \to \mathrm{C}_N + \mathrm{Lck},$
	$\mathrm{C}_N \to \mathrm{TCR} + \mathrm{pMHC}\}$.

Step 10: Add Shelving Inequalities.

According to the shelving just chosen, we construct the first inequalities and equalities for the system:

$$\begin{aligned}
\mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}} &> M_1 \\
\mu_{\mathrm{C}_0} &> M_1 \\
\mu_{\mathrm{C}_0} + \mu_{\mathrm{Lck}} = \mu_{\mathrm{C}_0.\mathrm{Lck}} &= M_1 \\
\mu_{\mathrm{C}_i} &= M_{2i} && \text{for } 1 \leq i \leq N-1 \\
\mu_{\mathrm{C}_i} + \mu_{\mathrm{Lck}} = \mu_{\mathrm{C}_i.\mathrm{Lck}} &= M_{2i+1} && \text{for } 1 \leq i \leq N-2 \\
\mu_{\mathrm{C}_{N-1}} + \mu_{\mathrm{Lck}} = \mu_{\mathrm{C}_{N-1}.\mathrm{Lck}} = \mu_{\mathrm{C}_N} &= M_{2N-1}.
\end{aligned}$$

Step 11: Add Upper and Lower Shelf Inequalities.

We add the upper shelf inequality for the shelf $\mathcal{U}_1$. Since the reaction $\mathrm{TCR} + \mathrm{pMHC} \to \mathrm{C}_0$ lies in $\mathcal{O}$, the following inequality has to be added to the system:

$$\mu_{\mathrm{C}_0} > \mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}}.$$

Step 12: Add Equalities for Colinearity Classes with Zero Sign.

There are no colinearity classes with zero sign. Thus, we can skip this step.

Step 13: Add M Inequalities and Equalities.

Having assigned a positive sign to each colinearity class, the equalities formed by the colinearity class vectors lying in the same two-dimensional subspace, $w_{2i} + w_{2i+1} = w_{2i-1}$ for all $1 \leq i \leq N-1$,

give the possible relations for the M_i's of each colinearity class. We can add one of the following three (in)equalities to the system:

$$M_{2i} > M_{2i-1} > M_{2i+1}$$
$$M_{2i} = M_{2i-1} = M_{2i+1}$$
$$M_{2i} < M_{2i-1} < M_{2i+1}.$$

Again, we choose the first inequalities.

Step 14: Check for Solutions for the Inequality System.

We seek a vector $\mu \in \mathbb{R}^{\mathcal{S}}$ that is sign compatible with the stoichiometric subspace and satisfies the system of equalities and inequalities produced, called a pre-signature. Since we determined the system to be linear, every pre-signature is also a signature. As our network follows mass action kinetics, we can conclude from finding a signature that the network has the capacity for multiple positive steady states. Conversely, if none of the equality and inequality systems has a solution as we proceed with the algorithm, the network cannot support multiple positive steady states. The stoichiometric subspace S is generated by the span of the linearly independent column vectors of the stoichiometric matrix N, thus

$$S = \operatorname{im}(L_{\mathcal{O}})$$

$$= \operatorname{span}\left\{ \begin{pmatrix} -1 \\ -1 \\ 1 \\ \hline \\ \\ \\ \end{pmatrix}, \begin{pmatrix} \\ -1 \\ -1 \\ 1 \\ \hline \\ \\ \\ \end{pmatrix}, \begin{pmatrix} \\ 1 \\ -1 \\ 1 \\ \hline \\ \\ \\ \end{pmatrix}, \begin{pmatrix} -1 \\ \\ -1 \\ 1 \\ \hline \\ \\ \end{pmatrix}, \begin{pmatrix} 1 \\ \\ \\ -1 \\ 1 \\ \hline \\ \end{pmatrix}, \ldots, \begin{pmatrix} -1 \\ \\ \\ \\ -1 \\ 1 \\ \hline \end{pmatrix}, \begin{pmatrix} -1 \\ \\ \\ \\ \\ -1 \\ 1 \end{pmatrix}, \ldots, \begin{pmatrix} -1 \\ \\ \\ \\ \\ -1 \\ 1 \end{pmatrix}, \begin{pmatrix} -1 \\ \\ \\ \\ \\ \\ -1 \end{pmatrix} \right\}$$

$$= \operatorname{span}\left\{ \begin{pmatrix} -1 \\ -1 \\ 1 \\ \hline \\ \\ \\ \end{pmatrix}, \begin{pmatrix} \\ -1 \\ -1 \\ 1 \\ \hline \\ \\ \\ \end{pmatrix}, \begin{pmatrix} \\ 1 \\ -1 \\ 1 \\ \hline \\ \\ \\ \end{pmatrix}, \underbrace{\begin{pmatrix} -1 \\ \\ \\ \\ \hline \vdots \\ \\ \\ -1 \\ 1 \\ \hline \vdots \end{pmatrix}, \begin{pmatrix} 1 \\ \\ \\ \\ \hline \vdots \\ \\ \\ -1 \\ 1 \\ \hline \vdots \end{pmatrix}}_{2 \le i \le N} \right\} \begin{matrix} \text{TCR} \\ \text{pMHC} \\ \text{C}_0 \\ \text{Lck} \\ \text{C}_0\text{.Lck} \\ \text{C}_1 \\ \\ \\ \\ \\ \text{C}_{i-1}\text{.Lck} \\ \text{C}_i \\ \\ \text{C}_N \end{matrix}.$$

Hence, the stoichiometric subspace is spanned by a total of $2N + 1$ linearly independent vectors. The vector $\mu \in \mathbb{R}^{\mathcal{S}}$ is sign compatible with the stoichiometric subspace S if there exist a vector

$\sigma \in S$ and a set of positive numbers $\{p_s\}$ such that $\mu_s = p_s\sigma_s$ for every species $s \in \mathcal{S}$. Thus, we are looking for a set of real numbers $\{a_1, a_2, \dots a_{2N+1}\}$ and a set of positive numbers $\{p_s : s \in \mathcal{S}\}$ such that

$$\begin{aligned}
p_{\mathrm{TCR}}\,(-a_1) &= \mu_{\mathrm{TCR}} \\
p_{\mathrm{pMHC}}(-a_1) &= \mu_{\mathrm{pMHC}} \\
p_{\mathrm{Lck}} \sum_{i=1}^{N} (-a_{2i} + a_{2i+1}) &= \mu_{\mathrm{Lck}} \\
p_{\mathrm{C}_i}\left(a_{2i+1} - a_{2(i+1)}\right) &= \mu_{\mathrm{C}_i} \qquad 0 \le i \le N-1 \\
p_{\mathrm{C}_i.\mathrm{Lck}}\left(a_{2(i+1)} - a_{2(i+1)+1}\right) &= \mu_{\mathrm{C}_i.\mathrm{Lck}} \qquad 0 \le i \le N-1 \\
p_{\mathrm{C}_N} a_{2N+1} &= \mu_{\mathrm{C}_N}.
\end{aligned}$$

The system of equalities and inequalities that we produced throughout the steps of the algorithm is given by

$$\begin{aligned}
&\mu_{\mathrm{C}_0} > \mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}} > M_1 = \mu_{\mathrm{C}_0} + \mu_{\mathrm{Lck}} = \mu_{\mathrm{C}_0.\mathrm{Lck}} \\
&\qquad\qquad M_{2i} = \mu_{\mathrm{C}_i} && 1 \le i \le N-1 \\
&\qquad\qquad M_{2i+1} = \mu_{\mathrm{C}_i} + \mu_{\mathrm{Lck}} = \mu_{\mathrm{C}_i.\mathrm{Lck}} && 1 \le i \le N-2 \\
&\qquad\qquad M_{2N-1} = \mu_{\mathrm{C}_{N-1}} + \mu_{\mathrm{Lck}} = \mu_{\mathrm{C}_{N-1}.\mathrm{Lck}} = \mu_{\mathrm{C}_N} \\
&\text{and} \qquad M_{2i+1} < M_{2i-1} < M_{2i} && 1 \le i \le N-1.
\end{aligned}$$

Summarizing this in one graphic, we have to find a solution for the following system:

$$\begin{array}{ccccccccccc}
 & & \mu_{\mathrm{C}_{N-1}} & & & & \mu_{\mathrm{C}_i} & & & & \mu_{\mathrm{C}_1} \\
 & & \| & & & & \| & & & & \| \\
 & & M_{2N-2} & & & & \vdots & & M_{2i} & & M_2 \\
 & & \vee & & & & \vee & & \vee & & \vee \\
M_{2N-1} & < & M_{2N-3} & < & \cdots & < & M_{2i+1} & < & M_{2i-1} & < \cdots < & M_1 \\
\| & & \| & & & & \| & & \| & & \| \\
\mu_{\mathrm{C}_{N-1}} + \mu_{\mathrm{Lck}} & & \mu_{\mathrm{C}_{N-2}} + \mu_{\mathrm{Lck}} & & & & \mu_{\mathrm{C}_i} + \mu_{\mathrm{Lck}} & & \mu_{\mathrm{C}_{i-1}} + \mu_{\mathrm{Lck}} & & \mu_{\mathrm{C}_0} + \mu_{\mathrm{Lck}} \\
\| & & \| & & & & \| & & \| & & \| \\
\mu_{\mathrm{C}_{N-1}.\mathrm{Lck}} & & \mu_{\mathrm{C}_{N-2}.\mathrm{Lck}} & & & & \mu_{\mathrm{C}_i.\mathrm{Lck}} & & \mu_{\mathrm{C}_{i-1}.\mathrm{Lck}} & & \mu_{\mathrm{C}_0.\mathrm{Lck}} \\
\| & & & & & & & & & & \wedge \\
\mu_{\mathrm{C}_N} & & & & & & & & & & \mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}} \\
 & & & & & & & & & & \wedge \\
 & & & & & & & & & & \mu_{\mathrm{C}_0}
\end{array}$$

Again, μ_{Lck} has to be negative. We choose M_{2N-1} to be negative and the other M_i to be positive. Specifically, we start with assigning a value to the M_i's with odd index and set

$$\begin{aligned}
M_{2N-1} &:= -1 \\
M_{2N-1-2i} &:= i, \qquad 1 \le i \le N-1.
\end{aligned}$$

That is, apart from M_{2N-1} we assign the M_i's with odd index the following values:

$$M_{2i-1} = M_{2N-1-2(N-i)} = N - i, \qquad 1 \le i \le N-1.$$

Knowing that $\mu_{\mathrm{Lck}} = \sum_{i=1}^{N} (-a_{2i} + a_{2i+1})$ has to be negative, but all μ_{C_i} and $\mu_{\mathrm{C}_i.\mathrm{Lck}}$ except for

μ_{C_N} and $\mu_{\mathrm{C}_{N-1}.\mathrm{Lck}}$ are positive, we set

$$a_j := -j, \qquad 1 \le j \le 2N$$
$$a_{2N+1} := -\left(2N - \frac{1}{2}\right).$$

This choice leads to the desired signs for the μ_s's:

- $-a_1 = -(-1) = +1 \quad \Rightarrow \quad \mu_{\mathrm{TCR/pMHC}} = p_{\mathrm{TCR/pMHC}}(+1) > 0$
- $$\sum_{i=1}^{N}(-a_{2i} + a_{2i+1}) = \sum_{i=1}^{N-1}(-a_{2i} + a_{2i+1}) + (-a_{2N} + a_{2N+1})$$
  $$= \sum_{i=1}^{N-1}(2i - (2i+1)) + \left(2N - \left(2N - \frac{1}{2}\right)\right) = \sum_{i=1}^{N-1}(-1) + \frac{1}{2} = -(N-1) + \frac{1}{2}$$
  $$\Rightarrow \quad \mu_{\mathrm{Lck}} = p_{\mathrm{Lck}}\left(-(N-1) + \frac{1}{2}\right) < 0$$
- $0 \le i \le N-1: \quad a_{2i+1} - a_{2(i+1)} = -(2i+1) + 2(i+1) = +1 > 0$

  $$\Rightarrow \quad \mu_{\mathrm{C}_i} = p_{\mathrm{C}_i}(+1) > 0 \quad \Rightarrow \quad \mu_{\mathrm{C}_0}, \ldots, \mu_{\mathrm{C}_{N-1}} > 0$$
- $a_{2N+1} = -\left(2N - \frac{1}{2}\right) < 0 \quad \Rightarrow \quad \mu_{\mathrm{C}_N} = p_{\mathrm{C}_N}\left(-\left(2N - \frac{1}{2}\right)\right) < 0$
- $0 \le i \le N-2: \quad a_{2(i+1)} - a_{2(i+1)+1} = -2(i+1) + 2(i+1) + 1 = +1 > 0$

  $$\Rightarrow \quad \mu_{\mathrm{C}_i.\mathrm{Lck}} = p_{\mathrm{C}_i.\mathrm{Lck}}(+1) > 0 \quad \Rightarrow \quad \mu_{\mathrm{C}_0.\mathrm{Lck}}, \ldots, \mu_{\mathrm{C}_{N-2}.\mathrm{Lck}} > 0$$
- $a_{2N} - a_{2N+1} = -2N + \left(2N - \frac{1}{2}\right) = -\frac{1}{2} < 0 \quad \Rightarrow \quad \mu_{\mathrm{C}_{N-1}.\mathrm{Lck}} = p_{\mathrm{C}_{N-1}.\mathrm{Lck}}\left(-\frac{1}{2}\right) < 0.$

With our choice for the a_j ,$1 \le j \le 2N+1$, the resulting signs for the μ_s's, $s \in \mathcal{S}$, coincide with the signs for the chosen M_i's, $1 \le i \le 2N-1$. In the next step, we determine the values for the positive numbers p_s belonging to the species that have to satisfy an equality with an M_i with odd index.

- $M_{2N-1} := -1$

  $$\mu_{\mathrm{C}_N} = p_{\mathrm{C}_N}\left(-\left(2N - \frac{1}{2}\right)\right) = -1 \quad \Leftrightarrow \quad p_{\mathrm{C}_N} = \frac{1}{2N - \frac{1}{2}} = \frac{2}{4N-1}$$
  $$\mu_{\mathrm{C}_{N-1}.\mathrm{Lck}} = p_{\mathrm{C}_{N-1}.\mathrm{Lck}}\left(-\frac{1}{2}\right) = -1 \quad \Leftrightarrow \quad p_{\mathrm{C}_{N-1}.\mathrm{Lck}} = 2$$
  $$\mu_{\mathrm{C}_{N-1}} + \mu_{\mathrm{Lck}} = p_{\mathrm{C}_{N-1}}(+1) + p_{\mathrm{Lck}}\left(-(N-1) + \frac{1}{2}\right) = -1$$

Setting $p_{\mathrm{Lck}} := 2$, we obtain:

$$\mu_{\mathrm{Lck}} = 2\left(-(N-1) + \frac{1}{2}\right) = -2N + 3$$
$$\mu_{\mathrm{C}_{N-1}} + \mu_{\mathrm{Lck}} = p_{\mathrm{C}_{N-1}}(+1) - 2N + 3 = -1 \quad \Leftrightarrow \quad p_{\mathrm{C}_{N-1}} = 2N - 4 = 2(N-2)$$

- $M_{2i-1} := N - i, \quad 1 \le i \le N-1$

  $$\mu_{\mathrm{C}_{i-1}.\mathrm{Lck}} = p_{\mathrm{C}_{i-1}.\mathrm{Lck}}(+1) = N - i \quad \Leftrightarrow \quad p_{\mathrm{C}_{i-1}.\mathrm{Lck}} = N - i$$
  $$\mu_{\mathrm{C}_{i-1}} + \mu_{\mathrm{Lck}} = p_{\mathrm{C}_{i-1}}(+1) - 2N + 3 = N - i \quad \Leftrightarrow \quad p_{\mathrm{C}_{i-1}} = 3N - 3 - i = 3(N-1) - i$$

This gives us the values for the M_i's with even index:

- $M_{2i} = \mu_{\mathrm{C}_i} = p_{\mathrm{C}_i}(+1) = 3(N-1) - (i+1) = 3N - i - 4 \quad \text{for } 1 \leq i \leq N-2$
- $M_{2(N-1)} = \mu_{\mathrm{C}_{N-1}} = 2N - 4$

We still have to assign a value to p_{TCR} and p_{pMHC} such that the (in)equalities hold:

- $M_1 = N - 1 < \mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}} = p_{\mathrm{TCR}}(+1) + p_{\mathrm{pMHC}}(+1) < \mu_{\mathrm{C}_0} = 3N - 4$

We set $p_{\mathrm{TCR}} := N - 1$ and $p_{\mathrm{pMHC}} := \frac{1}{2}$.

An overview over the values we assigned to the $\mu_s = p_s\sigma_s$ for $s \in \mathcal{S}$ and $\sigma_s \in S$ is given in the following table:

	μ_s	p_s	σ_s	value	sign
	μ_{TCR}	$N-1$	$+1$	$N-1$	>0
	μ_{pMHC}	$\frac{1}{2}$	$+1$	$\frac{1}{2}$	>0
	μ_{Lck}	2	$-(N-1)+\frac{1}{2}$	$-2N+3$	<0
$1 \leq i \leq N-1$	$\mu_{\mathrm{C}_{i-1}}$	$3(N-1)-i$	$+1$	$3(N-1)-i$	>0
	$\mu_{\mathrm{C}_{N-1}}$	$2N-4$	$+1$	$2N-4$	>0
	μ_{C_N}	$\frac{2}{4N-1}$	$-2N+\frac{1}{2}$	-1	<0
$1 \leq i \leq N-1$	$\mu_{\mathrm{C}_{i-1}.\mathrm{Lck}}$	$N-i$	$+1$	$N-i$	>0
	$\mu_{\mathrm{C}_{N-1}.\mathrm{Lck}}$	2	$-\frac{1}{2}$	-1	<0

It remains to verify that the chosen values really provide a solution $\mu \in \mathbb{R}^{\mathcal{S}}$ for the system:

$$
\begin{array}{ccccccccccc}
 & & \mu_{\mathrm{C}_{N-1}} & & & & \mu_{\mathrm{C}_i} & & & & \mu_{\mathrm{C}_1} \\
 & & =2N-4 & & & & =3(N-1)-(i+1) & & & & =3(N-1)-2 \\
 & & \| & & & & \| & & & & \| \\
 & & \boxed{2N-4} & & & & \boxed{3N-i-4} & & & & \boxed{3N-5} \\
 & & M_{2N-2} & & & & M_{2i} & & & & M_2 \\
 & & \vee & & & & \vee & & & & \vee \\
\boxed{-1} & < & \boxed{+1} & < \cdots < & \boxed{N-1-i} & < & \boxed{N-i} & < \cdots < & \boxed{N-1} \\
M_{2N-1} & & M_{2N-3} & & M_{2i+1} & & M_{2i-1} & & M_1 \\
\| & & \| & & \| & & \| & & \| \\
\mu_{\mathrm{C}_{N-1}} + \mu_{\mathrm{Lck}} & & \vdots & & \mu_{\mathrm{C}_i} + \mu_{\mathrm{Lck}} & & \mu_{\mathrm{C}_{i-1}} + \mu_{\mathrm{Lck}} & & \mu_{\mathrm{C}_0} + \mu_{\mathrm{Lck}} \\
=2N-4-2N+3 & & & & =3(N-1)-(i+1) & & =3(N-1)-i & & =3(N-1)-1 \\
=-1 & & & & -2N+3 & & -2N+3 & & -2N+3 \\
 & & & & =N-1-i & & =N-i & & =N-1 \\
\| & & & & \| & & \| & & \| \\
\mu_{\mathrm{C}_{N-1}.\mathrm{Lck}} & & & & \mu_{\mathrm{C}_i.\mathrm{Lck}} & & \mu_{\mathrm{C}_{i-1}.\mathrm{Lck}} & & \mu_{\mathrm{C}_0.\mathrm{Lck}} \\
=-1 & & & & =N-1-i & & =N-i & & =N-1 \\
\| & & & & & & & & \wedge \\
\mu_{\mathrm{C}_N} & & & & & & & & \mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}} \\
=-1 & & & & & & & & =N-1+\frac{1}{2} \\
 & & & & & & & & \wedge \\
 & & & & & & & & \mu_{\mathrm{C}_0} \\
 & & & & & & & & =3(N-1)-1 \\
 & & & & & & & & =3N-4
\end{array}
$$

We see that with the choices made throughout the algorithm, we have found a solution vector $\mu \in \mathbb{R}^{\mathcal{S}}$ that is sign compatible with the stoichiometric subspace and solves the system of equalities and inequalities produced. Thus, we have found a pre-signature. As the system fulfills the linearity conditions, every pre-signature is also a signature. Consequently, we have shown that the kinetic proofreading system with Lck and an arbitrary number of phosphorylation steps $N > 2$ supports multistationarity. That is, there exist positive reaction rates and initial concentrations such that the network exhibits at least two distinct positive steady states. Furthermore, we have shown the same result for $N = 2$ in the last section. Thus, we identified the kinetic proofreading system with Lck and at least two phosphorylation steps to be capable of exhibiting multiple positive steady states.

Theorem 3. *The kinetic proofreading system with Lck (2.2) and at least two phosphorylation steps, $N \geq 2$, supports multistationarity within the same positive stoichiometric compatibility class. That is, there exist positive reaction constants and initial concentrations such that the system exhibits more than one positive steady state.*

3.4 Multistationarity of the Kinetic Proofreading System with Lck and ZAP-70

In this section, we apply the Advanced Deficiency Algorithm to the kinetic proofreading system with Lck and ZAP-70. Hence, the model involves an even number of phosphorylation steps, $N = 2n$, $n \geq 1$. We show that the kinetic proofreading system with Lck and ZAP-70 supports multiple positive steady states within the same stoichiometric compatibility class for an arbitrary $n \in \mathbb{N}$, that is, for an arbitrary but even number of phosphorylation steps N.

Before starting with the Advanced Deficiency Algorithm, we identify the number of species, complexes, reactions and linkage classes to get an overview of the system (cf. Figure 2.9 and 3.5). The set of species for the kinetic proofreading system with Lck and ZAP-70 is given by

$$\begin{aligned}\mathcal{S} = \big\{&\text{TCR, pMHC, Lck, ZAP,}\\ &\text{C}_0\text{, C}_0\text{.Lck, C}_1\text{, C}_1\text{.Lck, C}_2\text{,}\\ &\text{C}_{2i}.i\text{ZAP, TCR}.i\text{ZAP, C}_{2i}.i\text{ZAP.Lck, C}_{2i+1}.i\text{ZAP, C}_{2i+1}.i\text{ZAP.Lck, C}_{2(i+1)}.i\text{ZAP,}\\ &\quad \text{where } 1 \leq i \leq n-1,\\ &\text{C}_{2n}.n\text{ZAP, TCR}.n\text{ZAP,}\\ &\text{C}_{2n}.n\text{ZAP}_i\text{.Lck, C}_{2n}.n\text{ZAP}_{i+1}, \quad \text{where } 0 \leq i \leq n-1\big\}.\end{aligned}$$

Here we listed the species in the order of their appearance in the reactions, and we impose this ordering for the species. Consequently, the model involves a number of

$$m = 4 + 5 + 6(n-1) + 2 + 2n = 8n + 5$$

species. We take the number of complexes, denoted by $\overline{n}$, reactions, denoted by r, reactions in orientation, denoted by $\overline{r}$, and linkage classes, denoted by l, from Figure 3.5:

$$\begin{aligned}\overline{n} &= 4 + 5n + 5(n-1) + (3+n) + (2n+1) + 2n = 15n + 3\\ r &= 4 + 6n + 6(n-1) + (4+n) + 3n + n = 17n + 2\\ \overline{r} &= 3 + 4n + 4(n-1) + (2+n) + 2n + n = 12n + 1\\ l &= 1 + n + (n-1) + 1 + 1 + n = 3n + 2.\end{aligned}$$

We now apply the Advanced Deficiency Algorithm to the kinetic proofreading system with Lck and ZAP-70.

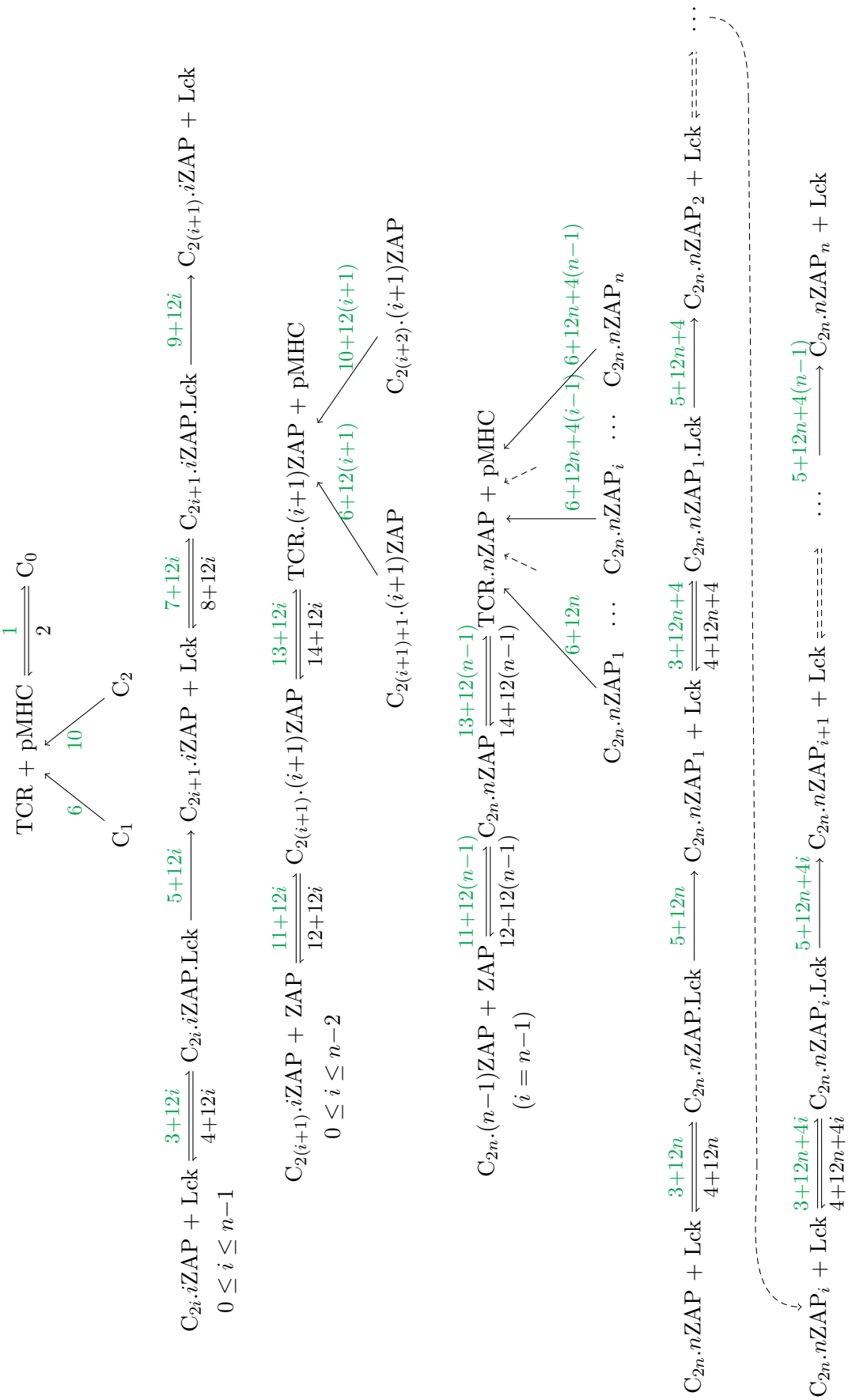


Figure 3.5: Kinetic proofreading with Lck and ZAP-70 and $N = 2n$ phosphorylation steps. The numbering above the reaction arrows indicates an order for the reactions. The i-th reaction is assigned the reaction rate k_i. The reactions that we choose in the first step of the Advanced Deficiency Algorithm to be in orientation are colored.

Step 1: Choose an Initial Orientation.

For the reversible reactions, we choose the binding of complexes to be the reaction in orientation. The reactions in orientation are colored green in Figure 3.5.

Step 2: Find the Colinearity Classes.

After having chosen the initial orientation, we can set up the stoichiometric matrix restricted to the reactions in orientation, $N|_{\mathcal{O}}$, with which we can identify the linear map $L_{\mathcal{O}} : \mathbb{R}^{\mathcal{O}} \to \mathbb{R}^{\mathcal{S}}$. For the sake of clarity, the entries of the matrix are presented in form of a table on the following double-page spread. The column numbers refer to the respective reactions. The brown colored reaction numbers play a role in a later step. We can eliminate four rows of this table, the rows corresponding to the species TCR, pMHC, Lck and ZAP. For the sake of clarity, we use the name of a species as an abbreviation for the corresponding row in the table to describe the elimination of these rows:

- $\mathrm{TCR} + \text{all rows from } \mathrm{C}_0 = 0$
- $-\mathrm{pMHC} + \mathrm{TCR} + \sum_{i=1}^{n} \mathrm{TCR}.i\mathrm{ZAP} = 0$
- $\mathrm{Lck} + \mathrm{C}_0.\mathrm{Lck} + \mathrm{C}_1.\mathrm{Lck} + \sum_{i=1}^{n} \mathrm{C}_{2i}.i\mathrm{ZAP}.\mathrm{Lck} + \sum_{i=1}^{n-1} \mathrm{C}_{2i+1}.i\mathrm{ZAP}.\mathrm{Lck} + \sum_{i=1}^{n-1} \mathrm{C}_{2n}.n\mathrm{ZAP}_i.\mathrm{Lck} = 0$
- $$\begin{aligned}\mathrm{ZAP} + \sum_{i=1}^{n-1} i\Big(&\mathrm{C}_{2i}.i\mathrm{ZAP} + \mathrm{TCR}.i\mathrm{ZAP} + \mathrm{C}_{2i}.i\mathrm{ZAP}.\mathrm{Lck} + \mathrm{C}_{2i+1}.i\mathrm{ZAP} + \mathrm{C}_{2i+1}.i\mathrm{ZAP}.\mathrm{Lck} \\ &+ \mathrm{C}_{2(i+1)}.i\mathrm{ZAP}\Big) \\ + n\left(\mathrm{C}_{2n}.n\mathrm{ZAP} + \mathrm{TCR}.n\mathrm{ZAP} + \sum_{i=1}^{n-1}\Big(\mathrm{C}_{2n}.n\mathrm{ZAP}_i + \mathrm{C}_{2n}.n\mathrm{ZAP}_i.\mathrm{Lck}\Big) + \mathrm{C}_{2n}.n\mathrm{ZAP}_n\right) &= 0.\end{aligned}$$

The elimination of the first four rows, corresponding to the species TCR, pMHC, Lck and ZAP, results in a row echelon form. Thus, the rank of the matrix $N|_{\mathcal{O}}$ is

$$\operatorname{rank}(N|_{\mathcal{O}}) = m - 4 = (8n + 5) - 4 = 8n + 1.$$

The reduction of the rank by four corresponds to our expectations according to the number of conserved quantities (2.6) involved in the system. Consequently, the dimension of the kernel of the linear map $L_{\mathcal{O}}$ is given by

$$\dim(\ker(L_{\mathcal{O}})) = \dim(\ker(N|_{\mathcal{O}})) = \overline{r} - \operatorname{rank}(N|_{\mathcal{O}}) = (12n + 1) - (8n + 1) = 4n.$$

We determine the following vectors to form a basis for the kernel:

$$
\begin{array}{r}
1\\3\\5\\6\\\hline 7\\9\\10\\\hline 11\\13\\16n+3\\\hline 12+3\\12+5\\12+6\\\hline \vdots
\end{array}
\begin{pmatrix} 1\\1\\1\\1 \end{pmatrix},
\begin{pmatrix} \\ -1\\1\\1\\1 \end{pmatrix},
\begin{pmatrix} \\ \\ -1\\1\\1\\1 \end{pmatrix},
\begin{pmatrix} \\ \\ \\ -1\\0\\1\\1\\1 \end{pmatrix},
\ldots,
\begin{array}{r}
\vdots\\\hline 3\\5\\6\\\hline 7\\9\\10\\\hline 11\\13\\16n+3+i\\\hline 3\\5\\6\\\hline \vdots
\end{array}
\begin{pmatrix} \\ \\ \\ \\ -1\\1\\1\\1 \end{pmatrix},
\begin{pmatrix} \\ \\ \\ \\ \\ -1\\1\\1\\1 \end{pmatrix},
\begin{pmatrix} \\ \\ \\ \\ \\ -1\\0\\1\\1\\1 \end{pmatrix},
\begin{pmatrix} \\ \\ \\ \\ \\ \\ -1\\1\\1\\1 \end{pmatrix},
\ldots,
\begin{array}{r}
\vdots\\\hline 12(n-1)+\\ \vdots\\ 13\\ 16n+3+(n-1)\\\hline 3\\12n+\quad 5\\ 6\\\hline 3\\12n+4+\quad 5\\ 6\\\hline \vdots
\end{array}
\begin{pmatrix} \\ \\ \\ \\ \\ \\ -1\\0\\1\\1\\1 \end{pmatrix},
\begin{pmatrix} \\ \\ \\ \\ \\ \\ \\ -1\\1\\1\\1 \end{pmatrix},
\ldots,
\begin{array}{r}
\vdots\\\hline \\ \vdots\\12n+4i+\\ 6\\\hline 3\\12n+4(i+1)+\quad 5\\ 6\\\hline \vdots
\end{array}
\begin{pmatrix} \\ \\ \\ \\ \\ \\ \\ \\ -1\\1\\1\\1 \end{pmatrix},
\ldots,
\begin{pmatrix} \\ \\ \\ \\ \\ \\ \\ \\ \\ -1\\1\\1\\1 \end{pmatrix},
$$

$1 \le i \le n-1$ $\qquad$ $1 \le i \le n-3$

where the green numbers indicate the reactions to which the respective rows correspond. The gray-colored vectors illustrate how the following or previous set of vectors continues. We listed a set of four vectors, $(n-1)$-times a set of three vectors and $(n-1)$-times a single vector, which sums up to a number of $4n$ vectors. Since these vectors are linearly independent, they form a basis for the kernel of the linear map $L_{\mathcal{O}}$. This provides the following w-vectors. To simplify the assignment of the reactions, we specify in the index both the reaction and the reaction number. We abbreviate

	1	3	5	6	7	9	10	11	13	$16n+3$	$\cdots$
TCR	-1			1			1			1	
pMHC	-1			1			1		1		
Lck		-1	1		-1	1					
ZAP								-1		1	
C_0	1	-1									
C_0.Lck		1	-1								
C_1			1	-1	-1						
C_1.Lck					1	-1					
C_2						1	-1	-1			
C_2.ZAP								1	-1		
TCR.ZAP									1	-1	
$\vdots$											
C_{2i}.iZAP											1 -1
TCR.iZAP											1 -1
C_{2i}.iZAP.Lck											
C_{2i+1}.iZAP											
C_{2i+1}.iZAP.Lck											
$C_{2(i+1)}$.iZAP											
$C_{2(i+1)}$.$(i+1)$ZAP											
TCR.$(i+1)$ZAP											
$C_{2(i+1)}$.$(i+1)$ZAP.Lck											
$C_{2(i+1)+1}$.$(i+1)$ZAP											
$C_{2(i+1)+1}$.$(i+1)$ZAP.Lck											
$C_{2(i+2)}$.$(i+1)$ZAP											
$C_{2(i+2)}$.$(i+2)$ZAP											
TCR.$(i+2)$ZAP											
$\vdots$											
C_{2n}.nZAP											
TCR.nZAP											
C_{2n}.nZAP.Lck											
C_{2n}.nZAP$_1$											
$\vdots$											
C_{2n}.nZAP$_i$.Lck											
C_{2n}.nZAP$_{i+1}$											
C_{2n}.nZAP$_{i+1}$.Lck											
C_{2n}.nZAP$_{i+2}$											
$\vdots$											
C_{2n}.nZAP$_n$											

$+12i$										$+12(i+1)$										$+12(n-1)$				$+12n$			$+12n$ $+4$			⋯	$+12n$ $+4i$			$+12n$ $+4(i+1)$			⋯	$+12n$ $+4(n-1)$		
3	5	6	7	9	10	11	13	$16n+3+i$	3	5	6	7	9	10	11	13	$16n+3+i+1$	⋯	11	13	$16n+3+(n-1)$	3	5	6	3	5	6	⋯	3	5	6	3	5	6	⋯	3	5	6
		1			1		1				1			1		1				1				1			1					1			1				1
-1	1		-1	1					-1	1		-1	1									-1	1		-1	1			-1	1		-1	1			-1	1	
						-1		1								-1	1		-1		1																		
-1																																							
		1			1			1																															
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																																				1	-1		
																																					1	-1	
																																						1	-1

this reaction number with the letter "R" and the corresponding reaction number to avoid confusion with the introduced numbering of the w-vectors. For the sake of clarity, we identify the complexes not carrying a ZAP-70 molecule with complexes ending in $.i\text{ZAP}$ where $i = 0$.

- $w_{\,1} := w_{\substack{\text{R1,R2}\\ \text{TCR+pMHC}\rightleftharpoons\text{C}_0}} = w_{\substack{\text{R3,R4}\\ \text{C}_0+\text{Lck}\rightleftharpoons\text{C}_0.\text{Lck}}} = w_{\substack{\text{R5}\\ \text{C}_0.\text{Lck}\rightarrow\text{C}_1+\text{Lck}}} = (1, 0, \ldots, 0)^T$

- $w_{\substack{1+6i\\ 1\le i\le n-1}} := w_{\substack{\text{R(3+12}i\text{),R(4+12}i\text{)}\\ \text{C}_{2i}.i\text{ZAP+Lck}\rightleftharpoons\text{C}_{2i}.i\text{ZAP.Lck}\\ 1\le i\le n-1}} = w_{\substack{\text{R(5+12}i\text{)}\\ \text{C}_{2i}.i\text{ZAP.Lck}\rightarrow\text{C}_{i+1}.i\text{ZAP+Lck}\\ 1\le i\le n-1}} = (0, \ldots, \underset{\substack{\uparrow\\ 1+3i}}{1}, \ldots, 0)^T$

- $w_{\substack{2+6i\\ 0\le i\le n-1}} := w_{\substack{\text{R(6+12}i\text{)}\\ \text{C}_{i+1}.i\text{ZAP}\rightarrow\text{TCR}.i\text{ZAP+pMHC}\\ 0\le i\le n-1}} = (0, \ldots, \underset{\substack{\uparrow\\ 1+3i}}{1}, -1, \ldots, 0)^T$

- $w_{\substack{3+6i\\ 0\le i\le n-1}} := w_{\substack{\text{R(7+12}i\text{),R(8+12}i\text{)}\\ \text{C}_{2i+1}.i\text{ZAP+Lck}\rightleftharpoons\text{C}_{2i+1}.i\text{ZAP.Lck}\\ 0\le i\le n-1}} = w_{\substack{\text{R(9+12}i\text{)}\\ \text{C}_{2i+1}.i\text{ZAP.Lck}\rightarrow\text{C}_{2(i+1)}.i\text{ZAP+Lck}\\ 0\le i\le n-1}} = (0, \ldots, \underset{\substack{\uparrow\\ 2+3i}}{1}, \ldots, 0)^T$

- $w_{\substack{4+6i\\ 0\le i\le n-1}} := w_{\substack{\text{R(10+12}i\text{)}\\ \text{C}_{2(i+1)}.i\text{ZAP}\rightarrow\text{TCR}.i\text{ZAP+pMHC}\\ 0\le i\le n-1}} = (0, \ldots, \underset{\substack{\uparrow\\ 2+3i}}{1}, -1, \ldots, 0)^T$

- $w_{\substack{5+6i\\ 0\le i\le n-1}} := w_{\substack{\text{R(11+12}i\text{),R(12+12}i\text{)}\\ \text{C}_{2(i+1)}.i\text{ZAP+ZAP}\rightleftharpoons\text{C}_{2(i+1)}.(i+1)\text{ZAP}\\ 0\le i\le n-1}} = w_{\substack{\text{R(16}n+3+i\text{)}\\ \text{TCR}.(i+1)\text{ZAP}\rightarrow\text{TCR}.i\text{ZAP+ZAP}\\ 0\le i\le n-1}} = (0, \ldots, \underset{\substack{\uparrow\\ 3+3i}}{1}, \ldots, 0)^T$

- $w_{\substack{6+6i\\ 0\le i\le n-1}} := w_{\substack{\text{R(13+12}i\text{),R(14+12}i\text{)}\\ \text{C}_{2(i+1)}.(i+1)\text{ZAP}\rightleftharpoons\text{TCR}.(i+1)\text{ZAP+pMHC}\\ 0\le i\le n-1}} = (0, \ldots, \underset{\substack{\uparrow\\ 3+3i}}{1}, -1, \ldots, 0)^T$

- $w_{\substack{6n+1+2i\\ 0\le i\le n-2}} := w_{\substack{\text{R(3+12}n\text{+4}i\text{),R(4+12}n\text{+4}i\text{)}\\ \text{C}_{2n}.n\text{ZAP}_i\text{+Lck}\rightleftharpoons\text{C}_{2n}.n\text{ZAP}_i.\text{Lck}\\ 0\le i\le n-2}} = w_{\substack{\text{R(5+12}n\text{+4}i\text{)}\\ \text{C}_{2n}.n\text{ZAP}_i.\text{Lck}\rightarrow\text{C}_{2n}.n\text{ZAP}_{i+1}\text{+Lck}\\ 0\le i\le n-2}} = (0, \ldots, \underset{\substack{\uparrow\\ 3n+1+i}}{1}, \ldots, 0)^T$

- $w_{\substack{6n+2+2i\\ 0\le i\le n-2}} := w_{\substack{\text{R(6+12}n\text{+4}i\text{)}\\ \text{C}_{2n}.n\text{ZAP}_{i+1}\rightarrow\text{TCR}.n\text{ZAP+pMHC}\\ 0\le i\le n-2}} = (0. \ldots, \underset{\substack{\uparrow\\ 3n+1+i}}{1}, -1, \ldots, 0)^T$

- $w_{\substack{6n+1+2(n-1)\\ =8n-1\\ (i=n-1)}} := w_{\substack{\text{R(3+12}n\text{+4}(n-1)\text{),R(4+12}n\text{+4}(n-1)\text{)}\\ \text{C}_{2n}.n\text{ZAP}_{n-1}\text{+Lck}\rightleftharpoons\text{C}_{2n}.n\text{ZAP}_{n-1}.\text{Lck}\\ (i=n-1)}} = w_{\substack{\text{R(5+12}n\text{+4}(n-1)\text{)}\\ \text{C}_{2n}.n\text{ZAP}_{n-1}.\text{Lck}\rightarrow\text{C}_{2n}.n\text{ZAP}_n\text{+Lck}\\ (i=n-1)}} = w_{\substack{\text{R(6+12}n\text{+4}(n-1)\text{)}\\ \text{C}_{2n}.n\text{ZAP}_n\rightarrow\text{TCR}.n\text{ZAP+pMHC}\\ (i=n-1)}} = (0, \ldots, \underset{\substack{\uparrow\\ 4n}}{1})^T.$

Hence, we get a number of $4n$ distinct w-vectors of the form $(\ldots, 1, \ldots)^T$ and a number of $4n-1$ w-vectors of the form $(\ldots, 1, -1, \ldots)^T$. None of these w-vectors are colinear with each other, and

there is no zero w-vector. Consequently, the system has a total of $8n-1$ colinearity classes, which we denote by $CC_1, \ldots, CC_{8n-1}$. Each colinearity class contains precisely the reactions whose w-vectors coincide. Thus, the two necessary conditions for the colinearity classes are fulfilled.

Step 3: Find the Colinkage Sets.

Colinearity Class	Colinkage Sets
CC_1	$\mathrm{TCR} + \mathrm{pMHC} \underset{2}{\overset{1}{\rightleftharpoons}} \mathrm{C}_0$ $\mathrm{C}_0 + \mathrm{Lck} \underset{4}{\overset{3}{\rightleftharpoons}} \mathrm{C}_0.\mathrm{Lck} \overset{5}{\longrightarrow} \mathrm{C}_1 + \mathrm{Lck}$
CC_{1+6i} $1 \leq i \leq n-1$	$\mathrm{C}_{2i}.i\mathrm{ZAP} + \mathrm{Lck} \underset{4+12i}{\overset{3+12i}{\rightleftharpoons}} \mathrm{C}_{2i}.i\mathrm{ZAP}.\mathrm{Lck} \overset{5+12i}{\longrightarrow} \mathrm{C}_{2i+1}.i\mathrm{ZAP} + \mathrm{Lck}$
CC_{2+6i} $0 \leq i \leq n-1$	$\mathrm{C}_{2i+1}.i\mathrm{ZAP} \overset{6+12i}{\longrightarrow} \mathrm{TCR}.i\mathrm{ZAP} + \mathrm{pMHC}$
CC_{3+6i} $0 \leq i \leq n-1$	$\mathrm{C}_{2i+1}.i\mathrm{ZAP} + \mathrm{Lck} \underset{8+12i}{\overset{7+12i}{\rightleftharpoons}} \mathrm{C}_{2i+1}.i\mathrm{ZAP}.\mathrm{Lck} \overset{9+12i}{\longrightarrow} \mathrm{C}_{2(i+1)}.i\mathrm{ZAP} + \mathrm{Lck}$
CC_{4+6i} $0 \leq i \leq n-1$	$\mathrm{C}_{2(i+1)}.i\mathrm{ZAP} \overset{10+12i}{\longrightarrow} \mathrm{TCR}.i\mathrm{ZAP} + \mathrm{pMHC}$
CC_{5+6i} $0 \leq i \leq n-1$	$\mathrm{C}_{2(i+1)}.i\mathrm{ZAP} + \mathrm{ZAP} \underset{12+12i}{\overset{11+12i}{\rightleftharpoons}} \mathrm{C}_{2(i+1)}.(i{+}1)\mathrm{ZAP}$ $\mathrm{TCR}.(i+1)\mathrm{ZAP} \overset{16n+3+i}{\longrightarrow} \mathrm{TCR}.i\mathrm{ZAP} + \mathrm{ZAP}$
CC_{6+6i} $0 \leq i \leq n-1$	$\mathrm{C}_{2(i+1)}.(i{+}1)\mathrm{ZAP} \underset{14+12i}{\overset{13+12i}{\rightleftharpoons}} \mathrm{TCR}.(i{+}1)\mathrm{ZAP} + \mathrm{pMHC}$
$CC_{6n+1+2i}$ $0 \leq i \leq n-2$	$\mathrm{C}_{2n}.n\mathrm{ZAP}_i + \mathrm{Lck} \underset{4+12n+4i}{\overset{3+12n+4i}{\rightleftharpoons}} \mathrm{C}_{2n}.n\mathrm{ZAP}_i.\mathrm{Lck} \overset{5+12n+4i}{\longrightarrow} \mathrm{C}_{2n}.n\mathrm{ZAP}_{i+1} + \mathrm{Lck}$
$CC_{6n+2+2i}$ $0 \leq i \leq n-2$	$\mathrm{C}_{2n}.n\mathrm{ZAP}_{i+1} \overset{6+12n+4i}{\longrightarrow} \mathrm{TCR}.n\mathrm{ZAP} + \mathrm{pMHC}$
CC_{8n-1}	$\mathrm{C}_{2n}.n\mathrm{ZAP}_{n-1} + \mathrm{Lck} \underset{4+12n+4(n-1)}{\overset{3+12n+4(n-1)}{\rightleftharpoons}} \mathrm{C}_{2n}.n\mathrm{ZAP}_{n-1}.\mathrm{Lck} \overset{5+12n+4(n-1)}{\longrightarrow} \mathrm{C}_{2n}.n\mathrm{ZAP}_n + \mathrm{Lck}$ $\mathrm{C}_{2n}.n\mathrm{ZAP}_n \overset{6+12n+4(n-1)}{\longrightarrow} \mathrm{TCR}.n\mathrm{ZAP} + \mathrm{pMHC}$

The terminal strong colinkage sets are shaded gray. There are several nontrivial terminal strong

colinkage sets. The binding and unbinding of the unphosphorylated complex C_0 in the first colinearity class forms a nontrivial terminal strong colinkage set, as is the case for the kinetic proofreading not including ZAP-70. Another nontrivial terminal strong colinkage set is given by the binding and unbinding of ZAP-70 in the colinearity classes CC_{5+6i}, $0 \leq i \leq n-1$. Additionally, the colinearity classes CC_{6+6i}, $0 \leq i \leq n-1$, are reversible and contain a single reversible reaction that forms a nontrivial terminal strong colinkage set. These colinearity classes are the only reversible colinearity classes.

Step 4: Choose Colinearity Class Vectors.

Every colinearity class of the system is associated to a single w-vector. For the reversible colinearity classes CC_{6+6i}, $0 \leq i \leq n-1$, we can pick any nonzero multiple of the respective w-vector to be the colinearity class vector. The other colinearity classes are irreversible, and the respective colinearity class vector has to be a positive multiple of the associated w-vector. We choose for all colinearity classes the w-vectors from Step 2 as colinearity class vectors.

Step 5: Realign the Orientation.

A realigning of the orientation is not necessary since the colinearity class vectors coincide with the w-vectors.

Step 6: Find Coplanar Sets and Connected Classes.

We identify the two-dimensional subspaces that contain three colinearity class vectors. Each of these subspaces contains precisely three colinearity class vectors. Thus, the three colinearity classes associated to these colinearity class vectors are contained in a coplanar set. We find a total of $4n-1$ coplanar sets:

$$\begin{aligned}
w_{1+6i} &= w_{2+6i} + w_{3+6i} && 0 \leq i \leq n-1 && \rightarrow && \mathcal{T}_{1+3i} \\
w_{3+6i} &= w_{4+6i} + w_{5+6i} && 0 \leq i \leq n-1 && \rightarrow && \mathcal{T}_{2+3i} \\
w_{5+6i} &= w_{6+6i} + w_{1+6(i+1)} && 0 \leq i \leq n-1 && \rightarrow && \mathcal{T}_{3+3i} \\
w_{6n+1+2i} &= w_{6n+2+2i} + w_{6n+1+2(i+1)} && 0 \leq i \leq n-2 && \rightarrow && \mathcal{T}_{1+3n+i}.
\end{aligned}$$

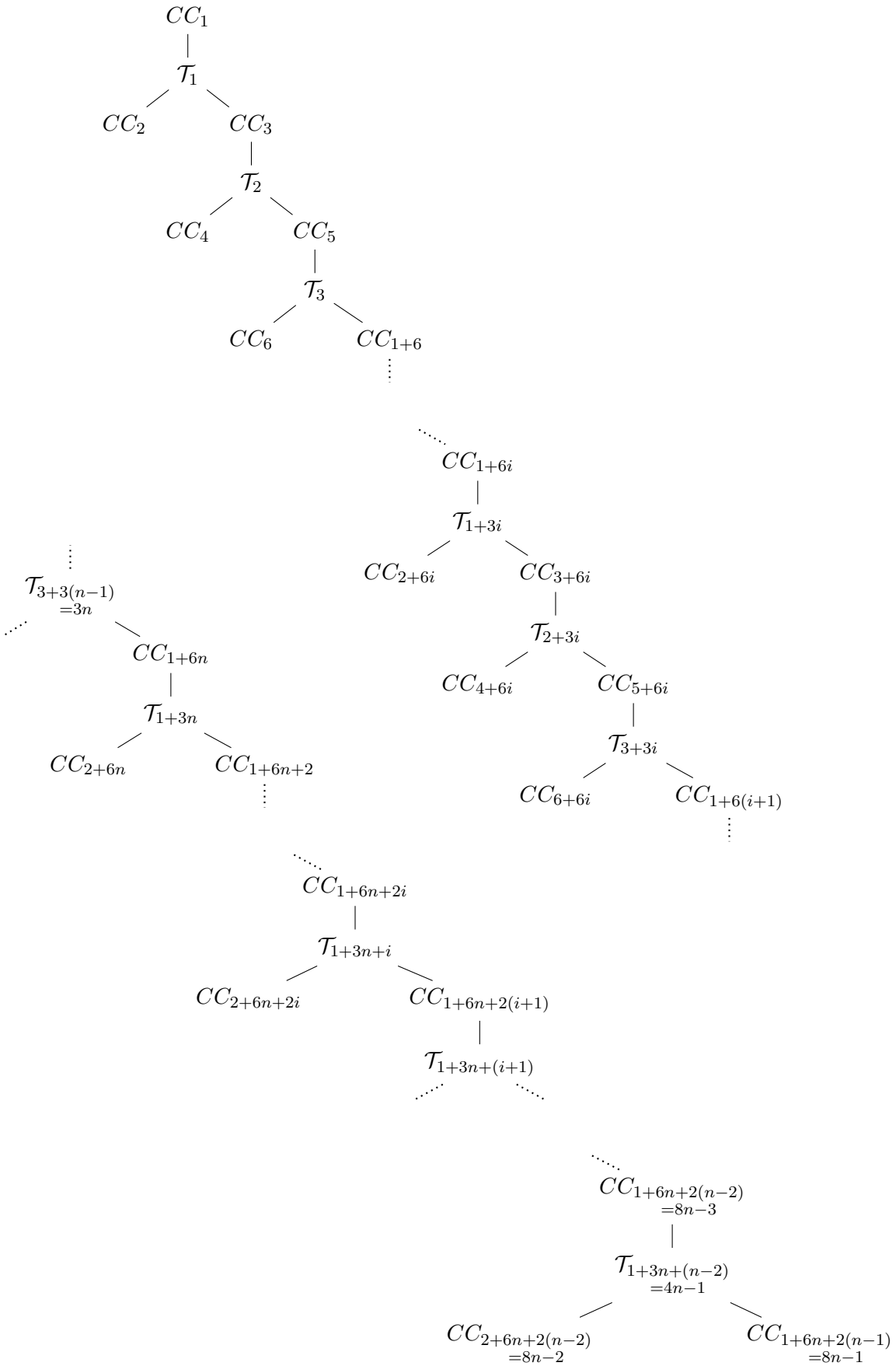
Again, all colinearity classes with an even index lie only in a single coplanar set, while those with an odd index lie in two distinct coplanar sets, linking these to a single connected class. The connected graph is displayed on the next page.

Step 7: Determine Linearity.

The Independence Linearity Condition is satisfied since the number of coplanar sets and connected classes adds up to the dimension of the kernel of the mapping $L|_{\mathcal{O}}$:

$$(4n-1)+1 = 4n = \dim\left(\ker\left(L_{\mathcal{O}}\right)\right).$$

Furthermore, each coplanar set contains exactly three colinearity classes. Hence, the Triplet Linearity Condition holds as well. Since both linearity conditions are satisfied, the generated inequality system is completely linear in μ. That is, the Advanced Deficiency Algorithm provides a definitive answer to whether the system is able to support multiple steady states within the same positive stoichiometric compatibility class. If we find a signature for the network, then the system supports multiple positive steady states. If we have gone through all possible choices in the algorithm and have not found a signature, then the system is not able to support multiple positive steady states in the same stoichiometric compatibility class.


CC_1
$\mathcal{T}_1$
CC_2
CC_3
$\mathcal{T}_2$
CC_4
CC_5
$\mathcal{T}_3$
CC_6
CC_{1+6}
CC_{1+6i}
$\mathcal{T}_{1+3i}$
CC_{2+6i}
CC_{3+6i}
$\mathcal{T}_{2+3i}$
CC_{4+6i}
CC_{5+6i}
$\mathcal{T}_{3+3i}$
CC_{6+6i}
$CC_{1+6(i+1)}$
$\mathcal{T}_{3+3(n-1)}$ $=3n$
CC_{1+6n}
$\mathcal{T}_{1+3n}$
CC_{2+6n}
CC_{1+6n+2}
$CC_{1+6n+2i}$
$\mathcal{T}_{1+3n+i}$
$CC_{2+6n+2i}$
$CC_{1+6n+2(i+1)}$
$\mathcal{T}_{1+3n+(i+1)}$
$CC_{1+6n+2(n-2)}$ $=8n-3$
$\mathcal{T}_{1+3n+(n-2)}$ $=4n-1$
$CC_{2+6n+2(n-2)}$ $=8n-2$
$CC_{1+6n+2(n-1)}$ $=8n-1$

Step 8: Choose Signs for Colinearity Classes.

Among the colinearity classes only CC_{6+6i}, $0 \leq i \leq n-1$, are reversible. All other colinearity classes are irreversible and are assigned a positive sign. The reversible colinearity classes CC_{6+6i} can be assigned a positive, negative or zero sign as long as the respective conditions are met. The colinearity class vector of the colinearity class CC_{6+6i} belongs to the coplanar set $\mathcal{T}_{3+3i}$ and satisfies the equation

$$w_{5+6i} = w_{6+6i} + w_{1+6(i+1)}.$$

If we assign a nonzero sign to CC_{6+6i}, it must not be possible to find positive values for c_{5+6i} and $c_{1+6(i+1)}$ that satisfy

$$\begin{aligned} & c_{5+6i}w_{5+6i} + c_{6+6i}w_{6+6i} + c_{1+6(i+1)}w_{1+6(i+1)} = 0 \\ \Leftrightarrow \quad & \underbrace{c_{5+5i}}_{>0}(\ldots,1,0,\ldots)^T + c_{6+6i}(\ldots,1,-1,\ldots)^T + \underbrace{c_{7+7i}}_{>0}(\ldots,0,1,\ldots)^T = (\ldots,0,0,\ldots)^T. \end{aligned}$$

The given equation cannot be satisfied for any $c_{5+6i}, c_{1+6(i+1)} \in \mathbb{R}_+$ and $c_{6+6i} \in \mathbb{R}$. Likewise, there cannot be positive values for c_{5+6i} and $c_{1+6(i+1)}$ that would make $c_{5+6i}w_{5+6i} + c_{1+6(i+1)}w_{1+6(i+1)}$ a multiple of w_{6+6i}, which is a necessary condition if we assign a zero sign to CC_{6+6i}. Thus, we can choose any sign (positive, negative or zero) for each colinearity class CC_{6+6i}, $0 \leq i \leq n-1$. We choose a positive sign for all colinearity classes.

Step 9: Choose Shelving for Reactions.

Reversible reactions whose reactant complexes lie in a terminal strong colinkage set can be placed on an arbitrary shelf, provided all other reactions of this colinkage set are placed on the same shelf. All other reactions have to be placed on the middle shelves of their respective colinearity class. These rules provide the following shelving options:

Colinearity Class	Shelves
CC_1	$\{\mathrm{TCR} + \mathrm{pMHC} \rightleftharpoons \mathrm{C}_0\}$ *arbitrary shelf* $(= \mathcal{L}_1, \subset \mathcal{M}_1$ or $= \mathcal{U}_1)$ $\{\mathrm{C}_0 + \mathrm{Lck} \rightleftharpoons \mathrm{C}_0.\mathrm{Lck} \to \mathrm{C}_1.\mathrm{Lck}\} \subset \mathcal{M}_1$
CC_{1+6i} $1 \leq i \leq n-1$	$\{\mathrm{C}_{2i}.i\mathrm{ZAP} + \mathrm{Lck} \rightleftharpoons \mathrm{C}_{2i}.i\mathrm{ZAP}.\mathrm{Lck} \to \mathrm{C}_{2i+1}.i\mathrm{ZAP} + \mathrm{Lck}\} = \mathcal{M}_{1+6i}$
CC_{2+6i} $0 \leq i \leq n-1$	$\{\mathrm{C}_{2i+1}.i\mathrm{ZAP} \to \mathrm{TCR}.i\mathrm{ZAP} + \mathrm{pMHC}\} = \mathcal{M}_{2+6i}$
CC_{3+6i} $0 \leq i \leq n-1$	$\{\mathrm{C}_{2i+1}.i\mathrm{ZAP} + \mathrm{Lck} \rightleftharpoons \mathrm{C}_{2i+1}.i\mathrm{ZAP}.\mathrm{Lck} \to \mathrm{C}_{2(i+1)}.i\mathrm{ZAP} + \mathrm{Lck}\} = \mathcal{M}_{3+6i}$
CC_{4+6i} $0 \leq i \leq n-1$	$\{\mathrm{C}_{2(i+1)}.i\mathrm{ZAP} \to \mathrm{TCR}.i\mathrm{ZAP} + \mathrm{pMHC}\} = \mathcal{M}_{4+6i}$
CC_{5+6i} $0 \leq i \leq n-1$	$\{\mathrm{C}_{2(i+1)}.i\mathrm{ZAP} + \mathrm{ZAP} \rightleftharpoons \mathrm{C}_{2(i+1)}.(i+1)\mathrm{ZAP}\}$ *arbitrary shelf* $\{\mathrm{TCR}.(i+1)\mathrm{ZAP} \to \mathrm{TCR}.i\mathrm{ZAP} + \mathrm{ZAP}\} \subset \mathcal{M}_{5+6i}$
CC_{6+6i} $0 \leq i \leq n-1$	$\{\mathrm{C}_{2(i+1)}.(i+1)\mathrm{ZAP} \rightleftharpoons \mathrm{TCR}.(i+1)\mathrm{ZAP} + \mathrm{pMHC}\}$ *arbitrary shelf*

$$
\begin{array}{ll}
CC_{6n+1+2i} \\ 0 \leq i \leq n-2 & \{\mathrm{C}_{2n}.n\mathrm{ZAP}_i + \mathrm{Lck} \rightleftharpoons \mathrm{C}_{2n}.n\mathrm{ZAP}_i.\mathrm{Lck} \rightarrow \mathrm{C}_{2n}.n\mathrm{ZAP}_{i+1} + \mathrm{Lck}\} = \mathcal{M}_{6n+1+2i} \\
CC_{6n+2+2i} \\ 0 \leq i \leq n-2 & \{\mathrm{C}_{2n}.n\mathrm{ZAP}_{i+1} \rightarrow \mathrm{TCR}.n\mathrm{ZAP} + \mathrm{pMHC}\} = \mathcal{M}_{6n+2+2i} \\
CC_{8n-1} & \{\mathrm{C}_{2n}.n\mathrm{ZAP}_{n-1} + \mathrm{Lck} \rightleftharpoons \mathrm{C}_{2n}.n\mathrm{ZAP}_{n-1}.\mathrm{Lck} \rightarrow \mathrm{C}_{2n}.n\mathrm{ZAP}_n + \mathrm{Lck}, \\
& \quad \mathrm{C}_{2n}.n\mathrm{ZAP}_n \rightarrow \mathrm{TCR}.n\mathrm{ZAP} + \mathrm{pMHC}\} = \mathcal{M}_{8n-1}.
\end{array}
$$

We listed only the nonempty shelves. The lower and upper shelves not listed are empty. Since we modeled the binding of a ZAP molecule as a reversible reaction and allowed a rebinding of the antigen-presenting MHC molecule to a T cell receptor whose phosphorylated ITAMs are all bound to a ZAP-70 molecule and therefore protected from direct dephosphorylation, two additional colinearity classes contain a colinearity set that can be placed in an arbitrary shelf. This results in additional inequalities in the inequality system that have to be satisfied. We choose to assign all still open colinearity sets to the upper shelf of their respective colinearity class.

Step 10: Add Shelving Inequalities.

According to the shelving just chosen, we get the following shelving inequalities and equalities, which form the first part of the inequality system in μ:

$$
\begin{array}{rl}
(i=1) & \mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}} > M_1 \\
& \mu_{\mathrm{C}_0} > M_1 \\
& \mu_{\mathrm{C}_0} + \mu_{\mathrm{Lck}} = \mu_{\mathrm{C}_0.\mathrm{Lck}} = M_1 \\
1 \leq i \leq n-1 & \mu_{\mathrm{C}_{2i}.i\mathrm{ZAP}} + \mu_{\mathrm{Lck}} = \mu_{\mathrm{C}_{2i}.i\mathrm{ZAP}.\mathrm{Lck}} = M_{1+6i} \\
0 \leq i \leq n-1 & \mu_{\mathrm{C}_{2i+1}.i\mathrm{ZAP}} = M_{2+6i} \\
0 \leq i \leq n-1 & \mu_{\mathrm{C}_{2i+1}.i\mathrm{ZAP}} + \mu_{\mathrm{Lck}} = \mu_{\mathrm{C}_{2i+1}.i\mathrm{ZAP}.\mathrm{Lck}} = M_{3+6i} \\
0 \leq i \leq n-1 & \mu_{\mathrm{C}_{2(i+1)}.i\mathrm{ZAP}} = M_{4+6i} \\
0 \leq i \leq n-1 & \mu_{\mathrm{C}_{2(i+1)}.i\mathrm{ZAP}} + \mu_{\mathrm{ZAP}} > M_{5+6i} \\
& \mu_{\mathrm{C}_{2(i+1)}.(i+1)\mathrm{ZAP}} > M_{5+6i} \\
& \mu_{\mathrm{TCR}.(i+1)\mathrm{ZAP}} = M_{5+6i} \\
0 \leq i \leq n-1 & \mu_{\mathrm{C}_{2(i+1)}.(i+1)\mathrm{ZAP}} > M_{6+6i} \\
& \mu_{\mathrm{TCR}.(i+1)\mathrm{ZAP}} + \mu_{\mathrm{pMHC}} > M_{6+6i} \\
0 \leq i \leq n-2 & \mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_i} + \mu_{\mathrm{Lck}} = \mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_i.\mathrm{Lck}} = M_{6n+1+2i} \\
0 \leq i \leq n-2 & \mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_{i+1}} = M_{6n+2+2i} \\
(i=n-1) & \mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_{n-1}} + \mu_{\mathrm{Lck}} = \mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_{n-1}.\mathrm{Lck}} = \mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_n} = M_{8n-1}.
\end{array}
$$

Step 11: Add Upper and Lower Shelf Inequalities.

We assigned all colinearity classes a positive sign. Therefore, we add for every reaction in an upper shelf which is in orientation, $y \rightarrow y' \in \mathcal{O}$, the inequality $y' \cdot \mu > y \cdot \mu$ to the system:

$$
\begin{array}{l}
\mu_{\mathrm{C}_0} > \mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}} \\
\mu_{\mathrm{C}_{2(i+1)}.(i+1)\mathrm{ZAP}} > \mu_{\mathrm{C}_{2(i+1)}.i\mathrm{ZAP}} + \mu_{\mathrm{ZAP}} \\
\mu_{\mathrm{TCR}.(i+1)\mathrm{ZAP}} + \mu_{\mathrm{pMHC}} > \mu_{\mathrm{C}_{2(i+1)}.(i+1)\mathrm{ZAP}}.
\end{array}
$$

Step 12: Add Equalities for Colinearity Classes with Zero Signs.

As no colinearity class was assigned a zero sign, we skip this step.

Step 13: Add M Inequalities and Equalities

We have assigned all colinearity classes a zero sign, and the two-dimensional subspace spanned by the colinearity class vectors belonging to the same coplanar set is given by

$$w_{2+2i} + w_{1+2(i+1)} = w_{1+2i}$$

for all coplanar sets, $0 \leq i \leq 4n-2$ (cf. Step 6). Consequently, we can either choose $M_{2+2i} > M_{1+2i} > M_{1+2(i+1)}$, $M_{2+2i} = M_{1+2i} = M_{1+2(i+1)}$ or $M_{2+2i} < M_{1+2i} < M_{1+2(i+1)}$ as M inequalities. We choose the first option:

$$M_{2+2i} > M_{1+2i} > M_{1+2(i+1)}$$

where $0 \leq i \leq 4n-2$.

Step 14: Check for Solutions for the Inequality System.

The inequality system we constructed throughout the algorithm is displayed on the following double-page spread. Since the system satisfies the linearity conditions, the inequality system constructed is complete. Consequently, if we find a nonzero solution vector μ that is sign compatible with the stoichiometric subspace, then we have found a signature for the inequality system, which implies that the kinetic proofreading system with Lck and ZAP-70 supports multiple positive steady states within the same stoichiometric compatibility class. The vector $\mu \in \mathbb{R}^{\mathcal{S}}$ is sign compatible with the stoichiometric subspace $S = \operatorname{span}\{y' - y : y \to y' \in \mathcal{R}\}$ if there exists a vector $\sigma \in S$ and a set of positive numbers $\{p_s : s \in \mathcal{S}\}$ such that $\mu_s = p_s\sigma_s$ for all $s \in \mathcal{S}$. We denote by v_i the column vector of the stoichiometric matrix N associated to the i-th reaction. Thus, with $a_i \in \mathbb{R}$ we construct a sign compatible vector $\sigma = \sum a_i v_i$. The column vectors that are sufficient to construct a suitable vector σ are indicated by brown colored reaction numbers in the matrix $N|_{\mathcal{O}}$ displayed in Step 2. The chosen column vectors v_i lead to the following entries for the sign compatible vector σ:

s	$\sigma_s = \left(\sum a_i v_i\right)_s$	
TCR	$-a_1$	< 0
pMHC	$-a_1 + \sum_{i=0}^{n-1} a_{13+12i}$	> 0
Lck	$\sum_{i=0}^{n-1}(-a_{3+12i} + a_{5+12i} - a_{7+12i} + a_{9+12i})$ $+ \sum_{i=0}^{n-1}(-a_{3+12n+4i} + a_{5+12n+4i})$	< 0
ZAP	$\sum_{i=0}^{n-1} -a_{11+12i}$	< 0
C_0	$a_1 - a_3$	> 0

$0 \leq i \leq n-1$:		
$C_{2i}.i$ZAP.Lck	$a_{3+12i} - a_{5+12i}$	> 0
$C_{2i+1}.i$ZAP	$a_{5+12i} - a_{7+12i}$	> 0
$C_{2i+1}.i$ZAP.Lck	$a_{7+12i} - a_{9+12i}$	> 0
$C_{2(i+1)}.i$ZAP	$a_{9+12i} - a_{11+12i}$	> 0
$C_{2(i+1)}.(i+1)$ZAP	$a_{11+12i} - a_{13+12i} - a_{3+12(i+1)}$	> 0
TCR.$(i+1)$ZAP	a_{13+12i}	> 0
$0 \leq i \leq n-1$:		
$C_{2n}.n$ZAP$_i$.Lck	$a_{3+12n+4i} - a_{5+12n+4i}$	< 0
$0 \leq i \leq n-2$:		
$C_{2n}.n$ZAP$_{i+1}$	$a_{5+12n+4i} - a_{3+12n+4(i+1)}$	< 0
$C_{2n}.n$ZAP$_n$	$a_{5+12n+4(n-1)}$	< 0

The negative sign of σ_{Lck} is a direct consequence of the M inequalities we chose, which is clear when looking at the displayed inequality system. The other signs result from the choices made for the M_i and a_i, which we will address now. We choose M_1 to be positive. Consequently, μ_{C_0}, $\mu_{C_0.\text{Lck}}$, μ_{C_1} and μ_{TCR} or μ_{pMHC} have to be positive and thereby σ_{C_0}, $\sigma_{C_0.\text{Lck}}$, σ_{C_1} and σ_{TCR} or σ_{pMHC} as well. Regarding σ_{C_0}, we choose a_1 to be positive, which implies that σ_{TCR} is negative and σ_{pMHC} has to be positive. We choose the σ_s of the second section ($C_{2i}.i$ZAP.Lck to TCR.$(i+1)$ZAP for $0 \leq i \leq n-1$) to be positive as well. Thus, the first sum in σ_{Lck} is negative. We assign the σ_s of the third section ($C_{2n}.n$ZAP$_i$.Lck to $C_{2n}.n$ZAP$_n$ for $0 \leq i \leq n-1$) a negative sign. The choices made so far can be met by choosing a_1 to $a_{13+12(n-1)}$ positive, where the latter is assigned a relatively high value to ensure that σ_{pMHC} is positive, and a_{3+12n} to $a_{5+12n+4(n-1)}$ negative:

$$\sum_{i=0}^{n-1} a_{13+12i} > a_1 > a_3 > a_3 > a_5 > a_7 > a_9 > a_{11} > (a_{13} + a_{3+12}) > a_{3+12} > \dots$$

$$\dots > a_{3+12i} > a_{5+12i} > a_{7+12i} > a_{9+12i} > a_{11+12i} > \left(a_{13+12i} + a_{3+12(i+1)}\right) > a_{3+12(i+1)} > \dots$$

$$\dots > \underbrace{a_{11+12(n-1)}}_{>0} > (\underbrace{a_{13+12(n-1)}}_{>0} + \underbrace{a_{3+12n}}_{<0}) > \underbrace{a_{3+12n}}_{<0} < a_{5+12n} < a_{3+12n+4} < \dots$$

$$\dots < a_{3+12n+4i} < a_{5+12n+4i} < a_{3+12n+4(i+1)} < \dots < a_{5+12(n-2)} < a_{3+12n+4(n-1)} < a_{5+12n+4(n-1)}.$$

The first two inequalities are a consequence of σ_{pMHC} and σ_{C_0} being positive. The following inequalities $a_i > a_{i+1}$ follow from the choice of the second section of σ_s to be positive. The positive signs for a_1 to $a_{13+12(n-1)}$ imply in particular that all a_{11+12i} are positive and therefore σ_{ZAP} is negative. To ensure that the first inequality is satisfied, we assign $a_{13+12(n-1)}$ a sufficiently high positive value and a_{13+12n} a negative value. The further a_i are assigned a negative sign and a value satisfying the inequality $a_i < a_{i+1}$. It follows from our choices that M_1 to M_{6n} are positive and M_{6n+1} to M_{8n-1} are negative. In addition to satisfying the given chain of inequalities, we must also ensure that σ_{Lck} is negative when assigning the values of the a_i. To satisfy the chain of inequalities, we assign the following values for the a_i:

$$0 \leq i \leq n-2: \quad a_{13+12i} = 1$$

$$
\begin{array}{ccccccccc}
 & & \mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_{n-1}} & & \mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_{i+2}} & & \mu_{\mathrm{C}_n.n\mathrm{ZAP}_{i+1}} & & \\
 & & \| & & \| & & \| & & \\
 & & M_{6n+2+2(n-2)} & & M_{6n+2+2(i+1)} & & M_{6n+2+2i} & & \\
 & & =8n-2 & & & & & & \\
 & & \vee & & \vee & & \vee & & \\
M_{8n-1} & < & M_{6n+1+2(n-2)} & < \cdots < & M_{6n+1+2(i+1)} & < & M_{6n+1+2i} & < & \cdots \\
 & & =8n-3 & & & & & & \\
\| & & \| & & \| & & \| & & \\
\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_{n-1}} + \mu_{\mathrm{Lck}} & & \mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_{n-2}} + \mu_{\mathrm{Lck}} & & \mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_{i+1}} + \mu_{\mathrm{Lck}} & & \mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_i} + \mu_{\mathrm{Lck}} & & \\
\| & & \| & & \| & & \| & & \\
\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_{n-1}.\mathrm{Lck}} & & \mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_{n-2}.\mathrm{Lck}} & & \mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_{i+1}.\mathrm{Lck}} & & \mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_i.\mathrm{Lck}} & & \\
\| & & & & & & & & \\
\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_n} & & & & & & & &
\end{array}
$$

$$
\begin{array}{ccccccccc}
 & & & & \mu_{\mathrm{TCR}.(i+1)\mathrm{ZAP}} + \mu_{\mathrm{pMHC}} & & & & \\
 & & & & \vee & & & & \\
 & & \mu_{\mathrm{C}_{2(i+1)+1}.(i+1)\mathrm{ZAP}} & & \mu_{\mathrm{C}_{2(i+1)}.(i+1)\mathrm{ZAP}} & & \mu_{\mathrm{C}_{2(i+1)}.i\mathrm{ZAP}} & & \mu_{\mathrm{C}_{2i+1}.i\mathrm{ZAP}} \\
 & & \| & & \vee & & \| & & \| \\
 & & M_{2+6(i+1)} & & M_{6+6i} & & M_{4+6i} & & M_{2+6i} \\
 & & \vee & & \vee & & \vee & & \vee \\
< \cdots < & & M_{1+6(i+1)} & < & M_{5+6i} & < & M_{3+6i} & < & M_{1+6i} & < \cdots \\
 & & \| & & \| & & \| & & \| \\
 & & \mu_{\mathrm{C}_{2(i+1)}.(i+1)\mathrm{ZAP}} + \mu_{\mathrm{Lck}} & & \mu_{\mathrm{TCR}.(i+1)\mathrm{ZAP}} & & \mu_{\mathrm{C}_{2i+1}.i\mathrm{ZAP}} + \mu_{\mathrm{Lck}} & & \mu_{\mathrm{C}_{2i}.i\mathrm{ZAP}} + \mu_{\mathrm{Lck}} \\
 & & \| & & \wedge & & \| & & \| \\
 & & \mu_{\mathrm{C}_{2(i+1)}.(i+1)\mathrm{ZAP.Lck}} & & \mu_{\mathrm{C}_{2(i+1)}.i\mathrm{ZAP}} + \mu_{\mathrm{ZAP}} & & \mu_{\mathrm{C}_{2i+1}.i\mathrm{ZAP.Lck}} & & \mu_{\mathrm{C}_{2i}.i\mathrm{ZAP.Lck}} \\
 & & & & \wedge & & & & \\
 & & & & \mu_{\mathrm{C}_{2(i+1)}.(i+1)\mathrm{ZAP}} & & & &
\end{array}
$$

$$
\begin{array}{ccccccccc}
 & & \mu_{\mathrm{TCR}.n\mathrm{ZAP}} + \mu_{\mathrm{pMHC}} & & & & & & \\
 & & \vee & & & & & & \\
 & & \mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}} & & \mu_{\mathrm{C}_{2n}.(n-1)\mathrm{ZAP}} & & \mu_{\mathrm{C}_{2(n-1)+1}.(n-1)\mathrm{ZAP}} & & \vdots \\
 & & \vee & & \| & & \| & & \vee \\
\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_1} & & M_{6+6(n-1)=6n} & & M_{4+6(n-1)=6n-2} & & M_{2+6(n-1)=6n-4} & & M_{6+6(n-2)=6n-6} \\
\| & & \vee & & \vee & & \vee & & \vee \\
M_{6n+2} & & M_{5+6(n-1)=6n-1} & & M_{3+6(n-1)=6n-3} & & M_{1+6(n-1)=6n-5} & & M_{5+6(n-2)=6n-7} \\
\vee & & \| & & \| & & \| & & \| \\
M_{6n+1} & & \mu_{\mathrm{TCR}.n\mathrm{ZAP}} & & \mu_{\mathrm{C}_{2(n-1)+1}.(n-1)\mathrm{ZAP}} + \mu_{\mathrm{Lck}} & & \mu_{\mathrm{C}_{2(n-1)}.(n-1)\mathrm{ZAP}} + \mu_{\mathrm{Lck}} & & \vdots \\
\| & & \wedge & & \| & & \| & & \\
\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}} + \mu_{\mathrm{Lck}} & & \mu_{\mathrm{C}_{2n}.(n-1)\mathrm{ZAP}} + \mu_{\mathrm{ZAP}} & & \mu_{\mathrm{C}_{2(n-1)+1}.(n-1)\mathrm{ZAP.Lck}} & & \mu_{\mathrm{C}_{2(n-1)}.(n-1).\mathrm{ZAP.Lck}} & & \\
\| & & \wedge & & & & & & \\
\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP.Lck}} & & \mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}} & & & & & & \\
< & & < & & < & & < & & < \quad \cdots
\end{array}
$$

$$
\begin{array}{ccccccc}
 & \mu_{\mathrm{TCR.ZAP}} + \mu_{\mathrm{pMHC}} & & & & & \\
 & \vee & & & & & \\
\mu_{\mathrm{C}_3.\mathrm{ZAP}} & \mu_{\mathrm{C}_2.\mathrm{ZAP}} & & \mu_{\mathrm{C}_2} & & \mu_{\mathrm{C}_1} & \\
\| & \vee & & \| & & \| & \\
M_{2+6=8} & M_6 & & M_4 & & M_2 & \\
\vee & \vee & & \vee & & \vee & \\
M_{1+6=7} & M_5 & & M_3 & & M_1 & \\
\| & \| & & \| & & \| & \\
\mu_{\mathrm{C}_2.\mathrm{ZAP}} + \mu_{\mathrm{Lck}} & \mu_{\mathrm{TCR.ZAP}} & & \mu_{\mathrm{C}_1} + \mu_{\mathrm{Lck}} & & \mu_{\mathrm{C}_0} + \mu_{\mathrm{Lck}} & \\
\| & \wedge & & \| & & \| & \\
\mu_{\mathrm{C}_2.\mathrm{ZAP.Lck}} & \mu_{\mathrm{C}_2} + \mu_{\mathrm{ZAP}} & & \mu_{\mathrm{C}_1.\mathrm{Lck}} & & \mu_{\mathrm{C}_0.\mathrm{Lck}} & \\
 & \wedge & & & & \wedge & \\
 & \mu_{\mathrm{C}_2.\mathrm{ZAP}} & & & & \mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}} & \\
 & & & & & \wedge & \\
 & & & & & \mu_{\mathrm{C}_0} & \\
< & < & < & & < & &
\end{array}
$$

$$
\begin{aligned}
0 \le i \le n-1: \quad & a_{11+12i} = 1 + 6(n-1-i) \\
& a_{9+12i} = 2 + 6(n-1-i) \\
& a_{7+12i} = 3 + 6(n-1-i) \\
& a_{5+12i} = 4 + 6(n-1-i) \\
& a_{3+12i} = 5 + 6(n-1-i)
\end{aligned}
$$

$$
\begin{aligned}
& a_1 = 1 + a_3 = 1 + 5 + 6(n-1) = 6n \\
& a_{13+12(n-1)} = 1 + \left(a_1 - \sum_{i=0}^{n-2} a_{13+12i} \right) = 1 + 6n - \sum_{i=0}^{n-2} 1 = 1 + 6n - (n-1) = 5n + 2 \\
& a_{3+12n} = -1 + \left(a_{11+12(n-1)} - a_{13+12(n-1)} \right) = -1 + 1 - (5n+2) = -2 - 5n
\end{aligned}
$$

$$
\begin{aligned}
1 \le i \le n-1: \quad & a_{3+12n+4i} = a_{3+12n} + 2i = -2 - 5n + 2i \\
0 \le i \le n-1: \quad & a_{5+12n+4i} = a_{3+12n} + 1 + 2i = -2 - 5n + 1 + 2i = -1 - 5n + 2i.
\end{aligned}
$$

For the sake of clarity, we list the values assigned to the a_i in order of their index:

$$
\begin{array}{lll}
 & a_1 = 6n & > 0 \\
0 \le i \le n-1: & a_{3+12i} = 5 + 6(n-1-i) & > 0 \\
 & a_{5+12i} = 4 + 6(n-1-i) & > 0 \\
 & a_{7+12i} = 3 + 6(n-1-i) & > 0 \\
 & a_{9+12i} = 2 + 6(n-1-i) & > 0 \\
 & a_{11+12i} = 1 + 6(n-1-i) & > 0 \\
0 \le i \le n-2: & a_{13+12i} = 1 & > 0 \\
(i = n-1) & a_{13+12(n-1)} = 5n + 2 & > 0 \\
0 \le i \le n-1: & a_{3+12n+4i} = -2 - 5n + 2i & < 0 \\
 & a_{5+12n+4i} = -1 - 5n + 2i & < 0.
\end{array}
$$

With the chosen a_i we calculate the values of the σ_s, $s \in \mathcal{S}$:

s	$\sigma_s = \left(\sum a_i v_i \right)_s$
TCR	$-a_1 = -6n < 0$
pMHC	$-a_1 + \sum_{i=0}^{n-1} a_{13+12i} = -6n + \sum_{i=0}^{n-2} 1 + 5n + 2 = -6n + n - 1 + 5n + 2$ $= 1 > 0$
Lck	$\sum_{i=0}^{n-1} \left(-\left(a_{3+12i} - a_{5+12i}\right) - \left(a_{7+12i} - a_{9+12i}\right) - \left(a_{3+12n+4i} - a_{5+12n+4i}\right) \right)$ $= \sum_{i=0}^{n-1} (-1 - 1 + 1) = -n < 0$
ZAP	$\sum_{i=0}^{n-1} -a_{11+12i} = \sum_{i=0}^{n-1} -\left(1 + 6(n-1-i)\right) = -n\left(1 + 6(n-1)\right) + 6\frac{(n-1)n}{2}$ $= -n(3n-2) < 0$

C_0	$a_1 - a_3 = 6n - (5 + 6(n-1)) = 1 > 0$

$0 \leq i \leq n-1$:

$C_{2i}.i\text{ZAP.Lck}$	$a_{3+12i} - a_{5+12i} = 5 + 6(n-1-i) - (4 + 6(n-1-i)) = 1 > 0$
$C_{2i+1}.i\text{ZAP}$	$a_{5+12i} - a_{7+12i} = 4 + 6(n-1-i) - (3 + 6(n-1-i)) = 1 > 0$
$C_{2i+1}.i\text{ZAP.Lck}$	$a_{7+12i} - a_{9+12i} = 3 + 6(n-1-i) - (2 + 6(n-1-i)) = 1 > 0$
$C_{2(i+1)}.i\text{ZAP}$	$a_{9+12i} - a_{11+12i} = 2 + 6(n-1-i) - (1 + 6(n-1-i)) = 1 > 0$

$0 \leq i \leq n-2$:

$C_{2(i+1)}.(i+1)\text{ZAP}$	$a_{11+12i} - a_{13+12i} - a_{3+12(i+1)}$ $= 1 + 6(n-1-i) - 1 - (5 + 6(n-1-(i+1))) = -5 + 6 = 1 > 0$
$\text{TCR}.(i+1)\text{ZAP}$	$a_{13+12i} = 1 > 0$
$C_{2n}.n\text{ZAP}$	$a_{11+12(n-1)} - a_{13+12(n-1)} - a_{3+12n}$ $= 1 + 6(n-1-(n-1)) - (5n+2) - (-2-5n) = 1 > 0$
$\text{TCR}.n\text{ZAP}$	$a_{13+12(n-1)} = 5n + 2 > 0$

$0 \leq i \leq n-1$:

$C_{2n}.n\text{ZAP}_i\text{.Lck}$	$a_{3+12n+4i} - a_{5+12n+4i} = (-2-5n+2i) - (-1-5n+2i) = -1 < 0$

$0 \leq i \leq n-2$:

$C_{2n}.n\text{ZAP}_{i+1}$	$a_{5+12n+4i} - a_{3+12n+4(i+1)} = -1 - 5n + 2i - (-2 - 5n + 2(i+1))$ $= -1 < 0$
$C_{2n}.n\text{ZAP}_n$	$a_{5+12n+4(n-1)} = -1 - 5n + 2(n-1) = -3n - 3 = -3(n+1) < 0$

The signs of the σ_s determine the signs of the μ_s and thus the M_i. M_1 to M_{6n} are positive and M_{6n+1} to M_{8n-1} are negative. In particular, no M_i may be assigned a zero sign. First, we assign a value to the M_i with odd index since these form a continuous chain of inequalities. We set:

$$\begin{aligned} \underset{0 \leq i \leq 3n-1}{M_{2i+1}} &:= 3n - i \\ \underset{=6n+1}{M_{2(3n)+1}} &:= -2 \\ \underset{3n+1 \leq i \leq 4n-1}{M_{2i+1}} &:= -4 + 3n - i. \end{aligned}$$

The first jump is necessary so that M_{6n+1} is not assigned the value zero. Instead, M_{6n+1} should be negative. We choose the value -2 such that $M_{2(3n)+1} < M_{2(3n+1)} < 0$ can be satisfied conveniently. The second jump is a consequence of the value assigned to $M_{2(3n+1)}$ and the value we choose for μ_{Lck}, as we will see in the following. The assignment of the values for the M_i with odd index determines the values of μ_s for the species where μ_s equals some M_i. These are the species $C_{2i}.i\text{ZAP.Lck}$, $C_{2i+1}.i\text{ZAP.Lck}$, $\text{TCR}.(i+1)\text{ZAP}$ and $C_{2n}.n\text{ZAP}_i\text{.Lck}$ for $0 \leq i \leq n-1$ and $C_{2n}.n\text{ZAP}_n$. The M inequalities

$$\underset{=2(3i+1)+1}{M_{3+6i}} < \underset{=2(3i)+1}{M_{1+6i}} < \underset{=2(3i+1)}{M_{2+6i}}$$

imply in particular that

$$\begin{aligned} &\mu_{\mathrm{C}_{2i+1}.i\mathrm{ZAP}} + \mu_{\mathrm{Lck}} = 3n - 3i - 1 < 3n - 3i < \mu_{\mathrm{C}_{2i+1}.i\mathrm{ZAP}} \\ \text{and} \quad &\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_1} + \mu_{\mathrm{Lck}} = -5 < -2 < \mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_1} \end{aligned}$$

hold for $0 \leq i \leq n-2$. To meet these inequalities, we set

$$\mu_{\mathrm{Lck}} := -4.$$

This determines the values of μ_s for the species $\mathrm{C}_{2i+1}.i\mathrm{ZAP}$ and $\mathrm{C}_{2(i+1)}.(i+1)\mathrm{ZAP}$ for $0 \leq i \leq n-1$ and for $\mathrm{C}_{2n}.n\mathrm{ZAP}_i$ for $1 \leq i \leq n$. The values for the former and the latter equal an M_i with even index and thus determine its value. Consequently, we set

$$\underset{\substack{=2(3i+1)\\0\leq i\leq n-1}}{M_{2+6i}} := 3n - 3i + 3 \qquad \text{and} \qquad \underset{3n+1\leq i\leq 4n-1}{M_{2i}} := 3n - i.$$

On the other hand, the value assigned to $\mu_{\mathrm{C}_{2(i+1)}.(i+1)\mathrm{ZAP}}$, $0 \leq i \leq n-1$, gives an upper bound for the value of M_{6+6i} since

$$\begin{aligned} &\mu_{\mathrm{C}_{2(i+1)}.(i+1)\mathrm{ZAP}} + \mu_{\mathrm{Lck}} = \underset{=2(3i+3)+1}{M_{1+6(i+1)}} < \underset{=2(3i+2)+1}{M_{5+6i}} < \underset{=2(3i+3)}{M_{6+6i}} < \mu_{\mathrm{C}_{2(i+1)}.(i+1)\mathrm{ZAP}} \\ \Leftrightarrow \quad &3n - 3i + 1 - 4 = 3n - 3i - 3 < 3n - 3i - 2 < \underset{=2(3i+2)}{M_{6+6i}} < 3n - 3i + 1 \quad \text{for} \quad 0 \leq i \leq n-2 \\ &\text{and} \quad 2 - 4 = -2 < 1 < \underset{=2(3n)}{M_{6+6(n-1)}} < 2 \quad \text{for} \quad i = n-1. \end{aligned}$$

We set

$$\underset{=2(3i+3)}{M_{6+6i}} := 3n - 3i \quad \text{for} \quad 0 \leq i \leq n-2 \qquad \text{and} \qquad \underset{=2(3n)}{M_{6+6(n-1)}} := 1.5.$$

Similarly, the value of $\mu_{\mathrm{C}_{2(i+1)}.(i+1)\mathrm{ZAP}}$, $0 \leq i \leq n-1$, gives an upper bound for $\mu_{\mathrm{C}_{2(i+1)}.i\mathrm{ZAP}}$, which in turn determines the value of $\underset{=2(3i+2)}{M_{4+6i}}$:

$$\begin{aligned} &\mu_{\mathrm{C}_{2(i+1)}.(i+1)\mathrm{ZAP}} > \mu_{\mathrm{C}_{2(i+1)}.i\mathrm{ZAP}} + \mu_{\mathrm{ZAP}} = \underset{=2(3i+2)+1}{M_{5+6i}} < \underset{=2(3i+1)+1}{M_{3+6i}} < \underset{=2(3i+2)}{M_{4+6i}} = \mu_{\mathrm{C}_{2(i+1)}.i\mathrm{ZAP}} \\ \Leftrightarrow \quad &3n - 3i + 1 > \mu_{\mathrm{C}_{2(i+1)}.i\mathrm{ZAP}} + \mu_{\mathrm{ZAP}} = 3n - 3i - 2 < 3n - 3i - 1 < \underset{=2(3i+2)}{M_{4+6i}} = \mu_{\mathrm{C}_{2(i+1)}.i\mathrm{ZAP}} \\ &\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \text{for} \quad 0 \leq i \leq n-2 \\ &\text{and} \quad 2 > \mu_{\mathrm{C}_{2n.(n-1)}\mathrm{ZAP}} + \mu_{\mathrm{ZAP}} = 1 < 2 < \underset{=2(3n-1)}{M_{4+6(n-1)}} = \mu_{\mathrm{C}_{2n}.(n-1)\mathrm{ZAP}} \quad \text{for} \quad i = n-1. \end{aligned}$$

These inequalities are satisfied by setting

$$\underset{=2(3i+2)}{M_{4+6i}} := 3n - 3i + 2 \quad \text{for} \quad 0 \leq i \leq n-2, \quad \underset{=2(3n-1)}{M_{4+6(n-1)}} := 3.5 \quad \text{and} \quad \mu_{\mathrm{ZAP}} := -2.$$

We have now assigned a value to every M_i, $1 \leq i \leq 8n-1$, and most of the μ_s. It remains to assign values for the species TCR and pMHC. For these species, the following inequalities have to be satisfied:

$$\begin{aligned} &\mu_{\mathrm{C}_0} > \mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}} > M_1 \\ \text{and} \quad &\mu_{\mathrm{TCR}.(i+1)\mathrm{ZAP}} + \mu_{\mathrm{pMHC}} > \mu_{\mathrm{C}_{2(i+1)}.(i+1)\mathrm{ZAP}} \quad \text{for} \quad 0 \leq i \leq n-1, \end{aligned}$$

which translates into

$$\begin{aligned} & 3n+4 > \mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}} > 3n \\ \text{and} \quad & 3n-3i-2+\mu_{\mathrm{pMHC}} > 3n-3i+1 \quad \text{for} \quad 0 \le i \le n-1. \end{aligned}$$

With regard to the calculated σ_s, we assign the following values to μ_{TCR} and μ_{pMHC}:

$$\mu_{\mathrm{TCR}} := -6n \qquad \text{and} \qquad \mu_{\mathrm{pMHC}} := 9n+1.$$

We have now assigned a value to every M_i and every μ_s. For the sake of clarity we summarize them again. The M_i were assigned the following values:

$$\underset{0\le i\le 3n-1}{M_{2i+1}} = 3n-i \qquad M_{2(3n)+1} = -2 \qquad \underset{3n+1\le i\le 4n-1}{M_{2i+1}} = -4+3n-i$$

$$\underset{\substack{=2(3i+1)\\ 0\le i\le n-1}}{M_{2+6i}} = 3n-3i+3$$

$$\underset{\substack{=2(3i+2)\\ 0\le i\le n-2}}{M_{4+6i}} = 3n-3i+2 \qquad M_{4+6(n-1)} = 3.5$$

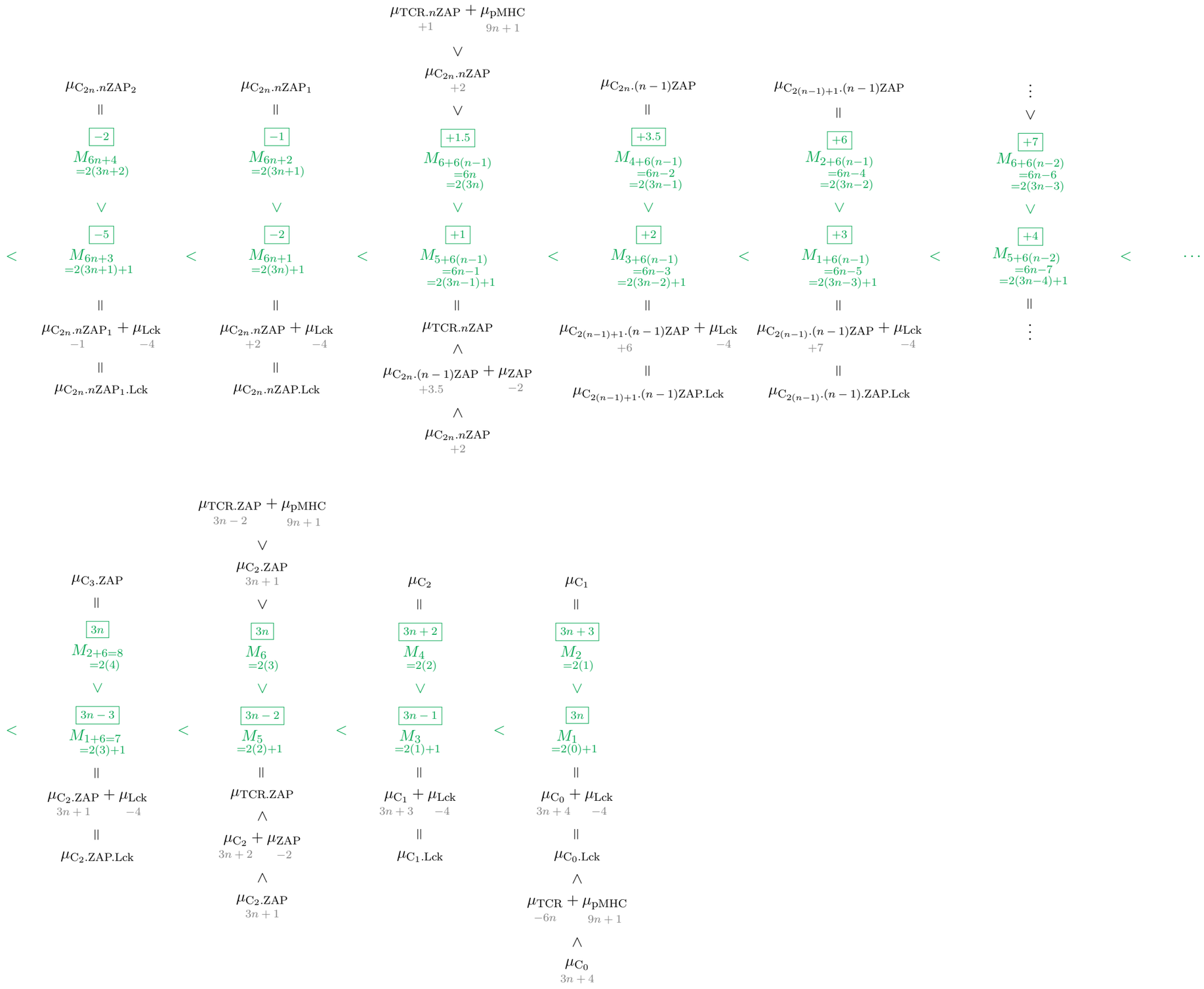
$$\underset{\substack{=2(3i+3)\\ 0\le i\le n-2}}{M_{6+6i}} = 3n-3i \qquad M_{6+6(n-1)} = 1.5$$

$$\underset{3n+1\le i\le 4n-1}{M_{2i}} = 3n-i.$$

For the solution vector μ to be sign compatible with the stoichiometric subspace, $\mu_s = p_s\sigma_s$ has to hold for some $\sigma \in S$ and $p_s > 0$ for all $s \in \mathcal{S}$. Since the sign of the value μ_s agrees with the sign of the respective σ_s, there exists for every species a positive value p_s such that $\mu_s = p_s\sigma_s$ holds. The table below lists the values of p_s, σ_s and their product μ_s for every species $s \in \mathcal{S}$. To verify that we have indeed found a signature with these values, we display all assigned values in the complete inequality system on the following double-page spread.

s	p_s	σ_s	μ_s
TCR	1	$-6n$	$-6n$
pMHC	$9n+1$	$+1$	$9n+1$
Lck	$\frac{4}{n}$	$-n$	-4
ZAP	$\frac{2}{n(3n-2)}$	$-n(3n-2)$	-2
C_0	$3n+4$	$+1$	$3n+4$
$0 \le i \le n-1$:			
C_{2i}.iZAP.Lck	$3n-3i$	$+1$	$3n-3i$
C_{2i+1}.iZAP	$3n-3i+3$	$+1$	$3n-3i+3$
C_{2i+1}.iZAP.Lck	$3n-3i-1$	$+1$	$3n-3i-1$
$0 \le i \le n-2$:			
$C_{2(i+1)}$.iZAP	$3n-3i+2$	$+1$	$3n-3i+2$
$C_{2(i+1)}$.$(i+1)$ZAP	$3n-3i+1$	$+1$	$3n-3i+1$

$\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_{n-1}}$
$-(n-1)$
$M_{6n+2+2(n-2)}$
$=8n-2$
$=2(4n-1)$
$-(n+3)$
M_{8n-1}
$=2(4n-1)+1$
$-(n+2)$
$M_{6n+1+2(n-2)}$
$=8n-3$
$=2(4n-2)+1$
$\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_{n-1}} + \mu_{\mathrm{Lck}}$
$-(n-1)$ -4
$\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_{n-2}} + \mu_{\mathrm{Lck}}$
$-(n-2)$ -4
$\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_{n-1}.\mathrm{Lck}}$
$\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_{n-2}.\mathrm{Lck}}$
$\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_{n}}$
$\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_{i+2}}$
$-(i+2)$
$M_{6n+2+2(i+1)}$
$=2(3n+i+2)$
$-(i+5)$
$M_{6n+1+2(i+1)}$
$=2(3n+i+1)+1$
$\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_{i+1}} + \mu_{\mathrm{Lck}}$
$-(i+1)$ -4
$\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_{i+1}.\mathrm{Lck}}$
$\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_{i+1}}$
$-(i+1)$
$M_{6n+2+2i}$
$=2(3n+i+1)$
$-(i+4)$
$M_{6n+1+2i}$
$=2(3n+i)+1$
$\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_{i}} + \mu_{\mathrm{Lck}}$
$-i$ -4
$\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_{i}.\mathrm{Lck}}$
$\mu_{\mathrm{TCR}.(i+1)\mathrm{ZAP}} + \mu_{\mathrm{pMHC}}$
$3n-3i-2$ $9n+1$
$\mu_{\mathrm{C}_{2(i+1)+1}.(i+1)\mathrm{ZAP}}$
$3n-3i$
$M_{2+6(i+1)}$
$=2(3i+4)$
$3n-3i-3$
$M_{1+6(i+1)}$
$=2(3i+3)+1$
$\mu_{\mathrm{C}_{2(i+1)}.(i+1)\mathrm{ZAP}} + \mu_{\mathrm{Lck}}$
$3n-3i+1$ -4
$\mu_{\mathrm{C}_{2(i+1)}.(i+1)\mathrm{ZAP}.\mathrm{Lck}}$
$\mu_{\mathrm{C}_{2(i+1)}.(i+1)\mathrm{ZAP}}$
$3n-3i+1$
$3n-3i$
M_{6+6i}
$=2(3i+3)$
$3n-3i-2$
M_{5+6i}
$=2(3i+2)+1$
$\mu_{\mathrm{TCR}.(i+1)\mathrm{ZAP}} \wedge \mu_{\mathrm{C}_{2(i+1)}.i\mathrm{ZAP}} + \mu_{\mathrm{ZAP}}$
$3n-3i+2$ -2
$\wedge \mu_{\mathrm{C}_{2(i+1)}.(i+1)\mathrm{ZAP}}$
$3n-3i+1$
$\mu_{\mathrm{C}_{2(i+1)}.i\mathrm{ZAP}}$
$3n-3i+2$
M_{4+6i}
$=2(3i+2)$
$3n-3i-1$
M_{3+6i}
$=2(3i+1)+1$
$\mu_{\mathrm{C}_{2i+1}.i\mathrm{ZAP}} + \mu_{\mathrm{Lck}}$
$3n-3i+3$ -4
$\mu_{\mathrm{C}_{2i+1}.i\mathrm{ZAP}.\mathrm{Lck}}$
$\mu_{\mathrm{C}_{2i+1}.i\mathrm{ZAP}}$
$3n-3i+3$
M_{2+6i}
$=2(3i+1)$
$3n-3i$
M_{1+6i}
$=2(3i)+1$
$\mu_{\mathrm{C}_{2i}.i\mathrm{ZAP}} + \mu_{\mathrm{Lck}}$
$3n-3(i-1)+1$ -4
$\mu_{\mathrm{C}_{2i}.i\mathrm{ZAP}.\mathrm{Lck}}$


$\mu_{\mathrm{TCR}.n\mathrm{ZAP}} + \mu_{\mathrm{pMHC}}$
$+1$ $9n+1$
$\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}}$
$+2$
$+1.5$
$M_{6+6(n-1)}$ $=6n$ $=2(3n)$
$+1$
$M_{5+6(n-1)}$ $=6n-1$ $=2(3n-1)+1$
$\mu_{\mathrm{TCR}.n\mathrm{ZAP}}$
$\mu_{\mathrm{C}_{2n}.(n-1)\mathrm{ZAP}} + \mu_{\mathrm{ZAP}}$
$+3.5$ -2
$\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}}$
$+2$
$\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_2}$
-2
M_{6n+4} $=2(3n+2)$
-5
M_{6n+3} $=2(3n+1)+1$
$\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_1} + \mu_{\mathrm{Lck}}$
-1 -4
$\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_1.\mathrm{Lck}}$
$\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}_1}$
-1
M_{6n+2} $=2(3n+1)$
-2
M_{6n+1} $=2(3n)+1$
$\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}} + \mu_{\mathrm{Lck}}$
$+2$ -4
$\mu_{\mathrm{C}_{2n}.n\mathrm{ZAP}.\mathrm{Lck}}$
$\mu_{\mathrm{C}_{2n}.(n-1)\mathrm{ZAP}}$
$+3.5$
$M_{4+6(n-1)}$ $=6n-2$ $=2(3n-1)$
$+2$
$M_{3+6(n-1)}$ $=6n-3$ $=2(3n-2)+1$
$\mu_{\mathrm{C}_{2(n-1)+1}.(n-1)\mathrm{ZAP}} + \mu_{\mathrm{Lck}}$
$+6$ -4
$\mu_{\mathrm{C}_{2(n-1)+1}.(n-1)\mathrm{ZAP}.\mathrm{Lck}}$
$\mu_{\mathrm{C}_{2(n-1)+1}.(n-1)\mathrm{ZAP}}$
$+6$
$M_{2+6(n-1)}$ $=6n-4$ $=2(3n-2)$
$+3$
$M_{1+6(n-1)}$ $=6n-5$ $=2(3n-3)+1$
$\mu_{\mathrm{C}_{2(n-1)}.(n-1)\mathrm{ZAP}} + \mu_{\mathrm{Lck}}$
$+7$ -4
$\mu_{\mathrm{C}_{2(n-1)}.(n-1).\mathrm{ZAP}.\mathrm{Lck}}$
$\vdots$
$+7$
$M_{6+6(n-2)}$ $=6n-6$ $=2(3n-3)$
$+4$
$M_{5+6(n-2)}$ $=6n-7$ $=2(3n-4)+1$
$\vdots$
$\cdots$
$\mu_{\mathrm{TCR}.\mathrm{ZAP}} + \mu_{\mathrm{pMHC}}$
$3n-2$ $9n+1$
$\mu_{\mathrm{C}_2.\mathrm{ZAP}}$
$3n+1$
$\mu_{\mathrm{C}_3.\mathrm{ZAP}}$
$3n$
$M_{2+6=8}$ $=2(4)$
$3n-3$
$M_{1+6=7}$ $=2(3)+1$
$\mu_{\mathrm{C}_2.\mathrm{ZAP}} + \mu_{\mathrm{Lck}}$
$3n+1$ -4
$\mu_{\mathrm{C}_2.\mathrm{ZAP}.\mathrm{Lck}}$
$3n$
M_6 $=2(3)$
$3n-2$
M_5 $=2(2)+1$
$\mu_{\mathrm{TCR}.\mathrm{ZAP}}$
$\mu_{\mathrm{C}_2} + \mu_{\mathrm{ZAP}}$
$3n+2$ -2
$\mu_{\mathrm{C}_2.\mathrm{ZAP}}$
$3n+1$
μ_{C_2}
$3n+2$
M_4 $=2(2)$
$3n-1$
M_3 $=2(1)+1$
$\mu_{\mathrm{C}_1} + \mu_{\mathrm{Lck}}$
$3n+3$ -4
$\mu_{\mathrm{C}_1.\mathrm{Lck}}$
μ_{C_1}
$3n+3$
M_2 $=2(1)$
$3n$
M_1 $=2(0)+1$
$\mu_{\mathrm{C}_0} + \mu_{\mathrm{Lck}}$
$3n+4$ -4
$\mu_{\mathrm{C}_0.\mathrm{Lck}}$
$\mu_{\mathrm{TCR}} + \mu_{\mathrm{pMHC}}$
$-6n$ $9n+1$
μ_{C_0}
$3n+4$

TCR.$(i+1)$ZAP	$3n-3i-2$	$+1$	$3n-3i-2$
$C_{2n}.(n-1)$ZAP	3.5	$+1$	$+3.5$
$C_{2n}.n$ZAP	2	$+1$	$+2$
TCR.nZAP	$\frac{1}{5n+2}$	$+5n+2$	$+1$
$C_{2n}.n$ZAP.Lck	2	-1	-2
$1 \leq i \leq n-1$:			
$C_{2n}.n\text{ZAP}_i$.Lck	$i+4$	-1	$-(i+4)$
$0 \leq i \leq n-2$:			
$C_{2n}.n\text{ZAP}_{i+1}$	$i+1$	-1	$-(i+1)$
$C_{2n}.n\text{ZAP}_n$	$\frac{n+3}{3(n+1)}$	$-3(n+1)$	$-(n+3)$

On the double-page spread, we can see that every inequality is satisfied. Thus, we have found a signature for the kinetic proofreading system with Lck and ZAP-70. Consequently, the system is able to support multiple positive steady states within the same stoichiometric compatibility class.

Theorem 4. *The kinetic proofreading system with Lck and ZAP-70 (2.5) and an even number of phosphorylation steps, $N = 2n > 0$, supports multistationarity within the same positive stoichiometric compatibility class. That is, there exist positive reaction constants and initial concentrations such that the system exhibits more than one positive steady state.*

4 Persistence

In this chapter, we show that both the kinetic proofreading system with Lck (2.2) and the one with Lck and ZAP-70 (2.5) do not support any boundary steady states or even ω-limit points on the boundary. As the trajectories of both models stay within a compact subset of the nonnegative orthant, it follows from Brouwer's fixed point theorem that at least one positive steady state in each stoichiometric compatibility class exists. The fixed point theorem of Brouwer states that every continuous self-map on a nonempty convex subset of a finite dimensional normed vector space has a fixed point [107]. Since the kinetic proofreading systems follow some general kinetics, that is, mass action kinetics is assumed and the kinase Lck brings an enzyme-substrate reaction to the model, we will start by formulating some general properties of this type of systems and then proceed to take advantage of the specific features of the kinetic proofreading systems.

4.1 Persistence for Dynamical Systems

In this section we investigate the networks from the perspective of dynamical systems. That is, we are looking at an autonomous system of differential equations

$$\dot{x} := \frac{dx}{dt} = f(x)$$

where $x(t) \in \mathbb{R}^m$ is a vector-valued function of an independent time-variable t and $f : U \to \mathbb{R}^m$ is a continuously differentiable function defined on an open subset $U \subset \mathbb{R}^m$. Thus f is locally Lipschitz and the existence and uniqueness of a solution for every initial value problem

$$\dot{x} = f(x) \quad \text{with} \quad x(t_0) = x_0$$

on a maximal interval of existence $I(x_0)$ follows. If f satisfies a global Lipschitz condition, then the initial value problem has a unique solution defined for all $t \in \mathbb{R}$. Let $\phi(t, x_0)$ be the solution of the initial value problem defined on its maximal interval of existence. We call the mapping $\phi(\cdot, x_0) : I(x_0) \to U$ a trajectory through the point $x_0 \in U$. The set of mappings ϕ_t defined by $\phi_t(x) = \phi(t, x)$ is referred to as the *flow* of the differential equation that is generated by the vector field f. The flow ϕ_t of the system satisfies the properties $\phi_0(x) = x$ and $\phi_{t+s}(x) = \phi_t(\phi_s(x))$ for all $x \in U$. We call such a function $\phi : \mathbb{R} \times U \to U$ that is continuously differentiable on an open subset $U \subset \mathbb{R}^m$ and satisfies the properties of a flow just given a *dynamical system.* Thus, a dynamical system is a function defined for all $t \in \mathbb{R}$ which describes how points in U move with respect to time [79, 37].

As we are focusing on dynamical systems that arise from chemical reaction networks, the function f satisfies certain properties. Since none of the species can reach a negative concentration, all solutions starting in the nonnegative orthant have to remain there for all time. Under the assumption of mass action kinetics, it follows that the term that determines the decrease of the concentration of a species involves exactly this species' concentration as a factor. Thus, the concentration of a species cannot decay when the species is extinct.

Lemma 1. [84] *Let x be a solution of a dynamical system $\dot{x} = f(x)$ in $\mathbb{R}^m$ where $f_i(x) = f_{i,1}(x) - x_i f_{i,2}(x)$ for each component, $1 \leq i \leq m$, and the functions $f_{i,1}$ and $f_{i,2}$ are continuously differentiable and nonnegative. Then the nonnegative and the positive orthant are positively invariant.*

That is, if $x_i(t_0) > 0$ *for all* $1 \leq i \leq m$*, then* $x_i(t) > 0$ *for all* $1 \leq i \leq m$ *and all times* $t \geq t_0$*, and if* $x_i(t_0) \geq 0$ *for all* $1 \leq i \leq m$*, then* $x_i(t) \geq 0$ *for all* $1 \leq i \leq m$ *and all* $t \geq t_0$*.*

Proof. [84] Suppose the first statement were false. Then there would be a smallest number $t_1 > t_0$ with $\min_{1\leq i\leq m}(x_i(t_1)) = 0$. Let i be an index for which this minimum is attained, then $x_i(t_1) = 0$. Thus, for $t_0 \leq t \leq t_1$ the inequality

$$\dot{x}_i(t) \geq -x_i(t) f_{i,2}(x(t))$$

holds. With $C := \sup_{t\in[t_0,t_1]}(f_{i,2}(x(t)))$ it follows that:

$$\frac{d}{dt}\ln(x_i(t)) \geq -f_{i,2}(x(t)) \geq -C \quad \Rightarrow \quad x_i(t_1) \geq x_i(t_0)e^{-C(t_1-t_0)} > 0,$$

a contradiction. Consequently, every solution starting in the positive orthant will stay within the positive orthant for all time. The second statement, that every solution starting within the nonnegative orthant will stay there, follows from the continuous dependence of the solution on the initial data. □

Thus, we have just shown that for every system arising from mass action kinetics, the positive and nonnegative orthant are positively invariant. Since the assumption of mass action kinetics is the foundation of Michaelis-Menten kinetics, the statements hold as well for Michaelis-Menten kinetics. Michaelis-Menten kinetics models enzyme-catalyzed reactions and is derived from mass action kinetics under the additional assumption of a quasi-steady state of the enzyme-substrate complex.

Dynamical systems associated to chemical reaction networks display a certain structure. In chemical reactions a set of substances is converted into another set of substances. A substance can be for example a molecule or an individual chemical element. Chemical elements are substances that cannot be broken down into other substances. These are the smallest compounds that build molecules, which in turn can form macromolecules and so forth. These elements cannot disappear or appear out of nowhere. They are either present as an individual substance or bound as a component in another substance. This principle is referred to as the law of conservation of mass. Chemical reactions do not change the total mass of substances involved. If this principle is violated in a chemical equation, then the equation does not include all substances involved in the reaction. For example, the autocatalytic reaction $'A \to 2A'$ does not include all substances involved as mass cannot be created. A simple autocatalytic reaction satisfying the law of conservation of mass is for example given by the reaction $'A + B \to 2A'$, where the substances A and B could for example represent isomers. In chemical reactions, components are transferred from one substance to another, and if there is no external supply or removal, the total amount of the components involved remains constant over time. Such quantities are called *conserved quantities*. In the example of the kinetic proofreading models, these are the total amount of T cell receptors, pMHC molecules, Lck and ZAP-70 (cf. equations (2.3 and (2.6)). Here the substances correspond to proteins. In the kinetic proofreading models, we do not include phosphate groups that are attached to a complex by the enzyme Lck as an individual substance or species, respectively, but assume they are present all the time at a sufficiently high concentration. Nevertheless, the conservation of mass is fulfilled for the T cell receptor, the pMHC molecule and the enzyme Lck, as well as the ZAP-70 proteins. Thus, the conserved quantities depend on the variables included in the model.

Having another look at the reaction graphs of the kinetic proofreading systems (cf. Figure 2.8 and Figure 2.9), we note that all reaction arrows start and end at a complex. Hence, there is no supply or removal of any of the complexes. This is not generally the case for chemical reaction networks. Some networks include for example a constant supply rate of certain reactants or the removal of a product. Such reactions can be graphically represented by reactions that do not start or end at a

complex or, in terms of the CRNT, a zero-complex can be added. For example, a graph for the external supply of a species could be displayed as "$\to S$" or "$0 \to S$".

For all chemical reaction networks without external supply or removal of a species which respect the principle of conservation of mass, every species contributes to a conserved quantity. Thus, the concentrations of the substances are bounded by the conserved quantities, which means that the dynamical system associated to such a chemical reaction network is defined on a neighborhood of an invariant compact subset of the nonnegative orthant. This is the case for any chemical reaction network in which each species contributes to a conserved quantity. Thus, even an elaborate model such as the one from Altan-Bonnet and Germain (cf. Section 2.2) has this property. In the following, we will therefore focus on dynamical systems defined on a neighborhood of an invariant compact subset of the nonnegative orthant.

Lemma 2. *Let $\dot{x} = f(x)$ be a dynamical system defined on a neighborhood of the nonnegative orthant in $\mathbb{R}^m$ which leaves the nonnegative orthant positively invariant. Given that every component x_i, $1 \leq i \leq m$, contributes to a conserved quantity consisting of at least two components, there exist nonnegative values $x_{1,\max}, \ldots, x_{m,\max}$ such that the compact subset $[0, x_{1,\max}] \times \cdots \times [0, x_{m,\max}] =: E$ is left invariant by the system, that is, positively and negatively invariant. In particular, for every $x_i \in \{0, x_{i,\max}\}$ the equation $f_i(x) = 0$ holds.*

Proof. Since the nonnegative orthant is positively invariant for the system $\dot{x} = f(x)$ in $\mathbb{R}^m$, every solution starting within the nonnegative orthant will stay there for all time. That is, if $x_i(t_0) \geq 0$ holds for all $1 \leq i \leq m$, then it follows that $x_i(t) \geq 0$ for all $t > t_0$. Thus, every conserved quantity is the sum of nonnegative summands. Hence, the value of every x_i, $1 \leq i \leq m$, contributing to a conserved quantity cannot exceed the value of the conserved quantity. That is, there exists a $x_{i,\max}$ such that $0 \leq x_i \leq x_{i,\max}$ and the compact subset $E = [0, x_{1,\max}] \times \cdots \times [0, x_{m,\max}]$ is left positively invariant by the system. Suppose C_{tot} is a conserved quantity, then $x_{i,\max} = C_{\text{tot}}$ for some $1 \leq i \leq m$. Let I_C be the index set of components that contribute to the conserved quantity C_{tot}, that is,

$$C_{\text{tot}} = \sum_{j \in I_C} x_j, \quad \text{which implies} \quad \sum_{j \in I_C} \dot{x}_j = 0.$$

Suppose now $x_i = 0$, then

$$C_{\text{tot}} = \sum_{\substack{j \in I_C \\ j \neq i}} x_j, \quad \text{which implies} \quad \sum_{\substack{j \in I_C \\ j \neq i}} \dot{x}_j = 0.$$

Thus, we get

$$\dot{x}_i\big|_{x_i=0} = 0 \quad \Rightarrow \quad f_i(x) = 0,$$

which means that the nonnegative orthant is not only positively but also negatively invariant since for the orthant to have an inflow there has to be a point on the boundary, that is, $x_i = 0$ for some $1 \leq i \leq m$ where $f_i(x) > 0$, which contradicts $f_i(x) = 0$ whenever $x_i = 0$. Suppose $x_i = x_{i,\max} = C_{\text{tot}}$, then

$$\dot{x}_i\big|_{x_i=x_{i,\max}} = 0 \quad \Rightarrow \quad f_i(x) = 0$$

follows. Consequently, the compact subset E is also positively and negatively invariant and thus left invariant by the dynamical system. □

Since the kinetic proofreading systems with Lck (2.2) and ZAP-70 (2.5) follow mass action kinetics, the decay of every species is proportional to its current concentration. Thus, we know from Lemma 1

that the nonnegative and positive orthant are positively invariant for the solutions starting in it. Furthermore, no species is added to or withdrawn from the system, and the model follows the principle of the conservation of mass for all species included in the model. Hence, all species contribute to a conserved quantity. It follows from Lemma 2 that all solutions starting in the nonnegative orthant are bounded within an invariant nonnegative compact subset. With the knowledge that a solution starting in the positive orthant stays there for all time $t > t_0$, the question arises if this solution can approach the boundary. The property that initially positive solutions do not approach the boundary of the positive orthant is often referred to as *persistence*. The term was first used in systems modeling population dynamics, where a solution tending to the boundary of the positive orthant corresponds to a population approaching extinction. In this context, a system is called persistent if

$$\liminf_{t\to\infty} x_i(t) > 0 \quad \text{for all } 1 \leq i \leq m$$

for every trajectory with positive initial conditions [34, 106]. In population dynamics, the birth and decay of a species depend on its own present population size, that is, the differential equations for the species take the form $\dot{x}_i = x_i f(x)$ where $1 \leq i \leq m$ and f a continuously differentiable function. The boundary of the positive orthant is invariant for such a system. If the size of a population reaches zero, the population is extinct and will never arise again. In a more general setting, a locally compact metric space (X, d) is considered together with a closed subset $E \subset X$. Given a dynamical system ϕ defined on E that leaves the boundary ∂E invariant, it is said to be persistent if

$$\liminf_{t\to\infty} d(\phi(x,t), \partial E) > 0$$

for all x starting in the interior of E [9, 106]. As we are looking at molecular concentrations instead of populations, a solution reaching the boundary of the positive orthant does not necessarily mean the extinction of a species. If a species of a chemical reaction network is nonexistent at some time, it can be present at a later time if it is the product of a chemical reaction. Hence, in a chemical reaction network, the boundary of the positive orthant is not necessarily invariant, and neither is the boundary of a compact subset. Thus, we define persistence with regard to a chemical reaction network whose solutions are bounded within the nonnegative orthant.

Definition 25. Let x be a trajectory of a dynamical system that is defined on a neighborhood of a closed subset E of the nonnegative orthant $\overline{\mathbb{R}}^m_+$ that is left positively invariant by the system. The system is called *persistent* if

$$\liminf_{t\to\infty} d\left(x(t), \partial E\right) > 0$$

for all x starting in the interior of E.

For a system with bounded solutions, being persistent is thus equivalent to not allowing any ω-limit points on the boundary for solutions starting in the interior. A point $x^* \in U$ of a trajectory $\phi(\cdot, x)$ of a dynamical system is an *ω-limit point* if there is a sequence $t_n \to \infty$ such that $\lim_{n\to\infty} \phi\left(t_n, x\right) = x^*$ [79]. Persistence is thus the property that positive solutions do not approach the boundary as $t \to \infty$. A weakening of this property is *weak persistence*, which implies that positive solutions do not asymptotically approach the boundary. That is, that $\limsup_{t\to\infty}(d(\phi(x,t), \partial E) > 0$ for all solutions x of a dynamical system ϕ defined on a closed subset E of a locally compact metric space (X, d) that start in the interior of E [9]. On the other hand, we can define a stronger version of persistence, *uniform persistence*, which describes systems whose positive solutions are eventually uniformly bounded away from the boundary [9]. Having its origin in the modeling of population dynamics, a uniformly persistent system is also called permanent, permanent coexistent or cooperative [47, 48, 39, 89].

Definition 26. Let x be a trajectory of a dynamical system that is defined on a neighborhood of a closed subset E of the nonnegative orthant $\overline{\mathbb{R}}^m_+$ that is left positively invariant by the system. The system is called *uniformly persistent* if there exists an $\varepsilon > 0$ such that

$$\liminf_{t\to\infty} d\left(x(t), \partial E\right) \geq \varepsilon$$

for all x starting in the interior of E.

We will show that the kinetic proofreading systems are uniformly persistent. We begin by proving that any ω-limit point of a nonnegative solution is positive given that the conserved quantities of the kinetic proofreading system are positive. This property is slightly stronger than persistence, which refers to positive solutions, stating that any ω-limit point of a positive solution is positive. We show that even for nonnegative solutions, we will not find any ω-limit points on the boundary.

Lemma 3. *Let $\dot{x} = f(x)$ be a dynamical system defined on a neighborhood of a compact subset E of the nonnegative orthant $\overline{\mathbb{R}}^m_+$ which leaves E positively invariant. If no solution starting in E possesses an ω-limit point on the boundary ∂E, then there exists an $\varepsilon > 0$ such that*

$$\liminf_{t\to\infty} d\left(x(t), \partial E\right) \geq \varepsilon$$

for all trajectories $x(t)$ starting in the E. In particular, the system is uniformly persistent.

Proof. Suppose the claim were wrong. That is, for every $\varepsilon > 0$, there exists a solution $x(t)$ starting in E such that $\liminf_{t\to\infty} d\left(x(t), \partial E\right) < \varepsilon$. Thus, for any $\varepsilon > 0$, there exists an ω-limit point x^*_ε such that $d\left(x^*_\varepsilon, \partial E\right) < \varepsilon$. Hence, in particular for every natural number $n \in \mathbb{N}$, there exists a nonnegative ω-limit point x^*_n such that $d\left(x^*_n, \partial E\right) < \frac{1}{n}$. Since the solutions are bounded, the sequence $(x^*_n)_{n\in\mathbb{N}}$ possesses a convergent subsequence converging to a limit point on the boundary $x^* \in \partial E$. Thus, $x^* \in \partial E$ corresponds to an ω-limit point for the sequence $(x^*_n)_{n\in\mathbb{N}}$, which is a contradiction to the absence of ω-limit points on the boundary. If the statement is true for any solution starting in E, then it is in particular true for any solution starting in the interior of E. Consequently, the system is uniformly persistent. □

Lemma 4. *Let $\dot{x} = f(x)$ be a dynamical system defined on a neighborhood of a compact subset $E = [0, x_{1,\max}] \times \cdots \times [0, x_{m,\max}]$ of the nonnegative orthant $\overline{\mathbb{R}}^m_+$ that leaves E invariant and where every component x_i, $1 \leq i \leq m$, contributes to a conserved quantity that consists of at least two different components. If no solution starting in E possesses an ω-limit point x^* with $x^*_i = 0$ for some $1 \leq i \leq m$, then the system does not have any ω-limit points on the boundary ∂E, and the system is uniformly persistent on E.*

Proof. Since every component x_i, $1 \leq i \leq m$, is part of a conserved quantity, every component is bounded by the starting concentration of the conserved quantities it is involved in. Hence, $x_{i,\max}$ corresponds to the smallest value of conserved quantities to which x_i contributes. Therefore, the other components of this conserved quantity take the value zero whenever $x_i = x_{i,\max}$. That means, there is at least one $j \neq i$ with $x_j = 0$. As it is assumed that the system does not have any ω-limit points x^* where $x^*_k = 0$ for some $1 \leq k \leq m$ for solutions starting in E, it follows that the system cannot possess any ω-limit points on the boundary where $x^*_j = x_{j,\max}$ for any $1 \leq j \leq m$. Hence, the system does not have any ω-limit points on the boundary ∂E for all solutions starting in E which implies uniform persistence by Lemma 3. □

The kinetic proofreading systems respect the principle of conservation of mass such that all species included contribute to a conserved quantity, and following mass action kinetics, the decay of a species depends on its own present concentrations such that $\dot{x}_i = f_i(x) = f_{1,i}(x) - x_i f_{i,2}(x)$ for every species concentration x_i. Thus, it follows from Lemma 1 that the nonnegative orthant is positively invariant

and from Lemma 2 that there is a compact invariant set of the form $E = [0, x_{1,\max}] \times \cdots \times [0, x_{m,\max}]$. From Lemma 4 it follows that, if the systems do not have any ω-limit points on the boundary with $x_i^* = 0$ for some $1 \leq i \leq m$ for a solution starting in E, then the systems do not possess any ω-limit points on the boundary ∂E for any solution starting in E and are, in particular, uniformly persistent on E.

Corollary 1. *If the kinetic proofreading systems with Lck (2.2) and ZAP-70 (2.5) do not possess any ω-limit point with $x_i^* = 0$ for some component for a solution starting in the compact invariant set associated to a choice of positive conserved quantities, then no solution within the compact set possesses an ω-limit point on the boundary, and the systems are uniformly persistent.*

Thus, it remains to show that the kinetic proofreading systems do not possess an ω-limit point x^* on the boundary of the nonnegative orthant. From Lemma 2 we know that if $x_i^* = 0$ for some $1 \leq i \leq m$, then $f_i(x^*) = 0$ holds. We will use this property to show that the kinetic proofreading systems do not possess any ω-limit points on the boundary and are uniformly persistent. Both kinetic proofreading models include the enzyme Lck, which catalyzes the transfer of phosphate groups to the ITAMs of the T cell receptor complex that is bound to an antigen-presenting cell. We will show that given enzyme-substrate reactions where the total enzyme concentration is a conserved quantity and there is no overlap between the set of substrates and the enzyme, the system possesses no ω-limit points on the boundary where the enzyme concentration is zero or maximal [83]. To see this we are looking at the following enzyme-substrate reaction

$$\mathrm{S}_i + \mathrm{E} \underset{k_{i,\mathrm{off}}}{\overset{k_{i,\mathrm{on}}}{\rightleftharpoons}} \mathrm{X}_i \xrightarrow{k_{i,\mathrm{cat}}} \mathrm{P}_i + \mathrm{E}$$

where S_i is a substrate that is converted by the enzyme E via an enzyme-substrate complex X_i to a product P_i. Let I be the index set of all substrates whose conversion is catalyzed by the same enzyme E. Then the total enzyme concentration is a conserved quantity and corresponds to the sum of the free enzyme concentration and the concentrations of all enzymes bound in an enzyme-substrate complex:

$$E_{\mathrm{tot}} = E + \sum_{i \in I} X_i.$$

Whereas the free enzyme concentration only contributes to the conserved quantity E_{tot}, the enzyme-substrate complexes are also part of the conserved quantity for the substrate in the case that the latter contributes to such a quantity. With the assumption that the enzyme E only takes part in the above reactions catalyzing the conversion of a substrate, the differential equation for its concentration change and the concentration change of the enzyme-substrate complexes are given by

$$\begin{aligned} \dot{E} &= -\sum_{i \in I} k_{i,\mathrm{on}} S_i E + \sum_{i \in I} \left(k_{i,\mathrm{off}} + k_{i,\mathrm{cat}}\right) X_i \\ \dot{X}_i &= k_{i,\mathrm{on}} S_i E - \left(k_{i,\mathrm{off}} + k_{i,\mathrm{cat}}\right) X_i, \quad i \in I. \end{aligned}$$

We know from Lemma 2 that for a nonnegative solution for a positive conserved quantity E_{tot} to have an ω-limit point with $E^* = 0$, the equality $\dot{E}|_{E^*=0} = 0$ has to hold. Hence, we get:

$$\dot{E}\big|_{E^*=0} = \sum_{i \in I} \left(k_{i,\mathrm{off}} + k_{i,\mathrm{cat}}\right) X_i^* = 0 \quad \Leftrightarrow \quad X_i^* = 0 \text{ for all } i \in I \quad \Leftrightarrow \quad E_{\mathrm{tot}} = 0,$$

a contradiction to the condition that E_{tot} is supposed to be positive. Applying the same lemma to the enzyme-substrate complexes X_i, we get:

$$\dot{X}_i\big|_{X_i^*=0} = k_{i,\mathrm{on}} S_i^* E^* = 0 \quad \Leftrightarrow \quad S_i^* = 0$$

as $E^* > 0$ for every potential ω-limit point. Thus, the following relation holds for every $i \in I$ for an ω-limit point of an enzyme-substrate reaction:

$$X_i^* = 0 \quad \Rightarrow \quad S_i^* = 0.$$

Transferred to the kinetic proofreading systems, the enzyme E equals the kinase Lck, which only takes part in the reactions where it catalyzes the phosphorylation of a substrate and thus acts as the enzyme that emerges unchanged from the reaction. Therefore, the same argumentation via the conserved quantity of total enzyme concentration as above is true for Lck, and we can conclude that for both kinetic proofreading systems, the one including only Lck and the one including Lck and ZAP-70, it is not possible for a nonnegative solution to have an ω-limit point where no free Lck is present. That is, $L^* \neq 0$ holds given that the conserved quantities are positive. The substrates S_i interacting with the kinase Lck are the complexes C_i, $1 \leq i \leq n-1$, and for the system with ZAP-70 additionally the complexes C_{2n}^n and D_i, $1 \leq i \leq n-1$, (cf. Figures 2.8 and 2.10), and the enzyme-substrate complexes X_i correspond to the complexes B_i, $0 \leq i \leq n-1$, and including ZAP-70 to B_{2i}^i and B_{2i+1}^i for $0 \leq i \leq n-1$ and E_i for $0 \leq i \leq n-1$ as well. For the kinetic proofreading system with Lck follows the relation

$$B_i^* = 0 \quad \Rightarrow \quad C_i^* = 0 \tag{4.1}$$

for all $0 \leq i \leq n-1$, and for the kinetic proofreading system with Lck and ZAP-70 we get

$$\begin{aligned} B_j^{i*} = 0 \quad &\Rightarrow \quad C_j^{i*} = 0 \\ \text{and} \quad E_i^* = 0 \quad &\Rightarrow \quad D_i^* = 0 \end{aligned} \tag{4.2}$$

for $0 \leq i \leq n-1$ with $D_0 := C_{2n}^n$. The kinetic proofreading mechanism of multiple phosphorylation steps implies that the product P_i in the example of an enzyme-substrate reaction above equals the next substrate for the enzyme, that is, $\mathrm{P}_i = \mathrm{S}_{i+1}$, $0 \leq i \leq n-1$. In fact, for the kinetic proofreading systems, the conversion for the above conclusions is true as well, as we will show in the following sections. If the concentration of one of the substrates of the enzyme-substrate reaction with Lck is zero at an ω-limit point, it follows that the concentration of the enzyme-substrate complex has to be zero as well. In the next sections, we will show that, given positive conserved quantities, no nonnegative solution of the kinetic proofreading systems possesses any ω-limit point on the boundary of the nonnegative orthant.

4.2 Uniform Persistence of the Kinetic Proofreading System with Lck

We will now show that the kinetic proofreading system with Lck does not possess any ω-limit points on the boundary and is uniformly persistent by taking advantage of the relations shown in the last section. We directly continue with the relation between the substrate and the enzyme-substrate complex in the specific case of the kinetic proofreading system with Lck (2.2). We know already that a nonnegative solution cannot have an ω-limit point where the concentration of free enzyme is zero given the conserved quantities are positive, thus $L^* \neq 0$. Furthermore, we know assuming the enzyme-substrate complex concentration to be zero for an ω-limit point entails that the substrate concentration is zero as well. Looking at the system of differential equations describing the kinetic proofreading with Lck and a number of N phosphorylation steps (2.2), we note that the assumption of an ω-limit point where the substrate concentration is zero implies that the concentration of the phosphorylated enzyme-substrate complexes is zero vice versa:

$$\begin{aligned} \dot{C}_i\big|_{C_i^*=0} = k_{5+4(i-1)}B_{i-1}^* + k_{4+4i}B_i^* = 0 \quad &\Leftrightarrow \quad B_i^* = B_{i-1}^* = 0, \quad 1 \leq i \leq N-1 \\ \dot{C}_i\big|_{C_N^*=0} = k_{5+4(N-1)}B_{N-1}^* = 0 \quad &\Leftrightarrow \quad B_{N-1}^* = 0. \end{aligned} \tag{4.3}$$

Here and in the following we use again Lemma 2, that is, for an ω-limit point x^* of the system holds the consequence that $x_i^* = 0$ implies $f_i(x^*) = 0$. Given positive conserved quantities, L_{tot}, R_{tot}, M_{tot}, suppose a nonnegative solution has an ω-limit point $(R^*, M^*, L^*, C_0^*, C_1^*, \dots C_N^*, B_0^*, B_1^*, \dots, B_{N-1}^*)$ on the boundary of the nonnegative orthant. Suppose $R^* = 0$. Hence we get:

$$\begin{aligned}
\dot{R}\big|_{R^*=0} = k_2 C_0^* + \sum_{i=0}^{N-1} k_{6+4i} C_{i+1}^* = 0 \quad &\Leftrightarrow \quad C_i^* = 0 \quad \text{for all } 0 \le i \le N \\
\Rightarrow \quad 0 \overset{!}{=} \dot{C}_i\big|_{R^*=C_i^*=0} &= \begin{cases} k_4 B_0^* & \text{for } i = 0 \\ k_{5+4(i-1)} B_{i-1}^* + k_{4+4i} B_i^* & \text{for } 1 \le i \le N-1 \\ k_{5+4(N-1)} B_{N-1}^* & \text{for } i = N \end{cases} \\
\Rightarrow \quad B_i^* = 0 \text{ for all } 0 \le i \le N-1 \\
\Rightarrow \quad R_{\text{tot}} = R^* + \sum_{i=0}^{N} C_i^* + \sum_{i=0}^{N-1} B_i^* &= 0,
\end{aligned}$$

a contradiction to the assumed positivity of the conserved quantities. Hence $R^* \neq 0$. Since $\dot{R}\big|_{R^*=0} = \dot{M}\big|_{M^*=0}$ it follows analogously that $M^* \neq 0$. Suppose now $C_0^* = 0$, then

$$\dot{C}_0\big|_{C_0^*=0} = k_1 M^* R^* + k_4 B_0^* = 0 \quad \Leftrightarrow \quad (M^* = 0 \text{ or } R^* = 0) \quad \text{and} \quad B_0^* = 0,$$

a contradiction since R^* and M^* cannot equal zero as shown above. Assuming $C_i^* = 0$ for some $1 \le i \le N$ entails that B_{i-1}^* equals zero as determined in (4.3). Together with the previously determined relation that C_j^* vanishes whenever B_j^* vanishes (4.1), it follows by induction that $C_0^* = 0$ has to be zero, which is, as we already know, not possible. Thus, none of the complex concentrations C_i^*, $0 \le i \le N$, can be zero. From relation (4.3), it follows directly that if none of the receptor-antigen complexes C_i^* can equal zero, then none of the complexes B_i^* formed by the receptor-antigen complex and the enzyme Lck can be zero either at an ω-limit point. That is $B_i^* \neq 0$ for all $0 \le i \le N-1$. Hence, we have shown that given positive conserved quantities no nonnegative solution possesses an ω-limit point on the boundary of the nonnegative orthant. With Corollary 1 it follows that the kinetic proofreading system with Lck does not possess any ω-limit points on the boundary of the compact invariant set associated to a choice of positive conserved quantities. Furthermore, we know that the system exhibits at least one positive steady state. Thus, we have just proved the following theorem about the kinetic proofreading system with Lck:

Theorem 5. *Every choice of positive conserved quantities of the kinetic proofreading system with Lck (2.2) induces the invariance of a compact subset of the nonnegative orthant which does not possess any ω-limit points on the boundary for any solution starting within that subset. In particular, the system is uniformly persistent on the invariant compact subset and admits at least one positive steady state.*

4.3 Uniform Persistence of the Kinetic Proofreading System with Lck and ZAP-70

The extension of the kinetic proofreading system with Lck by the ζ-chain-associated kinase ZAP-70 (2.5) does not change the answer to the question of ω-limit points on the boundary. The kinetic proofreading system with Lck and ZAP-70 likewise does not possess any ω-limit points on the boundary for nonnegative solutions given positive conserved quantities. The kinetic proofreading system with Lck and ZAP-70 is modeled with an even number of $N = 2n$, $n \ge 1$, phosphorylation steps and has an additional conserved quantity, the total amount of ZAP-70 molecules Z_{tot} (2.6). Again, the assumption that the enzyme-substrate complex concentration with the enzyme Lck is

zero at an ω-limit point not only implies the substrate concentration to be zero (4.2), but the converse is also true. Suppose there is an ω-limit point on the boundary where a substrate of one of the enzyme-substrate reactions with Lck is zero. That is, C_{2i}^{i*} , $C_{2i+1}^{i^*}$ or $C_{2(i+1)}^{i^*}$ with $0 \leq i \leq n-1$, C_{2n}^{n*} or D_i^* with $1 \leq i \leq n-1$ is zero. With Lemma 2 it follows from the differential equations for these substrates that the concentrations of the enzyme-substrate complexes, B_{2i}^{i*}, B_{2i+1}^{i*} and E_i^* with $0 \leq i \leq n-1$, have to be zero as well:

$$
\begin{aligned}
&\dot{C}_0^0\big|_{C_0^{0*}=0} = k_1 R^{0*} M^* + k_4 B_0^{0*} = 0 \\
&\qquad \Leftrightarrow \quad \big(R^{0*} = 0 \text{ or } M^* = 0\big) \quad \text{and} \quad B_0^{0*} = 0 \\
&\dot{C}_{2i}^i\big|_{C_{2i}^{i*}=0} = k_{4+12i} B_{2i}^{i*} + k_{11+12(i-1)} C_{2i}^{i-1*} Z^* + k_{14+12(i-1)} R^{i*} M^* = 0, \qquad 1 \leq i \leq n-1 \\
&\qquad \Leftrightarrow \quad B_{2i}^{i*} = 0 \quad \text{and} \quad \big(C_{2i}^{i-1*} = 0 \text{ or } Z^* = 0\big) \quad \text{and} \quad \big(R^{i*} = 0 \text{ or } M^* = 0\big) \\
&\dot{C}_{2n}^n\big|_{C_{2n}^{n*}=0} = k_{4+12n} E_0^* + k_{11+12(n-1)} C_{2n}^{n-1*} Z^* + k_{14+12(n-1)} R^{n*} M^* = 0 \\
&\qquad \Leftrightarrow \quad E_0^* = 0 \quad \text{and} \quad \big(C_{2n}^{n-1*} = 0 \text{ or } Z^* = 0\big) \quad \text{and} \quad (R^{n*} = 0 \text{ or } M^* = 0) \\
&\dot{C}_{2i+1}^i\big|_{C_{2i+1}^{i*}=0} = k_{5+12i} B_{2i}^{i*} + k_{8+12i} B_{2i+1}^{i*} = 0, \qquad 0 \leq i \leq n-1 \\
&\qquad \Leftrightarrow \quad B_{2i}^{i*} = B_{2i+1}^{i*} = 0 \qquad (4.4) \\
&\dot{C}_{2(i+1)}^i\big|_{C_{2(i+1)}^{i*}=0} = k_{9+12i} B_{2i+1}^{i*} + k_{12+12i} C_{2(i+1)}^{i+1*} = 0, \qquad 0 \leq i \leq n-1 \\
&\qquad \Leftrightarrow \quad B_{2i+1}^{i*} = C_{2(i+1)}^{i+1*} = 0 \\
&\dot{D}_i\big|_{D_i^*=0} = k_{5+12n+4(i-1)} E_{i-1}^* + k_{4+12n+4i} E_i^* = 0, \qquad 1 \leq i \leq n-1 \\
&\qquad \Leftrightarrow \quad E_{i-1}^* = E_i^* = 0 \\
&\dot{D}_n\big|_{D_n^*=0} = k_{5+12n+4(n-1)} E_{n-1}^* = 0 \\
&\qquad \Leftrightarrow \quad E_{n-1}^* = 0.
\end{aligned}
$$

Hence, with (4.2) for $0 \leq i \leq n-1$, it holds in particular:

$$
\begin{aligned}
B_{2i}^{i*} = 0 \quad &\Leftrightarrow \quad C_{2i}^{i*} = 0 \\
B_{2i+1}^{i*} = 0 \quad &\Leftrightarrow \quad C_{2i+1}^{i*} = 0 \qquad (4.5) \\
\text{and} \qquad D_i^* = 0 \quad &\Leftrightarrow \quad E_i^* = 0
\end{aligned}
$$

with the notation $D_0 := C_{2n}^n$. Given positive conserved quantities, L_{tot}, R_{tot}, M_{tot} and Z_{tot}, suppose a nonnegative solution has an ω-limit point on the boundary of the nonnegative orthant denoted by

$$
\begin{aligned}
\Big(&R^{0*}, R^{1*}, \ldots, R^{i*}, \ldots, R^{n*}, M^*, L^*, Z^*, \\
&C_0^{0*}, C_2^{1*}, \ldots, C_{2i}^{i*}, \ldots, C_{2n}^{n*}, C_1^{0*}, C_3^{1*}, \ldots, C_{2i+1}^{i*}, \ldots, C_{2n-1}^{n-1*}, C_2^{0*}, C_4^{1*}, \ldots, C_{2(i+1)}^{i*}, \ldots, C_{2n}^{n-1*}, \\
&B_0^{0*}, B_2^{1*}, \ldots, B_{2i}^{i*}, \ldots, B_{2(n-1)}^{n-1*}, B_1^{0*}, B_3^{1*}, \ldots, B_{2i+1}^{i*}, \ldots, B_{2n-1}^{n-1*}, \\
&D_1^*, D_2^*, \ldots, D_n^*, E_0^*, E_1^*, \ldots, E_{n-1}^*\Big).
\end{aligned}
$$

We know already from the explanations about general enzyme-substrate reactions that the system cannot possess an ω-limit point where the enzyme Lck vanishes. Suppose now the concentration of ZAP-70 is zero for an ω-limit point, that is $Z^* = 0$. The ζ-chain-associated kinase ZAP-70 is also an enzyme, but acts as a substrate for the kinase Lck in the kinetic proofreading mechanism. As soon as an ITAM is doubly phosphorylated, a ZAP-70 molecule binds to it. Once all ITAMs are doubly phosphorylated and occupied by a ZAP-70 molecule, the latter ones are phosphorylated and thereby activated by the kinase Lck, which is associated to a co-receptor (cf. Figure 1.3). The activated ZAP-70 molecules then take on the role of an enzyme and phosphorylate other intracellular signaling molecules in turn, resulting in a response of the immune system. Thus, ZAP-70 is not involved in

an enzyme-substrate reaction as the enzyme Lck, and we have to check separately if an ω-limit point with vanishing ZAP-70 concentration may appear. Suppose $Z^* = 0$, then

$$\begin{aligned}\dot{Z}\big|_{Z^*=0} &= \sum_{i=0}^{n-1} k_{12+12i} C_{2(i+1)}^{i+1*} + \sum_{i=1}^{n} k_{16n+2+i} R^{i*} = \sum_{i=1}^{n} \left(k_{12+12(i-1)} C_{2i}^{i*} + k_{16n+2+i} R^{i*}\right) = 0 \\ &\Leftrightarrow \quad C_{2i}^{i*} = R^{i*} = 0 \quad \text{for all} \quad 1 \le i \le n.\end{aligned}$$

Thus, we get for R^{n*} the following relation:

$$\dot{R}^n\big|_{R^{n*}=0=C_{2n}^{n*}} = \sum_{i=1}^{n} k_{6+12n+4(i-1)} D_i^* = 0 \quad \Leftrightarrow \quad D_i^* = 0 \quad \text{for all} \quad 1 \le i \le n,$$

and for all other receptors R^{i*} with $1 \le i \le n-1$ bound ZAP-70 molecules:

$$\begin{aligned}\dot{R}^i\big|_{R^{i*}=0=C_{2i}^{i*}} &= k_{6+12i} C_{2i+1}^{i*} + k_{10+12i} C_{2(i+1)}^{i*} + k_{16n+2+i+1} R^{i+1*} = 0 \\ &\Leftrightarrow \quad C_{2i+1}^{i*} = C_{2(i+1)}^{i*} = R^{i+1*} = 0 \quad \text{for all} \quad 1 \le i \le n-1.\end{aligned}$$

This implies together with (4.5) that

$$B_{2i}^{i*} = B_{2i+1}^{i*} = E_i^* = 0 \quad \text{for all} \quad 1 \le i \le n-1.$$

With $\dot{D}_i\big|_{D_i^*=0} = 0$ precisely when $E_{i-1}^* = E_i^* = 0$, in particular for $i = 1$ as shown above (4.4), we conclude $E_0^* = 0$ as well. Thus we get

$$\begin{aligned}Z_{\text{tot}} = Z^* &+ \sum_{i=1}^{n} i \left(C_{2i}^{i*} + R^{i*}\right) + \sum_{i=1}^{n-1} i \left(C_{2i+1}^{i*} + C_{2(i+1)}^{i*}\right) + \sum_{i=1}^{n-1} i \left(B_{2i}^{i*} + B_{2i+1}^{i*}\right) \\ &+ \sum_{i=1}^{n} n D_i^* + \sum_{i=0}^{n-1} n E_i^* = 0,\end{aligned}$$

a contradiction to the supposed positivity of the conserved quantities. Hence $Z^* \neq 0$. Suppose now $M^* = 0$, then

$$\begin{aligned}\dot{M}\big|_{M^*=0} &= k_2 C_0^{0*} + \sum_{i=1}^{n} k_{13+12(i-1)} C_{2i}^{i*} + \sum_{i=0}^{n-1} \left(k_{6+12i} C_{2i+1}^{i*} + k_{10+12i} C_{2(i+1)}^{i*}\right) + \sum_{i=1}^{n} D_i^* = 0 \\ &\Leftrightarrow \quad C_{2i}^{i*} = 0 \quad \text{for all} \quad 0 \le i \le n, \quad C_{2i+1}^{i*} = C_{2(i+1)}^{i*} = 0 \quad \text{for all} \quad 0 \le i \le n-1 \\ &\qquad \text{and} \quad D_i^* = 0 \quad \text{for all} \quad 1 \le i \le n.\end{aligned}$$

Together with (4.5) and $E_0^* = E_1^* = 0$ when $D_1^* = 0$ (4.4) we get

$$B_{2i}^{i*} = B_{2i+1}^{i*} = E_i^* = 0 \quad \text{for all} \quad 0 \le i \le n-1,$$

which leads again to a contradiction of the supposed positive conserved quantities:

$$M_{\text{tot}} = M^* + \sum_{i=0}^{n} C_{2i}^{i*} + \sum_{i=0}^{n-1} \left(C_{2i+1}^{i*} + C_{2(i+1)}^{i*}\right) + \sum_{i=0}^{n-1} \left(B_{2i}^{i*} + B_{2i+1}^{i*}\right) + \sum_{i=1}^{n} D_i^* + \sum_{i=0}^{n-1} E_i^* = 0.$$

Thus $M^* \neq 0$. Suppose now $R^{0*} = 0$, then

$$\begin{aligned}\dot{R}^0\big|_{R^{0*}=0} &= k_2 C_0^{0*} + k_6 C_1^{0*} + k_{10} C_2^{0*} + k_{16n+2+1} R^{1*} = 0\\ \Leftrightarrow\quad & C_0^{0*} = C_1^{0*} = C_2^{0*} = R^{1*} = 0.\end{aligned}$$

Furthermore, we have

$$\begin{aligned}\dot{R}^i\big|_{R^{i*}=0} &= k_{13+12(i-1)} C_{2i}^{i*} + k_{6+12i} C_{2i+1}^{i*} + k_{10+12i} C_{2(i+1)}^{i*} + k_{16n+2+i+1} R^{i+1*}\\ \Leftrightarrow\quad & C_{2i}^{i*} = C_{2i+1}^{i*} = C_{2(i+1)}^{i*} = R^{i+1*} = 0\end{aligned}$$

for $1 \leq i \leq n-1$. Hence, by induction we get $R^{i*} = 0$ for all $0 \leq i \leq n$ and therefore $C_{2i}^{i*} = C_{2i+1}^{i*} = C_{2(i+1)}^{i*} = 0$ for all $0 \leq i \leq n-1$. For the receptor where the total number of n ZAP-70 molecules is bound, we find

$$\begin{aligned}\dot{R}^n\big|_{R^{n*}=0} &= k_{13+12(n-1)} C_{2n}^{n*} + \sum_{i=1}^{n} k_{6+12n+4(i-1)} D_i^* = 0\\ \Leftrightarrow\quad & C_{2n}^{n*} = 0 \quad \text{and} \quad D_i^* = 0 \quad \text{for all} \quad 1 \leq i \leq n.\end{aligned}$$

With (4.5) and $E_0^* = E_1^* = 0$ when $D_0^* = 0$ (4.4), it follows again that

$$B_{2i}^{i*} = B_{2i+1}^{i*} = E_i^* = 0 \quad \text{for all} \quad 0 \leq i \leq n-1.$$

Hence we get

$$R_{\text{tot}} = \sum_{i=0}^{n} R^{i*} + \sum_{i=0}^{n} C_{2i}^{i*} + \sum_{i=0}^{n-1} \left(C_{2i+1}^{i*} + C_{2(i+1)}^{i*} + B_{2i}^{i*} + B_{2i+1}^{i*} \right) + \sum_{i=1}^{n} D_i^* + \sum_{i=0}^{n-1} E_i^* = 0.$$

Thus $R^{0*} \neq 0$. Suppose now $C_{2(i+1)}^{i*}$, C_{2i+1}^{i*} or C_{2i}^{i*} would equal zero for some $0 \leq i \leq n-1$ or $0 \leq i \leq n$, respectively. For C_0^{0*} the contradiction follows directly from $R^{0*} \neq 0 \neq M^*$ as

$$\dot{C}_0^0\big|_{C_0^{0*}=0} = k_1 R^{0*} M^* + k_4 B_0^{0*} = 0 \quad \Leftrightarrow \quad \left(R^{0*} = 0 \text{ or } M^* = 0\right) \quad \text{and} \quad B_0^{0*} = 0.$$

Taking in addition to $M^* \neq 0$ into account that $Z^* \neq 0$ as well, it follows for $1 \leq i \leq n-1$ that

$$\begin{aligned}\dot{C}_{2i}^i\big|_{C_{2i}^{i*}=0} &= k_{4+12i} B_{2i}^{i*} + k_{11+12(i-1)} C_{2i}^{i-1*} Z^* + k_{14+12(i-1)} R^{i*} M^* = 0\\ \Leftrightarrow\quad & B_{2i}^{i*} = C_{2i}^{i-1*} = R^{i*} = 0,\end{aligned}$$

and for $C_{2n}^{n*} = 0$ we get

$$\begin{aligned}\dot{C}_{2n}^n\big|_{C_{2n}^{n*}=0} &= k_{4+12n} E_0^* + k_{11+12(n-1)} C_{2n}^{n-1*} Z^* + k_{14+12(n-1)} R^{n*} M^*\\ \Leftrightarrow\quad & E_0^* = C_{2n}^{n-1*} = R^{n*} = 0.\end{aligned}$$

For C_{2i+1}^{i*} and $C_{2(i+1)}^{i*}$, $0 \leq i \leq n-1$, which represent the complexes with a singly phosphorylated ITAM and a doubly phosphorylated ITAM right before a ZAP-70 molecule binds to it, we get

$$\begin{aligned}\dot{C}_{2i+1}^i\big|_{C_{2i+1}^{i*}=0} &= k_{5+12i} B_{2i}^{i*} + k_{8+12i} B_{2i+1}^{i*} = 0 \quad \Leftrightarrow \quad B_{2i}^{i*} = B_{2i+1}^{i*} = 0\\ \dot{C}_{2(i+1)}^i\big|_{C_{2(i+1)}^{i*}=0} &= k_{9+12i} B_{2i+1}^{i*} + k_{12+12i} C_{2(i+1)}^{i+1*} = 0 \quad \Leftrightarrow \quad B_{2i+1}^{i*} = C_{2(i+1)}^{i+1*} = 0.\end{aligned}$$

With (4.5) the following three relations hold:

$$\begin{aligned}
&C_{2i}^{i*} = 0 && \Rightarrow && C_{2i}^{i-1*} = 0 && \text{for} \quad 1 \le i \le n \\
&C_{2i+1}^{i*} = 0 && \Rightarrow && B_{2i}^{i*} = C_{2i}^{i*} = 0 && \text{for} \quad 0 \le i \le n-1 \\
&C_{2(i+1)}^{i*} = 0 && \Rightarrow && B_{2i+1}^{i*} = C_{2i+1}^{i*} = 0 && \text{for} \quad 0 \le i \le n-1,
\end{aligned}$$

which leads to the following chain of implications:

$$C_{2(i+1)}^{i*} = 0 \quad \Rightarrow \quad C_{2i+1}^{i*} = 0 \quad \Rightarrow \quad C_{2i}^{i*} = 0 \quad \Rightarrow \quad C_{2i}^{i-1*} = 0.$$

Thus, we get by induction that every $C_{2(i+1)}^{i*} = 0$ or $C_{2i+1}^{i*} = 0$ for some $0 \le i \le n-1$ or $C_{2i}^{i*} = 0$ for some $1 \le i \le n$ leads to the conclusion that C_0^{0*} has to be zero, which is not possible as already shown above. Hence $C_{2i}^{i*} \neq 0$ for all $0 \le i \le n$ and $C_{2i+1}^{i*} \neq 0 \neq C_{2(i+1)}^{i*}$ for all $0 \le i \le n-1$. With relation (4.5) follows directly that $B_{2i}^{i*} \neq 0 \neq B_{2i+1}^{i*}$ for all $0 \le i \le n-1$ as well. Suppose now $R^{i*} = 0$ with $1 \le i \le n$. We know from the considerations for $R^{0*} \neq 0$ that $R^{i*} = 0$ for some $1 \le i \le n$ implies in particular $C_{2i}^{i*} = 0$, which is not possible as just shown. Thus $R^{i*} \neq 0$ for all $0 \le i \le n$. Assume $E_0^* = 0$. Then

$$\dot{E}_0\big|_{E_0^*=0} = k_{3+12n} C_{2n}^{n*} L^* = 0 \quad \Leftrightarrow \quad C_{2n}^{i*} = 0 \quad \text{or} \quad L^* = 0,$$

which is both not possible as already shown above. Hence $E_0^* \neq 0$ as well. Suppose $D_i^* = 0$ for some $1 \le i \le n$, then

$$\begin{aligned}
&\dot{D}_i\big|_{D_i^*=0} = k_{5+12n+4(i-1)} E_{i-1}^* + k_{4+12n+4i} E_i^* = 0 && \Leftrightarrow && E_{i-1}^* = E_i^* = 0 \quad \text{for} \quad 1 \le i \le n-1 \\
&\dot{D}_n\big|_{D_n^*=0} = k_{5+12n+4(n-1)} E_{n-1}^* = 0 && \Leftrightarrow && E_{n-1}^* = 0.
\end{aligned}$$

For $i = 1$ follows an immediate contradiction as $E_0^* \neq 0$ as just noted. For $i \ge 2$ we conclude with relation (4.5) the implication

$$D_i^* = 0 \quad \Rightarrow \quad E_{i-1}^* = 0 \quad \Rightarrow \quad D_{i-1}^* = 0.$$

Thus, by induction we get $E_0^* = 0$, which is not possible. Hence, we have shown that $D_i^* \neq 0$ for all $1 \le i \le n$. Again with relation (4.5), it follows that $E_i^* \neq 0$ for all $1 \le i \le n-1$. In this way, we have considered every entry of a potential ω-limit point on the boundary and shown that the system cannot possess an ω-limit point on the boundary of the nonnegative orthant by taking advantage of Lemma 2. From Corollary 1 it follows that the kinetic proofreading system with Lck and ZAP-70 does not possess any ω-limit points on the boundary of the compact invariant set associated to a choice of positive conserved quantities. Additionally, we know that the system admits at least one positive steady state. Thus, we have just proved the following theorem about the kinetic proofreading system with Lck and ZAP-70:

Theorem 6. *Every choice of positive conserved quantities of the kinetic proofreading system with Lck and ZAP-70 (2.5) induces the invariance of a compact subset of the nonnegative orthant which does not possess any ω-limit points on the boundary for any solution starting within that subset. In particular, the system is uniformly persistent on the invariant compact subset and admits at least one positive steady state.*

5 Stability under Single Phosphorylation

In this chapter, we will focus on the kinetic proofreading model with Lck (2.2) and just a single phosphorylation step, that is $N = 1$. As addressed in Chapter 2, multiple phosphorylation steps are necessary for the kinetic proofreading model to satisfy the phenomenon of antigen discrimination. Thus, reducing the number of phosphorylation steps to a single one drastically diminishes the quality of the antigen discrimination since the specificity of the process is decreased. After binding to a receptor, a self antigen then only has to survive one phosphorylation step to evoke an immune response, which could be fatal as the immune system should not fight self-peptides but exclusively foreign proteins, in particular infectious pathogens that invaded the body (cf. Chapter 1). Thus, we are not reducing the kinetic proofreading system with Lck to a single phosphorylation step because it is of biological relevance for a healthy body, but to get a deeper understanding of the kinetic proofreading mechanism. Here we only consider the kinetic proofreading model with Lck since the smallest model with Lck and ZAP-70 already incorporates two phosphorylation steps (cf. Section 2.4).

In Section 3.1 we have shown by applying the Deficiency One Algorithm that the kinetic proofreading system with Lck and a single phosphorylation step, $N = 1$, is not able to support multiple steady states within the same stoichiometric compatibility class. Furthermore, we have stated in Chapter 4 the existence of at least one positive steady state in each stoichiometric compatibility class for the kinetic proofreading systems as a consequence of Brouwer's fixed point theorem. Thus, the kinetic proofreading system with Lck and one phosphorylation step possesses a unique positive steady state. It is the only system considered here that does not support multistationarity. The kinetic proofreading system with Lck and the kinetic proofreading system with Lck and ZAP-70 are both able to support multiple steady states if the system incorporates at least two phosphorylation steps (cf. Sections 3.3 and 3.4). Therefore, having a unique steady state is a feature of the system with only one phosphorylation step. It is also possible to show the uniqueness of this steady state directly from the differential equations. Assuming that two different steady states exist for the same conserved quantities results in a contradiction [83]. We will not carry out these calculations here since we already determined the positive steady state to be unique. The steady state being unique raises the question if this steady state can be globally stable. In the next section, we will give general criteria for the local and global stability of steady states of a dynamical system. In the subsequent section, we will then apply these criteria to the kinetic proofreading system with Lck and $N = 1$ to determine the local stability of the steady state and find conditions under which it is globally stable. The last section of this chapter gives a supplementary result for the kinetic proofreading systems with two or three phosphorylation steps, addressing the flow under a quasi-steady state assumption.

5.1 Stability Criteria for Steady States of Dynamical Systems

In this section, we will first give an overview of common stability criteria for the local and global stability of a steady state of a dynamical system and the nonexistence of periodic solutions. Following that, we will introduce a further generalization of these criteria given by Li and Muldowney involving compound matrices and a matrix measure.

A steady state x^* of a dynamical system $\dot{x} = f(x)$ (cf. Section 4.1) is called *stable* if every solution $x(t)$ starting close to x^* stays close for all time, that is, for every neighborhood V of x^* there exists a neighborhood $U \subset V$ such that every solution with $x(t_0) \in U$ fulfills $x(t) \in V$ for all time $t \geq t_0$. A

steady state x^* is called *asymptotically stable* if U can be chosen such that $x(t) \to x^*$ as $t \to \infty$ [37]. A point x^* where the latter property is fulfilled, that is, there is a neighborhood around x^* such that all solutions in this neighborhood tend to x^* as $t \to \infty$, is called *attractive*. Thus, a steady state is asymptotically stable if it is stable and attractive. A steady state is *unstable* if it is not stable. The stability of a *hyperbolic* steady state, that is, a steady state in which the Jacobian matrix has no eigenvalue with zero real part, can be determined by its linearization (Hartman-Grobman theorem) [37]. A hyperbolic steady state is asymptotically stable precisely if all eigenvalues of the Jacobian matrix at this point have negative real part [79].

A criterion that ensures that all eigenvalues have negative real part was given by Routh and Hurwitz independently [86, 46]. The *Routh-Hurwitz criterion* states that an equation $a_m\lambda^m + a_{m-1}\lambda^{m-1} + \cdots + a_0 = 0$ with real coefficients a_i, $0 \leq i \leq m$, and $a_m > 0$ has only roots with negative real part if and only if all Hurwitz determinants D_k, $k = 1, \ldots, m$, are positive. The Hurwitz determinants are given by the following determinants of a $k \times k$ matrix:

$$D_k = \begin{vmatrix} a_{m-1} & a_{m-3} & a_{m-5} & \cdots & a_{m-(2k-1)} \\ a_m & a_{m-2} & a_{m-4} & \cdots & a_{m-(2k-2)} \\ 0 & a_{m-1} & a_{m-3} & \cdots & a_{m-(2k-3)} \\ \vdots & \vdots & \vdots & \ddots & \\ 0 & \cdot & \cdot & & a_{m-k} \end{vmatrix}$$

where a_i is to be set zero if $i > m$ [46]. Thus, applying the Routh-Hurwitz criterion to the characteristic polynomial of the Jacobian matrix at a steady state can answer the question whether this steady state is asymptotically stable. More precisely, a steady state is hyperbolic and asymptotically stable if and only if all Hurwitz determinants are positive.

Whereas the Routh-Hurwitz criterion only applies to hyperbolic steady states, a Lyapunov function approach can be applied to hyperbolic and nonhyperbolic steady states and identify not only asymptotically stable but also stable steady states that are not attractive. A Lyapunov function is a positive definite scalar function which decreases along the curves of the differential equation [37]. The Lyapunov function approach generalizes the idea of an energy function that is decreasing in the neighborhood of a stable steady state [60]. Let x^* be a steady state of a dynamical system defined on an open set $U \subset \mathbb{R}^m$. A *Lyapunov function* is defined as a differentiable function $V : W \to \mathbb{R}$ that is defined on some neighborhood $W \subset U$ of the steady state x^* such that V is positive definite, $V(x^*) = 0$ and $V(x) > 0$ if $x \neq x^*$, and its derivative along the solution curves is nonpositive, $\dot{V}(x) \leq 0$, where

$$\dot{V}(x) = \frac{d}{dt}V(x) = \sum_{i=1}^{m} \frac{\partial V}{\partial x_i}\dot{x}_i = (\mathrm{grad}V(x))^T f(x).$$

A *strict Lyapunov function* is given if the derivative is negative, $\dot{V}(x) < 0$ in $W \backslash \{x^*\}$. If a Lyapunov function for a steady state can be found, then this steady state is stable. The existence of a strict Lyapunov function implies the asymptotic stability of the steady state [37]. With a strict Lyapunov function, it is furthermore possible to prove global stability. Suppose $x^* = 0$ were a steady state and V a strict Lyapunov function. If the additional condition $V(x) \to \infty$ with $||x|| \to \infty$ holds, then the origin is globally stable, that is, it is asymptotically stable and attractive for all solutions. The condition for global stability ensures that every level set of V is bounded [60, 55]. A specific Lyapunov function is a strong and sufficient condition for the stability of a steady state. Indeed, the existence of a Lyapunov function is a necessary condition for stability [60]. However, it is often not evident how to find such a Lyapunov function. Thus, another method to establish global stability of a unique steady state would be desirable. Furthermore, it would be nice to know if the system is able to support a periodic solution if the steady state is not globally stable.

A trajectory which is not a steady state is a *periodic solution* if there exists a $T > 0$ such that $x(t) = x(t+T)$ for all times t. For planar systems, the absence of periodic solutions can be shown by *Bendixson's* or *Dulac's criterion*. Bendixson's criterion states that a continuously differentiable function which is defined on a simply connected open subset D of $\mathbb{R}^2$ does not possess a periodic solution lying entirely in D if $\operatorname{div}(f)$ is not identically zero and does not change sign on D. Dulac generalized this criterion proving that, under the same conditions, a system does not have a periodic solution lying entirely in D if $\operatorname{div}(\alpha f)$ is not identically zero and does not change sign on D, where α is a continuously differentiable function on D [79, 63]. For planar systems, Poincaré-Bendixson Theory gives further information about the nature of ω-limit points within compact subsets. The *Poincaré-Bendixson theorem* for continuously differentiable planar systems that are defined on an open subset of the plane $\mathbb{R}^2$ states that if the positive half-trajectory of a system is contained in a compact subset of the open subset it is defined on and this compact subset has only a finite number of steady states, it follows that the ω-limit set of the trajectory is either a steady state, a periodic solution or consists of a finite number of steady states and a countable number of trajectories connecting them [79, 37]. The latter are referred to as *heteroclinic* or *homoclinic orbits* depending on whether they connect distinct points or a point to itself [37].

Smith was able to find conditions under which a generalization of Bendixson's and Dulac's criterion for higher dimensions is possible [92, 93]. Whereas Bendixson's and Dulac's criterion apply for both signs of $\operatorname{div}(f)$ or $\operatorname{div}(\alpha f)$, respectively, Smith developed two different criteria which correspond for planar systems to the positive or negative sign of the divergences just considered. The former includes, amongst other conditions, the existence of a quadratic form that fulfills certain characteristics [92]. For the latter a quadratic form of this kind does not have to be found, but the criterion can be applied directly to the given dynamical system $\dot{x} = f(x)$ defined on $\mathbb{R}^m$. Let $\lambda_1(x), \ldots, \lambda_m(x)$ denote the eigenvalues of the symmetric matrix $\frac{1}{2}\left(\left(\frac{\partial f}{\partial x}\right)^T + \frac{\partial f}{\partial x}\right)$ arranged so that $\lambda_1 \geq \lambda_2 \geq \ldots \geq \lambda_m$. Here $\frac{\partial f}{\partial x}$ denotes the Jacobian matrix of f and T the transpose. Smith proved that if $\lambda_1(x) + \lambda_2(x) < 0$ on $\mathbb{R}^m$, then all bounded semi-orbits converge to a steady state [93]. That is, every nonconstant solution $x(t)$ converges for $t \to \infty$ and $t \to -\infty$. In particular, it follows that no periodic solution can exist. Smith extended the criterion also to simply-connected subsets of $\mathbb{R}^m$. Muldowney gave a generalized Bendixson criterion involving a quantity that we refer to as matrix measure and compound matrices. It states that if the matrix measure of the second additive compound matrix associated to a dynamical system is strictly negative or strictly positive for all $x \in \mathbb{R}^m$, then the system has no periodic solution [73]. The matrix measure itself is not a norm but involves the choice of a vector norm. If the Euclidean norm is chosen, then the criterion for a negative matrix measure of the second additive compound matrix reduces to Smith's criterion. Li and Muldowney further generalized the criterion involving the matrix measure and were able to make statements not only about the nonexistence of periodic solutions but also about global stability given that the system possesses a unique steady state which is locally asymptotically stable [63, 65, 64].

The stability of a steady state is determined by the real part of the eigenvalues of the Jacobian matrix in this steady state. More precisely, a steady state can only be stable if all eigenvalues have a nonpositive real part. The steady state is asymptotically stable, if all real parts are negative. The characteristic polynomial of an $(m \times m)$-matrix A is given by $\det(A - \lambda I_m) = (-1)^m\lambda^m + (-1)^{m-1}\operatorname{tr}(A)\lambda^{m-1} + \cdots + \det(A) = \prod_{i=1}^{m}(\lambda_i - \lambda) = \sum_{k=0}^{m}(-1)^k b_k \lambda^k$ where $b_k = \sum_{1 \leq i_1 < \cdots < i_k \leq m} \prod_{j=1}^{k} \lambda_{i_j}$ and $\lambda_1, \ldots, \lambda_m$ denote the eigenvalues of A, thus in particular $\operatorname{tr}(A) = \sum_{k=1}^{m} \lambda_k$ and $\det(A) = \prod_{k=1}^{m} \lambda_i$ [43]. Hence, the information about the stability of a steady state is stored in the signs of the sum and the different products of the eigenvalues. For planar systems, this gives the familiar criterion for hyperbolic steady states that the stability is determined by the signs of the determinant and the trace of the Jacobian matrix at the steady state. If the determinant is positive and the trace negative, then the hyperbolic steady state is asymptotically stable. A negative determinant or a positive trace in turn imply instability. For higher dimensional systems, the additional information of the sign of the various products of eigenvalues is necessary

to determine the stability of the steady state. This leads to *compound matrices*, more precisely additive and multiplicative compound matrices, which store information about the sum and the product of eigenvalues.

Definition 27. [7, 73] Let $A \in \mathbb{C}^{n\times m}$ and $k \in \{1,\dots,\min(n,m)\}$ be fixed. The *k-th multiplicative compound* of A, denoted $A^{(k)}$, is the $\binom{n}{k} \times \binom{m}{k}$ matrix that contains all the k-minors of A ordered lexicographically.

For example the second multiplicative matrix of a 3×3 matrix $A = (a_{ij})$ is given by

$$A^{(2)} = \begin{pmatrix} \begin{vmatrix} a_{11} & a_{12} \\ a_{21} & a_{22} \end{vmatrix} & \begin{vmatrix} a_{11} & a_{13} \\ a_{21} & a_{23} \end{vmatrix} & \begin{vmatrix} a_{12} & a_{13} \\ a_{22} & a_{23} \end{vmatrix} \\ \begin{vmatrix} a_{11} & a_{12} \\ a_{31} & a_{32} \end{vmatrix} & \begin{vmatrix} a_{11} & a_{13} \\ a_{31} & a_{33} \end{vmatrix} & \begin{vmatrix} a_{12} & a_{13} \\ a_{32} & a_{33} \end{vmatrix} \\ \begin{vmatrix} a_{21} & a_{22} \\ a_{31} & a_{32} \end{vmatrix} & \begin{vmatrix} a_{21} & a_{23} \\ a_{31} & a_{33} \end{vmatrix} & \begin{vmatrix} a_{22} & a_{23} \\ a_{32} & a_{33} \end{vmatrix} \end{pmatrix}.$$

In particular, if A is an $m \times m$ square matrix, then the m-th multiplicative compound matrix of A is just its determinant, that is, $A^{(m)} = \det(A)$. The term multiplicative results from the property

$$(AB)^{(k)} = A^{(k)}B^{(k)}$$

for two matrices $A \in \mathbb{C}^{n\times m}$ and $B \in \mathbb{C}^{m\times p}$ with $k \in \{1,\dots,\min(n,m,p)\}$, which follows from the Binet-Cauchy formula [73, 7]. The Binet-Cauchy formula states that the determinant of the product of an $m \times n$ matrix A and an $n \times m$ matrix B equals the sum of all products of m-minors of A with the corresponding minors of B, that is [35],

$$\det(AB) = \sum_{1\le k_1<k_2<\cdots<k_m\le n} \det \begin{pmatrix} a_{1k_1} & \cdots & a_{1k_m} \\ \vdots & & \vdots \\ a_{mk_1} & \cdots & a_{mk_m} \end{pmatrix} \det \begin{pmatrix} b_{1k_1} & \cdots & b_{1k_m} \\ \vdots & & \vdots \\ b_{mk_1} & \cdots & b_{mk_m} \end{pmatrix}.$$

A direct consequence of this property is that the eigenvalues of $A^{(k)}$ are just the products of all combinations of k different eigenvalues of A. Let $A \in \mathbb{C}^{m\times m}$ and let λ_i, $i = 1,\dots,m$, denote the eigenvalues of A. Denote by v^i, $i = 1,\dots,m$, the eigenvector corresponding to λ_i. Then

$$A \begin{pmatrix} v^{i_1} & \cdots & v^{i_k} \end{pmatrix} = \begin{pmatrix} v^{i_1} & \cdots & v^{i_k} \end{pmatrix} \operatorname{diag}(\lambda_{i_1},\dots,\lambda_{i_k})$$

holds. With the multiplicative property resulting from the Binet-Cauchy formula follows [7]

$$\begin{aligned} A^{(k)} \begin{pmatrix} v^{i_1} & \cdots & v^{i_k} \end{pmatrix}^{(k)} &= \begin{pmatrix} v^{i_1} & \cdots & v^{i_k} \end{pmatrix}^{(k)} \operatorname{diag}(\lambda_{i_1},\dots,\lambda_{i_k})^{(k)} \\ &= \left(\prod_{l=1}^{k} \lambda_{i_l} \right) \begin{pmatrix} v^{i_1} & \cdots & v^{i_k} \end{pmatrix}^{(k)}. \end{aligned}$$

Thus, the eigenvalues of $A^{(k)}$ are the $\binom{m}{k}$ products [7]

$$\left\{ \prod_{l=1}^{k} \lambda_{i_l} : 1 \le i_1 < i_2 < \cdots < i_k \le m \right\}.$$

The geometric interpretation of a k-th multiplicative compound matrix is linked to the evolution of a k-dimensional parallelotope subject to a linear time-varying system [7]. Let $a_1,\dots,a_k$ be vectors

in $\mathbb{R}^m$ with $1 \leq k \leq m$. Then the parallelotope spanned by these vectors is given by the compact set

$$P(a_1, \dots a_k) = \left\{ \sum_{i=1}^{k} r_i a_i : r_i \in [0,1] \right\}.$$

Let $A = (a_1 \cdots a_k)$ be the matrix consisting of the vectors generating this parallelotope. Given $\text{rank}(A) = k$, then the volume of the parallelotope is given by the root of the Gram determinant:

$$\text{vol}\left(P(a_1, \dots, a_k)\right) = \sqrt{\det\left(A^T A\right)}.$$

Since $A^T A$ is a $k \times k$ square matrix, its determinant corresponds to the k-th multiplicative compound matrix. With the multiplicative property resulting from the Binet-Cauchy formula, we get

$$\det\left(A^T A\right) = \left(A^T A\right)^{(k)} = \left(A^T\right)^{(k)} A^{(k)} = \left(A^{(k)}\right)^T A^{(k)}.$$

Thus, the volume of a k-dimensional parallelotope is given by the Gram matrix of the k-th multiplicative compound matrix:

$$\text{vol}\left(P(a_1, \dots, a_k)\right) = \sqrt{\left(A^{(k)}\right)^T A^{(k)}}.$$

For $k = m$ this reduces to the familiar formula $\text{vol}\left(P(a_1, \dots, a_k)\right) = |\det(A)|$.

We now want to examine how such a parallelotope evolves over time under the dynamics of a linear dynamical system. To do this, we consider a three-dimensional linear dynamical system, $\dot{x} = Ax$, where A has diagonal entries only, that is,

$$\dot{x} = \text{diag}(\lambda_1, \lambda_2, \lambda_3)\, x$$

with $\lambda_i \in \mathbb{R}$. The solutions of a linear system $\dot{x} = Ax$ with $x(0) = x_0$ are given by $e^{At}x_0$ where $e^{At} = \sum_{n=0}^{\infty} \frac{A^n t^n}{n!}$. For x_0 being a vector of the canonical basis in $\mathbb{R}^3$, denoted $\hat{e}_i$, $i = 1, 2, 3$, the solution of the simple linear system above is given by $x(t) = e^{\lambda_i t}\hat{e}_i$. Thus $e^{\lambda_i t}$ describes the evolution of the line given by the vector $\hat{e}_i$ [7]. Suppose now $x_0 = \hat{e}_i + \hat{e}_j$ with $i \neq j$, that is, we span a square generated by two canonical vectors. The differential equation we are investigating then describes the evolution of this square over time. Instead of the length of a line, we are now looking at the surface of a rectangle. A solution is then given by $x(t) = e^{\lambda_i t}\hat{e}_i + e^{\lambda_j t}\hat{e}_j$. That is, the initial rectangle changes with $e^{\lambda_i t}$ and $e^{\lambda_j t}$ in the directions of $\hat{e}_i$ and $\hat{e}_j$. The area of the rectangle is then given by $e^{\lambda_i t} \cdot e^{\lambda_j t} = e^{(\lambda_i + \lambda_j)t}$. If we span a three-dimensional cube with all three canonical basis vectors, the evolution of the volume of this cube is given by $e^{(\lambda_1 + \lambda_2 + \lambda_3)t}$. Thus, a matrix that stores the sums of any two eigenvalues and one that gives the sum of all three eigenvalues would provide information about the evolution of the square and the cube, special cases of a two-dimensional and a three-dimensional parallelotope [7]. This leads to the additive compound matrices, whose eigenvalues are just the sums of eigenvalues of the original matrix.

Definition 28. [7, 72] Let $A \in \mathbb{C}^{m \times m}$ and $k \in \{1, \dots, m\}$ be fixed. The *k-th additive compound* of A, denoted $A^{[k]}$, is the $\binom{m}{k} \times \binom{m}{k}$ matrix defined by

$$A^{[k]} := \frac{d}{dt}\left(I_m + tA\right)^{(k)}\Big|_{t=0} = \lim_{h \to 0} \frac{1}{h}\left(\left(I_m + hA\right)^{(k)} - I_m^{(k)}\right) = \lim_{h \to 0} \frac{1}{h}\left(\left(I_m + hA\right)^{(k)} - I_r\right)$$

where I_m denotes the $m \times m$ identity matrix and $r = \binom{m}{k}$.

Thus for $k = 1$, the additive compound matrix is just the matrix itself, $A^{[1]} = A$, and the m-th additive compound matrix corresponds to the trace of the matrix since the m-th multiplicative

compound matrix equals the determinant,

$$A^{[m]} = \frac{d}{dt}\left(I_m + tA\right)^{(m)}\Big|_{t=0} = \frac{d}{dt}\det(I_m + tA)\Big|_{t=0} = \operatorname{tr}(A).$$

The term additive results from the property that

$$(A+B)^{[k]} = A^{[k]} + B^{[k]}$$

for two square matrices $A, B \in \mathbb{C}^{m\times m}$, which results from the fact that $A^{[k]}$ is the coefficient of the first-order term in the Taylor expansion of $(I+tA)^{(k)}$ [7]:

$$(I_m + tA)^{(k)} = I_m^{(k)} + \frac{d}{dt}\left(I_m + tA\right)^{(k)}\Big|_{t=0} \cdot t + \mathcal{O}(t^2) = I_r + tA^{[k]} + \mathcal{O}(t^2)$$

where $r = \binom{m}{k}$. This implies that the map from a matrix to its additive compound is additive [7]:

$$\begin{aligned} I_r + t(A+B)^{[k]} &= (I_m + t(A+B))^{(k)} + \mathcal{O}(t^2) = \left((I_m + tA)\left(I_m + tB\right)\right)^{(k)} + \mathcal{O}(t^2) \\ &= (I_m + tA)^{(k)}\left(I_m + tB\right)^{(k)} + \mathcal{O}(t^2) = \left(I_r + tA^{[k]}\right)\left(I_r + tB^{[k]}\right) + \mathcal{O}(t^2) \\ &= I_r + t\left(A^{[k]} + B^{[k]}\right) + \mathcal{O}(t^2). \end{aligned}$$

Thus, by the continuity of the mapping $A \to A^{[k]}$, it follows that $(A+B)^{[k]} = A^{[k]} + B^{[k]}$ [7]. Let again λ_i, $i = 1, \ldots, m$, denote the eigenvalues of A and v^i, $i = 1, \ldots, m$, the eigenvector corresponding to λ_i. The eigenvalues of the additive compound matrix $A^{[k]}$ can be identified by using the multiplicative property of the multiplicative compound matrices:

$$\begin{aligned} & A\begin{pmatrix} v^{i_1} & \cdots & v^{i_k}\end{pmatrix} = \begin{pmatrix} v^{i_1} & \cdots & v^{i_k}\end{pmatrix} \operatorname{diag}\left(\lambda_{i_1}, \ldots, \lambda_{i_k}\right) \\ \Rightarrow \quad & (I + tA)\begin{pmatrix} v^{i_1} & \cdots & v^{i_k}\end{pmatrix} = \begin{pmatrix} v^{i_1} & \cdots & v^{i_k}\end{pmatrix}\left(I + t\operatorname{diag}\left(\lambda_{i_1}, \ldots, \lambda_{i_k}\right)\right) \\ \Rightarrow \quad & (I + tA)^{(k)}\begin{pmatrix} v^{i_1} & \cdots & v^{i_k}\end{pmatrix}^{(k)} = \begin{pmatrix} v^{i_1} & \cdots & v^{i_k}\end{pmatrix}^{(k)}\left(I + t\operatorname{diag}\left(\lambda_{i_1}, \ldots, \lambda_{i_k}\right)\right)^{(k)} \\ \Rightarrow \quad & A^{[k]}\begin{pmatrix} v^{i_1} & \cdots & v^{i_k}\end{pmatrix}^{(k)} = \begin{pmatrix} v^{i_1} & \cdots & v^{i_k}\end{pmatrix}^{(k)}\left(\operatorname{diag}\left(\lambda_{i_1}, \ldots, \lambda_{i_k}\right)\right)^{[k]} \\ & \qquad\qquad = \begin{pmatrix} v^{i_1} & \cdots & v^{i_k}\end{pmatrix}^{(k)} \operatorname{tr}\left(\operatorname{diag}\left(\lambda_{i_1}, \ldots, \lambda_{i_k}\right)\right). \end{aligned}$$

Thus, the eigenvectors of the additive compound matrix $A^{[k]}$ and the multiplicative compound matrix $A^{(k)}$ are the same, and the eigenvalues of $A^{[k]}$ are the $\binom{m}{k}$ sums of the eigenvalues λ_i of A [72, 7]:

$$\left\{\sum_{l=1}^{k}\lambda_{i_l} : 1 \le i_1 < i_2 < \cdots < i_k \le m\right\}.$$

Definition 28 of the additive compound matrix gives the following formula for the entries of the matrix. For an integer, $i \in \{1, \ldots, \binom{m}{k}\}$ let $(i) = (i_1, \ldots, i_k)$ be the i-th member in lexicographic ordering of all k-tuples with $1 \le i_1 < i_2 < \cdots < i_k \le m$. Then the entries b_{ij} for the additive compound matrix $B = A^{[k]}$ are given by [73]:

$$b_{ij} = \begin{cases} \sum_{l=1}^{k} a_{i_l i_l}, & \text{if } (i) = (j), \\ (-1)^{l+s} a_{i_l j_s}, & \text{if } (i) \text{ differs from } (j) \text{ in exactly one entry } i_l \neq j_s \\ 0, & \text{if } (i) \text{ differs from } (j) \text{ in two or more entries.} \end{cases} \tag{5.1}$$

Thus, the second additive compound matrix of a 3×3 matrix A is for example given by

$$A^{[2]} = \begin{pmatrix} a_{11}+a_{22} & a_{23} & -a_{13} \\ a_{32} & a_{11}+a_{33} & a_{12} \\ -a_{31} & a_{21} & a_{22}+a_{33} \end{pmatrix}.$$

We now want to consider multiplicative and additive compound matrices in the context of linear time-varying dynamical systems

$$\dot{x} = A(t)x$$

where $x(t) \in \mathbb{R}^m$ and $A(t)$ is a continuous $m \times m$ real or complex matrix-valued function. Let $X(t)$ be a fundamental matrix for the linear dynamical system. Thus X is an $m \times m$ matrix. Then $Y(t) = X^{(k)}(t)$ with $1 \leq k \leq m$ is a $\binom{m}{k} \times \binom{m}{k}$ fundamental matrix for the *k-th compound system* [72]:

$$\dot{y} = A^{[k]}(t)y.$$

This follows from the Taylor expansion of the fundamental matrix,

$$X(t+h) = (I_m + hA(t))\, X(t) + \mathcal{O}(h^2),$$

and by applying the Taylor expansion for the multiplicative compound matrix $(I + hA)^{(k)}$:

$$\begin{aligned} X^{(k)}(t+h) &= (I_m + hA(t))^{(k)}\, X^{(k)}(t) + \mathcal{O}(h^2) = \left(I_m^{(k)} + hA^{[k]}(t)\right) X^{(k)} + \mathcal{O}(h^2) \\ &= X^{(k)}(t) + hA^{[k]}(t)X^{(k)}(t) + \mathcal{O}(h^2). \end{aligned}$$

Thus, the multiplicative compound of the fundamental matrix, $X^{(k)}$, is a matrix solution for the linear system $\dot{y} = A^{[k]}y$:

$$\frac{d}{dt}\left(X^{(k)}(t)\right) = \lim_{h\to 0} \frac{X^{(k)}(t+h) - X^{(k)}(t)}{h} = \lim_{h\to 0}\left(A^{[k]}(t)X^{(k)}(t) + \mathcal{O}(h)\right) = A^{[k]}(t)X^{(k)}(t).$$

In particular, a fundamental matrix for the linear system $\dot{x} = Ax$, where $A(t) = A$ is a constant matrix and not time-varying, is given by $X(t) = \exp(At)$, and a fundamental matrix for the k-th compound system $\dot{y} = A^{[k]}y$ is given by $Y(t) = \exp\left(A^{[k]}t\right)$. Hence, setting $t = 1$ implies the equation [72]

$$(\exp(A))^{(k)} = \exp\left(A^{[k]}\right).$$

For a steady state of the linear system $\dot{x} = Ax$ to be stable, solutions $x(t) = c\exp(At)$, where $c \in \mathbb{R}$, starting close to the steady state have to stay close for all time. Thus, $\exp(At)$ has to be bounded for all $t \geq t_0$, which translates into the characterization for hyperbolic steady states that the real part of all eigenvalues has to be negative. The definition of the exponential function implies the upper estimate

$$||\exp(tA)|| \leq \exp(t||A||)$$

for all $t \geq 0$. However, this is just a rough estimate. Instead of $||A||$ a smaller constant can be found such that the inequality still holds. The least constant for which the estimate holds is given by the following *matrix measure* [57].

Definition 29. [7, 14] Let A be real or complex $m \times m$ matrix and let $||\cdot||$ be an induced matrix norm, $||A|| = \max_{||x||=1} ||Ax||$ for some vector norm $||\cdot||$. Then the corresponding *matrix measure*

is defined by

$$\mu(A) := \lim_{h \searrow 0} \frac{||I + hA|| - 1}{h} = D_+ \, ||I + tA||_{t=0} \, .$$

The quantity was introduced independently by Dahlquist and Lozinskii [16, 67]. In the literature this matrix measure is also referred to as *Lozinskii measure*, *logarithmic norm* or *Lozinskii logarithmic norm* [63, 99, 94, 57]. Note that the matrix measure is neither a norm nor a measure. The matrix measure can take on negative values. Some basic properties that follow from the definition of the matrix measure are the positive homogeneity and subadditivity [14, 18]:

$$\begin{aligned} \mu(cA) &= c\mu(A) \quad \text{for all } c \geq 0, \\ \mu(A + B) &\leq \mu(A) + \mu(B). \end{aligned}$$

Looking at the left-hand derivative instead of the right-hand derivative, that is a negative h, leads to an expression corresponding to the negative matrix measure of the negative matrix:

$$\lim_{h \nearrow 0} \frac{||I + hA|| - 1}{h} = \lim_{h \searrow 0} \frac{||I - hA|| - 1}{-h} = -\mu(-A).$$

In particular, the following estimates hold:

$$-||A|| \leq -\mu(-A) \leq \mathrm{Re}\,(\lambda_i) \leq \mu(A) \leq ||A||$$

where λ_i, $1 \leq i \leq m$, denote the eigenvalues of A [18]. Thus, given that $\mu(A)$ is negative, all eigenvalues of A have negative real part, and the origin is a stable steady state of the linear system $\dot{x} = Ax$. The matrix measure may be applied not only to linear but also to time-varying linear systems, $\dot{x} = A(t)x$. Using $||x||$ as a Lyapunov function for the linear time-varying system $\dot{x} = A(t)x$ leads to an estimate for the right-hand derivative of $||x||$, whose negativity is ensured if the matrix measure $\mu(A(t))$ is negative [72]:

$$\begin{aligned} D_+||x(t)|| &= \lim_{h \searrow 0} \frac{||x(t+h)|| - ||x(t)||}{h} = \lim_{h \searrow 0} \frac{\big|\big|x(t) + hA(t)x(t) + \mathcal{O}(h^2)\big|\big| - ||x(t)||}{h} \\ &\leq \lim_{h \searrow 0} \frac{||I + hA(t)||\,||x(t)|| - ||x(t)||}{h} = \mu(A(t))\,||x(t)||. \end{aligned}$$

Analogously, we obtain

$$D_-||x(t)|| = \lim_{h \nearrow 0} \frac{||x(t+h)|| - ||x(t)||}{h} \geq -\mu(-A(t))\,||x(t)||.$$

Thus, we get the following upper and lower estimate:

$$||x(t_0)||\, e^{\int_{t_0}^{t} -\mu(-A(s))ds} \leq ||x(t)|| \leq ||x(t_0)||\, e^{\int_{t_0}^{t} \mu(A(s))ds}. \tag{5.2}$$

From the upper estimate it follows that the steady state at the origin of the linear time-varying system is stable if $\int_0^t \mu(A(s))ds$ is bounded for all $t \geq 0$ and asymptotically stable if $\int_0^t \mu(A(s))ds \to -\infty$ as $t \to \infty$ [72]. We already noticed that the multiplicative compound matrix $X^{(k)}$ is a fundamental matrix for the additive compound system $\dot{y} = A^{[k]}(t)y$, given that $X(t)$ is a fundamental matrix for the time-varying linear system $\dot{x} = A(t)x$. Thus, the estimates just given also hold for the compound system. Now we want to transfer the estimates made for the linear time-varying system to a general dynamical system in $\mathbb{R}^m$,

$$\dot{x} = f(x).$$

In this context, we consider the linear systems given by the linearization associated to a given solution $\phi(t) = \phi(t, x_0)$ of the system and its associated compound system [72]:

$$\dot{z} = \frac{\partial f}{\partial x}(\phi(t))\, z \quad \text{and} \quad \dot{y} = \frac{\partial f}{\partial x}^{[k]}(\phi(t))\, y$$

where $\frac{\partial f}{\partial x}$ and $\frac{\partial f}{\partial x}^{[k]}$ denote the Jacobian matrix and its k-th additive compound, respectively. We note that if $\phi(t)$ is a solution for the dynamical system, then $z = \dot{\phi}$ is a solution for the associated linearization. A fundamental matrix for the associated linearization is given by $Z = \frac{\partial \phi}{\partial x_0}(t, x_0)$ which satisfies $Z(0) = I_m$ [72]. Therefore, a fundamental matrix for the compound system is given by $Y = Z^{(k)} = \frac{\partial \phi}{\partial x_0}^{(k)}(t, x_0)$. Muldowney [73] used this relation to find a generalization of Bendixon's and Dulac's criterion for the nonexistence of periodic solutions for higher dimensional systems. The proof of the latter uses Green's theorem, which states that in the plane, the double integral of the divergence of a continuously differentiable function over a compact region with piecewise smooth boundary can be calculated via the line integral of the curve corresponding to the boundary of the compact set. Instead of curves, Muldowney considered surfaces. In that case, a *k-surface* in $\mathbb{R}^m$ is given by a continuously differentiable function $\psi_0 : D \to \mathbb{R}^m$, $u \mapsto \psi_0(u)$, where $D \subset \mathbb{R}^k$. A measure for the content of this k-surface is then given by [73, 72]

$$\int_D \left|\left| \frac{\partial \psi_0}{\partial u_1} \wedge \ldots \wedge \frac{\partial \psi_0}{\partial u_m} \right|\right| = \int_D \left|\left| \frac{\partial \psi_0}{\partial u}^{(k)} \right|\right|,$$

which corresponds to the discussion that the k-th multiplicative compound matrix stores the information about the time evolution of a parallelotope. To examine the time evolution of the k-surface ψ_0, we set $\psi_t(u) = \phi(t, \psi_0(u))$. This function is also a k-surface in $\mathbb{R}^m$ as long as it exists [72]. With $\psi_t(u) = \phi(t, \psi_0(u))$ being a solution for the dynamical system, an $m \times k$ matrix solution for the associated linear system is given by $\frac{\partial \psi_t}{\partial u} = \frac{\partial \phi}{\partial u}(t, \psi_0(u)) = \frac{\partial \phi}{\partial x_0}(t, \psi_0(u)) \frac{\psi_0}{\partial u}$. Therefore, $\frac{\partial \psi_t}{\partial u}^{(k)} = \frac{\partial \phi}{\partial x_0}^{(k)}(t, \psi_0(u)) \, \frac{\psi_0}{\partial u}^{(k)}$ is a matrix solution for the compound system. This holds for all k-surfaces $\psi_0(u)$, $u \in D$. By applying the above lower and upper estimates (5.2) to the solution of the compound system we get an estimate for the time evolution of the k-surface for $t \geq t_0$ [72]:

$$\left|\left| \frac{\partial \psi_{t_0}}{\partial u}^{(k)} \right|\right| e^{\int_{t_0}^t -\mu\left(-\frac{\partial f}{\partial x}^{[k]}(\phi(s,\psi_0))\right) ds} \leq \left|\left| \frac{\partial \psi_t}{\partial u}^{(k)} \right|\right| \leq \left|\left| \frac{\partial \psi_{t_0}}{\partial u}^{(k)} \right|\right| e^{\int_{t_0}^t \mu\left(\frac{\partial f}{\partial x}^{[k]}(\phi(s,\psi_0))\right) ds}.$$

Suppose now that the dynamical system $\dot{x} = f(x)$ possesses a periodic solution γ. Then the trajectory γ encloses a 2-surface that does not change over time. Thus, the area of this surface stays constant. Hence, if the area of all 2-surfaces decreases or increases over time, then the system cannot admit a periodic solution. In particular, the content of a 2-surface is strictly decreasing if $\mu\left(\frac{\partial f}{\partial x}^{[2]}\right) < 0$ and strictly increasing if $-\mu\left(-\frac{\partial f}{\partial x}^{[2]}\right) > 0$. This idea underlies the generalization of Bendixson's and Dulac's negative criterion by Muldowney.

Theorem 7. (Muldowney) [73] *Suppose that one of the inequalities*

$$\mu\left(\frac{\partial f}{\partial x}^{[2]}\right) < 0, \qquad \mu\left(-\frac{\partial f}{\partial x}^{[2]}\right) < 0$$

holds for all $x \in \mathbb{R}^m$. Then the dynamical system $\dot{x} = f(x)$ has no periodic solutions.

If the Euclidean norm is chosen, this corresponds to $\mu\left(\frac{\partial f}{\partial x}^{[2]}\right) = \lambda_1 + \lambda_2 < 0$ and $\mu\left(-\frac{\partial f}{\partial x}^{[2]}\right) = \lambda_{m-1} + \lambda_m < 0$ where $\lambda_1 \geq \lambda_2 \geq \cdots \geq \lambda_m$ are the eigenvalues of $\frac{1}{2}\left(\frac{\partial f}{\partial x}^T + \frac{\partial f}{\partial x}\right)$. Thus, for the Euclidean norm, the first criterion corresponds to Smith's criterion, stating that if $\lambda_1 + \lambda_2 < 0$, all bounded solutions converge to a steady state, which implies in particular the nonexistence of any periodic solution.

Li and Muldowney [63] were able to generalize Muldowney's criterion further. Instead of the evolution of areas of surfaces with a fixed boundary, they considered more general functionals. More precisely, they considered functionals on Lipschitz continuous functions mapping from the closed Euclidean unit ball in $\mathbb{R}^2$ into a subset of $\mathbb{R}^m$. In their work, Li and Muldowney refer to the latter functions as surfaces. The functionals they are considering are then given by the integral on the closed unit ball in $\mathbb{R}^2$, $\overline{U}$, over a real-valued function $S(x,y)$ with $x \in D \subset \mathbb{R}^m$ and $y \in \mathbb{R}^{\binom{m}{2}}$. For a surface ψ, the integral is then given by

$$\int_{\overline{U}} S\left(\psi(u), \frac{\partial \psi(u)}{\partial u_1} \wedge \frac{\partial \psi(u)}{\partial u_2}\right) du$$

where $u = (u_1, u_2)$ [63]. Let D be the domain of f for the dynamical system $\dot{x} = f(x)$. The requirements on the function S are then that it is locally Lipschitz continuous and that the limit defined by

$$\dot{S}(x,y) := \lim_{h \searrow 0} \frac{1}{h}\left(S\left(x + hf(x), y + h\frac{\partial f(x)}{\partial x}^{[2]} y\right) - S(x,y)\right)$$

exists for all $(x,y) \in D \times \mathbb{R}^{\binom{m}{2}}$. Li and Muldowney now considered in particular the class of functionals that is given by $S(x,y) = ||A(x)y||$, where $||\cdot||$ is a norm on $\mathbb{R}^{\binom{m}{2}}$ and $x \mapsto A(x)$ is a continuously differentiable nonsingular real $\binom{m}{2} \times \binom{m}{2}$ function [63]. That is, instead of using $||x||$ as a Lyapunov function for the linear time-varying system, the function $||A(x)y||$ is used as Lyapunov function for the $m + \binom{m}{2}$ dimensional system that is formed by the dynamical system and its compound system [65]:

$$\dot{x} = f(x), \quad \dot{y} = \frac{\partial f}{\partial x}^{[2]} y. \tag{5.3}$$

Again we can calculate the right-hand derivative of $||A(x)y||$ to get an upper estimate involving the matrix measure:

$$\begin{aligned}
D_+ ||A(x)y|| = D_+ ||A(x(t))\, y(t)|| &= \lim_{h \searrow 0} \frac{||A(x(t+h))\, y(t+h)|| - ||A(x(t))\, y(t)||}{h} \\
&= \lim_{h \searrow 0} \frac{1}{h}\left(\left|\left|A(x(t))\, y(t) + \frac{d}{dt}\left(A(x(t))\, y(t)\right) h + \mathcal{O}(h^2)\right|\right| - ||A(x(t))\, y(t)||\right) \\
&= \lim_{h \searrow 0} \frac{1}{h}\left(\left|\left|A(x)\, y + \left(\frac{\partial A(x)}{\partial x}\frac{dx}{dt} y + A(x)\frac{dy}{dt}\right) h\right|\right| - ||A(x)\, y||\right) \\
&= \lim_{h \searrow 0} \frac{1}{h}\left(\left|\left|A\, y + h\left(\frac{\partial A}{\partial x} f(x) A^{-1} + A\frac{\partial f}{\partial x}^{[2]} A^{-1}\right) Ay\right|\right| - ||A\, y||\right) \\
&\leq \lim_{h \searrow 0} \frac{1}{h}\left(\left|\left|I_{\binom{m}{2}} + h\left(\frac{\partial A}{\partial x} f(x) A^{-1} + A\frac{\partial f}{\partial x}^{[2]} A^{-1}\right)\right|\right| - 1\right) ||Ay|| \\
&= \mu\left(A_f A^{-1} + A\frac{\partial f}{\partial x}^{[2]} A^{-1}\right) ||Ay||
\end{aligned}$$

where A_f denotes the directional derivative in direction f, $A_f = \frac{\partial A}{\partial x} f(x) = \frac{\partial A}{\partial x}\frac{dx}{dt} = \frac{dA}{dt}$. Analogously, we get an estimate for a lower bound. This gives the following estimates for the limit of the function $S(x,y) = ||A(x)y||$:

$$-\mu(-B)S \leq \dot{S} \leq \mu(B)S$$

with $B := A_f A^{-1} + A\left(\frac{\partial f}{\partial x}\right)^{[2]} A^{-1}$ [63]. Note that the matrix A can be chosen as any nonsingular real

matrix such that $x \mapsto A(x)$ is continuously differentiable. For $A = I_r$ where $r = \binom{m}{2}$ this reduces to $B = \left(\frac{\partial f}{\partial x}\right)^{[2]}$, which corresponds to Muldowney's theorem (Theorem 7). Li and Muldowney showed that, given a differential equation $\dot{x} = f(x)$ defined on a simply connected set D whose solutions exist for all $t \geq 0$, if $\mu(B) \leq \delta < 0$ for some $\delta \in \mathbb{R}$ on a set which is absorbing with respect to the differential equation, then there exists no surface in D whose boundary is left invariant over time. That means, no periodic solution can exist. Here a set $U \subset D$ is said to be *absorbing* if each bounded subset $V \subset D$ satisfies $\phi(t, V) \subset U$ for all sufficiently large t with $\phi(t, x_0)$ being a solution of $\dot{x} = f(x)$ [63]. Similar to Smith's generalization of Bendixson's criterion, which implies that not only no periodic solution can exist, but all bounded solutions converge to a steady state, Li and Muldowney [65, 64] were able to extend the previous statements and relate it to the question of global stability of a unique steady state. Smith's theorem about the convergence of solutions of an autonomous differential equation, thus in particular of a dynamical system, is referred to as an autonomous convergence theorem [93, 65, 64]. Li and Muldowney were able to show a convergence theorem which generalizes Smith's theorem. In particular, this convergence theorem gives a global stability criterion for systems with a unique steady state.

Theorem 8. (Li and Muldowney) [64] *Let the map $x \mapsto f(x)$ from an open subset $D \subset \mathbb{R}^m$ to $\mathbb{R}^m$ be such that each solution $x(t)$ of the differential equation $\dot{x} = f(x)$ is uniquely determined by its initial value. Assume that D is simply connected, there exists a compact absorbing set $K \subset D$ and x^* is the only steady state of the system. If*

$$\mu\left(A_f A^{-1} + A\,\frac{\partial f}{\partial x}^{[2]} A^{-1}\right) \leq \delta < 0 \quad \text{on } K$$

for some $\delta \in \mathbb{R}$ and a continuously differentiable nonsingular $\binom{m}{2} \times \binom{m}{2}$ matrix function $x \mapsto A(x)$, then x^* is globally asymptotically stable in D.

For a dynamical system defined on a neighborhood of a compact subset in the nonnegative orthant that is left positively invariant by the system, showing the existence of a compact set which is absorbing in the interior of the compact subset is equivalent to proving that the system is uniformly persistent [64]. Suppose a dynamical system defined on the neighborhood of a compact subset E is uniformly persistent, that is, $\liminf_{t\to\infty} d\left(x(t), \partial E\right) \geq \varepsilon$ for some $\varepsilon > 0$ for all solutions starting in the interior of E (Definition 26). Then there exist numbers $0 < c \leq C < \infty$ such that for every solution x in the interior of E, denoted E°, there exists a time $t_0(x)$ such that $c \leq x_i(t) \leq C$ for all $t > t_0(x)$ and all $i = 1, \ldots, m$ [47]. That is, there is a compact region $K \subset E^\circ \subset \overline{\mathbb{R}}^m_+$ with $d(K, \partial E) > 0$ such that, given any solution $x \in E^\circ$, there is a $t_0(x)$ such that $x(t) \in K$ for all $t \geq t_0(x)$ [48]. Since any solution in E° eventually enters K, it true that $\phi(t, E^\circ) \subset K$ for t sufficiently large. Thus, it also holds that $\phi(t, V) \subset K$ for any bounded subset $V \subset E^\circ$ for t sufficiently large. Therefore K is a compact absorbing set in E°. On the other hand, for a compact set $E \subset \overline{\mathbb{R}}^m_+$ the existence of a compact absorbing set $K \subset E$ with $d(K, \partial E) > 0$ implies uniform persistence as E° is bounded and thus $\phi(t, E^\circ) \subset K$ for t sufficiently large.

Apart from the choice of some matrix function $A(x)$, the method of Li and Muldowney involves the choice of a norm for which the matrix measure is calculated. Setting $A = I_r$ where $r = \binom{m}{2}$ reduces the term for which the matrix measure has to be calculated to the second additive compound matrix, $\mu\left(\frac{\partial f}{\partial x}^{[2]}\right) \leq \delta < 0$, which of course still depends on the chosen norm. Given a real square matrix A, the following table gives the matrix measure of three commonly used norms [14, 7]:

Vector norm $\|\|x\|\|$	Induced matrix norm $\|\|A\|\| = \max_{\|\|x\|\|=1} \|\|Ax\|\|$	Induced matrix measure $\mu(A)$
$\sum_i \|x_i\|$	$\max_j \sum_i \|a_{ij}\|$	$\max_j \left\{ a_{jj} + \sum_{i \neq j} \|a_{ij}\| \right\}$
$\sqrt{\sum_i \|x_i\|^2}$	$\sqrt{\lambda_{1(A^T A)}}$	$\lambda_{1\left(\frac{1}{2}(A^T + A)\right)}$
$\max_i \|x_i\|$	$\max_i \sum_j \|a_{ij}\|$	$\max_i \left\{ a_{ii} + \sum_{j \neq i} \|a_{ij}\| \right\}$

where $\lambda_{1(B)}$ denotes the largest eigenvalue of the symmetric matrix B. For a complex square matrix A, the transpose matrix in the table above has to be replaced by the conjugate transpose. The consequence for the induced matrix measure is that the real parts of the diagonal entries have to be used, that is, $\mathrm{Re}\,(a_{ii})$ instead of a_{ii}. The matrix measure of the first and the third choice for a norm correspond to the upper bounds of the Geršgorin discs for the real parts of the eigenvalues of A [73]. The Geršgorin discs are the discs in the complex plane given by the estimates $|\lambda - a_{ii}| \leq \sum_{j \neq i} |a_{ij}|$ and $|\lambda - a_{jj}| \leq \sum_{i \neq j} |a_{ji}|$. Every eigenvalue of A lies in such a disc [35]. Thus, an upper bound for the real parts of the eigenvalues of A is given by the matrix measure associated to the l^1-norm or maximum norm, respectively.

Using the formula for the entries of the k-th additive compound matrix (5.1), we can extend the above table with the induced matrix measure of the k-th additive compound matrix $B = A^{[k]}$ [73]:

Vector norm $\|\|x\|\|$	Induced matrix measure $\mu\left(A^{[k]}\right)$
$\sum_i \|x_i\|$	$\max_{(j)} \left\{ (a_{j_1 j_1} + \cdots + a_{j_k j_k}) + \sum_{i \notin (j)}^{k} (\|a_{i j_1}\| + \cdots + \|a_{i j_k}\|) \right\}$
$\sqrt{\sum_i \|x_i\|^2}$	$\lambda_{1\left(\frac{1}{2}(A^T + A)\right)} + \cdots + \lambda_{k\left(\frac{1}{2}(A^T + A)\right)}$
$\max_i \|x_i\|$	$\max_{(i)} \left\{ (a_{i_1 i_1} + \cdots + a_{i_k i_k}) + \sum_{j \notin (i)}^{k} (\|a_{i_1 j}\| + \cdots + \|a_{i_k j}\|) \right\}$

where the entries a_{ij} denote again the matrix entries of the real square matrix A and $\lambda_{1(B)} \geq \lambda_{2(B)} \geq \cdots \geq \lambda_{m(B)}$ denote again the eigenvalues of the symmetric $m \times m$ matrix B and $(i) = (i_1, \ldots, i_k)$ is the k-tuple from (5.1). For example, the matrix measure of $\frac{\partial f}{\partial x}^{[2]}$ for the Euclidean norm is given by the sum of the two largest eigenvalues of $\frac{1}{2}\left(\left(\frac{\partial f}{\partial x}\right)^T + \frac{\partial f}{\partial x}\right)$ or by the largest eigenvalue of $\frac{1}{2}\left(\left(\frac{\partial f}{\partial x}^{[2]}\right)^T + \frac{\partial f}{\partial x}^{[2]}\right)$, respectively. In the case that A is a complex square matrix, the transponse has to be replaced by the conjugate transponse and instead of the sum of the diagonal entries, the real part of the sum of the diagonal entries has to be taken. The matrix measures just given are examples for commonly used norms, but any norm can be chosen. Considering that the stability theorem of Li and Muldowney (Theorem 8) is based on a Lyapunov function for the $m + \binom{m}{2}$ dimensional system (5.3), less conventional norms may prove to be effective. Li and Muldowney gave an example of a three-dimensional epidemiological model where the vector norm $\|\|x\|\| = \|\|(x_1, x_2, x_3)\|\| = \max\{|x_1|, |x_2| + |x_3|\}$ led to the desired result [64].

In the next section, we apply Li and Muldowney's theorem (Theorem 8) to the kinetic proofreading model with a single phosphorylation step. However, we will only be able to prove global stability of the steady state under certain restrictions on the kinetic parameters. Another approach to get information about the global stability of the unique steady state of the kinetic proofreading system with Lck and $N = 1$ is given by a quasi-steady state assumption. Since the quasi-steady state assumption reduces the system to a two-dimensional system, we can then apply Poincaré-Bendixson Theory for planar systems. The quasi-steady state assumption is an approximation which can be applied to differential equations resulting from Michaelis-Menten kinetics, that is, to chemical

reactions involving an enzyme that catalyses the conversion of a substrate to a certain product where mass action kinetics is assumed. Concretely, such an enzyme-substrate reaction is given by $\mathrm{E} + \mathrm{S} \rightleftharpoons \mathrm{X} \longrightarrow \mathrm{E} + \mathrm{P}$ where S denotes the substrate, E the enzyme, X the enzyme-substrate complex and P the product. The underlying assumption is that the substrate concentration is significantly higher than the enzyme concentration, and the affinity between substrate and enzyme is high such that as soon as the substrate in the enzyme-substrate complex is converted into the product, the enzyme immediately binds to another substrate molecule. Thus, we assume that the enzymes are always saturated. Hence, the concentration of the enzyme-substrate complexes, that is, the occupied enzymes, remains nearly constant over time, which translates into $\dot{X} \approx 0$ where X denotes the concentration of the enzyme-substrate complex. This quasi-steady state assumption reduces the number of differential equations that determine the dynamics of a system. Mathematically, the quasi-steady state assumption translates into a fast and a slow time scale, which can be dealt with using the *Geometric Singular Perturbation Theory*.

A system where the variables evolve on two different time scales, a *fast-slow system*, can be formulated by splitting the variables into two groups. An (m, n)-fast-slow system is given by a system of ordinary differential equations taking the form

$$\begin{aligned} \varepsilon\, \dot{x} &= f(x, y, \varepsilon) \\ \dot{y} &= g(x, y, \varepsilon) \end{aligned} \tag{5.4}$$

where $(x, y) \in \mathbb{R}^m \times \mathbb{R}^n$ and ε is a small parameter $0 < \varepsilon \ll 1$ representing the ratio of time scales. The functions f and g are assumed to be sufficiently smooth [58]. The variables x are here the variables associated with the fast time scale, whereas the variables y change more slowly. For the example of an enzyme-substrate reaction, sometimes also referred to as Michaelis-Menten reaction, the enzyme-substrate complex and the enzyme itself correspond to the fast variables, whereas substrate and product are slow variables. This classification results from transferring the differential equations derived from mass action kinetics into a fast-slow system by scaling the time with ε and the fast variables with $\frac{1}{\varepsilon}$, where ε is again a small parameter $0 < \varepsilon \ll 1$. The above system is the formulation in terms of the slow time t. Rescaling the system in terms of a fast time $\tau = \frac{t}{\varepsilon}$ gives the equivalent formulation

$$\begin{aligned} x' &= f(x, y, \varepsilon) \\ y' &= \varepsilon\, g(x, y, \varepsilon). \end{aligned}$$

In many situations, just as in the example of the enzyme-substrate reaction, f and g are independent of ε [58]. For $\varepsilon > 0$ both systems are equivalent. Setting $\varepsilon = 0$ in the slow-time formulation (5.4) results in the *reduced problem* or *slow subsystem*:

$$\begin{aligned} 0 &= f(x, y, 0) \\ \dot{y} &= g(x, y, 0). \end{aligned}$$

Similarly, setting ε to zero in the system formulated in terms of the fast time variable τ gives the *layer problem* or *fast subsystem*:

$$\begin{aligned} x' &= f(x, y, 0) \\ y' &= 0. \end{aligned}$$

Ideally, the two subsystems provide us with an approximation for the original system. Whether the reduced problem or the layer problem gives a suitable approximation depends on the region of the phase space. We expect that near the set described by the algebraic equation $f(x, y, 0) = 0$ a trajectory should be approximately represented by solutions of the reduced problem, whereas sufficiently far away from that set we assume the impact of the slow variables to be negligible and hope

to approximate the trajectories with the layer problem [58]. The set $C_0 = \{(x, y) : f(x, y, 0) = 0\}$ is called *critical set* or if C_0 is a submanifold of $\mathbb{R}^m \times \mathbb{R}^n$ *critical manifold*. Thus, the critical set consists of the steady states of the layer problem, whereas the reduced problem corresponds to a dynamical system on this critical set. If the Jacobian matrix $\frac{\partial}{\partial x} f(x, y, 0)$ is nonsingular in a neighborhood of a point in the critical set, then it follows from the implicit function theorem that there exists a function such that $x = h(y)$ holds in this neighborhood and the reduced problem is described by the equation $\dot{y} = g(h(y), y, 0)$. In particular, if this is the case for every point of the critical set C_0, then C_0 is a critical manifold. A subset $S \subset C_0$ is called *normally hyperbolic* if, for every point of it, the Jacobian matrix with respect to the fast variables, $\frac{\partial f}{\partial x}$, has no eigenvalues with zero real part. If all eigenvalues have negative real part, the normally hyperbolic subset is called *attracting*, and it is called *repelling* if all eigenvalues have positive real part. If the normally hyperbolic subset is neither attracting nor repelling, it is of *saddle type* [58]. An essential condition for the solutions of the reduced problem to be an approximation for the solutions of the original system close the critical set C_0 is that there is a subset $S \subset C_0$ which is a compact normally hyperbolic manifold $S = M_0$. The following theorem about the perturbation of such compact normally hyperbolic manifolds goes back to Fenichel [32, 31].

Theorem 9. (Fenichel) [58, 52] *Suppose M_0 is a compact normally hyperbolic submanifold (possibly with boundary) of the critical set C_0 of the fast-slow system (5.4) where $f, g \in C^r$ with $r < \infty$. Then there exists a locally invariant manifold M_ε diffeomorphic to M_0 that lies within $\mathcal{O}(\varepsilon)$ of M_0. Moreover, M_ε is normally hyperbolic and has the same stability properties with respect to the fast variables as M_0 (attracting, repelling or of saddle type) and the flow of M_ε converges to the flow of the reduced problem as $\varepsilon \to 0$.*

Local invariance means here that trajectories can enter or leave M_ε only by crossing its boundaries. More precisely, there exists a neighborhood V for M_ε such that no trajectory can leave M_ε without leaving V [52]. That is, M_ε is locally invariant if, for all $(x, y) \in M_\varepsilon$, $(x(t), y(t)) \in V$ for $t \in [t_0, t_1]$ implies $(x(t), (y(t)) \in M_\varepsilon$ for all $t \in [t_0, t_1]$. The manifold M_ε is called *slow manifold* and is usually not unique [58]. After finding conditions under which the unique steady state of the kinetic proofreading system with Lck and a single phosphorylation step is globally stable via Li and Muldowney's method, we will apply Fenichel's theorem and Poincaré-Bendixson Theory to show global stability for the system when the enzyme Lck is almost always saturated.

5.2 Kinetic Parameters for Global Stability under Single Phosphorylation

In this section, we will first show that the unique steady state of the kinetic proofreading system with Lck and a single phosphorylation step is asymptotically stable and then apply Li and Muldowney's theorem to find conditions under which it is even globally asymptotically stable. To identify further parameters under which global stability holds, we will finally assume a quasi-steady state for the reaction catalyzed by Lck and apply Geometric Singular Perturbation Theory.

By taking into account that there are three conserved quantities, R_{tot}, M_{tot} and L_{tot}, we can eliminate three variables and three differential equations. We eliminate R, M and L and get the following three differential equations involving the species C_0, B_0 and C_1:

$$\begin{aligned}
\dot{C}_0 &= k_1 \left(R_{\text{tot}} - C_0 - C_1 - B_0\right)\left(M_{\text{tot}} - C_0 - C_1 - B_0\right) \\
&\quad - k_2 C_0 - k_3 C_0 \left(L_{\text{tot}} - B_0\right) + k_4 B_0 \\
\dot{B}_0 &= k_3 C_0 \left(L_{\text{tot}} - B_0\right) - \left(k_4 + k_5\right) B_0 \\
\dot{C}_1 &= k_5 B_0 - k_6 C_1.
\end{aligned} \tag{5.5}$$

To simplify the following calculations, we set

$$\Theta := R_{\text{tot}} + M_{\text{tot}} - 2C_0 - 2B_0 - 2C_1$$

and note that this quantity is always nonnegative. We denote by A the matrix resulting from linearization and its entries by a_{ij}. With $\Theta \geq 0$ as just defined we get [83]

$$\begin{aligned} A &= \begin{pmatrix} -k_1\Theta - k_2 - k_3\left(L_{\text{tot}} - B_0\right) & -k_1\Theta + k_3C_0 + k_4 & -k_1\Theta \\ k_3\left(L_{\text{tot}} - B_0\right) & -k_3C_0 - k_4 - k_5 & 0 \\ 0 & k_5 & -k_6 \end{pmatrix} \\ &= \begin{pmatrix} -k_1\Theta - k_2 - k_3L & -k_1\Theta + k_3C_0 + k_4 & -k_1\Theta \\ k_3L & -k_3C_0 - k_4 - k_5 & 0 \\ 0 & k_5 & -k_6 \end{pmatrix} \end{aligned}$$

where $L = L_{\text{tot}} - B_0$. We apply the Routh-Hurwitz criterion to the characteristic polynomial of the Jacobian matrix A to show that it possesses only eigenvalues with negative real part at any steady state, which implies the asymptotic stability of the unique steady state. The characteristic polynomial $\det\left(\lambda I_3 - A\right)$ is given by

$$\begin{aligned} &\lambda^3 - \operatorname{tr}(A)\lambda^2 + \left(\begin{vmatrix} a_{11} & a_{12} \\ a_{21} & a_{22} \end{vmatrix} + \begin{vmatrix} a_{11} & a_{13} \\ a_{31} & a_{33} \end{vmatrix} + \begin{vmatrix} a_{22} & a_{23} \\ a_{32} & a_{33} \end{vmatrix}\right)\lambda - \det(A) \\ =&\lambda^3 + \left(k_1\Theta + k_2 + k_3L + k_3C_0 + k_4 + k_5 + k_6\right)\lambda^2 \\ &+ \left[\left(k_1\Theta + k_2\right)\left(k_3C_0 + k_4 + k_5\right) + k_3L\left(k_5 + k_1\Theta\right)\right. \\ &\quad \left. + k_6\left(k_1\Theta + k_2 + k_3L\right) + k_6\left(k_3C_0 + k_4 + k_5\right)\right]\lambda \\ &+ k_1\Theta k_3Lk_5 + k_6\left(k_1\Theta + k_2\right)\left(k_3C_0 + k_4 + k_5\right) + k_6k_3L\left(k_5 + k_1\Theta\right). \end{aligned}$$

Denoting the coefficients by b_i, that is, $\lambda^3 + b_2\lambda^2 + b_1\lambda + b_0$, the determinant whose minors must be calculated to apply the Routh-Hurwitz criterion is given by

$$D_3 = \begin{vmatrix} b_2 & b_0 & 0 \\ 1 & b_1 & 0 \\ 0 & b_2 & b_0 \end{vmatrix}.$$

Since all coefficients are positive and all summands of b_0 are also terms in the product b_1b_2, we get

$$\begin{aligned} D_1 &= b_2 > 0 \\ D_2 &= b_1b_2 - b_0 > 0 \\ D_3 &= b_0D_2 > 0. \end{aligned}$$

Thus, it follows from the Routh-Hurwitz criterion that all eigenvalues of the Jacobian matrix A have a negative real part, which implies the asymptotic stability of the unique steady state of the kinetic proofreading system with $N = 1$ [83].

Lemma 5. *The unique positive steady state of the kinetic proofreading system with $N = 1$ (2.2) is always hyperbolic and asymptotically stable.*

As a consequence, there are no bifurcations, that is, in a neighborhood of the steady state the qualitative behavior of the system does not change. In particular, it follows from the negativity of all real parts of the eigenvalues that there is no Hopf bifurcation, which occurs if a pair of complex eigenvalues passes through the imaginary axis and generates a periodic orbit. With the asymptotic stability of the unique steady state, the question arises if the steady state may even be globally asymptotically stable. Applying the theorem of Li and Muldowney (Theorem 8) we want to find conditions under which this global stability of the steady state is ensured.

The matrix measure of $A^{[2]}$ depends on the norm we choose. We will start with the l^1-norm, $||x|| = |x_1| + |x_2| + |x_3|$. From the last section we know that the matrix measure of the second additive matrix with respect to the l^1-norm is given by

$$\begin{aligned}
\mu\left(A^{[2]}\right) &= \max_{(j)=(j_1,j_2)} \left\{ (a_{j_1j_1} + a_{j_2j_2}) + \sum_{i\notin(j)}^{2} (|a_{ij_1}| + |a_{ij_2}|) \right\} \\
&= \max\left\{a_{11} + a_{22} + |a_{31}| + |a_{32}|, a_{11} + a_{33} + |a_{21}| + |a_{23}|, a_{22} + a_{33} + |a_{12}| + |a_{13}|\right\} \\
&= \max\{-k_1\Theta - k_2 - k_3L - k_3C_0 - k_4 - k_5 + |k_5|, \; -k_1\Theta - k_2 - k_3L - k_6 + |k_3L|, \\
&\qquad -k_3C_0 - k_4 - k_5 - k_6 + |-k_1\Theta + k_3C_0 + k_4| + |-k_1\Theta|\} \\
&= \max\{-k_1\Theta - k_2 - k_3L - k_3C_0 - k_4, -k_1\Theta - k_2 - k_6, \\
&\qquad k_1\Theta - k_3C_0 - k_4 - k_5 - k_6 + |-k_1\Theta + k_3C_0 + k_4|\}.
\end{aligned}$$

The first two terms are negative. Thus, the question if $\mu\left(A^{[2]}\right)$ is negative is determined by the third term:

$$\begin{aligned}
& k_1\Theta - k_3C_0 - k_4 - k_5 - k_6 + |-k_1\Theta + k_3C_0 + k_4| < 0 \\
\Leftrightarrow \quad & k_3C_0 + k_4 \geq k_1\Theta \quad \text{or} \quad \left(k_3C_0 + k_4 < k_1\Theta \quad \text{and} \quad 2\left(k_1\Theta - k_3C_0 - k_4\right) < k_5 + k_6\right).
\end{aligned}$$

Hence, the matrix measure of the second additive matrix is negative if

$$k_1\Theta - k_3C_0 - k_4 < \frac{k_5 + k_6}{2}.$$

This is fulfilled for all concentrations if

$$k_1\left(R_{\text{tot}} + M_{\text{tot}}\right) < k_4 + \frac{k_5 + k_6}{2} \tag{5.6}$$

holds. This translates into a biological setting where a quantity describing the binding of the antigen to the T cell receptor is small compared to the catalysis and decomposition rates of the enzyme-substrate complex, k_5 and k_4, and the decomposition rate of the phosphorylated complex, k_6. That is, either the total amount of receptor and antigen or its binding rate has to be rather small, or one of the rates associated to the enzyme-substrate complex has to be sufficiently high, or the decomposition of the phosphorylated complex happens very fast. Enzymes act as catalysts, that is, they increase the rate of a reaction. Therefore, it is biologically reasonable to assume that the catalysis rate, k_5, is high compared to the other reaction rates, in particular to k_1, the binding of the antigen to the T cell receptor.

Muldowney's criterion (Theorem 7) states that if the matrix measure for the second additive compound matrix is negative, which is the case for the kinetic proofreading system with Lck and a single phosphorylation step if condition (5.6) is satisfied, then there cannot exist any periodic solution. More precisely, there cannot exist any periodic solution within the invariant compact subset created by the conserved quantities, E, which we identified in Section 4.2. We know from Theorem 5 that the kinetic proofreading system is uniformly persistent on this nonnegative invariant compact subset. Consequently, there exists a compact absorbing set within this subset E as discussed after the introduction of Li and Muldowney's theorem (Theorem 8). Since the steady state is unique, all assumptions for Li and Muldowney's theorem are fulfilled. Specifically, we identify the open subset D in the theorem with E°, and K with the compact absorbing subset whose existence follows from the uniform persistence. Note that we cannot identify D with an open neighborhood of E since E is not absorbing for this neighborhood, but invariant. Setting the matrix A in Li and Muldowney's theorem (Theorem 8) equal to the identity matrix, we get the above calculations for the matrix measure of the second additive compound of the Jacobian matrix. We have to ensure that this matrix measure is smaller or equal to some negative constant. As condition (5.6) only involves

constants, the difference $\overline{\delta} := k_1 (R_{\text{tot}} + M_{\text{tot}}) - k_4 - \frac{k_5+k_6}{2}$ equals a negative constant provided that the condition holds. Consequently, we get

$$\mu\left(A^{[2]}\right) \leq \max\left\{-k_2 - k_4, -k_2 - k_6, -k_5 - k_6, 2\overline{\delta}\right\} =: \delta < 0.$$

Hence, under the condition that (5.6) holds, all assumptions for Li and Muldowney's theorem (Theorem 8) are satisfied. Setting $D = E^\circ$, the theorem implies the global stability of the unique positive steady state in E°. Furthermore, we know from Theorem 5 that no solution starting in E possesses an ω-limit point on the boundary ∂E and that E is invariant. Consequently, we can conclude that the unique positive steady state is globally asymptotically stable in all E.

Thus, we have found a condition for the parameters such that the kinetic proofreading system with Lck and one phosphorylation step has a globally asymptotically stable steady state. However, as the matrix measure depends on the choice of a norm, other norm choices might lead to less restrictive conditions. Hence, we will also apply other norms to check if we can improve the condition. If we choose the l^2-norm, $||x|| = \sqrt{x_1^2 + x_2^2 + x_3^2}$, then the value of the matrix measure for the second additive compound matrix corresponds to the largest eigenvalue of the symmetric matrix $\frac{1}{2}\left(\left(A^{[2]}\right)^T + A^{[2]}\right)$ or the sum of the two largest eigenvalues of $\frac{1}{2}\left(A^T + A\right)$, as seen in the last section. Consequently, if the largest eigenvalue of $\left(A^{[2]}\right)^T + A^{[2]}$ or the sum of the two largest eigenvalues of $A^T + A$ is negative, then the matrix measure of the second additive compound matrix, $\mu\left(A^{[2]}\right)$, is also negative. The characteristic polynomials for these matrices do not yield coefficients with fixed signs or give rise to easy conditions which would ensure the negativity of the largest eigenvalues. Though for the characteristic polynomial $\det\left(\frac{1}{2}\left(\left(A^{[2]}\right)^T + A^{[2]}\right) - \lambda I_3\right)$ all coefficients except the constant one, which corresponds to the determinant, are negative, the latter has several positive terms which do not cancel, and the conditions to ensure their negativity are more complicated and restrictive than the one we got from the l^1-norm. Thus, the l^2-norm does not offer an improvement. Next, we will try the l^∞-norm, $||x|| = \max\{|x_1|, |x_2|, |x_3|\}$.

$$\begin{aligned}
\mu\left(A^{[2]}\right) &= \max_{(i)=(i_1,i_2)} \left\{ (a_{i_1 i_1} + a_{i_2 i_2}) + \sum_{j \notin (i)}^{2} \left(|a_{i_1 j}| + |a_{i_2 j}|\right) \right\} \\
&= \max\left\{a_{11} + a_{22} + |a_{13}| + |a_{23}|, a_{11} + a_{33} + |a_{12}| + |a_{32}|, a_{22} + a_{33} + |a_{21}| + |a_{31}|\right\} \\
&= \max\left\{-k_1\Theta - k_2 - k_3 L - k_3 C_0 - k_4 - k_5 + |-k_1\Theta|, \right. \\
&\qquad - k_1\Theta - k_2 - k_3 L - k_6 + |-k_1\Theta + k_3 C_0 + k_4| + |k_5|, \\
&\qquad \left. -k_3 C_0 - k_4 - k_5 - k_6 + |k_3 L|\right\} \\
&= \max\left\{-k_2 - k_3 L - k_3 C_0 - k_4 - k_5, \right. \\
&\qquad - k_1\Theta - k_2 - k_3 L - k_6 + |-k_1\Theta + k_3 C_0 + k_4| + k_5, \\
&\qquad \left. -k_3 C_0 - k_4 - k_5 - k_6 + k_3 L\right\}.
\end{aligned}$$

In addition to controlling the term $|-k_1\Theta + k_3 C_0 + k_4|$, we have to ensure here that k_5 and $k_3 L$ are small enough. Concretely, this leads to the following three conditions:

$$k_3 \min\left\{R_{\text{tot}}, M_{\text{tot}}\right\} + k_4 + k_5 < k_2 + k_6, \quad k_5 < k_2 + k_4 + k_6 \quad \text{and} \quad k_3 L_{\text{tot}} < k_4 + k_5 + k_6.$$

Thus, the l^∞-norm does not improve the condition for globally stability either. However, we see that the two additional positive terms, k_5 and $k_3 L$, would cancel if we were to look at the sum of the second and third expression. This motivates the choice of the vector norm $||x|| = \max\left\{|x_1|, |x_2| + |x_3|\right\}$, which was also used in an example of Li and Muldowney [64]. Therefore, we first calculate the

second additive compound of the Jacobian matrix (cf. formula (5.1)):

$$A^{[2]} = \begin{pmatrix} -k_1\Theta - k_2 - k_3L - k_3C_0 - k_4 - k_5 & 0 & k_1\Theta \\ k_5 & -k_1\Theta - k_2 - k_3L - k_6 & -k_1\Theta + k_3C_0 + k_4 \\ 0 & k_3L & -k_3C_0 - k_4 - k_5 - k_6 \end{pmatrix}.$$

Now we can calculate the matrix norm of $I_3 + hA^{[2]}$ for some small $h > 0$ for the chosen norm $||x|| = \max\{|x_1|, |x_2| + |x_3|\}$:

$$\begin{aligned}
\left|\left|I_3 + hA^{[2]}\right|\right| &= \max_{||x||=1} \left\{ \left|\left| \left(I_3 + hA^{[2]}\right) x \right|\right| \right\} \\
&= \max \left\{ \left|\left| \begin{pmatrix} x_1 + h\left(\left(-k_1\Theta - k_2 - k_3L - k_3C_0 - k_4 - k_5\right)x_1 + k_1\Theta x_3\right) \\ x_2 + h\left(k_5x_1 + \left(-k_1\Theta - k_2 - k_3L - k_6\right)x_2 + \left(-k_1\Theta + k_3C_0 + k_4\right)x_3\right) \\ x_3 + h\left(k_3Lx_2 + \left(-k_3C_0 - k_4 - k_5 - k_6\right)x_3\right) \end{pmatrix} \right|\right| , \right. \\
&\qquad \left. ||x|| = \max\{|x_1|, |x_2| + |x_3|\} = 1 \right\} \\
&= \max \Big\{ \max \Big\{ \left|x_1 + h\left(\left(-k_1\Theta - k_2 - k_3L - k_3C_0 - k_4 - k_5\right)x_1 + k_1\Theta x_3\right)\right|, \\
&\qquad \left|x_2 + h\left(k_5x_1 + \left(-k_1\Theta - k_2 - k_3L - k_6\right)x_2 + \left(-k_1\Theta + k_3C_0 + k_4\right)x_3\right)\right| \\
&\qquad + \left|x_3 + h\left(k_3Lx_2 + \left(-k_3C_0 - k_4 - k_5 - k_6\right)x_3\right)\right| \Big\}, \\
&\qquad ||x|| = \max\{|x_1|, |x_2| + |x_3|\} = 1 \Big\}
\end{aligned}$$

As $h > 0$ is assumed to be small, the absolute value of the expressions is dominated by the terms without h as a factor, that is, by x_1, x_2 or x_3, respectively. There are no summands involving the product x_2x_3, thus to maximize the expression we consider either $|x_2| = 1$ and $x_3 = 0$ or $|x_3| = 1$ and $x_2 = 0$:

$$\begin{aligned}
&= \max \Big\{ 1 + h\left(-k_1\Theta - k_2 - k_3L - k_3C_0 - k_4 - k_5 + k_1\Theta\right), \\
&\qquad 1 + h\left(k_5 - k_1\Theta - k_2 - k_3L - k_6 + k_3L\right), \\
&\qquad 1 + h\left(k_5 + \left|-k_1\Theta + k_3C_0 + k_4\right| - k_3C_0 - k_4 - k_5 - k_6\right) \Big\} \\
&= \max \Big\{ 1 + h\left(-k_2 - k_3L - k_3C_0 - k_4 - k_5\right), 1 + h\left(k_5 - k_1\Theta - k_2 - k_6\right), \\
&\qquad 1 + h\left(\left|-k_1\Theta + k_3C_0 + k_4\right| - k_3C_0 - k_4 - k_6\right) \Big\}.
\end{aligned}$$

Whereas the term associated to h is strictly negative in the first expression since the positive summand cancels, we need assumptions to ensure that this term is negative in the second and third expression. The second expression is negative if $k_5 < k_2 + k_6$ holds. The sign of the third expression depends again on the sign of the term $-k_1\Theta + k_3C_0 + k_4$. Its negativity is ensured if $k_1\left(R_{\text{tot}} + M_{\text{tot}}\right) < 2k_4 + k_6$ is fulfilled. Thus, if both conditions,

$$k_5 < k_2 + k_6 \quad \text{and} \quad k_1\left(R_{\text{tot}} + M_{\text{tot}}\right) < 2k_4 + k_6, \tag{5.7}$$

hold, the negativity of the matrix measure of the second additive compound of the Jacobian matrix is ensured:

$$\mu\left(A^{[2]}\right) = \lim_{h \searrow 0} \left(\frac{1}{h} \left(\left|\left| I_3 + hA^{[2]} \right|\right| - 1 \right) \right)$$

$$
\begin{aligned}
&= \lim_{h \searrow 0} \left(\frac{1}{h} \Big(1 + h \max \big\{ -k_2 - k_3 L - k_3 C_0 - k_4 - k_5,\, k_5 - k_1 \Theta - k_2 - k_6, \right. \\
&\qquad \left. |-k_1 \Theta + k_3 C_0 + k_4| - k_3 C_0 - k_4 - k_6 \big\} - 1 \Big) \right) \\
&= \max \big\{ \underbrace{-k_2 - k_3 L - k_3 C_0 - k_4 - k_5}_{<0},\, \underbrace{k_5 - k_1 \Theta - k_2 - k_6}_{<0 \text{ if } k_5 < k_2 + k_6}, \\
&\qquad \underbrace{|-k_1 \Theta + k_3 C_0 + k_4| - k_3 C_0 - k_4 - k_6}_{<0 \text{ if } k_1 (R_{\mathrm{tot}} + M_{\mathrm{tot}}) < 2k_4 + k_6} \big\}.
\end{aligned}
$$

Setting $\delta := \max \{-k_2 - k_4 - k_5,\, k_5 - k_2 - k_6,\, -k_6,\, k_1 (R_{\mathrm{tot}} + M_{\mathrm{tot}}) - 2k_4 - k_6\}$ gives $\mu \left(A^{[2]} \right) \leq \delta < 0$ if both conditions (5.7) are fulfilled. As already discussed, the kinetic proofreading system with Lck and a single phosphorylation step satisfies the assumptions for Li and Muldowney's theorem for global stability (Theorem 8). Namely, the system is defined on an open and simply connected subset, the interior of the compact subset defined by the conserved quantities, E°, and has a unique steady state. Furthermore, there exists a compact absorbing set within this open set E°, which is a consequence of the uniform persistence of the system. Thus, if both conditions (5.7) are satisfied, the matrix measure of the second additive compound of the Jacobian matrix is less or equal to a negative constant on E° and therefore in particular on the compact absorbing set, and the theorem implies the global stability of the unique steady state in E°. Together with the knowledge about the nonexistence of any ω-limit points on the boundary for any solutions starting in E, this ensures the global stability in E. Hence, we have found another condition for the global stability of the steady state where again the total concentration of antigen and receptor or the binding rate of T cell receptor and antigen has to be sufficiently small compared to the combined rates with which the enzyme-substrate complex and the phosphorylated complex decompose. Additionally, the catalysis rate, k_5, with which the phosphate residue is transferred has to be smaller than the sum of the rates with which the unphosphorylated and the phosphorylated complex decompose. A common assumption for enzyme-catalyzed reactions is that the catalysis reaction takes place quickly, meaning that the rate k_5 is relatively high. However, the situation where the kinetic proofreading system is reduced to a single phosphorylation step is not what we expect to find in a healthy body. Thus, the globally stable steady state under this condition might rather correspond to a pathological steady state. Furthermore, a relatively high catalysis rate may satisfy condition (5.6). Hence, we have a condition for a high (5.6) and one for a low catalysis rate (5.7) under which the global stability of the unique steady state is ensured by Li and Muldowney's theorem (Theorem 8).

In the calculations above we chose for the matrix A of Li and Muldowney's theorem the identity matrix, thus we reduced the calculations of the matrix measure to the second additive compound of the Jacobian matrix. Therefore, the question arises if we could improve the condition for global stability with a clever choice for the matrix A. However, no obvious choice emerges as the improvement of the condition entails controlling the term $|-k_1 \Theta + k_3 C_0 + k_4|$, which appears not to be eliminable but rather essential for the dynamics of the system. To see this, we will examine the matrix norm of the matrix $B := D_f D^{-1} + D A^{[2]} D^{-1}$ where D is a diagonal matrix, $D = \mathrm{diag}\{d_1, d_2, d_3\}$, and $A^{[2]}$ denotes the second additive compound of the Jacobian matrix as in the previous calculations and a_{ij} its entries:

$$
\begin{aligned}
||I_3 + hB|| &= \max_{||x||=1} \left\{ \left|\left| \left(I_3 + h \left(D_f D^{-1} + h D A^{[2]} D^{-1} \right) \right) x \right|\right| \right\} \\
&= \max_{||x||=1} \left\{ \left|\left| \left(I_3 + h \begin{pmatrix} \frac{(d_1)_f}{d_1} + a_{11} & \frac{d_1}{d_2} a_{12} & \frac{d_1}{d_3} a_{13} \\ \frac{d_2}{d_1} a_{21} & \frac{(d_2)_f}{d_2} + a_{22} & \frac{d_2}{d_3} a_{23} \\ \frac{d_3}{d_1} a_{31} & \frac{d_3}{d_2} a_{32} & \frac{(d_3)_f}{d_3} + a_{33} \end{pmatrix} \right) x \right|\right| \right\}.
\end{aligned}
$$

After inserting the entries of the second additive compound of the Jacobian matrix of the kinetic proofreading system with a single phosphorylation step, we have to maximize the following norm for some vector $||x|| = 1$:

$$\left|\left|\begin{pmatrix} x_1 + h\left(\left(\frac{(d_1)_f}{d_1} - k_1\Theta - k_2 - k_3L - k_3C_0 - k_4 - k_5\right)x_1 + \frac{d_1}{d_3}k_1\Theta x_3\right) \\ x_2 + h\left(\frac{d_2}{d_1}k_5x_1 + \left(\frac{(d_2)_f}{d_2} - k_1\Theta - k_2 - k_3L - k_6\right)x_2 + \frac{d_2}{d_3}\left(-k_1\Theta + k_3C_0 + k_4\right)x_3\right) \\ x_3 + h\left(\frac{d_3}{d_2}k_3Lx_2 + \left(\frac{(d_3)_f}{d_3} - k_3C_0 - k_4 - k_5 - k_6\right)x_3\right) \end{pmatrix}\right|\right|.$$

The entry $a_{23} = -k_1\Theta + k_3C_0 + k_4$ of $A^{[2]}$ is the only entry that does not have a fixed sign. Any choice for some constant entries d_2 and d_3 cannot avoid the necessity of the case distinction whether $k_1\Theta$ or $k_3C_0 + k_4$ is greater. Opting for the nonconstant choice $d_3 = -k_1\Theta + k_3C_0 + k_4$ to eliminate this case distinction only results in additional case distinctions for the terms involving d_3 as a factor. Since a_{23} is an off-diagonal entry, it contributes with its absolute value to the norms considered. Neither for the l^1-norm nor for the l^∞-norm or the norm $||x|| = \max\{|x_1|, |x_2| + |x_3|\}$ there is a choice for constant entries of the diagonal matrix D which would improve the previously found conditions. For example, for the l^1-norm the matrix norm $||I_3 + hB||$ is given by

$$\begin{aligned} \max\Bigg\{ & 1 + h\left(\frac{(d_1)_f}{d_1} - k_1\Theta - k_2 - k_3L - k_3C_0 - k_4 - k_5 + \left|\frac{d_1}{d_3}\right|k_1\Theta + \left|\frac{d_2}{d_1}\right|k_5\right), \\ & 1 + h\left(\frac{(d_2)_f}{d_2} - k_1\Theta - k_2 - k_3L - k_6 + \left|\frac{d_3}{d_2}\right|k_3L\right), \\ & 1 + h\left(\left|\frac{d_1}{d_3}\right|k_1\Theta + \left|\frac{d_2}{d_3}\right|\left|-k_1\Theta + k_3C_0 + k_4\right| + \frac{(d_3)_f}{d_3} - k_3C_0 - k_4 - k_5 - k_6\right)\Bigg\}. \end{aligned}$$

In particular, we see that most terms cancel for the choice $d_1 = d_2 = d_3$, which corresponds to our calculations where $D = I_3$. For any constant entry d_i, the differential vanishes, $(d_i)_f = \frac{d}{dt}d_i = 0$, which avoids an additional positive term $\left|\frac{(d_i)_f}{d_i}\right|$. Hence, we see that choosing different constants where $d_i \neq d_j$ does not seem to be helpful, and any nonconstant terms do not appear beneficial either as they produce new positive summands. Thus, to find more conditions under which the unique steady state of the kinetic proofreading system with Lck is globally asymptotically stable, we need a new approach.

We will now focus on the scenario where the enzyme Lck is nearly saturated at all times, allowing us to make the quasi-steady state assumption and apply Geometric Singular Perturbation Theory. A fast-slow system for the three-dimensional kinetic proofreading system with Lck and a single phosphorylation step, where R, M and L are expressed in terms of the conserved quantities, R_{tot}, M_{tot} and L_{tot}, and the other variables (5.5), is obtained by rescaling the time with a small parameter $0 < \varepsilon \ll 1$ and scaling the fast variables as well as the kinetic rates associated to mass action kinetics involving only slow variables with $\frac{1}{\varepsilon}$. Concretely, this means we define $\tilde{t} = \varepsilon t$ and $\tilde{L} = \frac{1}{\varepsilon}L$, $\tilde{B}_0 = \frac{1}{\varepsilon}B_0$ as well as $\tilde{k}_1 = \frac{1}{\varepsilon}k_1$, $\tilde{k}_2 = \frac{1}{\varepsilon}k_2$, $\tilde{k}_6 = \frac{1}{\varepsilon}k_6$. Since the conserved quantity L_{tot} consists only of fast variables, we redefine $\tilde{L}_{\text{tot}} = \varepsilon L_{\text{tot}}$. Dropping the tildes, we get the following system [83]:

$$\begin{aligned} C_0' &= k_1RM - k_2C_0 - k_3C_0L + k_4B_0 \\ \varepsilon B_0' &= k_3C_0L - (k_4 + k_5)B_0 \\ C_1' &= k_5B_0 - k_6C_1 \end{aligned} \tag{5.8}$$

where $R = R_{\text{tot}} - C_0 - \varepsilon B_0 - C_1$, $M = M_{\text{tot}} - C_0 - \varepsilon B_0 - C_1$ and $L = L_{\text{tot}} - B_0$ result from the expressions for the conserved quantities and the rescaling of the fast variables. Setting $\varepsilon = 0$

translates the system into the reduced problem. The critical set is then given by the points that satisfy the equation $k_3 C_0 L - (k_4 + k_5) B_0 = 0$, that is, the points where $C_0 = 0 = B_0$ or $C_0 \neq 0$ and $L = \frac{k_4+k_5}{k_3 C_0} B_0 = L_{\text{tot}} - B_0$, which translates into $B_0 = \frac{k_3 C_0}{k_4+k_5+k_3 C_0} L_{\text{tot}}$ and $L = \frac{k_4+k_5}{k_4+k_5+k_3 C_0} L_{\text{tot}}$. Since the above system consists of a single fast variable, B_0, and two slow variables, C_0 and C_1, the Jacobian matrix of the right-hand side of the differential equations for the fast variables with respect to these fast variables reduces to the partial derivative of $\varepsilon B_0'$ with respect to B_0:

$$\frac{\partial}{\partial B_0}\big(k_3 C_0 (L_{\text{tot}} - B_0) - (k_4 + k_5) B_0\big) = -k_3 C_0 - k_4 - k_5$$

which is strictly negative for arbitrary positive kinetic parameters and a nonnegative complex concentration C_0. In particular, the Jacobian matrix for $\varepsilon B_0'$ has full rank one, thus the critical set is a two-dimensional submanifold of $\mathbb{R}^3$. The eigenvalue of the Jacobian matrix is real and negative. Hence, there is no eigenvalue with vanishing real part and the critical manifold is normally hyperbolic, more precisely, it is an attracting manifold. We know from the last chapter that the solutions for the kinetic proofreading system with Lck lie within an invariant compact subset (Theorem 5), thus the normally hyperbolic critical manifold is compact, and we can apply Fenichel's theorem (Theorem 9) to the critical manifold. Therefore, we know that for a sufficiently small $\varepsilon > 0$ the flow of the kinetic proofreading system with Lck and one phosphorylation step converges to the flow of the reduced system. Thus, we can save ourselves additional calculations and transfer the characteristics we found for the full system to the reduced system. In particular, this means that the reduced system has a unique positive steady state which is hyperbolic and asymptotically stable, and does not possess any ω-limit points on the boundary. The flow of the reduced system, C_0' and C_1' on the critical manifold, corresponds to a two-dimensional differential system. Thus, we can apply Poincaré-Bendixson Theory for planar systems. Since the steady state is hyperbolic and asymptotically stable, all eigenvalues have a negative real part. Hence, the trace of the Jacobian matrix at the steady state, which corresponds to the sum of the eigenvalues, is negative. Consequently, the divergence of the function defining the reduced system is negative. Therefore, Bendixson's criterion implies that no periodic solution exists in $\mathbb{R}^2$ and hence, in particular, not in an open neighborhood of the nonnegative compact subset defined by the conserved quantities. The compact subset is invariant, thus all solutions starting in the compact subset stay there for all time. Consequently, it follows from the Poincaré-Bendixson theorem that any ω-limit points of the trajectories in the compact subset are either steady states or belong to a periodic solution or a homo- or heteroclinic orbit. Since the unique steady state is asymptotically stable, there cannot exist any homo- or heteroclinic trajectories. Furthermore, we just argued that no periodic solution can exist. Thus, the unique positive steady state is the only ω-limit point for all solutions in the compact subset defined by the conserved quantities. Hence, all trajectories converge towards this steady state. The steady state is therefore globally asymptotically stable [83]. Since the critical manifold is normally hyperbolic and attracting, the steady state has to be also globally stable for the full system for $\varepsilon > 0$ sufficiently small.

Lemma 6. *For $\varepsilon > 0$ sufficiently small in the fast-slow formulation (5.8) of the kinetic proofreading system with Lck and $N = 1$ (2.2), the system has a unique positive steady state within the invariant compact subset associated to a choice of positive conserved quantities which is globally stable with respect to this subset.*

Thus, we have found various conditions under which the global stability of the unique steady state of the kinetic proofreading system with one phosphorylation step, which is always asymptotically stable (Lemma 5), is ensured. We summarize the results obtained by Geometric Singular Perturbation Theory (Lemma 6) and by the application of Li and Muldowney's criterion (conditions (5.6) and (5.7)) to get one theorem including all conditions obtained that ensure global stability:

Theorem 10. *The kinetic proofreading system with Lck and $N = 1$ (2.2) has a unique positive steady state which is asymptotically stable within the invariant compact subset associated to the choice of positive conserved quantities. This steady state is globally asymptotically stable if one of the following conditions holds:*

(i) $k_1 (R_{\text{tot}} + M_{\text{tot}}) < k_4 + \frac{k_5+k_6}{2}$.

(ii) $k_1 (R_{\text{tot}} + M_{\text{tot}}) < 2k_4 + k_6$ *and* $k_5 < k_2 + k_6$.

(iii) The rate of change of the concentration of the enzyme-substrate complex B_0 has to be sufficiently small compared to the rates of change of the concentration of the substrate C_0 and the product C_1. This translates into $\varepsilon > 0$ has to be small enough for the fast-slow system (5.8).

The last criterion that $\varepsilon > 0$ has to be sufficiently small for the fast-slow system resulted from the introduction of fast variables for the enzyme and the enzyme-substrate complex concentration, which implies that the concentration of enzymes and enzyme-substrate complexes has to be sufficiently small. That is, the concentration of free and bound Lck molecules has to be small compared to the concentration of T cell receptor complexes, unphosphorylated and phosphorylated, which is a realistic assumption. Enzymes are highly specific and have a strong affinity to their substrates. Thus, if the substrate concentration is significantly higher than the enzyme concentration, the assumption that the enzymes are almost always saturated is reasonable, which corresponds to the quasi-steady state assumption. Whereas the reduction resulting from the quasi-steady state assumption, also referred to as Michaelis-Menten reduction, is based on a realistic biological situation for a healthy body, we recall that the reduction of the kinetic proofreading system to a single phosphorylation step is not a model for the triggering of the immune response of a healthy body as there should be more than a single phosphorylation step to optimize the discrimination process.

5.3 Fast-slow Formulation for the Kinetic Proofreading System with Lck

The kinetic proofreading system with Lck and at least two phosphorylation steps supports multiple steady states (Theorem 3). Thus, there exist kinetic parameters such that the system has more than one steady state. This does not preclude that under other parameters the system may only have a single steady state. Furthermore, for more than one phosphorylation step, we have no explicit expressions for the steady states and know nothing about their local stability properties. However, we can translate the system into a fast-slow system and show for a number of two or three phosphorylation steps that the reduction to the slow flow is regular. More precisely, the critical set is a normally hyperbolic manifold. Moreover, we can show that it is attracting. Thus, the stability of any steady state is determined by the dynamics of the slow variables, C_i, if the rate of change of the fast variables, the enzyme-substrate complexes B_i, is sufficiently small.

We can translate the kinetic proofreading system with Lck (2.2) and at least two phosphorylation steps, $N \geq 2$, into fast-slow systems by setting $\tilde{t} = \varepsilon t$, $\tilde{L} = \frac{1}{\varepsilon} L$ and $\tilde{B}_i = \frac{1}{\varepsilon} B_i$ for $0 \leq i \leq N-1$ as well as $\tilde{k}_1 = \frac{1}{\varepsilon} k_1$, $\tilde{k}_2 = \frac{1}{\varepsilon} k_2$ and $\tilde{k}_{6+4(i-1)} = \frac{1}{\varepsilon} k_{6+4(i-1)}$ for $1 \leq i \leq N$ where $\varepsilon > 0$. Furthermore, we set $\tilde{L}_{\text{tot}} = \varepsilon L_{\text{tot}}$. Here the enzyme-substrate complexes B_i, $0 \leq i \leq N-1$, are the fast variables and the complexes C_i, $0 \leq i \leq N$, are the slow variables. Dropping the tildes gives us the following fast-slow formulation for the kinetic proofreading systems with Lck and $N \geq 2$:

$$\begin{aligned}
C_0' &= k_1 RM - k_2 C_0 - k_3 C_0 L + k_4 B_0 \\
C_i' &= k_{5+4(i-1)} B_{i-1} - k_{6+4(i-1)} C_i - k_{3+4i} C_i L + k_{4+4i} B_i, \qquad 1 \leq i \leq N-1 \\
C_N' &= k_{5+4(N-1)} B_{N-1} - k_{6+4(N-1)} C_N \\
\varepsilon B_i' &= k_{3+4i} C_i L - (k_{4+4i} + k_{5+4i}) B_i, \qquad 0 \leq i \leq N-1
\end{aligned} \tag{5.9}$$

where $R = R_{\text{tot}} - \sum_{i=0}^{N} C_i - \varepsilon \sum_{i=0}^{N-1} B_i$, $M = M_{\text{tot}} - \sum_{i=0}^{N} C_i - \varepsilon \sum_{i=0}^{N-1} B_i$ and $L = L_{\text{tot}} - \sum_{i=0}^{N-1} B_i$. The critical set is given by the points where $\varepsilon B_i' = 0$ for all $0 \leq i \leq N-1$. The Jacobian matrix of the right-hand side of the differential equations for the fast variables with respect to these fast variables is then given by the matrix $\left(\frac{\partial(\varepsilon B_i')}{\partial B_j}\right)_{ij}$. Substituting the expression for L in terms of the conserved quantities gives the following expression for the differential equations of the fast variables B_i, $0 \leq i \leq N-1$:

$$\begin{aligned}\varepsilon B_i' &= k_{3+4i} C_i \left(L_{\text{tot}} - \sum_{j=0}^{N-1} B_j \right) - (k_{4+4i} + k_{5+4i}) B_i \\ &= k_{3+4i} C_i L_{\text{tot}} - \left(k_{3+4i} C_i + k_{4+4i} + k_{5+4i}\right) B_i - k_{3+4i} C_i \sum_{\substack{j=0 \\ j \neq i}}^{N-1} B_j.\end{aligned}$$

Thus, the entries of the Jacobian matrix $\left(\frac{\partial(\varepsilon B_i')}{\partial B_j}\right)_{ij}$ are given by

$$\begin{aligned}\frac{\partial\left(\varepsilon B_i'\right)}{\partial B_i} &= -\left(k_{3+4i} C_i + k_{4+4i} + k_{5+4i}\right) \\ \text{and} \quad \frac{\partial\left(\varepsilon B_i'\right)}{\partial B_j} &= -k_{3+4i} C_i \quad \text{for } j \neq i.\end{aligned}$$

The Jacobian matrix $\left(\frac{\partial(\varepsilon B_i')}{\partial B_j}\right)_{ij}$ has full rank for all complex concentrations. Hence, the same is true for the Jacobian matrix with respect to all variables. Thus, the critical set described by the points where $\varepsilon B_i' = 0$ for all $0 \leq i \leq N-1$ is a submanifold. Furthermore, it is compact. Setting $x_i = k_{3+4i} C_i$ and $a_i = k_{4+4i} + k_{5+4i}$ for all $0 \leq i \leq N-1$, the characteristic polynomial of the Jacobian matrix associated to the fast variables is given by

$$\det\left(\lambda \mathrm{I}_N - \left(\frac{\partial\left(\varepsilon B_i'\right)}{\partial B_j}\right)_{ij}\right) = \begin{vmatrix} \lambda + x_0 + a_0 & x_0 & x_0 & \cdots & x_0 \\ x_1 & \lambda + x_1 + a_1 & x_1 & \cdots & x_1 \\ x_2 & x_2 & \lambda + x_2 + a_2 & & x_2 \\ \vdots & & & \ddots & \\ x_{N-1} & x_{N-1} & \cdots & & \lambda + x_{N-1} + a_{N-1} \end{vmatrix}$$

$$= \lambda^N + \lambda^{N-1} \underbrace{\left(\underbrace{\sum_{i=0}^{N-1} x_i}_{\geq 0} + \underbrace{\sum_{j=0}^{N-1} a_j}_{>0} \right)}_{=: b_{N-1}}$$

$$+ \sum_{k=2}^{N} \lambda^{N-k} \underbrace{\left(\underbrace{\sum_{\substack{i \in \{0,\ldots,N-1\} \\ \{j_1,\ldots,j_{k-1}\} \subseteq \{0,\ldots,N-1\} \setminus \{i\} \\ j_l \neq j_r}} \left(x_i \prod_{l=1}^{k-1} a_{j_l} \right)}_{\geq 0} + \underbrace{\sum_{\substack{\{j_1,\ldots,j_k\} \subseteq \{0,\ldots,N-1\} \\ j_l \neq j_r}} \left(\prod_{l=1}^{k} a_{j_l} \right)}_{>0} \right)}_{=: b_{N-k}}.$$

See Proposition 1 and 2 in the appendix for explicit calculations. Since $x_i = k_{3+4i} C_i \geq 0$ and $a_i = k_{4+4i} + k_{5+4i} > 0$ for all $0 \leq i \leq N-1$, all coefficients b_i are positive. In particular $b_0 \neq 0$, which implies that zero is not an eigenvalue. However, to determine if the critical manifold is hyperbolic, we have to assure that no eigenvalue has a zero real part.

For $N = 2$ and $N = 3$ we can determine the critical manifold as hyperbolic and attracting. This can be shown with the Routh-Hurwitz criterion. For two phosphorylation steps, $N = 2$, the characteristic polynomial associated to the critical manifold is of second degree. Hence, for the critical manifold to be hyperbolic and attracting all minors of the determinant

$$D_2 = \begin{vmatrix} b_1 & 0 \\ 1 & b_0 \end{vmatrix}$$

have to be positive. Since $b_1 > 0$ and $D_2 = b_1 b_0 > 0$, this is true. For $N = 3$ the minors of the determinant

$$D_3 = \begin{vmatrix} b_2 & b_0 & 0 \\ 1 & b_1 & 0 \\ 0 & b_2 & b_0 \end{vmatrix}$$

have to be calculated. As $D_1 = b_2 > 0$ and $D_3 = (-1)^{3+3} b_0 D_2 = b_0 D_2$, the stability is determined by the sign of the minor $D_2 = b_1 b_2 - b_0$. This minor is also positive since b_0 consists of terms of three factors, x_i and a_i as defined above, with distinct indices, and $b_1 b_2$ is the product of terms with two or one factor, resulting in terms with three factors. This includes in particular the summands with distinct indices. Thus, any summand of b_0 is also a summand in the product $b_1 b_2$, and $D_2 = b_1 b_2 - b_0$ is positive. Hence, the critical manifold associated to the fast-slow system of the kinetic proofreading system with $N = 3$ is also attracting. For $N \geq 4$, it is not possible to continue the argument with the Routh-Hurwitz criterion since the positive and negative terms of the second minor differ in their number of factors. For example, for $N = 4$ the minors of the determinant

$$D_4 = \begin{vmatrix} b_3 & b_1 & 0 & 0 \\ 1 & b_2 & b_0 & 0 \\ 0 & b_3 & b_1 & 0 \\ 0 & 1 & b_2 & b_0 \end{vmatrix}$$

have to be calculated. Already the second minor $D_2 = b_2 b_3 - b_1$ can have a positive or negative sign or be of sign zero depending on the kinetic parameters and the slow variables C_i, $0 \leq i \leq N$. However, for $N = 2$ and $N = 3$, the critical manifold is hyperbolic and attracting, which implies that for $\varepsilon > 0$ small enough, the corresponding fast-slow system has the same stability property with respect to the fast variables. Thus, the stability of any steady state on the critical manifold, or sufficiently close to it as $\varepsilon \to 0$, is determined by the flow on the critical manifold.

Lemma 7. *For $\varepsilon > 0$ sufficiently small in the fast-slow formulation (5.9) of the kinetic proofreading systems with Lck and $N = 2$ or $N = 3$, the stability of the steady states is determined by the stability properties of these steady states with respect to the reduced system.*

Conclusion

In this work, we analyzed the system of ordinary differential equations associated to a kinetic proofreading system with explicit modeling of the kinase Lck and ZAP-70, respectively. We showed that including the kinase Lck in form of an enzyme-substrate reaction in the basic kinetic proofreading model suffices to generate multistationarity (Theorem 3) by applying the Advanced Deficiency Algorithm of Chemical Reaction Network Theory. This is a fundamental change of the characteristics of McKeithan's kinetic proofreading model, which is known to possess a unique positive steady state which is globally asymptotically stable [95]. This property remains when a dephosphorylation rate is included in the model [85]. Among the existing models, only the additional extension with the phosphatase Shp-1 contributing to the dephosphorylation through a negative feedback loop has been shown to enable the system to support multiple steady states. Specifically, multistationarity in this system has been established for the case of three phosphorylation steps [85]. In this work, we reveal that such a negative feedback is not necessary to create the capacity for multiple steady states for a model incorporating a kinetic proofreading mechanism. The explicit modeling of the kinase Lck by an enzyme-substrate reaction suffices to produce multistationarity. In other words, there exist positive kinetic parameters and nonnegative initial concentrations such that the system admits more than one positive steady state. We showed that the same is true for the additional extension by the molecule ZAP-70 (Theorem 4), following the core model of Altan-Bonnet and Germain [2]. Thus, this core model itself generates the ability to support multiple positive steady states, and a feedback loop, negative or positive, is not necessary for multistationarity in the kinetic proofreading mechanism. Altan-Bonnet and Germain's model is distinguished by its prediction of a digital response in T cells, manifesting at the population level as a bimodal distribution. The existence of multiple steady states within the same stoichiometric compatibility class may be a hint to this observed digital response. Furthermore, we showed that the solutions of both systems, the kinetic proofreading system with Lck and with ZAP-70, respectively, are contained in an invariant compact subset, and there do not exist any ω-limit points on this boundary (Theorem 5 and Theorem 6). This is true for any arbitrary number of phosphorylation steps, and implies in particular the existence of at least one positive steady state and the uniform persistence of the systems. The arguments for the existence of an invariant compact subset and the non-existence of boundary ω-limit points can be transferred to systems of a similar category (Lemma 2 and Lemma 4). That is, to systems which follow mass action kinetics and respect the conservation of mass and which arise from a chemical reaction network without external supply or removal of species or complexes, in other words, systems where every species contributes to a conserved quantity. While these characteristics hold for the kinetic proofreading systems with an arbitrary number of phosphorylation steps, there have to be at least two phosphorylation steps for the systems to support multistationarity. A single phosphorylation step in the kinetic proofreading system with Lck does not suffice to generate multistationarity. In the case of a single phosphorylation step, there exists a unique positive steady state which is asymptotically stable. We found conditions under which this steady state is also globally asymptotically stable (Theorem 10) by using a generalized Bendixson criterion, which involves a matrix measure and compound matrices, and Singular Perturbation Theory. To close with a first hint regarding the stability of the steady states in the case of multiple phosphorylation steps, we applied Geometric Singular Perturbation Theory to show that for two or three phosphorylation steps, the critical set is a normally hyperbolic manifold and attracting. Thus, in this case we identified the slow variables, which correspond to the phosphorylated complexes, as determining for the stability of the steady states (Lemma 7). This work emphasizes the significance of the kinetic proofreading mechanism. It reveals that important features can be generated by the inclusion of an enzyme in the basic kinetic proofreading model and do not require further model

extensions. Thus, this model serves as a bridge between minimal and more complex models, assisting with the identification of triggering mechanisms within the latter.

Certain fundamental aspects of the kinetic proofreading model with Lck and ZAP-70 remain unresolved by the end of this work. While we have identified the systems' ability to support multiple steady states, further investigations are needed to determine the stability of these states. In the case of multistationarity, it is possible to identify two exemplary steady states. However, the precise number of steady states is unknown. Furthermore, it is not clear which values of the kinetic parameters give rise to these multiple steady states. It is possible to determine specific kinetic parameters under which multistationarity is guaranteed, but it remains unclear which parameter ranges support or prevent multistationarity. Answers to these questions could potentially help to identify steady states associated to healthy and pathological conditions, and contribute to a deeper understanding of the mechanism of T cell activation.

Outlook

The activation and regulation of T cells are highly complex processes, involving multiple receptors, cell surface proteins and various intricate intracellular pathways. However, kinetic proofreading models that focus on the initial activation signal, achieved through the sustained binding of an antigen-presenting MHC molecule to the T cell receptor, provide robust foundational frameworks for understanding antigen discrimination. The lasting relevance of these relatively simple models is supported by recent mathematical and biological research, which emphasizes the critical role of the pMHC-TCR complex lifetime as a key kinetic parameter for initiating a T cell receptor activation signal.

In the quest to identify the most influential determining kinetic parameter for T cell activation, experiments have shown correlations between the strength of the activation signal and the binding constant, which is the ratio of binding rate to dissociation rate of the pMHC-TCR complex and sometimes referred to as affinity, and between the signal strength and the dissociation rate, leaving controversial discussions [40, 71, 101, 53, 10, 15]. Another study [1], addressing the role of the binding rate, found that for a larger variety of the binding rate, neither the dissociation constant nor the binding rate show a satisfying correlation to the strength of the activation signal, suggesting that both correlations may correspond to limit cases. Dushek et al. [20] addressed in 2011 this question which kinetic parameters are critical for the efficiency of T cell activation by comparing two models, emphasizing either the dissociation constant or the dissociation rate as critical parameter. The term efficiency refers here to the induced signal strength measured by downstream molecules. They compared two models, the "affinity model" also known as "occupancy model" and the "productive hit rate model". The former states that the number of pMHC-TCR complexes is the primary determinant of T cell response [20]. The latter assumes that the pMHC-TCR binding time is essential and a single pMHC can serially bind to multiple TCRs. The two assumptions of this model lead to a maximal T cell stimulation of intermediate dissociation rates for the pMHC-TCR complexes. For the analysis, the system is assumed to be in steady state. The authors then calculated the maximal response by considering the limit case for large pMHC concentrations. For the affinity model, the maximal response is assumed to be given by the product of the total number of receptors and the area of the contact interface. Apart from these two factors, the expression for the hit rate model involves the dissociation rate and the time as factors, as well as a decreasing function of the dissociation rate describing the probability to introduce a downstream signal. The probability that a pMHC-TCR complex passes all phosphorylation steps in the kinetic proofreading model can be such a function. Besides the value for maximal response, the value at which the antigen concentration gives rise to the half of the maximal response is used to characterize the models. This value is referred to as antigen potency. The authors found that both models share the same antigen potency. Experiments showed a correlation between the concentration for half-maximal response and dissociation constant, the ratio of binding rate to dissociation rate, as well as a correlation between the maximal response and the dissociation rate [20]. The former correlation agrees with previous reports and was used to support the affinity model [40, 71, 101]. However, the authors showed that this correlation is predicted by both models. On the other hand, the second correlation is only predicted by the productive hit rate model. Thus, the hit rate model could explain both correlations observed, whereas the affinity model only predicts the first but not the second correlation. The productive hit rate model predicts that the dissociation rate is the critical parameter. In the productive hit rate model, a decrease in the dissociation rate can lead to a decrease in the induced activation signal, as observed in several experiments [53, 10, 15]. This means a higher dissociation constant or lower affinity can reduce the activation signal. These findings were

used to support the suggestion that the dissociation rate is the determining parameter for T cell activation. Dushek et al. suggested determining antigens not only by their potency, that is, their half-maximal efficacy, but also by their maximal efficacy. With a view to the kinetic proofreading system this underlines that, apart from the affinity of the pMHC-TCR complex, the duration of the binding of the phosphorylated complexes and the dissociation rate are critical for the effectivity of the T cell activation.

In 2019 Tischer and Weiner [102] and Yousefi et al. [110] developed a new approach to show that the binding times of the complexes of T cell receptor and pMHC molecule are a key parameter for T cell activation. For their experiments, they used artificial T cell receptors not involving co-receptors. Using a technique where light stimulates different dissociation times of the complexes, they were able to control and vary the binding time of the complexes while holding all other parameters constant. Conventional methods rely on altered peptides to change the binding time. However, an altered peptide varies not only the binding time but also the mechanical stability of the complex due to the altered binding interface. The new approach of the authors using light stimulation enables the direct measurement of the influence of binding time of the complexes on the activation of the T cell. Hence, the approach can be used to test kinetic proofreading models. Tischer and Weiner [102] compared their experimental data to the prediction of the T cell activation signal of McKeithan's kinetic proofreading model. For the measurement of the activation signal serves a molecule whose accumulation is induced by the fully phosphorylated complexes. For the comparison with the kinetic proofreading model, they assumed that the concentration of these molecules is saturable and includes a basal signaling. The probability that a phosphorylation step is completed is assumed to be proportional to the half-life of the complexes to the receptor. Their fitting of McKeithan's kinetic proofreading model is in accordance with their experimental data, giving evidence that the binding time of the complexes is a key parameter for antigen discrimination. Furthermore, their experimental data revealed that ZAP-70 recruitment is independent of the binding time of the T cell receptor complex and the pMHC molecule. Yousefi et al. [110] used a different artificial T cell receptor than Tischer and Weiner. Their T cell receptor is soluble and needs a cross-link to another T cell receptor. The ligands, which bind bivalently to the two T cell receptors, are modeled as dimers. The steady state fraction of receptors exhibiting all phosphorylation steps is modeled according to McKeithan's proofreading model as the probability of a single phosphorylation step to be completed raised to the power of the number of phosphorylation steps. As a reference for the strength of the activation signal, the authors measured the concentration of calcium, which is involved in an intracellular signaling pathway induced by the fully phosphorylated complexes. The authors assume the calcium response to be proportional to the steady state fraction that completed all binding events and the number of T cell receptors that are cross-linked. They compared their experimental data with the mathematical model including kinetic proofreading and also with the model output when kinetic proofreading was absent. The authors found that only the model with kinetic proofreading yielded a satisfactory fit [110]. Furthermore, they showed that fast rebinding of just dissociated ligands, which can increase the effective binding time, can be neglected in their approach. The two approaches, using light stimulation techniques that keep all parameters constant except for the binding time of the T cell receptor and pMHC molecule, identify the binding time as the decisive factor in inducing intracellular signaling of the T cell receptor and thus substantiate the kinetic proofreading model. This underlines the importance and continuing relevance of the kinetic proofreading models based on McKeithan's model.

The ability of the T cell receptor for antigen discrimination is crucial for an intact immune system. Dysregulation of T cell receptor signaling can lead to the generation of various diseases [90]. T cell receptors that do not recognize foreign antigens or do not bind them long enough to induce the intracellular signaling pathway can result in an immune deficiency. Conversely, if the T cell receptors are hyperactive, potentially sending intracellular activation signals for self antigens, this can lead to an autoimmune disease. Both immune deficiency and autoimmunity have been associated to misexpressed tyrosine phosphatase CD45 [59, 100, 50, 78]. When this phosphatase, which is

responsible for the dephosphorylation of the ITAMs and thereby for the decomposition of the phosphorylated pMHC-TCR complexes, is dysfunctional, that is, up- or downregulated, then the complexes bound to a pathogenic antigen may not reach the fully phosphorylated state or, on the other hand, antigen that should not trigger an immune response may pass all phosphorylation steps. Another source for immune deficiency in humans is associated to a mutation in the gene for ZAP-70 [88, 108]. Thus, tightly related to the modeling of T cell activation are dysfunctional T cell receptors and the question of how these can be regulated and controlled.

Immunotherapies target the regulation of the immune response to treat diseases with the help of the body's own immune cells. T cell receptor engineering has provided new approaches in the fight against cancer, including adoptive cellular therapy, checkpoint blockade, tumor microenvironment regulation and cancer therapeutic vaccines [90]. A major breakthrough in the adoptive cellular therapy was the development of synthetic chimeric antigen receptors (CARs). These artificial T cell receptors recognize and lyse tumor antigens on the surface of the malignant cell independently of MHC presentation, which overcomes the barrier that tumor cells downregulate MHC molecules, preventing the cell activation [90]. For the CAR T cell therapy, the patient's own T cells are collected and genetically modified to express a synthetic receptor that binds to a specific tumor antigen. The engineered cells are then expanded in vivo and reinfused into the patient's body [90]. Another breakthrough in the field of cancer immunotherapy was achieved by the discovery of inhibitory checkpoints. Inhibitory checkpoints are co-signaling molecules that inhibit the activation signal. Co-stimulatory and co-inhibitory signaling contribute to the antigen discrimination mechanism of the T cell receptor to ensure that pathogens are detected and initiate an immune response and prevent the body's own cells from being attacked. Naive T cells need a second activation signal, in addition to the binding of the T cell receptor to the peptide-carrying MHC molecule. To activate naive T cells, antigen-presenting cells carry co-stimulatory molecules that interact with co-stimlatory receptors on the surface of naive T cells. One of these co-stimulatory receptors is the cell surface protein CD28. CD28 forms microclusters with the T cell receptor and these microclusters recruit and activate proteins which, in turn, recruit co-stimulatory molecules as well as co-inhibitory receptors to the synapse. Two of these co-inhibitory receptors are CTLA4, cytotoxic T lymphocyte antigen 4, and PD1, programmed cell death 1. CTLA4 displaces CD28 from the cell surface, preventing it from recruiting and activating a co-stimulatory molecule. CTLA4 and PD1 disrupt positive signaling through recruitment of phosphatases which dephosphorylate key signaling molecules [12]. One of the phosphatases activated by PD1 is Shp-1, inducing amongst other the dephosphorylation of the CD3 ζ chains and ZAP-70 (cf. Section 1.2). Thus, CTLA4 and PD1 both inhibit the T cell activation signal. In this way, CTLA4 acts as a brake for the co-stimulatory CD28 molecule through which it is activated. Besides the co-stimulatory receptor CD28 and the co-inhibitory receptors CTLA4 and PD1 there are further co-signaling molecules involved in the complex dynamic of T cell activation and regulation. In fact, co-signaling ligands and counter-receptors have been identified on nearly all cell types and have a crucial role in regulating T cell activation, subset differentiation, effector function and survival [12]. It remains challenging to characterize the effect of a single co-stimulatory or co-inhibitory receptor as there is a significant overlap in their downstream signaling pathways. The entangled mechanisms of co-stimulation and co-inhibition contribute significantly to the regulation and fine-tuning of the immune response, supporting the recognition and rapid response to pathogenic antigens and, on the other hand, ensuring that the immune response does not overshoot and is not directed against the body's own cells. Whereas the co-stimulatory molecule CD28 is necessary to evoke an immune response, the co-inhibitory molecules CTLA4 and PD1 serve as immune checkpoints. CTLA4 and PD1 are highly expressed by various types of cancers [90]. Expressing inhibitory molecules is a mechanism that tumor cells employ to evade detection and elimination by the immune system. Immune checkpoint therapy targets these co-inhibitory molecules and aims to prevent the inhibitory signals, that is, to release the brakes on the immune system. This recent approach to therapy cancer goes back to research on CTLA4 and PD1 by Allison and Honjo and was awarded with the Nobel Prize in Physiology or Medicine in 2018. Another approach in immunotherapy are cancer therapeutic vaccines. Combined with TCR-based vaccines using

tumor-specific antigens prove an efficient antitumor response. Upon vaccination, T cells binding to such tumor-specific antigens have been found to express a high amount of PD1 [90]. Consequently, a combination with PD1 blockade increases the effect of the vaccine. Including therapeutic vaccines in the therapy of cancer cells gives the potential of developing long-lasting memory T cells and thus protection against cancer recurrence. The remarkable advances in immunotherapy and in particular in immuno-checkpoint therapy call for an extension of the model examined in this work to include regulatory checkpoints in the kinetic proofreading model, raising the question of how these checkpoints change the dynamics of the system. Does expanding the model by the co-inhibitory molecule PD1 or by the combination of the co-stimulatory CD28 receptor and the inhibitory CTLA4 receptor change the ability of the system to support multistationarity? Is the system still able to exhibit multiple positive steady states, or do co-inhibitory molecules prevent multistationarity? The kinetic proofreading model examined in this work could serve as a basis to analyze the dynamics that regulatory checkpoints impose on the T cell activation process.

Notation

Kinetic Proofreading Models

N	Number of Phosphorylation Steps (pages 13,22)
$N = 2n$	Even Number of Phosphorylation Steps for the System including ZAP-70 (page 25)

Chemical Reaction Networks

$\mathcal{C}$	Set of Complexes (page 33)
CC_i	Colinearity Class (page 48)
δ	Deficiency (page 36)
δ_i	Deficiency of the i-th Linkage Class (page 37)
d	$\dim(\ker(L_{\mathcal{O}}))$ (page 48)
g	Confluence Vector (page 41)
$\kappa_{y\to y'}$	Rate Function (page 33)
k_i	Rate Constant for the i-th Reaction (page 32)
$\mathcal{K}$	Set of Rate Functions (page 33)
$\mathcal{K}_m$	Set of Rate Constants (page 34)
l	Number of Linkage Classes (page 35)
$\mathcal{L}_i$	Lower Shelf of CC_i (page 54)
$L_{\mathcal{O}}$	Linear Map from $\mathbb{R}^{\mathcal{O}}$ to $\mathbb{R}^{\mathcal{S}}$, corresponds to $N\|_{\mathcal{O}}$ (page 47)
μ	Signature (pages 42,57)
m	Number of Species (page 34)
$\mathcal{M}_i$	Middle Shelf of CC_i (page 54)
M_i	Value of $\mathcal{M}_i$ (page 55)
n, $\overline{n}$	Number of Complexes (pages 36,68)
n_i	Number of Complexes in the i-th Linkage Class (page 37)
N	Stoichiometric Matrix (page 34)
$\mathcal{O}$	Set of Reactions in Orientation (page 47)
r	Number of Reactions (page 34)
$\overline{r}$	Number of Reactions in Orientation (page 47)
$\mathcal{R}$	Set of Reactions (page 33)
$\mathbb{R}_+$	Positive Real Numbers (page 32)
$\overline{\mathbb{R}}_+$	Nonnegative Real Numbers (page 32)
$\mathbb{R}^{\mathcal{I}}$	$\mathbb{R}^{\|\mathcal{I}\|}$ where $\mathcal{I}$ is a Finite Set and $\|\mathcal{I}\|$ denotes its Cardinality (page 32)
s	$\operatorname{rank}(N)$, Rank of a Chemical Reaction Network (page 34)
s_i	Rank of the i-th Linkage Class (page 37)
S	Stoichiometric Subspace (page 34)
$\mathcal{S}$	Set of Species (page 33)
$\mathcal{T}$	Coplanar Set (page 49)

$\mathcal{U}_i$	Upper Shelf of CC_i (page 54)
v^i	Basis Vector of $\ker(L_{\mathcal{O}})$, $i \in \{1, \ldots, d\}$ (page 48)
$w_{y \to y'}$	w-vector $\left(\in \mathbb{R}^d\right)$ (page 48)
w_i	Colinearity Class Vector for CC_i (page 49)
ω_i	Characteristic Function of the i-th Species (page 32)

Glossary

Affinity Binding strength of a molecule to another, for example of a ligand to a receptor. (pages 1, 12)

Agonist Substance that binds to a receptor, initiating a signal that leads to a physiological response. In T cell activation, the agonist refers to the antigen intended to be recognized and bound by the T cell receptor, thereby triggering an immune response. (page 1)

Antagonist Substance that binds to a receptor without initiating a physiological response. Antagonists block receptors, thereby diminishing the response initiated by agonists intended to bind to the same receptor. In T cell activation, antagonists are antigens that are not supposed to trigger a T cell response but which possess epitopes that are similar enough to those of the antigen to bind to the T cell receptor when presented by an MHC molecule. (page 1)

Antigen Substance that can trigger an immune response. Antigens have molecular structures that can be recognized and bound by antibodies or T cell receptors. (pages 1, 4)

APC Antigen-presenting cell. APCs present antigens via MHC molecules on the cell surface. (page 5)

CD3 Protein of the T cell receptor complex with an intracellular domain that carries ITAMs. (page 9)

CD4 Co-receptor of the T cell receptor of helper T cells. CD4 recognizes regions on MHC class II proteins. (page 6)

CD8 Co-receptor of the T cell receptor of cytotoxic T cells. CD8 recognizes regions on MHC class I proteins. (page 6)

Cytokines Signal proteins. (page 5)

Epitope Portion of the antigen's molecular structure that is recognized by an antigen receptor. (page 6)

ERK Extracellular signal-related kinase. Enzyme involved in the intracellular signal transduction. Associated to a positive feedback in the T cell activation process. (pages 2, 14)

ITAM Immunoreceptor tyrosine-based activation motif. Intracellular regions of the invariant chains of the T cell receptor complex that can be phosphorylated. (page 9)

Kinase Enzyme that catalyzes the transfer of a phosphate group to a molecule. (pages 2, 9)

Lck Tyrosine kinase associated to the co-receptor of a T cell receptor complex. Primarily responsible for the phosphorylation of the ITAMs of the T cell receptor complex. (pages 3, 10)

MAPK cascade Mitogen-activated protein kinase cascade. A series of protein kinases of the intracellular signal pathway initiated by the T cell receptor upon antigen recognition. (page 14)

MHC Major histocompatibility complex. Protein for antigen presentation on the cell surface. MHC class I molecules are expressed on most of the body's own cells and serve also as self-identifying label. MHC class II molecules are found on the surface of "professional" APCs like macrophages, dendritic cells and B cells and are directed to helper T cells. (pages 1, 5)

Pathogen Microorganism that can cause a disease. (page 4)

Phosphatase Enzyme that removes phosphate groups from a molecule. (pages 2, 9)

pMHC Peptide-carrying MHC molecule. MHC molecule that has bound to an antigen and is presenting it. (pages 1, 13)

ppERK Doubly phosphorylated ERK. Activated form of the kinase ERK. (page 14)

Shp-1 Phosphatase. Activated Shp-1 can bind to the T cell receptor complex and dephosphorylate its ITAMs. Associated to a negative feedback in the T cell activation process. (pages 2, 15)

TCR T cell receptor. (pages 1, 9, 13)

ZAP-70 Tyrosine kinase associated to the T cell receptor complex. Activated by Lck. The activated ZAP-70 phosphorylates and thus activates other intracellular signaling molecules. (pages 3, 10)

List of Figures

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Appendix

Proposition 1. *For $n \geq 2$ the following statement where $\sum_{k=1}^{0} = 0$ holds:*

$$\begin{vmatrix} x_1 & x_1 & x_1 & \cdots & x_1 \\ x_2 & \lambda + x_2 + a_2 & x_2 & \cdots & x_2 \\ x_3 & x_3 & \lambda + x_3 + a_3 & \cdots & x_3 \\ \vdots & & & \ddots & \vdots \\ x_{n-1} & & & & \lambda + x_{n-1} + a_{n-1} \end{vmatrix}$$
$$= x_1 \left(\lambda^{n-2} + \sum_{k=1}^{n-2} \left(\lambda^{n-2-k} \sum_{\substack{\{i_1,\ldots,i_k\} \subseteq \{2,\ldots,n-1\} \\ i_j \neq i_l}} \left(\prod_{j=1}^{k} a_{i_j} \right) \right) \right).$$

Proof. We prove the proposition by induction. For $n = 2$ the statement holds. Assume now the induction hypothesis (IH) that the statement were true for $n-1$. For the induction step $n-1 \mapsto n$, the matrix whose determinant is to be calculated is an $n \times n$ matrix. We expand the determinant along the last row. As the minors associated to the expansion along the last row and the second to the $(n-1)$-th column have two identical columns, precisely the first and the last column, they do not contribute to the expansion:

$$\begin{vmatrix} x_1 & x_1 & x_1 & \cdots & x_1 \\ x_2 & \lambda + x_2 + a_2 & x_2 & \cdots & x_2 \\ x_3 & x_3 & \lambda + x_3 + a_3 & \cdots & x_3 \\ \vdots & & & \ddots & \vdots \\ x_n & \cdots & & & \lambda + x_n + a_n \end{vmatrix}$$
$$= (-1)^{n+1} x_n \begin{vmatrix} x_1 & x_1 & \cdots & x_1 \\ \lambda + x_2 + a_2 & x_2 & \cdots & x_2 \\ x_3 & \lambda + x_3 + a_3 & \cdots & x_3 \\ \vdots & & \ddots & \vdots \\ x_{n-1} & \cdots & \lambda + x_{n-1} + a_{n-1} & x_{n-1} \end{vmatrix}$$
$$+ (-1)^{n+n} (\lambda + x_n + a_n) \begin{vmatrix} x_1 & x_1 & x_1 & \cdots & x_1 \\ x_2 & \lambda + x_2 + a_2 & x_2 & \cdots & x_2 \\ x_3 & x_3 & \lambda + x_3 + a_3 & \cdots & x_3 \\ \vdots & & & \ddots & \vdots \\ x_{n-1} & \cdots & & & \lambda + x_{n-1} + a_{n-1} \end{vmatrix}$$

$$
\begin{aligned}
&= \Big((-1)^{(n+1)+(n-2)} x_n + (\lambda + x_n + a_n)\Big) \\
&\quad \cdot \begin{vmatrix} x_1 & x_1 & x_1 & \cdots & x_1 \\ x_2 & \lambda + x_2 + a_2 & x_2 & \cdots & x_2 \\ x_3 & x_3 & \lambda + x_3 + a_3 & \cdots & x_3 \\ \vdots & & & \ddots & \vdots \\ x_{n-1} & \cdots & & & \lambda + x_{n-1} + a_{n-1} \end{vmatrix} \\
&\overset{\text{IH}}{=} (\lambda + a_n)\, x_1 \left(\lambda^{n-2} + \sum_{k=1}^{n-2} \left(\lambda^{n-2-k} \sum_{\substack{\{i_1,\ldots,i_k\} \subseteq \{2,\ldots,n-1\} \\ i_j \neq i_l}} \left(\prod_{j=1}^{k} a_{i_j} \right) \right) \right) \\
&= x_1 \left[\lambda^{n-1} + \sum_{k=1}^{n-2} \left(\lambda^{n-1-k} \sum_{\substack{\{i_1,\ldots,i_k\} \subseteq \{2,\ldots,n-1\} \\ i_j \neq i_l}} \left(\prod_{j=1}^{k} a_{i_j} \right) \right) \right. \\
&\qquad \left. + a_n \lambda^{n-2} + \sum_{k=1}^{n-2} \left(\lambda^{n-2-k} \sum_{\substack{\{i_1,\ldots,i_k\} \subseteq \{2,\ldots,n-1\} \\ i_j \neq i_l}} a_n \left(\prod_{j=1}^{k} a_{i_j} \right) \right) \right] \\
&= x_1 \left[\lambda^{n-1} + \lambda^{n-2} \sum_{\{i_1\} \subseteq \{2,\ldots n-1\}} a_{i_1} + \sum_{k=2}^{n-2} \left(\lambda^{n-1-k} \sum_{\substack{\{i_1,\ldots,i_k\} \subseteq \{2,\ldots,n-1\} \\ i_j \neq i_l}} \left(\prod_{j=1}^{k} a_{i_j} \right) \right) + a_n \lambda^{n-2} \right. \\
&\qquad \left. + \sum_{k=2}^{n-2} \lambda^{n-1-k} \sum_{\substack{\{i_1,\ldots,i_{k-1}\} \subseteq \{2,\ldots,n-1\} \\ i_j \neq i_l}} a_n \left(\prod_{j=1}^{k-1} a_{i_j} \right) + \lambda^0 \sum_{\substack{\{i_1,\ldots,i_{n-2}\} \subseteq \{2,\ldots,n-1\} \\ i_j \neq i_l}} a_n \left(\prod_{j=1}^{n-2} a_{i_j} \right) \right] \\
&= x_1 \left(\lambda^{n-1} + \lambda^{n-2} \sum_{\{i_1\} \subseteq \{2,\ldots n\}} a_{i_1} + \sum_{k=2}^{n-2} \left(\lambda^{n-1-k} \sum_{\substack{\{i_1,\ldots,i_k\} \subseteq \{2,\ldots,n\} \\ i_j \neq i_l}} \left(\prod_{j=1}^{k} a_{i_j} \right) \right) + a_2 \cdots a_{n-1} a_n \right) \\
&= x_1 \left(\lambda^{n-1} + \sum_{k=1}^{n-1} \left(\lambda^{n-1-k} \sum_{\substack{\{i_1,\ldots,i_k\} \subseteq \{2,\ldots n\} \\ i_j \neq i_l}} \left(\prod_{j=1}^{k} a_{i_j} \right) \right) \right).
\end{aligned}
$$

□

Proposition 2. *For $n \geq 2$ the following statement holds:*

$$
\begin{vmatrix} \lambda + x_0 + a_0 & x_0 & x_0 & \cdots & x_0 \\ x_1 & \lambda + x_1 + a_1 & x_1 & \cdots & x_1 \\ x_2 & x_2 & \lambda + x_2 + a_2 & & x_2 \\ \vdots & & & \ddots & \\ x_{n-1} & x_{n-1} & \cdots & & \lambda + x_{n-1} + a_{n-1} \end{vmatrix}
$$

$$= \lambda^n + \lambda^{n-1}\left(\sum_{i=0}^{n-1} x_i + \sum_{j=0}^{n-1} a_j\right)$$
$$+ \sum_{k=2}^{n} \lambda^{n-k}\left(\sum_{\substack{i\in\{0,\dots,n-1\}\\ \{j_1,\dots,j_{k-1}\}\subseteq\{0,\dots,n-1\}\setminus\{i\}\\ j_l\neq j_r}} \left(x_i \prod_{l=1}^{k-1} a_{j_l}\right) + \sum_{\substack{\{j_1,\dots,j_k\}\subseteq\{0,\dots,n-1\}\\ j_l\neq j_r}} \left(\prod_{l=1}^{k} a_{j_l}\right)\right).$$

Proof. We prove the proposition again by induction. For $n = 2$ the statement is true as

$$\begin{vmatrix} \lambda + x_0 + a_0 & x_0 \\ x_1 & \lambda + x_1 + a_1 \end{vmatrix} = \lambda^2 + \lambda\left(x_0 + x_1 + a_0 + a_1\right) + x_0a_1 + x_1a_0 + a_0a_1.$$

The induction hypothesis is that the statement is true for some $n - 1 \geq 2$. The induction step $n - 1 \mapsto n$ gives an $(n + 1) \times (n + 1)$ matrix, which we calculate by expanding along the first row and applying Proposition 1.

$$\begin{vmatrix} \lambda + x_0 + a_0 & x_0 & x_0 & \cdots & x_0 \\ x_1 & \lambda + x_1 + a_1 & x_1 & \cdots & x_1 \\ x_2 & x_2 & \lambda + x_2 + a_2 & & x_2 \\ \vdots & & & \ddots & \\ x_n & x_n & \cdots & & \lambda + x_n + a_n \end{vmatrix}$$
$$= (-1)^{1+1}\left(\lambda + x_0 + a_0\right)\begin{vmatrix} \lambda + x_1 + a_1 & x_1 & \cdots & x_1 \\ x_2 & \lambda + x_2 + a_2 & & x_2 \\ \vdots & & \ddots & \\ x_n & \cdots & & \lambda + x_n + a_n \end{vmatrix}$$
$$+ (-1)^{1+2}x_0\begin{vmatrix} x_1 & x_1 & \cdots & x_1 \\ x_2 & \lambda + x_2 + a_2 & & x_2 \\ \vdots & & \ddots & \\ x_n & \cdots & & \lambda + x_n + a_n \end{vmatrix}$$
$$+ (-1)^{1+3}x_0\begin{vmatrix} x_1 & \lambda + x_1 + a_1 & x_1 & \cdots & x_1 \\ x_2 & x_2 & x_2 & & x_2 \\ x_3 & x_3 & \lambda + x_3 + a_3 & & x_3 \\ \vdots & & & \ddots & \\ x_n & \cdots & & & \lambda + x_n + a_n \end{vmatrix} + \cdots$$
$$+ (-1)^{1+(k+1)}x_0\begin{vmatrix} x_1 & & & & \cdots & x_1 \\ \vdots & \ddots & & & & \\ x_{k-1} & & \lambda + x_{k-1} + a_{k-1} & x_{k-1} & & \\ x_k & & x_k & x_k & & \\ x_{k+1} & & x_{k+1} & \lambda + x_{k+1} + a_{k+1} & & \\ \vdots & & & & \ddots & \\ x_n & \cdots & & & & \lambda + x_n + a_n \end{vmatrix}$$

$$
\begin{aligned}
&+\cdots+(-1)^{1+(n+1)}x_0\begin{vmatrix} x_1 & \lambda+x_1+a_1 & x_1 & \cdots & x_1 \\ x_2 & x_2 & \lambda+x_2+a_2 & & x_2 \\ \vdots & & & \ddots & \\ x_{n-1} & & & & \lambda+x_{n-1}+a_{n-1} \\ x_n & \cdots & & & x_n \end{vmatrix} \\
&\overset{\substack{\text{IH}\\ \text{Prop.1}}}{=}(\lambda+x_0+a_0)\left[\lambda^n+\lambda^{n-1}\left(\sum_{i=1}^{n}x_i+\sum_{j=1}^{n}a_j\right)\right. \\
&\qquad\left.+\sum_{k=2}^{n}\lambda^{n-k}\left(\sum_{\substack{i\in\{1,\dots,n\}\\ \{j_1,\dots,j_{k-1}\}\subseteq\{1,\dots,n\}\setminus\{i\}\\ j_l\neq j_r}}\left(x_i\prod_{l=1}^{k-1}a_{j_l}\right)+\sum_{\substack{\{j_1,\dots,j_k\}\subseteq\{1,\dots,n\}\\ j_l\neq j_r}}\left(\prod_{l=1}^{k}a_{j_l}\right)\right)\right] \\
&\quad-x_0x_1\left(\lambda^{n-1}+\sum_{k=1}^{n-1}\left(\lambda^{n-1-k}\sum_{\substack{\{j_1,\dots,j_k\}\subseteq\{2,\dots,n\}\\ j_l\neq j_r}}\left(\prod_{l=1}^{k}a_{j_l}\right)\right)\right) \\
&+(-1)^{1+3+1}x_0\begin{vmatrix} x_2 & x_2 & x_2 & & x_2 \\ x_1 & \lambda+x_1+a_1 & x_1 & \cdots & x_1 \\ x_2 & x_2 & \lambda+x_2+a_2 & & x_2 \\ \vdots & & & \ddots & \\ x_n & x_n & \cdots & & \lambda+x_n+a_n \end{vmatrix}+\cdots \\
&+(-1)^{1+k+1+(k-1)}x_0 \\
&\qquad\cdot\begin{vmatrix} x_k & x_k & \cdots & & & \cdots & x_k \\ x_1 & \lambda+x_1+a_1 & \cdots & & & \cdots & x_1 \\ \vdots & & \ddots & & & & \vdots \\ x_{k-1} & \cdots & & \lambda+x_{k-1}+a_{k-1} & x_{k-1} & \cdots & x_{k-1} \\ x_{k+1} & \cdots & & x_{k+1} & \lambda+x_{k+1}+a_{k+1} & \cdots & x_{k+1} \\ \vdots & & & & & \ddots & \vdots \\ x_n & \cdots & & & & & \lambda+x_n+a_n \end{vmatrix} \\
&+\cdots+(-1)^{1+n+1+(n-1)}x_0\begin{vmatrix} x_n & \cdots & & & x_n \\ x_1 & \lambda+x_1+a_1 & x_1 & \cdots & x_1 \\ x_2 & x_2 & \lambda+x_2+a_2 & & x_2 \\ \vdots & & & \ddots & \\ x_{n-1} & x_{n-1} & \cdots & & \lambda+x_{n-1}+a_{n-1} \end{vmatrix} \\
&\overset{\text{Prop.1}}{=}(\lambda+x_0+a_0)\left[\lambda^n+\lambda^{n-1}\left(\sum_{i=1}^{n}x_i+\sum_{j=1}^{n}a_j\right)\right.
\end{aligned}
$$

$$
\begin{aligned}
&\quad + \sum_{k=2}^{n} \lambda^{n-k} \left(\sum_{\substack{i \in \{1,\ldots,n\} \\ \{j_1,\ldots,j_{k-1}\} \subseteq \{1,\ldots,n\} \setminus \{i\} \\ j_l \neq j_r}} \left(x_i \prod_{l=1}^{k-1} a_{j_l} \right) + \sum_{\substack{\{j_1,\ldots,j_k\} \subseteq \{1,\ldots,n\} \\ j_l \neq j_r}} \left(\prod_{l=1}^{k} a_{j_l} \right) \right) \Bigg] \\
&\quad - x_0 \sum_{i=1}^{n} \left[x_i \left(\lambda^{n-1} + \sum_{k=1}^{n-1} \left(\lambda^{n-1-k} \sum_{\substack{\{j_1,\ldots,j_k\} \subseteq \{1,\ldots,n\} \setminus \{i\} \\ j_l \neq j_r}} \left(\prod_{l=1}^{k} a_{j_l} \right) \right) \right) \right] \\
&= \lambda^{n+1} + \lambda^n \left(\sum_{i=1}^{n} x_i + \sum_{j=0}^{n} a_j \right) \\
&\quad + \sum_{k=2}^{n} \lambda^{n+1-k} \left(\sum_{\substack{i \in \{1,\ldots,n\} \\ \{j_1,\ldots,j_{k-1}\} \subseteq \{1,\ldots,n\} \setminus \{i\} \\ j_l \neq j_r}} \left(x_i \prod_{l=1}^{k-1} a_{j_l} \right) + \sum_{\substack{\{j_1,\ldots,j_k\} \subseteq \{1,\ldots,n\} \\ j_l \neq j_r}} \left(\prod_{l=1}^{k} a_{j_l} \right) \right) \\
&\quad + a_0 \lambda^{n-1} \left(\sum_{i=1}^{n} x_i + \sum_{j=1}^{n} a_j \right) \\
&\quad + a_0 \sum_{k=3}^{n+1} \lambda^{n+1-k} \left(\sum_{\substack{i \in \{1,\ldots,n\} \\ \{j_1,\ldots,j_{k-2}\} \subseteq \{1,\ldots,n\} \setminus \{i\} \\ j_l \neq j_r}} \left(x_i \prod_{l=1}^{k-2} a_{j_l} \right) + \sum_{\substack{\{j_1,\ldots,j_{k-1}\} \subseteq \{1,\ldots,n\} \\ j_l \neq j_r}} \left(\prod_{l=1}^{k-1} a_{j_l} \right) \right) \\
&\quad + x_0 \Bigg[\lambda^n + \lambda^{n-1} \left(\sum_{i=1}^{n} x_i + \sum_{j=1}^{n} a_j \right) \\
&\qquad + \sum_{k=2}^{n} \lambda^{n-k} \left(\sum_{\substack{i \in \{1,\ldots,n\} \\ \{j_1,\ldots,j_{k-1}\} \subseteq \{1,\ldots,n\} \setminus \{i\} \\ j_l \neq j_r}} \left(x_i \prod_{l=1}^{k-1} a_{j_l} \right) + \sum_{\substack{\{j_1,\ldots,j_k\} \subseteq \{1,\ldots,n\} \\ j_l \neq j_r}} \left(\prod_{l=1}^{k} a_{j_l} \right) \right) \Bigg] \\
&\quad - x_0 \left[\lambda^{n-1} \sum_{i=1}^{n} x_i + \sum_{k=1}^{n-1} \lambda^{n-1-k} \left(\sum_{\substack{i \in \{1,\ldots,n\} \\ \{j_1,\ldots,j_k\} \subseteq \{1,\ldots,n\} \setminus \{i\} \\ j_l \neq j_r}} \left(x_i \prod_{l=1}^{k} a_{j_l} \right) \right) \right] \\
&= \lambda^{n+1} + \lambda^n \left(\sum_{i=0}^{n} x_i + \sum_{j=0}^{n} a_j \right) \\
&\quad + \sum_{k=2}^{n+1} \lambda^{n+1-k} \left(\sum_{\substack{i \in \{1,\ldots,n\} \\ \{j_1,\ldots,j_{k-1}\} \subseteq \{0,\ldots,n\} \setminus \{i\} \\ j_l \neq j_r}} \left(x_i \prod_{l=1}^{k-1} a_{j_l} \right) + \sum_{\substack{\{j_1,\ldots,j_k\} \subseteq \{0,\ldots,n\} \\ j_l \neq j_r}} \left(\prod_{l=1}^{k} a_{j_l} \right) \right)
\end{aligned}
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$$
\begin{aligned}
&+ x_0 \Bigg[\lambda^{n-1} \sum_{j=1}^{n} a_j + \sum_{k=2}^{n} \lambda^{n-k} \left(\sum_{\substack{i \in \{1,\ldots,n\} \\ \{j_1,\ldots,j_{k-1}\} \subseteq \{1,\ldots,n\} \setminus \{i\} \\ j_l \neq j_r}} \left(x_i \prod_{l=1}^{k-1} a_{j_l} \right) \right) \\
&\quad + \sum_{k=2}^{n} \lambda^{n-k} \left(\sum_{\substack{\{j_1,\ldots,j_k\} \subseteq \{1,\ldots,n\} \\ j_l \neq j_r}} \left(\prod_{l=1}^{k} a_{j_l} \right) \right) - \sum_{k=2}^{n} \lambda^{n-k} \left(\sum_{\substack{i \in \{1,\ldots,n\} \\ \{j_1,\ldots,j_{k-1}\} \subseteq \{1,\ldots,n\} \setminus \{i\} \\ j_l \neq j_r}} \left(x_i \prod_{l=1}^{k-1} a_{j_l} \right) \right) \Bigg] \\
&= \lambda^{n+1} + \lambda^n \left(\sum_{i=0}^{n} x_i + \sum_{j=0}^{n} a_j \right) \\
&\quad + \sum_{k=2}^{n+1} \lambda^{n+1-k} \left(\sum_{\substack{i \in \{1,\ldots,n\} \\ \{j_1,\ldots,j_{k-1}\} \subseteq \{0,\ldots,n\} \setminus \{i\} \\ j_l \neq j_r}} \left(x_i \prod_{l=1}^{k-1} a_{j_l} \right) + \sum_{\substack{\{j_1,\ldots,j_k\} \subseteq \{0,\ldots,n\} \\ j_l \neq j_r}} \left(\prod_{l=1}^{k} a_{j_l} \right) \right) \\
&\quad + x_0 \sum_{k=2}^{n+1} \lambda^{n+1-k} \left(\sum_{\substack{\{j_1,\ldots,j_{k-1}\} \subseteq \{1,\ldots,n\} \\ j_l \neq j_r}} \left(\prod_{l=1}^{k-1} a_{j_l} \right) \right) \\
&= \lambda^{n+1} + \lambda^n \left(\sum_{i=0}^{n} x_i + \sum_{j=0}^{n} a_j \right) \\
&\quad + \sum_{k=2}^{n+1} \lambda^{n+1-k} \left(\sum_{\substack{i \in \{0,\ldots,n\} \\ \{j_1,\ldots,j_{k-1}\} \subseteq \{0,\ldots,n\} \setminus \{i\} \\ j_l \neq j_r}} \left(x_i \prod_{l=1}^{k-1} a_{j_l} \right) + \sum_{\substack{\{j_1,\ldots,j_k\} \subseteq \{0,\ldots,n\} \\ j_l \neq j_r}} \left(\prod_{l=1}^{k} a_{j_l} \right) \right).
\end{aligned}
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□