The Oxygen Sensors FNR from *Escherichia coli* and NreABC from *Staphylococcus carnosus*

Dissertation

Zur Erlangung des Grades

“Doktor der Naturwissenschaften”

Am Fachbereich Biologie

der Johannes Gutenberg-Universität Mainz

Florian Reinhart

geb. am 25.01.1980 in Mainz

Mainz, 13.10.2010

Dekan: Prof. Dr. XXX

1. Berichterstatter: Prof. Dr. XXX
2. Berichterstatter: Prof. Dr. XXX

Tag der mündlichen Prüfung: November 2010

"Doing what little one can to increase the general stock of knowledge is a respectable object of life."

Charles Darwin

Contents

1. Abstract

Molecular oxygen is a widespread substrate and signal molecule in nature. Evolution has developed a large number of sensory devices in bacteria for controlling the expression of catabolic, biosynthetic and protective reactions in response to O_2. Under anoxic conditions, the oxygen sensor FNR from *Escherichia coli* is in the active state. Oxygen converts active $[4Fe4S]^{2+}$ FNR to $[2Fe2S]^{2+}$ FNR and further to apoFNR, which are both physiological inactive. The presence of apoFNR in aerobically and anaerobically growing *E. coli* was analyzed *in vivo* using thiol reagents. Alkylation of Cys residues in FNR and counting the labelled residues by mass spectrometry showed a form of FNR corresponding to apoFNR in aerobic bacteria. Exposure of anaerobic bacteria to oxygen caused conversion to apoFNR within 6 min.

The gram positive bacterium *Staphylococcus carnosus* is able to grow under anaerobic conditions by nitrate and nitrite respiration and by fermentation. The NreBC two-component system stimulates the expression of genes for nitrate respiration under anaerobic conditions. NreB is a cytoplasmic sensor histidine kinase using a PAS domain with a $[4Fe4S]^{2+}$ cofactor for sensing O_2. The state of NreB was studied *in vivo* and *in vitro* by measuring the reactivity and accessibility of Cys residues to alkylating agents. The change in Cys accessibility allowed determination of the half-time for the conversion of $[4Fe4S]^{2+}$ NreB to apoNreB after the addition of oxygen. In anaerobic bacteria most of the NreB exists as $[4Fe4S]^{2+}$ NreB, whereas in aerobic bacteria apoNreB is predominant and represents the physiological form. The number of accessible Cys residues was also determined by iodoacetate alkylation followed by mass spectrometry of Cys-containing peptides.

The function of NreA in the NreABC system is unclear. This study shows that NreA interacts with NreB and NreC. The presence of a GAF domain in NreA, which is known to bind small molecules as cofactors, qualifies NreA as a candidate for a nitrate sensor. Until now a nitrate sensor has not been described in *Staphylococcus carnosus.*

2. Introduction

2.1 Oxygen sensing by bacteria

Molecular oxygen is a widespread substrate and signal molecule in nature. Many bacteria grow in an environment with permanently or temporarily limiting O_2 concentrations and have to cope with microaerobic conditions. Evolution has developed a large number of sensory devices for controlling the expression of catabolic, biosynthetic and protective reactions in response to O_2 availability.

Due to the chemical versatility of O_2, many different sensing and signaling devices have been developed in bacteria (Bauer *et al*., 1999). Direct O_2 sensors control gene expression by direct reaction with O_2, whereas indirect O_2 sensors are able to respond to metabolic situations, e.g. the aerobic respiratory chain as signal for the presence of O_2. The combination of direct and indirect sensing allows efficient adaptation to various metabolic requirements. Direct O_2 sensors react with cytoplasmic O_2 using $[4Fe4S]^{2+}$, heme and potentially FAD as cofactors for interaction. The reaction with O_2 triggers a conformational change to transmit the signal to the output domains of the sensors. Whereas indirect sensors are often linked to electron transfer chains in the cytoplasmic membrane, direct O_2-sensors sense O_2 from the cytoplasm.

The diffusion rate of O_2 in water and through membranes is relatively high over small distances (µm range) when compared to the O_2 consumption by respiration (FIG.I1). This leads to a cytoplasmic O_2 tension that is comparable to the extracellular O_2 tension, even under microaerobic conditions. The rapid O_2 diffusion therefore supplies sufficient O_2 under aerobic and microaerobic conditions for regulatory and catabolic processes (Becker *et al*., 1996). Molecular oxygen functions as an electron acceptor and as a substrate for catabolism of aerobic bacteria. Under hyperoxic conditions, O_2 induces oxidative stress responses in aerobic bacteria. Facultative anaerobic bacteria also measure the presence of O_2 to adapt their metabolism. In general, O_2 represses anaerobic respiration and fermentation, which are both less efficient in energy conservation (Unden *et al*., 1995).

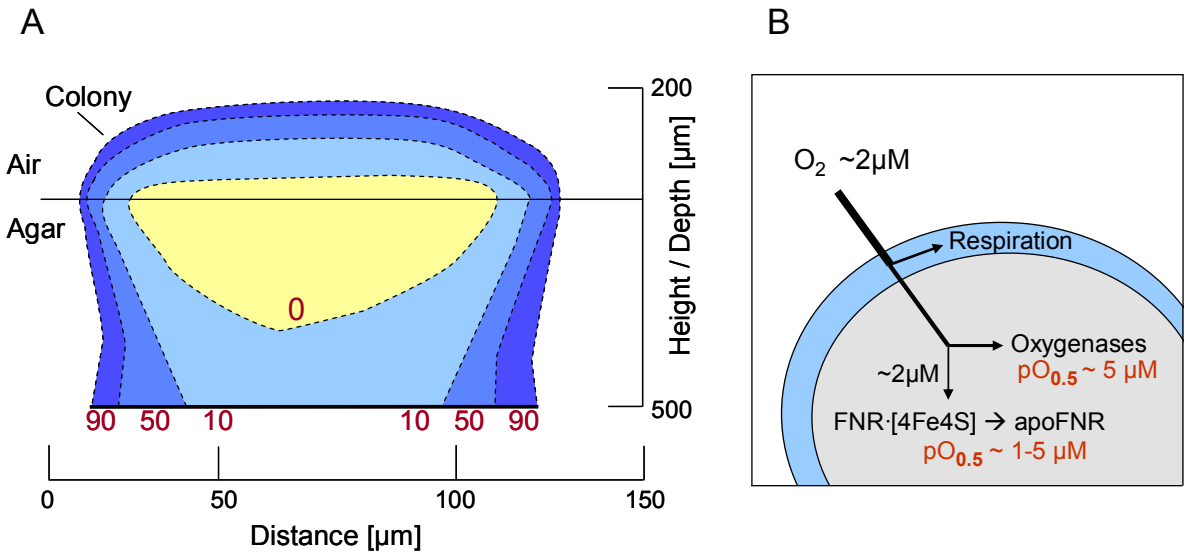


FIG.I1. O_2 gradients in surface colonies of bacteria (A), and diffusion of O_2 over short distances into the bacterial cytoplasm (B). Scheme (A) gives the contour map of O_2 concentration in a surface colony grown on an agar plate determined with microelectrodes. The contour lines give the O_2 content (% of air saturation in red). Scheme (B) shows the amount of O_2 which can be estimated to diffuse over short distances (< 1 µm) into the bacterial cells, amounting to approx. 7 µmol O_2 min^{-1} mg^{-1} protein under microaerobic conditions. The diffusion rate compares to O_2 consumption of about 0.5 µmol O_2 min^{-1} mg^{-1} protein by respiration of *E. coli* under the same conditions. Therefore O_2 diffuses rapidly through the cytoplasm membrane (blue) into the cytoplasm (grey) and the cytoplasmic concentration equals the extracellular O_2 concentration under both aerobic and microaerobic conditions. The O_2 supply for regulatory and catabolic purpose in the cytoplasm is sufficient under these conditions. The switch point ($pO_{0.5}$ or half-maximal activity) for regulation by the direct oxygen sensor FNR from *E. coli* and for cytoplasmic oxygenases is 1 to 5 µM, and 5 to 10 µM O_2, respectively. (Unden *et al*., 1995; 1997).

Bacteria contain a wide array of oxygen sensors for responding to the presence of O_2. Regulation occurs mostly at the transcriptional level, but sensors like Aer and WhiB3 control aerotaxis and metabolism at the protein level. Many bacteria contain more than one O_2-sensor which can function in different ways and dynamically regulate different target genes.

$[4Fe4S]^{2+}$ clusters are widely used cofactors for sensing of O_2. The cofactor is found in unrelated proteins, like the transcriptional regulators FNR_{Ec} from *Escherichia coli*, FNR_{Bs} from *Bacillus subtilis*, and the PAS domain of $NreB_{Sc}$ from *Staphylococcus carnosus*. The proteins contain three or four conserved Cys residues for binding of the $[4Fe4S]^{2+}$ cluster, but the sequences and the spacing of the Cys residues show

no similarities. It appears that the clusters have been acquired independently during evolution by different classes of proteins, a process which also has been described for other cofactors (Masip *et al*., 2004). As a result the same cofactor $[4Fe4S]^{2+}$ controls function by dimerisation (FNR_{Ec}), DNA binding (FNR_{Bs}) and kinase activity ($NreB_{Sc}$) in the respective sensor proteins.

2.2 FNR, a direct O_2 sensor from *Escherichia coli*

The transcriptional regulator FNR (*f*umarate *n*itrate reductase *r*egulator) of *Escherichia coli* functions as a direct O_2 sensor (Green *et al*., 1991; Unden & Schirawski, 1997). Under anoxic conditions, the protein is in the active state and is predominately found as a homodimer with one $[4Fe4S]^{2+}$ cluster per monomer (FIG.I2). The FeS cluster is bound by four cysteine residues (Cys20, Cys23, Cys29, and Cys122). The fifth Cys residue of FNR, Cys16, is not essential and not involved in binding of the FeS cluster (Sharrocks *et al*., 1990). The FeS cluster in FNR can be detected by Mössbauer spectroscopy, which has been essential for characterizing the FeS-containing FNR forms *in vitro* and *in vivo* (Popescu *et al*., 1998).

In the presence of O_2, $[4Fe4S]^{2+}$FNR is converted to monomeric $[2Fe2S]^{2+}$FNR, which is no longer active in gene regulation (Lazazzera *et al*.,1996). $[2Fe2S]^{2+}$FNR degrades further upon continued O_2 exposure (Sutton *et al*., 2004), and FeS depleted apoFNR is formed within a few minutes (FIG.I2). ApoFNR, like $[2Fe2S]^{2+}$FNR, is inactive in DNA binding and gene regulation (Trageser *et al.*, 1991). The conditions for apoFNR formation from $[4Fe4S]^{2+}$FNR have been characterized *in vitro* by Achebach *et al*., 2005.

Only indirect evidence for the formation of apoFNR *in vivo* has been provided so far: the $[4Fe4S]^{2+}$ and $[2Fe2S]^{2+}$ clusters of FNR are lacking in aerobically grown cells under conditions in which the FNR protein is still present (Sutton *et al*., 2004). It has been concluded that the remaining FNR is apoFNR, but its identity has not been directly demonstrated. It is not known whether *in vivo* apoFNR is of the same type as *in vitro*, where apoFNR contains two disulfides (Cys16/20 and Cys23/29), which are of unknown physiological significance (Achebach *et al*., 2004 and 2005).

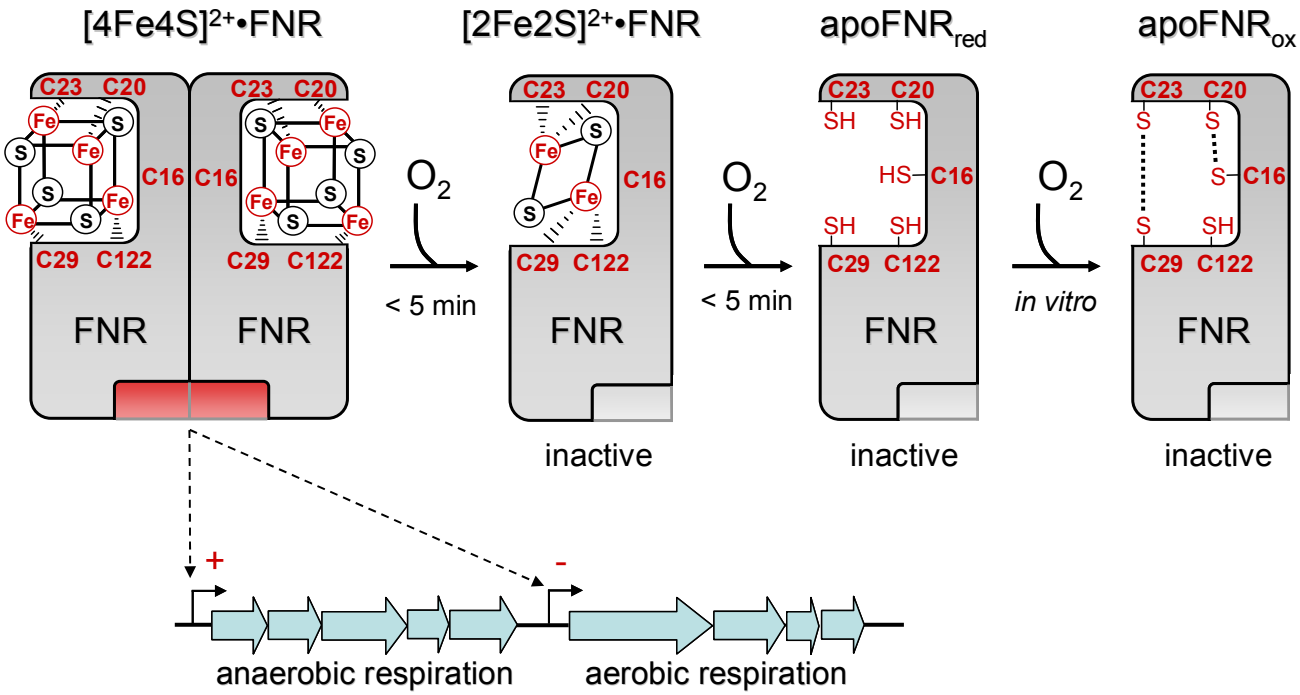


FIG.I2. Various functional forms of FNR and the accessibility and redox state of the five Cys residues. The state of the Cys residues in the N-terminus (C16, C20, C23, and C29) and the central part (C122) of $[4Fe4S]^{2+}$FNR, $[2Fe2S]^{2+}$FNR, reduced apoFNR$_{red}$ and oxidized apoFNR$_{ox}$ are shown (from left to right). ApoFNR$_{ox}$ indicates the oxidized form of apoFNR obtained after isolation of the protein under oxic conditions or after oxygen inactivation of reconstituted $[4Fe4S]^{2+}$FNR *in vitro*. The physiological relevance of apoFNR$_{red}$ and apoFNR$_{ox}$ was investigated in this study. ApoFNR$_{red}$ is the form found in aerobically grown bacteria or of isolated apoFNR after reduction by DTT. The broken lines in oxidized apoFNR represent the Cys disulfides C16/C20 and C23/C29. Active $[4Fe4S]^{2+}$FNR forms a dimer in anaerobic cells. Dimeric FNR acts as a gene regulator and induces the expression of genes for anaerobic respiration, while it represses the expression of genes for aerobic respiration. ApoFNR could be derived from $[2Fe2S]^{2+}$FNR or from *de novo* synthesis *in vivo*. The times for the interconversion of the different forms are approximate times from *in vivo* and *in vitro* experiments (Achebach *et al.*, 2005).

In this study the formation and presence of apoFNR in aerobically and anaerobically growing *E. coli* was investigated by differentiating apoFNR from FeS-containing FNR using a method for measuring the accessibility of the Cys residues to thiol reagents (Reinhart *et al.*, 2008). Up to five Cys residues are accessible to thiol reagents in apoFNR, whereas only Cys16 is accessible in $[4Fe4S]^{2+}$FNR and $[2Fe2S]^{2+}$FNR. The ligands of the FeS clusters in $[4Fe4S]^{2+}$FNR and $[2Fe2S]^{2+}$FNR are protected from labeling. The amount of accessible Cys residues was determined by the use of thiol-specific reagents which introduce a fluorescence label into FNR or change the apparent *Mr* of FNR in SDS gels. For labeling, membrane permeable and impermeable thiol reagents were used. Membrane permeable labels are supposed to

allow labeling of cytoplasmic proteins *in situ* without cell disruption (Rogers *et al*., 2006) and with minimal perturbation of cellular metabolism and functional state of FNR. The results demonstrate that the major form of FNR in aerobically growing *E. coli* is apoFNR and that the kinetics of apoFNR formation *in vivo* and *in vitro* (FIG.I2) are similar.

2.3. The NreBC two-component system from *Staphylococcus carnosus*

Staphylococcus carnosus is able to grow under anaerobic conditions by nitrate and nitrite respiration and by fermentation. The genome is well equipped with all functions necessary for a starter culture in food technology (Rosenstein *et al*., 2008). *S. carnosus* contains various oxygen sensors that control the switch from aerobic to anaerobic catabolism. SrrB-SrrA (*Staphylococcus r*espiratory *r*esponse) is a two-component system that controls the expression of genes for virulence factors and for enzymes of fermentation and the citric acid cycle (Ulrich et al., 2007; Throup *et al*., 2001). SrrB-SrrA is an indirect O_2 sensor system and responds to O_2 via the aerobic respiratory chain. *S. carnosus* also encodes a transcriptional regulator homologous to Rex. The Rex proteins of *Streptomyces* sp. and *Bacillus* sp. control the expression of genes for respiratory enzymes and use NAD^+ as a corepressor (Wang *et al*., 2008).

A third regulatory system, the NreBC two-component system, stimulates the expression of genes of nitrate respiration under anaerobic conditions (Fedtke *et al*., 2002). The target genes of NreBC are the genes for the nitrate reductase (*narGHJI*), nitrite reductase (*nirRBD* and *sirAB*), and the nitrate transporter NarT (*narT*) (FIG.I3). NreB is a cytoplasmic sensor histidine kinase using a PAS domain with a $[4Fe4S]^{2+}$ cofactor for sensing O_2 (FIG.I4). Reaction of purified NreB with O_2 causes degradation of the $[4Fe4S]^{2+}$ cluster to a $[2Fe2S]^{2+}$ cluster, with a half-time of 2.5 min. The $[2Fe2S]^{2+}$ cluster is further degraded, resulting in FeS-less apoNreB. NreB is a permanent dimer in the active and inactive state. Disintegration of the $[4Fe4S]^{2+}$ cluster is coupled to the loss of kinase activity of H159 and lead to inactivation of NreB. $[2Fe2S]^{2+}$NreB and apoNreB are both inactive (Müllner *et al*., 2008). *nreBC*

homologous genes were found in *Staphylococcus* strains and *Bacillus clausii*, and a modified form was found in *Lactobacillus* sp.

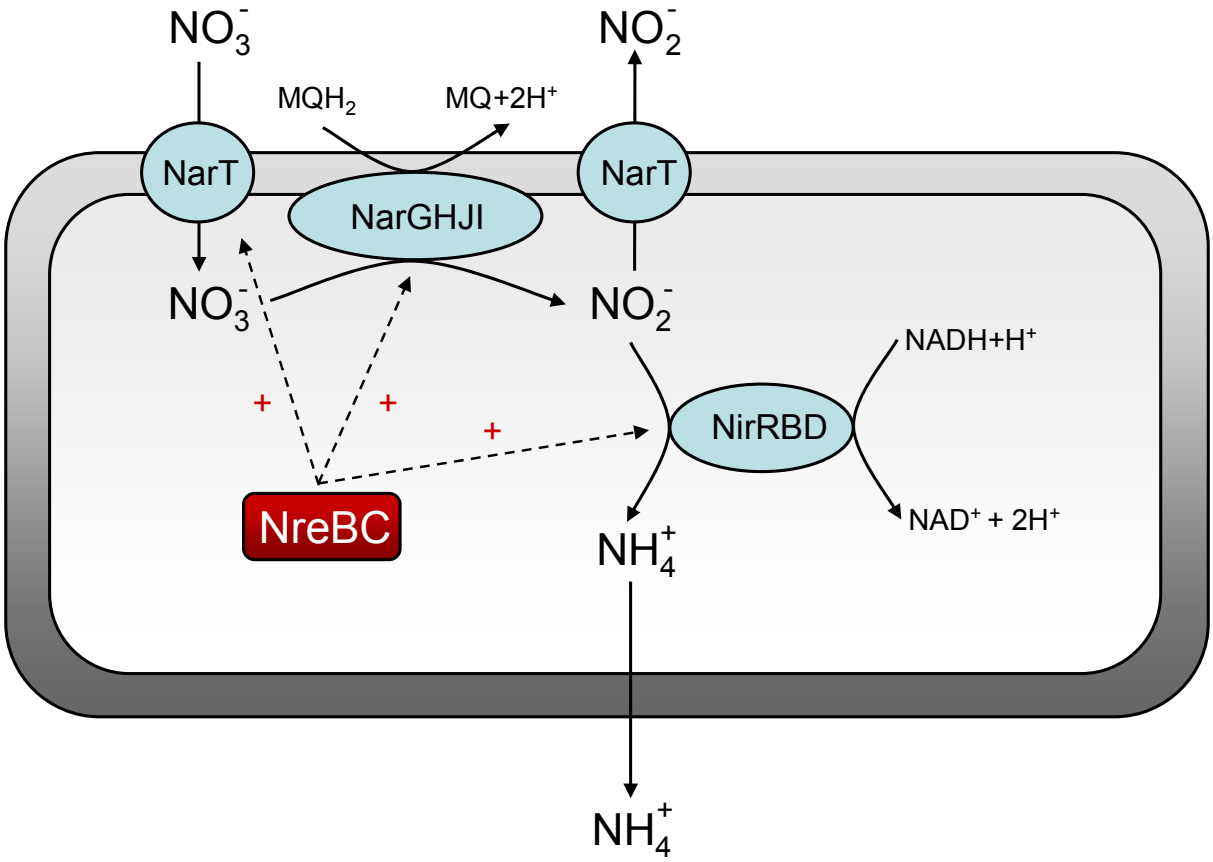


FIG.I3. Nitrate respiration of *Staphylococcus carnosus*. Under anaerobic conditions nitrate is transported into the cytoplasm by the nitrate-nitrite antiporter NarT and subsequently reduced to nitrite by the dissimilatoric nitrate reductase NarGHJI. This multimeric enzyme is membrane bound and plays a role in energy conservation. Nitrite is then reduced to ammonium by the cytoplasmatic nitrite reductase NirRBD. Ammonium is then exported. The expression of the antiporter narT, the nitrate reductase and the nitrite reductase is under positive control of the NreBC two-component system.

For NreB, the complex redox situation in the bacterial cell does not allow for a direct transfer of the *in vitro* data and redox reactions to the cellular situation. *Staphylococcus* sp. (similar to *Bacillus* sp.) contains various reducing and oxidizing agents, including low-molecular-weight thiols (Leichert *et al*., 2003) and quinones. These compounds are able to react with FeS clusters and thiol groups of proteins and could affect the redox state or the reaction of NreB with oxygen. In addition, the presence of the two inactivated forms, $[2Fe2S]^{2+}$NreB and apoNreB, *in vitro* raises

the question of the physiological relevant form of inactive NreB in aerobic bacteria. To summarize, understanding the function of NreB in O_2 sensing requires *in vivo* analysis of NreB in bacteria.

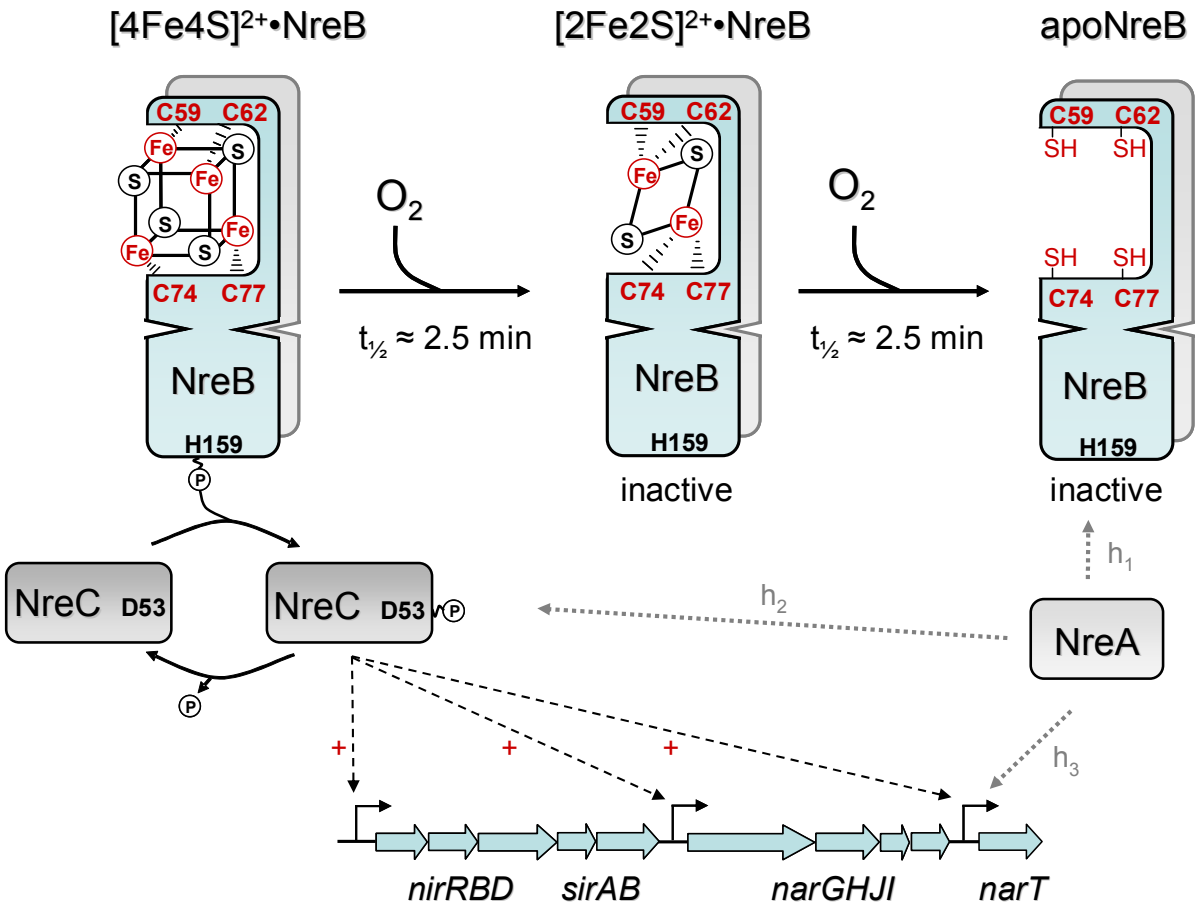


FIG.I4. Function of the NreBC two-component system as a direct O_2 sensor system. For NreB, the kinase domain with the conserved His residue (H159) and the PAS domain with the conserved Cys residues (C59, C62, C74, C77) are shown. The figure summarizes the reactions at the sensor kinase NreB and the FeS cluster during transition from anaerobic $[4Fe4S]^{2+}$NreB to the air-inactivated forms. NreB is a permanent dimer (Müllner, 2008). $[2Fe2S]^{2+}$NreB is formed *in vitro* as an intermediate, but the presence of this form has not been tested *in vivo*. The overall process of $[4Fe4S]^{2+}$ NreB to apoNreB conversion has a halftime of about 6 min, and the half-time of $[4Fe4S]^{2+}$NreB to $[2Fe2S]^{2+}$NreB conversion is about 2.5 min. The anaerobic $[4Fe4S]^{2+}$NreB protein has high kinase activity, resulting in the auto-phosphorylation of NreB and the transfer of the phosphate to NreC. Phosphorylated NreC is active as a gene regulator and activates the transcription of the target genes *narGHJI*, *narT*, and *nirRBD* & *sirAB*. The function of NreA, the third gene of the NreABC operon is unclear. Sequence analysis identified a GAF domain containing a putative helix-turn-helix motif. Three hypotheses for NreA function were tested in this study: Interaction at protein level with NreB (h_1), interaction with NreC (h_2) and interaction with regulatory target DNA (h_3).

The state of NreB was studied *in vivo* by measuring the reactivity and accessibility of Cys residues to alkylating agents. NreB contains four Cys residues, which are all ligands of the $[4Fe4S]^{2+}$ and $[2Fe2S]^{2+}$ clusters. Mutation of each of the four Cys residues inactivated the NreB function *in vivo.* This is in accordance with their role as ligands of the FeS clusters (Müllner *et al*., 2008).

The accessibility of the Cys residues should vary depending on the presence or absence of an FeS cluster and in this way allow differentiation of the FeS-containing forms from the apo form of NreB. The switch between aerobic and anaerobic NreB can be studied by measuring the accessibility of the Cys residues *in vivo*. The method depends on a procedure for quantitative fluorescence labeling, or for measuring the mass increase by mass spectrometry, similar to the method described for FNR. It turned out that only apoNreB is present in aerobically growing *S. carnosus*, whereas anaerobically growing bacteria carry essentially an FeS-containing form.

For NreB, an advanced two-step labeling procedure was used to study quantitatively the accessibility of the Cys residues *in vivo*. In the first step, accessible residues were incubated with iodoacetate (IAA), which is highly reactive to surface-exposed Cys residues. After denaturing of the protein, the residual (FeS-protected) residues were incubated with the fluorescent reagent monobromobimane (mBBr). By this procedure, the accessible Cys residues of NreB were determined *in vitro* and *in vivo* under both aerobic and anaerobic conditions. The fluorescence label results were also confirmed by MALDI-TOF studies on IAA labelled NreB.

2.4 Anaerobic NreB features a $[4Fe4S]^{2+}$ cluster which is required for kinase activity

NreB contains a sensory domain with similarity to heme B binding PAS domains. Anaerobically prepared NreB of *S. carnosus* features a diamagnetic $[4Fe4S]^{2+}$ cluster, which can be analyzed by Mossbauer spectroscopy (Müllner *et al*., 2008). Upon reaction with air, the cluster is degraded with a half-life of ~2.5 min (FIG.I5). Magnetic Mossbauer spectra reveal the formation of a diamagnetic $[2Fe2S]^{2+}$ cluster,

which is further degraded during oxygen exposure. After extended exposure to air (~5.5 min), NreB is devoid of a FeS cluster and the cluster decay product γ–FeOOH can be detected in Mössbauer spectroscopy (FIG.I5D).

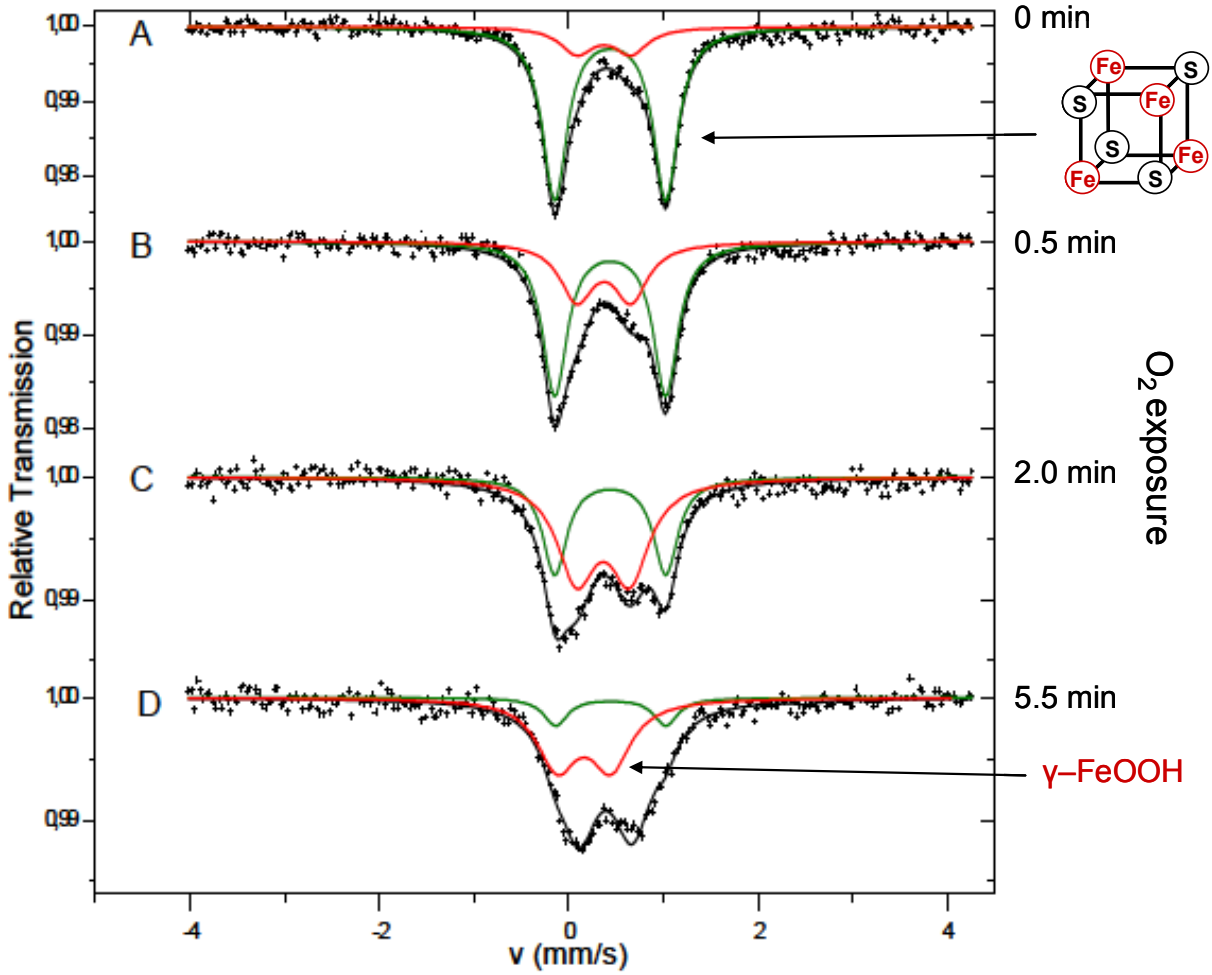


FIG.I5. Anaerobic NreB features an oxygen sensitive $[4Fe4S]^{2+}$ cluster. Mössbauer spectra of anaerobically prepared NreB after 0 min (A), 0.5 min (B), 2 min (C), and 5.5 min (D) of oxygen exposure are shown. The Mössbauer spectrum of anaerobic NreB (A) is characteristic for $[4Fe4S]^{2+}$ cluster containing proteins. After 5.5 min the $[4Fe4S]^{2+}$ cluster (green line) is oxidized almost completely to γ–FeOOH (red line). The black line is calculated as the overlay of the $[4Fe4S]^{2+}$ cluster signal (green) and the γ–FeOOH (red) signal. The intermediate product, the $[2Fe2S]^{2+}$ cluster, is EPR silent and was detected in magnetic Mössbauer spectroscopy (Müllner *et al.*, 2008).

Phosphorylated $[4Fe4S]^{2+}$NreB transfers a phosphate group to the response regulator NreC, which then acts a as transcription factor on target DNA. $[4Fe4S]^{2+}$ containing NreB shows high autophosphorylation activity. Exposure to oxygen decreases both the $[4Fe4S]^{2+}$ cluster content and the kinase activity, whereas the content of decay products increase simultaneously (FIG.I6). Loss of the $[4Fe4S]^{2+}$

cluster leads to inactivation of NreB, which finally results in a decreased autophosphorylation activity of histidine residue H159. However, magnetic Mössbauer spectroscopy showed that there is no correlation between the $[2Fe2S]^{2+}$ cluster content and the auto-phosphorylation activity of NreB.

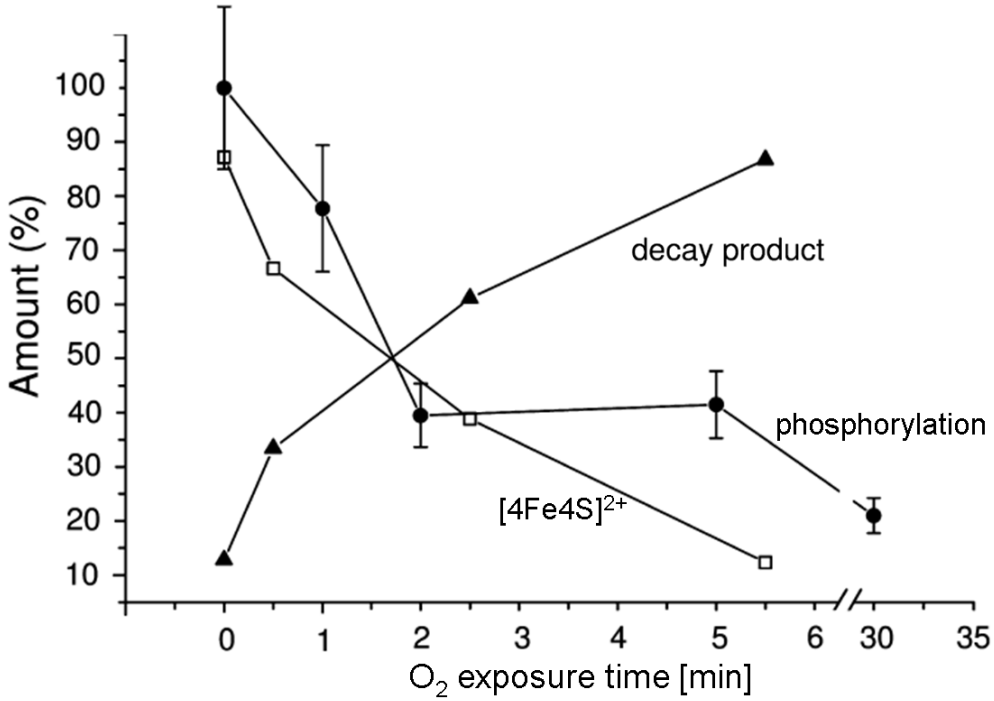


FIG.I6. A $[4Fe4S]^{2+}$ cluster is required for the kinase activity of NreB. Anaerobically isolated NreB was incubated with 33P-labeled ATP after different times of oxygen exposure. The autophosphorylation activity of NreB (filled circles) is correlated with the content of $[4Fe4S]^{2+}$ clusters (squares). The $[4Fe4S]^{2+}$ content data and the content of the decay product γ–FeOOH (filled triangles) was derived from Mössbauer experiments (Müllner *et al*., 2008).

2.5 NreA, a GAF domain protein with unknown function

The oxygen sensor NreB and the response regulator NreC are encoded in one operon with NreA. The function of NreA is unknown, but sequence analyses indicate the presence of a GAF domain. The GAF acronym comes from the names of the first three different classes of proteins identified to contain them: c*G*MP-regulated cyclic nucleotide phosphodiesterase, *A*denylyl cyclase, and the transcription factor *F*hlA (Arravind *et al.,* 1997). GAF domains show the same basic fold as PAS domains

(Anantharaman *et al*., 2001), and they are distantly related (Huang *et al*., 1993). GAF domains represent one of the largest families of small molecule binding units present in nature. The first GAF domains discovered were the cGMP-binding regulatory domains of several cyclic nucleotide phosphodiesterases. cGMP binds to a well-defined pocket in one of the two GAF domains that is analogous to the ligand-binding pocket of the distantly related PAS domains of FixL (Martinez *et al*., 2002). However, the consensus cGMP-binding motif suggests that only certain GAF domains will bind cGMP, although the detailed sites of cGMP binding to the GAF domain remains to be determined.

In this study the function of NreA was investigated by sequence analysis and comparison to other GAF domains with known functions, e.g. binding of isoleucine. NreA deletion mutants of *S. carnosus* perform nitrate respiration even under aerobic conditions (S. Schlag, personal communication). It can be speculated that NreA represses the NreBC system, e.g. in absence of nitrate or nitric oxide (a by-product of nitrate reductase). Until now it is not known if the NreBC system is modulated in presence or absence of nitrate. In this study NreA has been over-expressed and purified and three possible hypotheses regarding function of NreA were tested (FIG.I4). The DNA binding ability of NreA was tested in an electro mobility shift assay (EMSA) with putative target DNA (upstream regions of NreBC regulated genes), and the influence of NreA on the NreBC system was tested in protein interaction studies with NreB and NreC.

3. Materials & Methods

3.1 Strains and plasmids

3.1.1 Bacterial strains and plasmids

TAB.M1. Bacterial strains and plasmids used in this study.

Strain / Plasmid	Genotype / Characteristics	Reference
E. coli CAG627	$lacZ_{am}$, trp_{am}, pho_{am}, $supC^{ts}$, mal, rpsL, lon	J.R.Guest, JGM 133,3279
E. coli CAG627 (pMW32)	pMW32	Holighaus *et al.*, 1997
E. coli JM109	*endA1, glnV44, sbcB15, rpsL, thi-1, Δ(lac-proAB), [F' traD36 proAB$^+$ lacI^q lacZΔM15], hsdR4(rK$^-$mK$^+$)*	Yanisch-Perron, 1985
E. coli M15 (pQE31*nreA*)	pQE31*nreA, Amp^r,* Kan^r	Schlag *et al.*, 2007
E. coli JM105	pQE30; Qiagen HisTag protein expression system	Qiagen, IMW335
E. coli EC3 (pMal*nreC*)	expression strain for NreC-MalE fusion	Fedtke *et al.*, 2002
S. carnosus TM300	wildtype	F. Götz, 1995
S. carnosus m1	*nreABC*::*ermB,* Erm^r	Fedtke *et al.*, 2002
S. carnosus m1 (pCQE1*nreB)*	pCQE1*nreB*, Erm^r, Cam^r	Wieland *et al.*, 1995
S. carnosus m1 (pCQE1*nreB**)	pCQE1*nreB** (C59S C62S) , Erm^r, Cam^r	This study, IMW1884
pQE30*nreC*	*nreC* with N-terminal 5xHis-Tag (*Kpn*I; *Sal*I), Amp^r	This study
pQE31*nreA*	*nreA* with N-terminal 5xHis-Tag (*Bam*HI/*Hind*III), Amp^r	Schlag *et al.*, 2007
pMW32	pTrc99A with 750-bp *NcoI*/*Bam*HI *fnr* fragment of pGS199, Amp^r	Spiro & Guest, 1987
pMW418	pRB473*nreABC* (*Bam*HI; *Sal*I); shuttlevector, Cam^r in *S.carnosus*; Amp^r in *E.coli*	Müllner, 2008
pMW664	pMW418 but *nreB C59S C62S;* Cam^r in *S.carnosus*; Amp^r in *E.coli*	Staßen, 2008
pCQE1*nreB*	pCQE1 with wildtype *nreB* (*Bgl*II; *Ksp*I), Cam^r	Wieland *et al.*, 1995
pCQE1*nreB**	pCQE1 with *nreB** C59S C62S (*Bgl*II; *Ksp*I), Cam^r	This study, pMW876
pMal*nreC*	pMAL-c2X with *Bam*HI and *Xmn*I fragment of *nreC*	Fedtke *et al.*, 2002

3.1.2 Long-term storage of bacterial strains

To store a bacterial strain (*E .coli* or *S. carnosus*) for long term 5 ml LB medium was inoculated with one colony from an agar plate and incubated overnight at 37°C (160 rpm). Appropriate antibiotics were added. After centrifugation (5.000 g, 1 min, RT) the pellet was resuspended in 2 ml LB and 2 ml glycerol solution (50 %). Aliquots á 1 ml were frozen in 2.0-ml micro tubes with screw cups (Biologix) in liquid nitrogen and stored at -80 °C.

3.2 Bacterial growth and media

3.2.1 Growth of *Escherichia coli*

E. coli CAG627 carrying plasmid pMW32 (pTrc99A with the 750-bp *Nco*I / *BamH*I *fnr* fragment of pGS199) overproduces GST-FNR from the plasmid-encoded *fnr* gene. The strain was grown under oxic or anoxic conditions in 2x YT medium with glucose (100 mM), and the oxic and anoxic conditions were strictly maintained throughout the experiment procedures. At an OD_{578} of 0.6 to 0.8, the culture was diluted 1:10 in the same medium, and *fnr* transcription was induced by isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM final concentration). After 2 hours cells were harvested by centrifugation (10.000 g, 15 min, 4 °C).

3.2.3 Growth of *Staphylococcus carnosus*

S. carnosus m1 (*nreABC*::*ermB*) carrying plasmid pCQE1*nreB* for xylose-induced over-expression (*xylA* promoter) of *nreB* was grown at 37°C under oxic or anoxic conditions in yeast extract production medium (YEPD). The cells were grown under aerobic conditions (300 ml in a 2-liter flask with baffles) on a rotary shaker (250 rpm) to an OD_{578} of 0.5, and then 150 mM xylose was added for induction of NreB expression. Aerobic cells were harvested after 2 h by centrifugation (10.000 g, 15 min, 4 °C). To obtain anaerobic cells, the aerobically grown bacteria were incubated after induction of NreB synthesis as described above for 5 h in sealed serum bottles under N_2 (99.99%) at 37°C and were harvested subsequently.

3.2.4 Media and buffers for *Escherichia coli*

All media and buffers were autoclaved or filter-sterilised prior to use.

LB (Luria Bertani) broth (Sambrook and Russell, 2001)

10 g/l Casein (Select peptone Nr. 140, Gibco)

5 g/l Yeast extract (Servabacter 24540, Serva)

5 g/l NaCl (Roth)

15 g/l Agar-agar, Kobe I pulv. (Roth), optional

2x YTG media (Sambrook *et al*., 2001)

16 g/l Tryptone (Difco)

10 g/l Yeast extract (Servabacter 24540, Serva)

5 g/l NaCl (Roth)

2 % (w/v) Glucose (from 1 M ≈ 20 % stock solution)

PBS buffer (10x)

27 mM KCl (Roth)

100 mM Na_2HPO_4 (Roth)

18 mM NaH_2PO_4 (Roth)

SOC medium (Sambrook and Russel, 2001)

20 g/l Casein (Select peptone Nr. 140, Gibco)

5 g/l Yeast extract (Serverbacter 24540, Serva)

0.584 g/l NaCl (Roth)

0.19 g/l KCl (Roth)

2.46 g/l $MgSO_4$ x 7 H_2O (Fluka)

2.03 g/l $MgCl_2$ x 6 H_2O (Fluka)

3.96 g/l Glucose x H_2O (Roth)

Protein isolation buffer A

50 mM Sodium phosphate pH 7.2 (Serva)

300 mM NaCl (Roth)

20 % Glycerol (Roth)

10 mM Imidazole pH 7.2 (Roth)

Protein isolation buffer B

50 mM Sodium phosphate pH 7.2 (Serva)

1 M NaCl (Roth)

20 % Glycerol (Roth)

20 mM Imidazole pH 7.2 (Roth)

Protein isolation buffer C

50 mM Sodium phosphate pH 7.2 (Serva)

300 mM NaCl (Roth)

20 % Glycerol (Roth)

150 mM Imidazole pH 7.2 (Roth)

3.2.5 Media and buffers for *Staphylococcus carnosus*

All media and buffers were autoclaved or filter-sterilised prior to use.

BM (Basic media) pH 7.2 (Götz & Schumacher, 1987)

10 g/l Casein (Select peptone Nr. 140, Gibco)

5 g/l Yeast extract (Serverbacter 24540, Serva)

5 g/l NaCl (Roth)

1 g/l K_2HPO_4 (Roth)

1 g/l Glucose (Roth)*

optional for plates:

15 g/l Agar-agar, (Roth)

*glucose was not added to over-expressing cultures of plasmid pCQE1 since its *xylA* promoter is repressed by glucose

YEPD (Yeast extract production media) pH 7.2 (Kempf *et al*., 1999)

45 g/l Yeast extract (Serverbacter 24540, Serva)

50 mM Na_2PO_4 (Roth)

40 mM Glycerol (Roth)

10 mM $NaNO_3$ (Roth)

Lysis-buffer for isolation of genomic DNA (Graf, 2009)

25 mM Tris/HCl, pH 8 (Roth)

50 mM Na_2-EDTA (Roth)

50 mM Glucose x H_2O (Roth)

5 mg/ml Lysozym (Merck)

Solutions for protoplast transformation of *S. carnosus*:

SMMP-B buffer (50 ml)

37 ml 2x SMM

10 ml 4x PAB

2.5 ml BSA (5%) solution in 1x SMM

0.5 ml H_2O

2xSMM (Sterilisation by filtration)

1 M (D)-Saccharose (Roth)

40 mM Malenic acid (Roth)

40 mM $MgCl_2$ x $6H_2O$ (Roth)

pH adjusted to 6.8 with NaOH (Roth)

4x PAB (Sterilisation by filtration)

6 g/l Meat extract (Serva)

6 g/l Yeast extract (Serva)

20 g/l Peptone (Serva)

10 g/l Glucose (Roth)

14 g/l NaCl (Roth)

14.95 g/l K_2HPO_4 (Roth)

5.278 g/l KH_2PO_4 (Roth)

Fusogen (Sterilisation by filtration)

40 g Polyethylenglycol 6000 (Serva)

50 ml 2x SMM

100 ml H_2O

DM3 agar plates:

Solution 1:
15 g Agar (Serva)
200 ml H_2O

Solution 2:
135.08 g Na_2-succinat („Bernsteinsäure Dinatriumsalz Hexahydrat“)
500 ml H_2O
pH adjusted to 7.3 with succinic acid

Solution 3:
5 g Caseinhydrolysat (Serva)
5 g Yeast extract (Serva)
150 ml H_2O

Solution 4:
3.5 g K_2HPO_4 (Roth)
1.5 g KH_2PO_4 (Roth)
150 ml H_2O

Solution 5:
20 ml 1M $MgCl_2$ (Roth)

Solution 6: sterilised by filtration (0.45 µm pore size)
5 g Glucose (Roth)
10 ml H_2O

Solution 7: sterilised by filtration (0.45 µm pore size):
10 ml BSA 5% (w/v, Roth) in 1xSMM

For DM3 agar plates solutions 1 to 5 were autoclaved separately and cooled to 60°C before use. If the temperature is to high BSA will precipitate. If the temperature is to low the ager will agglomerate.

CY-3 soft-agar

DM3 agar plates were topped with 3 ml CY-3 soft-agar, respectively. CY-3 was autoclaved for no longer than 12 min

CY-3 (5 ml)
25 g/l Peptone (Serva)
25 g/l Yeast extract (Serva)
15 g/l NaCl (Roth)
10 g/l Agar (Serva)

1 M Na_2-succinat (pH 7.3, „Bernsteinsäure Dinatriumsalz Hexahydrat“, 5 ml)

Mix solution (0.8 ml)
500 ml/l 1.5 M Na-ß-Glycerolphosphat (Roth)
250 ml/l 1 M $MgCl_2$ (Roth)
125 ml/l Glucose 50% (w/v, Roth)
125 ml/l BSA 5% (w/v, Roth)
108 µl Chloramphenicol (10 mg/ml stock)

CY-3 soft-agar was homogenised by heating in a microwave oven and sub sequentially cooled to 60°C. Sodium-succinat is heated to 60°C. The solutions are carefully mixed at 60°C to avoid agglomeration of the agar and topped onto the DM3 agar plates.

3.2.6 Inducers

Stock solutions

1 M Isopropyl-β-D-thiogalactopyranoside (IPTG, Roth) in H_2O, stored at -20°C, 1 mM final concentration, e.g. for induction of GST-FNR (under control of the T5 promoter) from *E. coli* CAG627 (pMW68).

50 % (w/v) Xylose (≈ 3.3 M) (Roth, always fresh prepared) for induction of proteins under the control of the *xylA* promoter (Kloos *et al*., 1991). This promoter is repressed by glucose.

3.2.7 Antibiotics

TAB.M2. Stock solutions and final concentrations of antibiotics.

Antibiotic	Stock solution	Final concentration
Ampicillin (Roth)	50 mg/ml in H_2O	100 µg/ml
Kanamycinsulfat (Roth)	50 mg/ml in H_2O	50 µg/ml
Chloramphenicol (Fluka)	20 mg/ml in EtOH	10 µg/ml
Erythromycin (Fluka)	2,5 mg/ml in EtOH	2,5 µg/ml

3.3 Protein isolation procedures

3.3.1 Isolation of GST-FNR

FNR was produced and isolated as a GST-FNR fusion protein from *E. coli* CAG627 (pMW68) as described in Achebach *et al*., 2005. GST-FNR bound to glutathione-sepharose 4B (1.5 ml bed volume) was digested for 2 h at 20 °C with 20 U thrombin in 1 ml protein isolation buffer C (50 mm Tris / HCl, pH 7.6) and then eluted from the column in 3 ml protein isolation buffer C.

For the isolation of anaerobic apoFNR, anoxic buffers prepared and maintained in an anaerobic chamber were used and the whole procedure starting with incubation of the bacteria was performed under anoxic conditions. Aerobic apoFNR was prepared in the same way, but all buffers were air saturated and all steps were performed under air. FNR obtained from GST-FNR in this way contains a Gly-Ser extension in front of the N-terminal Met-Ile-Pro of wild-type FNR.

3.3.2 Isolation of (6x-His)-NreA

Aerobic NreA was prepared as (6x-His)-NreA from *E. coli* M15 (pQE31*nreA)*. 2-4 h after induction with 1 mM IPTG the *E. coli* cells were harvested by centrifugation (10.000 g, 15 min, 4 °C) and cell walls were disrupted in a French Pressure Cell (SLM-Aminco, 3 cycles with 1.38 x 10^5 kPa). Isolation of NreA was performed under air as described for NreB. The procedure for preparation of anaerobic NreA was the same as that for aerobic preparation, but all steps (including French Pressure cell) were performed under anoxic conditions in an anaerobic chamber (Coy), using anoxic buffers. (6xHis)-NreA was purified on a Ni-nitrilotriacetic acid (Ni-NTA) agarose column (Qiagen, Germany), yielding 5.1 mg (1.0 mg under anaerobic conditions) NreA protein in 2 ml of protein isolation buffer C.

3.3.3 Isolation of (6x-His)-NreB

Aerobic NreB was prepared as (6x-His)-NreB from *S. carnosus* strain m1 (pCQE1*nreB*) after growth and induction with xylose. Isolation of the protein was performed under air as described previously (Müllner *et al*., 2008)*,* but without β-mercaptoethanol in the buffers. The procedure for preparation of anaerobic NreB was the same as that for aerobic preparation, but all steps were performed under anoxic conditions in an anaerobic chamber (Coy), using anoxic buffers lacking β-mercaptoethanol. The isolation procedure included cell disruption for six cycles (40 s each) in an MP Fastprep24 system (0.1-mm beads; 5.0 m/s). The cell suspension was used to fill an airtight screw cup (2.0-ml micro tube; Biologix), transferred outside the chamber, and broken by the use of Fastprep24. (6xHis)-NreB was purified in the anaerobic chamber on a Ni-nitrilotriacetic acid (Ni-NTA) agarose column (Qiagen) as described in the manual, yielding 5 to 10 mg (25 to 50 µM) NreB protein in 5 ml of protein isolation buffer C.

3.3.4 Isolation of MalE-NreC

MalE-NreC was prepared from *E. coli* EC3 (pMal*nreC*) (Fedtke *et al*., 2002) after growth and induction with IPTG. The vector pMal*nreC*, a pMAL-c2X derivate, is designed to produce maltose-binding protein (MBP) fusions, where the protein of interest can be cleaved from MalE with the specific protease Factor *X*a (NEB

#P8010S). The *malE* gene on this vector is deleted for the export signal sequence, so the fusion protein produced remains in the cytoplasm. 10 mM glucose was added to the growth medium to repress the chromosomal maltose genes of *E. coli*, which can degrade the amylose on the affinity resin. Cells were harvested (4,000 g, 20 min, 4 °C) and resuspended in column buffer (20 mM Tris / HCl pH 7.4, 200 mM NaCl, 1 mM EDTA). Cell walls were disrupted in a French Pressure Cell (SLM-Aminco, 3 cycles with 1.38 x 10^5 kPa) and the supernatant was put an a amylose resin (New England Biolabs) column. After washing with 10 volumes of column buffer MalE-NreC was eluted with 10 mM maltose added to column buffer. Fractions were collected as 1 ml aliquots and subjected to Bradford protein assay. MalE-NreC fusion protein was cleaved by Factor Xa protease (New England Biolabs) as described in the NEB manual after addition of 0.2 % SDS and samples were checked on an SDS-PAGE. Isolated NreC was phosphorylated *in vitro* by incubation with 20 mM carbamoylphosphate for 60 min at RT. Alternatively, acetylphosphate can be used.

3.3.5 Immunoprecipitation of NreB from cell homogenates (pull-down assay)

For *in vivo* labeling experiments NreB was precipitated with polyclonal antiserum against NreB (Eurogentec) to reduce the background of fluorescence. After cell disruption by MP Fastprep 24 (0.1-mm beads; 5 m/s) for six intervals (40 s each), the debris was sedimented in a centrifuge at 10.000 g for 15 min. The supernatant (1 ml; 1 g protein / ml) was incubated on a rocking platform with polyclonal antibodies (5 µl polyclonal antiserum from rabbits, Eurogentec) against NreB for 1 h at 4°C, and then for another 2 h at 4 °C after the addition of 50 µl of a suspension of protein A coupled to agarose beads (Calbiochem). The beads were separated from the mixture by use of Qiaprep spin columns (Qiagen) and washed three times with 1 ml wash buffer (20 mM Tris / HCl, pH 8, 130 mM NaCl, 10% glycerol). Protein bound to the beads was eluted by the addition of 4% SDS in 20 mM Tris / HCl, pH 8, to the beads from the last wash step, followed by incubation for 15 min at room temperature. The beads were centrifuged in a spin column, and the flow through was subjected to SDS-PAGE and immunoblotting.

3.3.6 Bradford protein assay

200 µl Roti-Quant solution (Roth) was added to 780 ml H_2O and 20 µl protein sample (1-20 mg protein / ml endconcentration) and inverted several times. After 10 min the excitation at 595 nm was measured and compared to a freshly prepared calibration curve with samples containing 0, 2, 5, 10, 15 and 20 mg BSA (Roth) / ml. The r^2 value of the calibration curve was 0.99 or higher.

3.4 Thiol labeling methods

3.4.1 Cysteine labeling agents

TAB.M3. Thiol reagent solutions and final *in vivo* labeling concentrations. All solutions were stored at -20 °C and always protected from light. The pH of the IAA solution was adjusted with NH_3. IAA and NEM solutions were prepared freshly prior to use. mBBr, qBBr and AMS solutions were stored up to 6 months.

Thiol reagent	Stock solution	Labeling concentration
IAA (Sigma)	500 mM in H_2O pH 8.0	2 mM
mBBr (Invitrogen)	10 mM in acetonitrile	2 mM
qBBr (Invitrogen)	10 mM in acetonitrile	2 mM
AMS (Sigma)	500 mM in DMSO	8 mM
NEM (Sigma)	50 mM in HEPES pH 6.8	2 mM

3.4.2 *In vitro* thiol labeling procedures

In vitro labeling of FNR

FNR was isolated as apoFNR and reconstituted to $[4Fe4S]^{2+}$ FNR as described (Achebach *et al.*, 2005). mBBr binding was quantitatively calibrated using FNR with one, two, three, and five Cys residues in the thiol state. To obtain FNR with one, two, or three free Cys thiol groups, isolated FNR was incubated with 1, 2 or 3 mol N-ethylmaleimide (NEM) per mol FNR, respectively. FNR with five Cys thiols was

produced by reducing isolated FNR with an equimolar amount of DTT. Samples of the various forms of FNR in 50 mM Tris / HCl pH 7.6 were incubated with 2 mM mBBr for 5 min at RT and then subjected to SDS-PAGE, followed by western blotting.

For labeling of purified FNR with 4-Acetamido-4´-maleimidylstilbene-2,2´-disulfonic acid (AMS), reconstituted $[4Fe4S]^{2+}$ FNR was incubated with AMS (8 mM) in a glove box under N_2 for 20 min. For air inactivation, a solution (1 ml) of reconstituted FNR was spread onto a petri dish and shaken under air for 20 min at 130 rpm.

In vitro labeling of NreB

(6xHis)-NreB was purified from *S. carnosus* m1 (pCQE1*nreB*) under aerobic or anaerobic conditions, using a Ni-NTA agarose column as described. Aerobically or anaerobically purified NreB (25 to 50 µM) in protein isolation buffer C was incubated with mBBr (1 mM) for 10 min in the dark before the labeling reaction was stopped by adding 2 mM DTT. Structures and reaction mechanisms of thiol reagents used in this study are shown in FIG.M3.

3.4.3 *In vivo* thiol labeling procedures

In vivo thiol labeling of FNR

Cells of *E. coli* CAG627 (pMW32) were grown to an OD_{578} of 0.7 (mid-exponential phase) and 2 mM monobromobimane (mBBr, Invitrogen) from a freshly prepared stock solution (10 mM) was added (incubation time 5 min in the dark). The aerobic sample (1 ml in a 15-ml plastic tube) was shaken at 150 rpm under air. The anaerobic sample (1 ml in a 2-ml plastic tube) was handled and incubated in a glove box under N_2, and anoxic solutions were used throughout. Labeling reached maximal levels after 1 min. The labeling was stopped after 5 min by adding 2 mM dithiothreitol (DTT). The cells were collected by centrifugation (10.000 g, 15 min, RT) and resuspended in 2x SDS-loading buffer containing 5 % SDS.

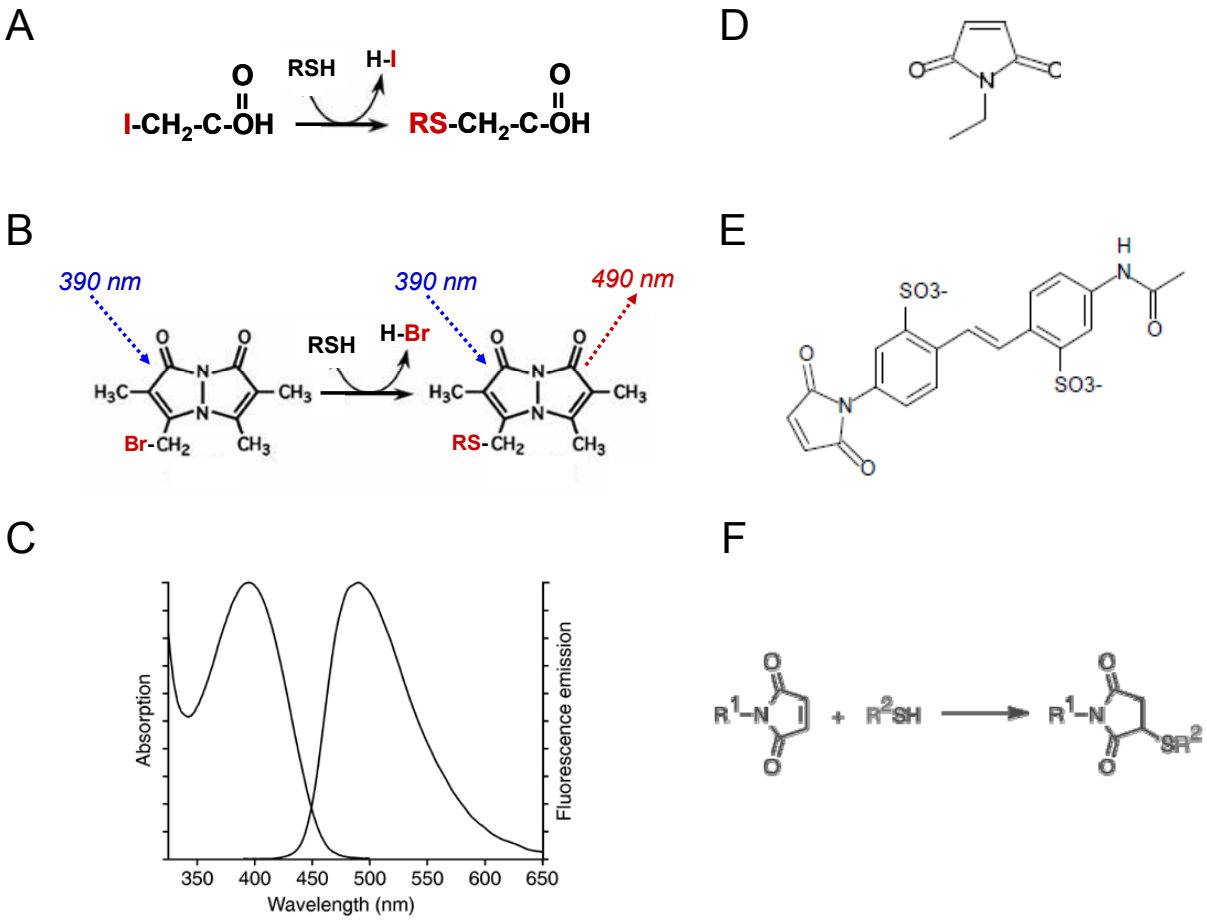


FIG.M3. Structures and alkylating mechanisms of Iodoacetate (IAA, A), Monobromobimane (mBBr, B), N-ethylmaleimide (NEM, D) and 4-Acetamido-4´-maleimidylstilbene-2,2´-disulfonic acid (AMS, E). IAA and mBBr are membrane permeable, NEM and AMS are not membrane permeable. After alkylation of a protein thiol with mBBr (or qBBr) the product can be excited at 390 nm to emit fluorescence at 490 nm (B, C), which was detected on a UV-bench and quantified with Gel Pro 6.0 software (Intas). Maleimide derivates (NEM and AMS) are suitable reagents for specific protein thiol modification. The favoured reaction is the addition of a cysteinyl thiolate anion to the maleimide double bound (Culham *et al*, 2003; F). In absence of thiols maleimides are also known to react with primary amines, e.g. lysine.

For measuring the kinetics of accessible Cys residues, *E. coli* CAG627 (pMW32) was grown anaerobically in the presence of IPTG to an OD_{578} of 1.0. Aliquots (1 ml) were transferred to petri dishes (diameter, 8.5 cm) and shaken under air (130 rpm). At different time points, 1 mM mBBr was added, and the suspensions were incubated for 1 min at RT. The reaction was stopped by the addition of 2 mM DTT, and the samples were prepared for SDS-PAGE as described.

Proteins were labelled with monobromotrimethylammoniobimane (qBBr, Calbiochem, discontinued) following the same method as for mBBr, except that cells were permeabilized with chloroform (50 µl) before adding qBBr (2 mM final concentration) from a qBBr stock solution (10 mM in acetonitrile). NreB was labelled with 4-acetamido-4´-maleimidylstilbene-2´2-disulfonic acid (AMS, 8 mM final concentration) in 1 ml of cell suspension (OD_{578} of 0.8) containing 50 µl chloroform for 30 min (similar to as described for qBBr).

In vivo thiol labeling of NreB

For labeling of NreB with mBBr *in vivo* aerobically growing *S. carnosus* m1 (pCQE1*nreB*) were induced at an OD_{578} of 1.0 by the addition of 150 mM xylose for synthesis of NreB. After 1 h, labeling was started by the addition of 0.2 U/ml of lysostaphin from a 0.5 U/µl stock solution, and 2 mM mBBr (Invitrogen) from a stock solution (10 mM in acetonitrile) was added. During labeling, the bacterial culture was protected from light and shaken at 200 rpm under air for 10 min before the labeling reaction was stopped with 10 mM dithiothreitol (DTT), followed by sedimentation of the cells by centrifugation (10.000 g, 15 min, 4 °C).

For anaerobic labeling, *S. carnosus* m1 (pCQE1*nreB*) was incubated after induction for 5 h in sealed serum bottles under an N_2 gas phase (99.99%) and subsequently labelled as described for the aerobic samples, but in a glove box under N_2 and using anoxic solutions throughout. The labeling was stopped after 10 min by adding 10 mM DTT. The labelled cells were collected by centrifugation (10.000 g, 15 min, 4 °C) and disrupted as described for the isolation of NreB.

The cleared cell homogenate was subjected to immunoprecipitation as described and dissolved in SDS sample buffer. Approximately 3 micrograms of purified protein, 100 µg of protein from cell homogenates, or 10 µg of precipitated protein was subjected to SDS-PAGE. The proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore Immobilon-FL; pore size, 0.45 µm) for further analysis as described.

3.4.4 Two-step thiol labeling procedure

In the first step, the native proteins (FNR or NreB) were labelled with IAA or (where indicated) with *N*-ethylmaleimide (NEM). Labeling with IAA was performed by adding 1 mM IAA from a 500 mM stock solution in H_2O, adjusted to pH 8 with NH_3 (or by 1 mM NEM from a 50 mM stock solution), to the sample (cell suspension or isolated protein). After 15 min, the reaction was stopped by adding 1 mM DTT, and the sample was mixed with guanidiniumhydrochloride (GnHCl, 2 M final concentration, from a 6 M stock solution). The Cys residues which became accessible after denaturing of the protein were then labelled with 2 mM mBBr as described.

For O_2 inactivation kinetics, FNR or NreB was purified under anoxic conditions in a glove box, using anoxic buffers (without β-mercaptoethanol). The anoxic protein was exposed to air by stirring 1 ml of the sample with a magnetic bar in a 15-ml Nalgene tube under air. Samples were withdrawn and labelled with IAA as described after various times of exposure to air. After stopping the reaction with excess DTT, the protein was denatured with 2 M (final concentration) guanidinium hydrochloride and the cysteine residues were labelled with mBBr as described.

3.4.5 SDS-PAGE

For casting and running SDS Gels the Mini-PROTEAN System (Bio-Rad), including 220V power supply was used.

<u>Resolving Gel (10%)</u>

3.33 ml 30 % acryl amid mix (Roth)
3.75 ml 1M Tris-HCl; pH 8.8 (Roth)
0.1 ml 10 % (w/v) SDS (Roth)
0.01 ml TEMED (Fluka)
0.2 ml 10% APS in H_2O (Fluka)
2.7 ml H_2O

<u>Resolving Gel (6%, EMSA)</u>

2.0 ml 30 % acryl amid mix (Roth)
3.75 ml 1M Tris/HCl; pH 8.8 (Roth)

0.1 ml 10 % (w/v) SDS (Roth)
0.01 ml TEMED (Fluka)
0.2 ml 10% APS in H_2O (Fluka)
4.03 ml H_2O

Stacking Gel (4%)
0.67 ml 30 % acryl amid mix (Roth)
1.25 ml 1M Tris/HCl; pH 6.8 (Roth)
0.05 ml 10 % (w/v) SDS (Roth)
0.005 ml TEMED (Fluka)
0.1 ml 10% APS in H_2O (Fluka)
2.98 ml H_2O

SDS Sample buffer (2x, Laemmli, 1970)
100 mM Tris/HCl, pH 6.8 (Roth)
200 mM Dithiothreitol (DTT, Sigma)
4 % (w/v) SDS (Roth)
0.2 % (w/v) Serva Blue G (Serva)
20 % glycerol (Roth)
or Laemmli sample buffer (Bio-Rad)

SDS Running buffer (10x)
250 mM Tris base (Roth)
1.92 M glycine (Roth)
1 % (w/v) SDS (Roth)

SDS samples were mixed with 2x SDS loading buffer and heated at 95°C for 5 min, if not described otherwise. To run SDS Gels the Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad) was used. 12 % gels were run for 60 min at 200 V and 6% gels were run at 175 V for 45 min. All steps were executed as described in the BioRad manual. Gels were sub sequentially used for western blotting or stained and protein fixated with Coomassie Blue (Serva), acetic acid (10%) and methanol (5%) in H_2O.

Coomassie staining procedure

2 g Coomassie Brillant Blue (Serva) was solved in 400 ml H_2O. 500 ml methanol and 400 ml acetic acid were carefully added. SDS-Gels were stained for 45 min and sub sequentially destained in H_2O in a microwave oven (800 W, 10 min). After destaining gels were dried by heating in a vacuum device (Biotec-Fischer).

3.4.6 Semi-dry Western Blotting

For protein blotting a custom built blotting device or the Mini Trans-blot Cell by Bio-Rad was used.

Transfer buffer (1x)

25 mM Tris base (Roth)

192 mM Glycine (Roth)

20 % (v/v) Methanol (Roth)

Washing buffer

0.1 % Tween in 1x PBS buffer

Antibody solutions

1x PBS buffer

0.1 % Tween 20

Antibody serum was added as described.

Primary antibodies

Polyclonal antibodies against FNR (*E. coli*) were produced in rabbit (Eurogentec) and diluted 1:1000 prior to use. Polyclonal antibodies against NreB (*S. carnosus*) were produced in rabbit, (Eurogentec) and diluted 1:10.000 prior to use. Polyclonal His-Tag antibodies were produced in mouse (Sigma) and diluted 1:10.000 prior to use. Primary antibodies were long-term stored at -20 °C as 20 µl aliquots.

Secondary antibodies

All secondary antibodies used in this study were coupled to the horseradish peroxidase (HRP). For primary antibodies produced in rabbit Anti-IgG-rabbit coupled to HRP (Bio-Rad) was used in a 1:10.000 dilution. For primary antibodies produced in mouse Anti-IgG-mouse coupled to HRP (Qiagen) was used in a 1:10.000 dilution. Secondary antibodies were long-term stored at -20 °C as 20 µl aliquots.

Developing solution for horseradish peroxidase

30 mg 4-Chlor-1-naphtol (Sigma), suspended in 20 ml Ethanol
80 ml 100 mM Tris / HCl pH 7.6
50 µl H_2O_2 (Roth)

Semi-dry blotting method

FNR, NreA or NreB protein samples were subjected to SDS-PAGE and subsequently transfered to a nitrocellulose (Protran, Whatman) or PVDF membrane (Millipore Immobilon-FL; pore size, 0.45 µm, Towbin *et al.*, 1979). Three layers of filter paper (Whatman) were soaked in methanol-containing transfer buffer. The PVDF membrane was equilibrated with methanol and then washed in H_2O. SDS gel, membrane and filter paper were piled to a sandwich with gel and membrane in the middle. The membrane-containing side was located on the anode of the blotting device and bubbles between the layers were removed carefully. The proteins were transferred to the membrane at a constant current of 5-10 V for 2 h (for smaller proteins shorter times may be sufficient).

For BSA-saturation, the blotted membrane was incubated in blocking buffer overnight at RT on a rocking platform. If required, the blotted SDS gel was checked by Coomassie Brilliant Blue (Serva) staining. The protein-saturated membrane was incubated with the primary antibody solution for 3 hours. The membrane was then washed for 2 x 3 min in 20 ml washing buffer and then incubated for 1.5 h with the secondary antibody solution. After removing of the secondary antibody and additional washing steps, protein bands were visualized by membrane-incubation with 4-Chlor-1-naphtol developing solution.

3.4.7 Quantitative evaluation of mBBr and qBBr labeling of FNR

The thiol labeling of FNR was quantitatively evaluated using the Western blots of the labelled proteins. For each set of experiments, the samples were run and evaluated on the same gel and blot. The fluorescence of protein bands labelled with mBBr or qBBr was measured with a fluorescence imager (Kodak ImageStation CF440) from 400 to 500 nm. The fluorescence of individual bands was quantified using Kodak software by integrating the amount of fluorescence of a specific band after subtraction of background fluorescence ("net intensity"). The integrated fluorescence is given in pixel intensity (AU, arbitrary units) by the software.

From the same blot, the FNR content was determined using anti-FNR serum and a secondary antibody coupled to horseradish peroxidase. The stain intensity was recorded by the imager. For quantitative experiments, all samples from one blot were treated the same, and at least four independent labeling experiments were performed. The amount of label is given as the pixel intensity of the fluorescence and of the absorption of the anti-FNR stain. FNR with one or five accessible Cys residues was always used as the reference (20% and 100% labeling, respectively).

For determining the amount of labelled thiols of FNR *in vivo*, the cells were labelled as described above. Samples were run on a gel with isolated FNR labelled on one or five Cys residues as the reference. After SDS-PAGE and blotting, mBBr fluorescence and FNR immunostaining were measured. The specific amount of labeling was calculated (pixel intensity of mBBr fluorescence per pixel intensity of FNR immunostain). For each sample, the ratio was determined and compared to the ratio of fivefold-labelled FNR as the reference.

3.4.8 Quantitative evaluation of mBBr labeling of NreB

After SDS-PAGE of the proteins and blotting to a PVDF membrane, mBBr fluorescence and NreB immunostaining were determined by recording the fluorescence of the corresponding bands on the PVDF membrane by an Intas video system (Sony IIDC camera with Heliopan S49 objective). Generally, the fluorescence on the PVDF membrane derived from mBBr was registered first.

After immunostaining, the immunostain from the same membrane was recorded. The video system was connected by a digital interface to a personal computer for data evaluation. The specific labeling was calculated from the pixel intensities of mBBr fluorescence (PI_{Fluor}) and of the NreB immunostain (PI_{Immuno}) for the total area of the protein bands. For the samples, the specific labeling (PI_{Fluor}/PI_{Immuno}) was compared to that of fourfold- and twofold- labelled NreB as a reference. Fourfold-labelled NreB was aerobic wild-type NreB that was denatured by the addition of 2 M guanidinium hydrochloride and reduced with DTT before being labelled with mBBr.

Twofold-labelled NreB was mutant NreB* (C59S C62S) that was labelled in the same way as aerobic wild-type NreB. For quantification of fluorescence and immunostaining signals, the Gel-Pro Analyzer 6.0 software of Media Cybernetics was used. After direct labeling of NreB and NreB* with mBBr, the fluorescence was used to calculate the specific amount of accessible cysteine residues by comparison to NreB labelled at 0, 2, and 4 Cys residues.

3.4.9 Electro mobility shift assay (EMSA)

Binding buffer
20 mM Tris/HCl, pH 7.5 (Roth)
0.5 mM EDTA (Roth)
5 % (v/v) glycerol (Roth)
1 mM DTT (Fluka)
0.005% (v/v) Triton X-100 (Fluka)
50 mM NaCl (Roth)
5 mM $MgCl_2$ (Roth)
2.5 mM $CaCl_2$ (Roth)
10 mM $NaNO_3$ (Roth, optional)

10x TBE buffer
890 mM Tris base (Roth)
890 mM boric acid (Fuka)
20 mM EDTA (Roth)

For band shift assays of NreA with putative target promoters of *narG*, *nirB* and *narT*, NreA protein was purified aerobically or anaerobically as described and mixed with sample DNA fragments (483–628 bp, final concentrations 8–13 nM) in a total volume of 20 µl. Primers used for amplification of the upstream DNA region of *narG*, *nirB* and *narT* from genomic *s. carnosus* TM300 DNA are shown in TAB.M3.

100 nM of random salmon DNA fragments (Calbiochem) was added as a negative control. After incubation for 30 min at room temperature, the samples were separated on a 10% native polyacrylamide gel (170 V) at room temperature using 1x TBE without SDS as electrophoresis buffer. The gels were subsequently stained with SYBR Green I according to the instructions of the supplier (1:10.000, Sigma). All PCR products used in the gel shift assays were purified with the PCR purification Kit (Qiagen, Hilden, Germany) and checked on an agarose gel prior to use.

The anaerobic EMSA experiments were conducted in an anoxic glove chamber using anaerobically isolated NreA protein and anoxid buffers.

3.4.10 Protein crosslink assay

NreA and NreB were aerobically or anaerobically isolated as described. 100 µl NreB (500 µg / ml) and 100 µl NreA (600 µg / ml) were mixed and formaldehyde (0.1–1.0% final concentration, from a 3.65% stock solution in H_2O, from Sigma) was added. The sample was incubated for 1 h (4 °C). Glycerol (0.3 M final concentration, from 2M stock solution) was added to stop the crosslink reaction.

The samples were mixed with 2x SDS-loading buffer and analyzed in an SDS-PAGE (10–12.5% resolving gels), followed by coomassie staining as described. To avoid damage to cross-linked proteins, samples were not heated to 99 °C. The anaerobic samples were prepared the same way, but under anaerobic conditions in a glove box until SDS-PAGE.

3.5 MALDI-TOF

3.5.1 *In vivo* alkylation of FNR by *N*-ethylmaleimide (NEM) or iodoacetate (IAA) for MALDI-TOF

FNR was produced as a GST-FNR (*G*lutathione-*S*-*t*ransferase) fusion in *E. coli* CAG627 (pMW68) growing under aerobic conditions as described previously. Two hours after start of induction, *N*-ethylmaleimide (NEM, 50 mM in H_2O_{dd}) or iodoacetate (IAA, 500 mM in H_2O_{dd}, pH 8.0) was added for 15 min (final label concentration 2 mM, respectively). The bacteria were harvested, and GST-FNR was prepared by chromatography on glutathione-Sepharose 4B. The GST tag was removed by incubation with thrombin for 2 h, and the isolated FNR (600 µg / ml) was analyzed by MALDI-TOF (Matrix-assisted laser desorption ionization–time-of-flight) mass spectrometry (MS).

All analyses were carried out on a Q-TOF II system (Waters Corp., Manchester, United Kingdom). Prior to mass measurements, the samples were separated by reversed-phase high-performance liquid chromatography (HPLC) (Waters Alliance 2795; Waters Corp., Manchester, United Kingdom) on a Vydac C18 column (250 by 2.1 mm) connected online to the MS instrument (Roche, Penzberg).

3.5.2 *In vivo* alkylation of NreB by iodoacetate (IAA) for MALDI-TOF

For mass spectrometry, purified native NreB in protein isolation buffer C was labelled with 5 mM IAA (from a 500 mM stock solution in H_2O, adjusted to pH 8 with NH_3) for 30 min at 4°C after fresh aerobic or anaerobic isolation. The labeling reaction was stopped by adding 10 mM DTT.

The labelled protein was then digested for 6 h (37°C) by a freshly prepared solution of Asp-N endoprotease (1 µg protease / 100 µg NreB) or for 6 h (25°C) by a freshly prepared solution of Glu-C endoprotease (1 µg protease / 40 µg NreB) (both sequencing grade; from Roche Diagnostics). All buffers were prepared without

glycerol to avoid disadvantages for MALDI-TOF. It was verified that NreB does not agglomerate.

Protein digestion was controlled by running a sample on the SDS-PAGE gel. The digested protein as well as labelled intact protein from aerobic and anaerobic bacteria was frozen in liquid N_2 and analyzed by mass spectrometry. The fragments were checked for the presence of the Cys-containing peptides by screening for peptides with masses corresponding to the requested peptide and the mass increases due to incorporation of the acetyl label (58 Da per Cys residue label) for the Asp-N and Glu-C peptides. The MALDI-TOF experiments were conducted by Roche, Penzberg.

3.5.3 MALDI-TOF Parameters

Sinapic acid (Sigma Aldrich, Taufkirchen, Germany) and α-cyano-4-hydroxy-cinnamic acid (Bruker Daltonics, Bremen, Germany) were used as matrices. External calibration of the spectra was performed using peptide calibration standard II (Bruker Daltonics, Bremen, Germany). The samples were desalted with ZipTip _C18 tips (Millipore, Billerica, MA), using ACN (BioSolve, Westford, MA), trifluoroacetic acid (TFA; Mallinckrodt Baker, Phillipsburg, NJ), and MilliQ water (prepared by a Millipore water purification system [Billerica, MA]).

To obtain better signal-to-noise levels, the samples were desalted with a pipette tip (ZipTip μC18; Millipore) and adjusted to 1.5 μl according to the following procedure. First, the ZipTip was washed two times with 50% aqueous ACN solution, and then it was washed two times with 0.1% TFA. Afterwards, 1.5 μl of sample was picked up, rinsed back and forth in the pipette tip, and pushed out.

This procedure was repeated 10 times, using a total volume of 15 μl of sample. After being washed with 0.1% TFA and MilliQ water three times, the samples were eluted on the target with the matrix. Sinapic acid was used (10 mg/ml solved in 2/1 ACN/0.1% TFA [v/v]) for all samples digested by endoproteinase Asp-N (Roche Diagnostics). α-Cyano-4-hydroxy-cinnamic acid was used (30 mg/ml solved in 2/1

ACN/0.1% TFA [v/v]) for all samples digested with endoproteinase Glu-C (Roche Diagnostics). The spots were allowed to dry at room temperature.

All MALDI-TOF mass spectra were acquired on a Bruker Reflex III mass spectrometer (Bruker Daltonics, Bremen, Germany). An MTP AnchorChip 600/ 384 (Bruker Daltonics, Bremen, Germany) was used as the target. The MALDI-TOF mass spectrometer utilizes a pulsed nitrogen laser, emitting at 337 nm. The extraction voltage was 20 kV, and gated matrix suppression was applied to prevent the saturation of the detector by matrix ions. Fifty single laser shots were averaged for each mass spectrum. The laser strength was kept at about 35% to obtain an optimum signal-to-noise ratio. To enhance the spectral resolution, all spectra were acquired in reflector mode. External calibration was used with a mass tolerance of < 4 Da.

3.6 Molecular genetic methods

Molecular genetic methods such as gene amplification via PCR, restriction and ligation of DNA fragments were performed by using standard procedures (Sambrook and Russell, 2001).

3.6.1 Agarose gel-electrophoresis

1% (w/v) Agarose was suspended in 1x TBE buffer and heated in a microwave oven. Gels were cast after addition of ethidiumbromid (Roth, 5 µl of stock solution to 200 ml agarose). Gels were run at 100 Volt for 30-50 min, depending on the sizes of DNA samples, and analyzed on a UV- bench.

3.6.2 Isolation of genomic DNA from *Staphylococcus carnosus*

Genomic DNA from *S. carnosus* was isolated using an altered phenol / chloroform extraction protocol (Zúñiga *et al.*, 1996) described in Graf, 2009 or with the Innu Speed Bacteria DNA Fungi Kit (Analytik Jena). The *pfu* polymerase (Stratagene) is inhibited by phenol and cannot be used for amplification of template DNA previously isolated with phenol / chloroform extraction.

5 ml overnight culture of *S. carnosus* TM300 were centrifuged (5.000 g, 10 min, 4°C), washed with H_2O and resuspended in 800 µl lysis buffer, followed by an incubation at 37°C (30 min) until the cell suspension turned clear. 20 µl Proteinase K (20 mg/ml), 25 µl 10 % SDS and 50 µl RNase A/T1-Mix (2 mg/ml, Fermentas) were added and incubated at 37°C for 1h. 500 µl phenol / chloroform / isoamylethanol solution (25:24:1) was added and the sample was inverted 5 times, followed by centrifugation (14.000 g, 30 min). The (upper) aqueous phase was carefully separated from the lower phase.

The phenol / chloroform extraction was repeated once, followed by a chloroform / isoamylethanol (500 µl, 24:1) extraction. The sample was centrifuged (14.000 g, 30 min) and the aqueous phase was carefully separated from the lower phase. 50 µl acetate (3 M, pH 5.2) was added and after carefully inversion (1 min) 1 ml cold ethanol (99.9 %, -20°C) was added and incubated overnight at -20°C. After centrifugation (14.000 g, 20 min, 4°C) the pellet was carefully washed with cold ethanol (75 %, -20°C) and centrifuged again. The pellet was dried (45 min on air or 10 min in SpeedVac concentrator 5301, Eppendorf) and resuspended in 20 µl elution buffer (25 mM Tris / HCl pH 7, Fermentas)

3.6.3 Isolation of plasmid DNA from *Staphylococcus carnosus*

10 µl Lysostaphin (0,5 U/ml, Genmedics) was added to a 5 ml overnight culture in BM media. Cells were incubated for 20 min at 37°C, followed by FastPrep 24 treatment (MP Biomedicals 4.5 m/s, 30 sec, 3 cycles) to break the cell wall. Plasmids were isolated using the QIAprep Spin Miniprep kit (Qiagen, Hilden) according to the manual and eluted in 30 µl H_2O. The dsDNA concentration was measured with the

bio-photometer (Eppendorf, Germany). Due to the high amount of shredded genomic DNA samples with less than 150 ng dsDNA / µl were discarded.

3.6.4 Isolation of plasmid DNA from *Escherichia coli*

Plasmids were isolated using the QIAprep Spin Miniprep kit (Qiagen, Hilden) according to the manual and eluted in 30 µl H_2O. The dsDNA concentration was measured with the bio-photometer (Eppendorf). Samples with less than 80 ng /µl were discarded.

3.6.5 Polymerase chain reaction (PCR)

In vitro gene amplification was carried out by the polymerase chain reaction (Mullis *et al.*, 1986). For all reactions with plasmid DNA and chromosomal DNA as a template the proof-reading polymerases *Pfu*Ultra (Stratagene) or *Phusion* Pol. (Finnzymes) were used. A list of all oligonucleotides used in this study is given in TAB.M3. The PCR protocols are listed in TAB.M4.

Components of a PCR reaction mixture (for 300 µl, 5-6 reactions)

190.5 µl PCR H_2O (Roth)
37.5 µl 1 M DMSO
30 µl 10x Phusion buffer (Finnzymes)
6 µl 10 mM dNTPs
15 µl forward primer 1:10 (10 pmol)
15 µl reverse primer 1:10 (10 pmol)
3 µl genomic DNA template (100-200 ng per reaction)
3 µl Phusion Polymerase

TAB.M4. Oligoneucleotides used in this study. Primers used to amplify *nreC* from genomic wildtype DNA: F nreC SalI and R nreC KpnI. Primers used to mutate pRB473 carrying *nreABC*: F nreB C59S C62S and R nreB C59S C62S. Primers used for amplification of mutant *nreB* from pRB473nreAB*C: F nreB mut BglII and R nreB mut KspAI. Primers used for amplification of upstream DNA regions of *narG* (F narG prom and R narG prom), *nirB* (F nirB prom and R nirB prom) and *narT* (F narT prom and R narT prom). For sequencing of pQE30nreC primer pQE30nreC seq was used. T_m : salt adjusted melting temperature (Oligo Calc). All oligonucleotides for gene amplification and sequencing were sythesized by MWG Biotech (Ebersberg, Germany) and HPSF-purified.

Primer	Sequence (5`→ 3`)	T_m [°C]
F nreC SalI	CCT GTC TTT GTC GAC TGA TTC C	62.1
R nreC KpnI	GGA GAC GGT ACC ATG AAG ATA G	62.1
pQE30nreC seq	TAG ATT CAA TTG TGA GCG G	53.0
F nreB C59S C62S	GAC GAA TGC AGT TTC TCG CAG ATC GGA AGG ATA TT	74.3
R nreB C59S C62S	AAT ATC CTT CCG ATC TGC GAG AAA CTG CAT TCG TC	74.3
F nreB mut BglII	GTC TCC TAA GAT CTA TCA GTT ATT GG	62.9
R nreB mut KspAI	CGG TTA GGG AGT TAA CCA GAT G	62.1
F nirB prom	GTT GGC AGC GCG ATT AAA TGA AGT TAA G	67.2
R nirB prom	CGT TGT TTA GAC ATC CGT TAT TCC CTC	66.6
F narG prom	CTG GCC ACC TTT CTG ATA TTG AG	62.9
R narG prom	GGC TTA AAA AAG TTC ATT CCA AAT TTC G	62.7
F narT prom	CGC GGC GAT ACG TAT ATT GAT CC	64.6
R narT prom	GGA CTG TTA ATT GTA AAC CGC CTT TTG	65.3

The annealing temperature for the amplification of DNA fragments was chosen 5°C below the melting temperature T_m of the primer pair and tested by step gradient PCR, if required. The elongation time depended on the size of the amplified DNA fragment and the polymerase (bp / sec information from supplier). PCR was performed in the iCycler (Bio-Rad) or MyCycler (Bio-Rad) thermocyclers. A typical PCR protocol is given in TAB.M5.

TAB.M5. PCR protocol for the Phusion Polymerase (Finnzymes). *The cycle (steps 2-4) was repeated 30 times

Step	Temp. / Duration
1. Initial denaturation	95°C, 3 min
2. Denaturation*	95°C, 30 sec
3. Annaeling*	T_m-5°C, 30 sec
4. Elongation*	72°C, 30 sec / kb template

3.6.6 DNA restriction, ligation and sequencing

DNA restriction fragments were purified with the QIAquick PCR purification kit (Qiagen, Hilden). Restriction endonucleases and T4 DNA ligase were applied from MBI Fermentas. Prior to ligation, dephosphorylation of the restricted vector was accomplished by incubation with CIAP-phosphatase (Fermentas). DNA concentrations were determined with the bio-photometer (Eppendorf). Ligations were incubated for 3 h at 37°C or overnight at 16°C and precipitated with butanol prior to transformation.

3.6.7 Construction of NreB mutant C59S C62S (IMW1884)

The C59S and C62S mutations were introduced into NreB by use of a QuikChange site-directed mutagenesis kit (Stratagene) and shuttle plasmid pRB473, carrying the *nreABC* operon (Staßen, 2008). The mutations were introduced using primers F nreB C59S C62S (5'-GAC GAA TGC AGT TTC TCG CAG ATC GGA AGG ATA TT-3') and R nreB C59S C62S (5'-AAT ATC CTT CCG ATC TGC GAG AAA CTG CAT TCG TC-3'). The *nreB* mutant encoding NreB (C59S C62S) on the resulting plasmid, pRB473nreAB*C, was amplified using primers F nreB mut BglII (5'-GTC TCC TAA GAT CTA TCA GTT ATT GG-3') and R nreB mut KspAI (5'-CGG TTA GGG AGT TAA CCA GAT G-3') and cloned into the *Bgl*II and *KspA*I sites of the staphylococcal expression plasmid pCQE1, yielding plasmid pCQE1nreB* (pMW876, FIG.M1.). *S. carnosus* m1 was transformed with the plasmid. Induction by xylose and

overproduction and isolation of NreB* (C59S C62S) were conducted as described for wild-type NreB.

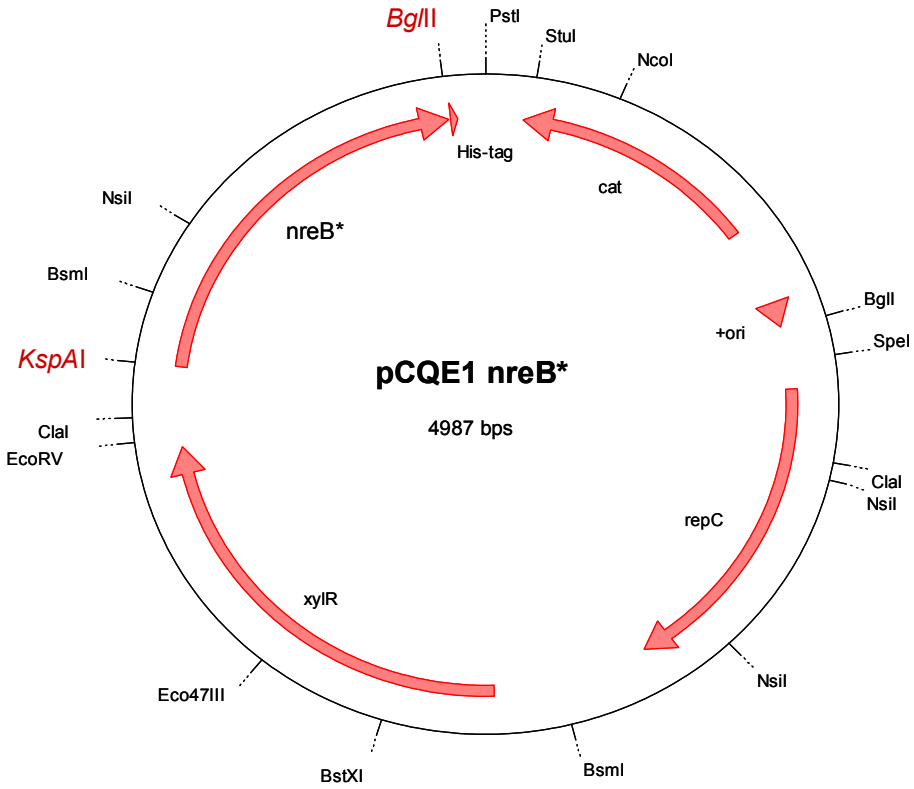


FIG.M1. Plasmid pQE1nreB* for over-expression and isolation of NreB C59S C62S. *nreB* was mutated in pRB473*nreABC* (*E. coli* shuttle vector) and the resulting *nreB** C59S C62S was cloned into the over-expression vector pCQE1 using the *Ksp*AI and *Bgl*II restriction sites. The resulting plasmid, pCQE1*nreB** carries *nreB** connected to a C-terminal His-tag under control of a xylose inducable promoter *xylR* and was verified by control restriction and DNA sequencing (StarSEQ, Mainz).

3.6.8. Construction of pQE30*nreC*

nreC was amplified from genomic DNA of *S. carnosus* TM300 via PCR using primers F nreC SalI (5'-CCT GTC TTT GTC GAC TGA TTC C-3') and R nreC KpnI (5'-GGA GAC GGT ACC ATG AAG ATA G-3'), introducing *Sal*I and *Kpn*I restriction sites. The amplificated DNA (677 bp) was purified using the Qiagen PCR purification kit. This purification step was repeated after *Sal*I and *Kpn*I restrictions, respectively. After the last purification step the dsDNA concentration was higher than 100 ng / µl.

pQE30 was isolated from *E. coli* JM105 (IMW335, from Qiagen), restricted with *Sal*I and *Kpn*I as described for *nreC* and dephosphorylated. 100 ng of pQE30 was mixed with 20, 60, 100 or 200 ng of restricted *nreC* and incubated overnight at 16 °C with T4 ligase.

pQE30*nreC* (FIG.M2) was transformed into *E. coli* JM109 using electro-transformation as described and cells were plated on LB agar plates containing ampicilin. pQE30*nreC* was purified and subjected to control restriction with *Pvu*II (Fermentas) and DNA sequencing (StarSEQ, Mainz).

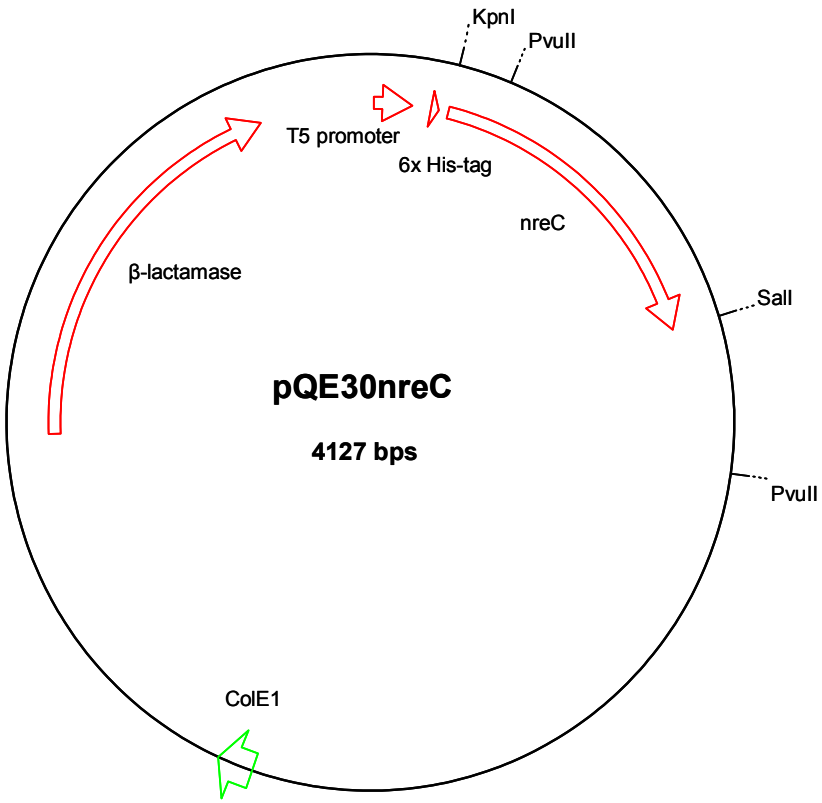


FIG.M2. Plasmid pQE30*nreC* for over-expression and isolation of NreC with N-terminal His-Tag. The restriction sites *Kpn*I and *Sal*I were used for cloning *nreC* into pQE30. *Pvu*II cuts *nreC* asymmetrically and can be used in a control digestion to verify the presence of *nreC*. IPTG induces protein expression at the T5 promoter. Transformed cells can be selected with ampicillin as pQE30*nreC* carries and expresses the β-lactamase encoding gene.

3.6.9 Protoplast-transformation of *Staphylococcus carnosus*

Preparation of protoplasts (Götz & Schumacher, 1987)

300 ml basic media (BM) containing erythromycin were inoculated with 1 ml of an overnight culture of *S. carnosus* m1 and incubated at 37 °C and 160 rpm. At an OD_{578} of 0.5 the cells were centrifuged (8.000 g, 15 min, 4 °C) and resuspended in 30 ml SMMP-B buffer. After addition of 25 µl lysostaphin (0,5 U/ml, Genmedics) solution the cells were incubated for 10-16 h at 30°C.

The formation of protoplasts was microscopically verified. Protoplasts appear as separated, single cells, whereas conventional cells of *S. carnosus* appear in packs of four or more cells.

The protoplasts were centrifuged (4.500 g, 25 min, RT) and the supernatant was carefully separated. The pellet was washed with 5 ml SMMP-B buffer and resuspended in 2 ml SMMP-B buffer. The protoplast suspension was aliquoted á 300 µl, frozen in liquid nitrogen and stored at -80°C.

Protoplast transformation (modified after Götz & Schumacher, 1987)

0.5 – 1.0 µg or more ligated DNA was added to 300 µl protoplast suspension and carefully thawed. Then 2 ml of fusogen solution was added to prevent protoplast lysis. The sample was carefully mixed for 2 min (not longer) and 7 ml SMMP-B buffer was added. The protoplasts were centrifuged (4.500 g, 25 min, RT) and the supernatant was discarded. The pellet was resuspended in ca. 300 µl supernatant and carefully plated on DM3 agar plates. After incubation (4 h, 37 °C) the DM3 agar plates were topped with CY3 soft agar and incubated for 1-2 days at 37°C.

Colonies were picked for overnight cultures and the sequence of the resulting construct was verified by control digestion or DNA sequencing (StarSEQ, Mainz). Colonies appearing after more than 2 days of incubation are mostly due to contamination and should be ignored.

3.6.10 Electro-transformation of *Escherichia coli*

E. coli JM109 was transformed with pQE30*nreC* by electroporation (Dower *et al.*, 1988). Preparation of electro-competent cells was performed according to Farinha *et al.* (1990). The sequences of resulting constructs were verified by DNA sequencing with self-designed primers (StarSEQ, Mainz).

3.7 Databases

TAB.M6. Databases used in this study.

Database	URL
NCBI, PubMed:	http://www.ncbi.nlm.nih.gov/pubmed/
University of Mainz Library	http://www.ub.uni-mainz.de/
Swiss-Prot and associated links	http://www.expasy.org/sprot/
PSIPRED	http://bioinf.cs.ucl.ac.uk/psipred/
Uniprot	http://www.uniprot.org/
SMART	http://smart.embl-heidelberg.de/
EMBL	http://www.ebi.ac.uk/
Pharyngula	http://scienceblogs.com/pharyngula/
KEGG	http://www.genome.jp/kegg/kegg2.html/
ClustalW	http://www.ebi.ac.uk/Tools/clustalw2/index.html/
OligoCalc	www.basic.northwestern.edu/biotools/oligocalc.html/
FeS cluster Info	http://www.ebi.ac.uk/Tools/clustalw2/index.html/
HeBIS	http://thea.hebis.de/thea/
Beolingus	http://dict.tu-chemnitz.de/
PREDICT	http://www.predictprotein.org/

4. Results

4.1 Labeling FNR Cys residues with mBBr or qBBr

4.1.1 Accessibility of FNR Cys residues to mBBr or qBBr

ApoFNR can be differentiated from FNR that contains a FeS cluster by measuring the accessibility of the Cys residues (Achebach *et al*., 2005). The accessible Cys residues of the cellular proteins of *E*. *coli*, including FNR, were labelled with the membrane-permeable thiol reagent monobromobimane (mBBr) and the impermeable thiol reagent monobromo-trimethylammonio-bimane (qBBr). mBBr and qBBr become fluorescent only after reaction with thiol groups. By penetrating the cell membrane without need of cell disruption or treatment with detergents or solvents (Kosower *et al*., 1979 and Baeyens *et al*., 1988), mBBr should allow labeling with minimal perturbation of the functional state of the cell and of FNR. *E*. *coli* CAG627 (pMW32) overproduces FNR to moderate levels which are sufficient for direct quantization of FNR from cell extracts by immunoblotting. In wildtype cells, on the other hand FNR contents are too low for direct quantization (Schirawski *et al*., 1997 and Reinhart 2006). FNR from bacteria with moderately overproduced FNR is supposed to show normal response to the presence of O_2 (Trageser *et al*., 1989).

E. *coli* CAG627 (pMW32) was grown aerobically or anaerobically under inducing or non-inducing conditions and then labelled with mBBr or qBBr. The cellular proteins were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. In the blots of non-induced aerobic or anaerobic *E*. *coli* CAG627 (pMW32), a limited number of bands with strong fluorescence was found; the bands were mainly in the range of 50 to 60 and around 90 kDa (FIG.R1; A and C, lane 1). After mBBr labelling, in induced cells of aerobically or anaerobically grown *E*. *coli*, an additional major fluorescent band at 30 kDa was observed. A similar labeling pattern was obtained when the bacteria were permeabilized by chloroform or after cell disruption before labeling. This demonstrates that mBBr gains sufficient access to the cytoplasm of *E*. *coli*, as suggested earlier for other types of cells (Kosower *et al*., 1995).

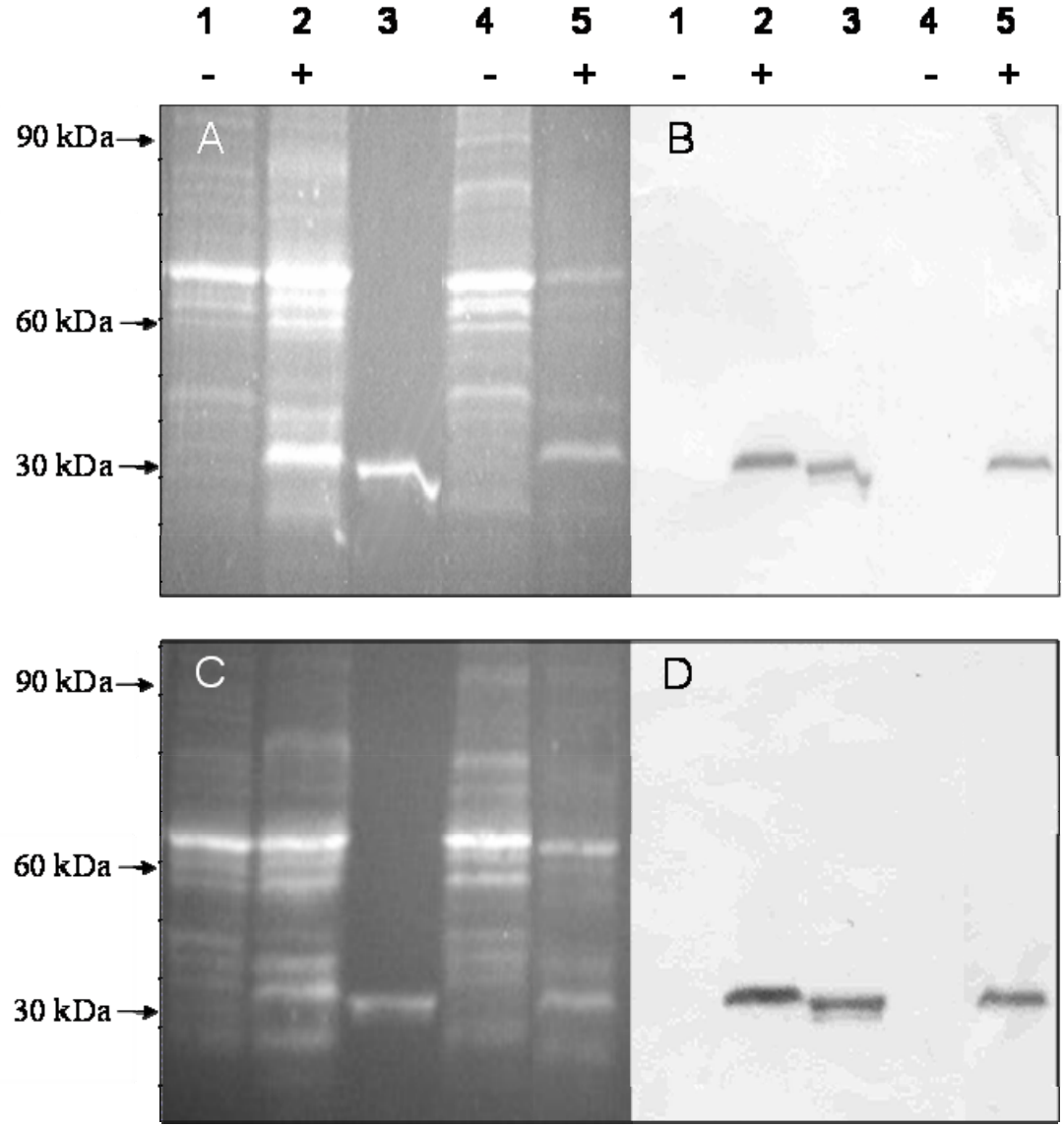


FIG.R1. *In vivo* labeling with mBBr (A and B) and qBBr (C and D) of proteins of aerobically and anaerobically grown *E. coli* CAG627 (pMW32). Detection of bound mBBr (A) and qBBr (C) by fluorescence and FNR by immunostaining (B and D). The bacteria were grown aerobically (lanes 1 and 2) or anaerobically (lanes 4 and 5) to an OD_{578} of 0.7 under *fnr*-inducing (+) (1 mM IPTG; lanes 2 and 5) or non-inducing (-) (lanes 1 and 4) conditions. Lane 3 contains 1.2 µg purified FNR. Cells were incubated for 5 min with 2 mM mBBr or qBBr, respectively. Before incubation with qBBr 2M guanidinium hydrochloride was added to the cells. Sedimented bacteria were resuspended and lysed in SDS-PAGE sample buffer (30 µg/lane); samples were subjected to SDS-PAGE and blotted onto nitrocellulose. Fluorescence was measured using a Kodak ImageStation CF440 with emission at 400 to 500 nm (A and C). The same blot was then used subsequently for immunostaining with anti-FNR (B and D). Staining was recorded as the absorbance at 400 to 500 nm. The positions of proteins corresponding to 30, 60 and 90 kDa (calculated from M_r markers) are shown to the left.

Purified and mBBr-labelled apoFNR formed a fluorescent band with similar mobility as the 30 kDa band (FIG.R1; A and C, lane 3). When proteins were labelled with the impermeable thiol reagent monobromo-trimethylammonio-bimane (qBBr, FIG.R1; C and D), a very similar pattern of fluorescence labeling was observed. Proteins were labelled only after permeabilization of the cells.

4.1.2 Detection of FNR with FNR-antiserum

The band corresponding to the fluorescent 30 kDa protein reacted specifically with anti-FNR serum (FIG.R1; B and D). When the expression of plasmid-encoded *fnr* was not induced, neither the fluorescent band (FIG.R1; A and C) nor the band responding to anti-FNR in the immunoblot was produced (FIG.R1; B and D). These findings identified the reactive 30 kDa protein as FNR and the corresponding fluorescence as FNR derived.

4.2 *In vivo* cysteine accessibility of aerobic and anaerobic FNR

4.2.1 FNR-specific fluorescence calibration

The specific amount of label incorporated into FNR was determined using purified apoFNR with five accessible Cys residues as a reference. In purified apoFNR, defined numbers of Cys residues were blocked by reaction with defined amounts of N-ethylmaleimide (NEM, Achebach *et al*., 2005). In this way, FNR with one, two, three, or five Cys residues in the thiol state was available, which then reacted with mBBr. The fluorescence of the proteins was determined in Western blots as described in FIG.R1 and normalized for the amount of FNR protein in immunoblots.

The specific amount of fluorescence per FNR protein showed a linear relationship to the number of accessible Cys residues (FIG.R2). The specific labeling varied by a factor of approximately 5 when FNR with one and five accessible Cys residues was labelled, confirming that by this method up to five Cys residues per FNR become labelled. The calibration curve was used to determine the specific content of mBBr-labelled FNR of *E. coli* cells. The label which was found in FNR from aerobically

(solid circle) and anaerobically (solid square) growing cells corresponded to approximately 80 % and 40 %, respectively, of FNR labelled at five Cys residues (FIG.R2). Therefore, in aerobically and anaerobically growing *E. coli*, on average four and two Cys residues are accessible to modification. In aerobic bacteria, labeling of five Cys residues is expected. The lower degree of labeling might be due to incomplete application of aerobic conditions or to incomplete chemical labeling *in vivo*.

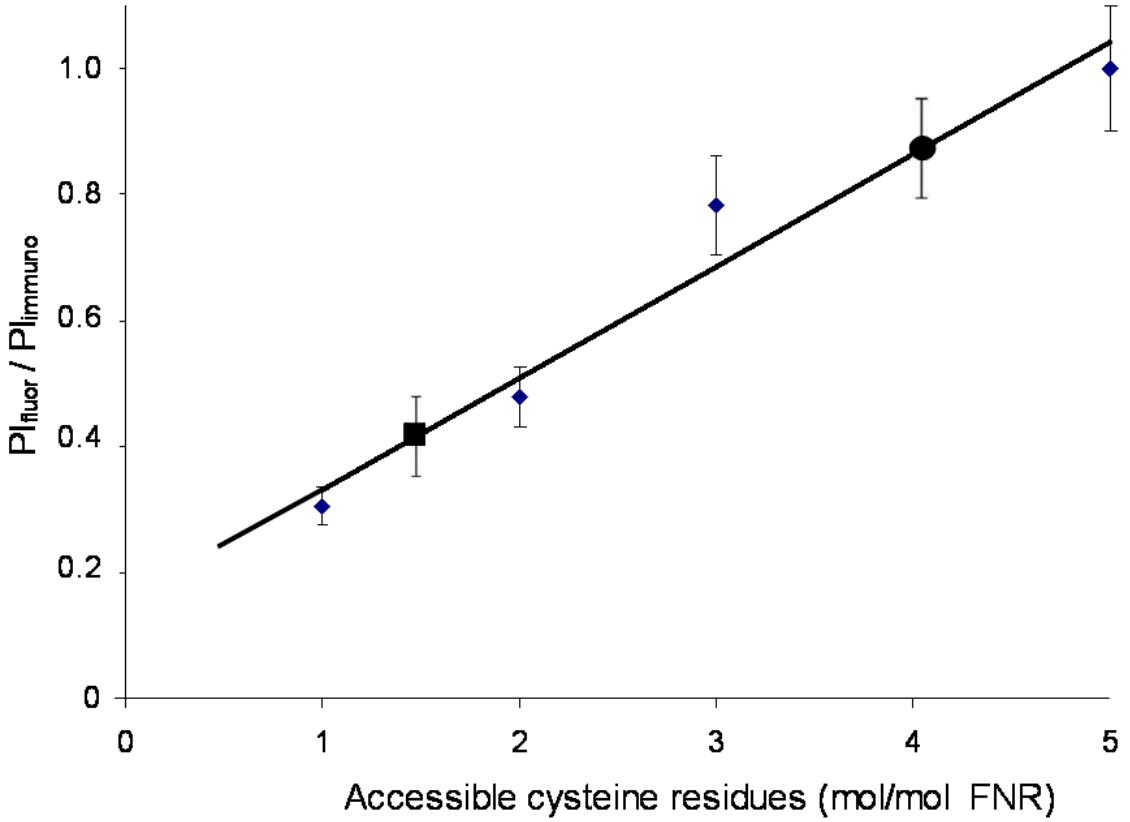


FIG.R2. Specific fluorescence of mBBr-labelled FNR in aerobically and anaerobically grown *E. coli* CAG627 (pMW32). The specific fluorescence was calibrated using purified FNR with one, two, three, and five Cys thiols/FNR (small solid diamonds) labelled with mBBr. After separation by SDS-PAGE and blotting onto nitrocellulose, mBBr fluorescence (integrated pixel intensity of fluorescence [PI_{fluor}]) and FNR immunostaining (integrated pixel intensity of immunostaining [PI_{immuno}]) were measured by fluorescence and absorption imaging as described in the legend to Fig. 1. The specific labeling of the different FNR species (PI_{fluor}/PI_{immuno}) was determined as described and the specific labeling of FNR with five accessible Cys residues was taken as 100%. Following the same method, the PI_{fluor}/PI_{immuno} of protein from aerobically grown cells (one solid circle) and anaerobically grown cells (one solid square) was determined. The mean experimental values are derived from four or more independent experiments.

4.2.2 Differentiation between aerobic and anaerobic FNR

To calculate the specific content of labelled Cys residues in FNR, the amounts of mBBr that was bound to different forms of FNR were determined by quantifying the fluorescence and immunostaining in Western blots, as shown in TAB.R1. The specific amount of fluorescence per FNR from aerobically grown *E. coli* was reproducibly twofold higher than that from anaerobically grown *E. coli*. The increased labeling was specific for FNR; the fluorescence of other labelled bands did not increase in the same way.

qBBr labelled proteins were evaluated in a similar way. Although the intensity of qBBr fluorescence was lower than that of mBBr fluorescence, aerobically grown cells again showed approximately a twofold increase in labeling of FNR compared to anaerobically grown bacteria (TAB.R1). $[4Fe4S]^{2+}$FNR and $[2Fe2S]^{2+}$FNR both require four Cys residues for coordination of the FeS cluster.

TAB.R1. *In vivo* labeling of FNR Cys residues with mBBr and qBBr in aerobically and anaerobically growing *E. coli* CAG627 (pMW32). The amount of label was determined from western blots by measuring the fluorescence of mBBr and qBBr by fluorescence imaging (integrated pixel intensity of fluorescence [PI_{fluor}] of the complete bands). The amount of FNR protein was determined from immunostaining with anti-FNR serum by absorption imaging with a Kodak ImageStation CF440 (integrated pixel intensity of immunostaining [PI_{immuno}] of the complete bands). The specific fluorescence label of FNR was calculated from the two values (PI_{fluor} / PI_{immuno}). All values are means ± standard deviations from at least four replicated and independent samples.

	Aerobic			Anaerobic		
	Fluorescence label	Anti-FNR stain	Specific FNR labeling	Fluorescence label	Anti-FNR stain	Specific FNR labeling
	PI_{fluor}	PI_{immuno}	PI_{fluor} / PI_{immuno}	PI_{fluor}	PI_{immuno}	PI_{fluor} / PI_{immuno}
mBBr	67,860 ± 7,000	113,230 ± 11,000	0.6 ± 0.06	30,530 ± 3,000	117,800 ± 12,000	0.3 ± 0.03
qBBr	2,520 ± 250	20,740 ± 2,100	0.12 ± 0.01	1,385 ± 0,140	24,890 ± 2,500	0.056 ± 0.006

4.2.3 Kinetics of $[4Fe4S]^{2+}$ FNR conversion to apoFNR upon exposure to air

Labeling of the FNR Cys residues by mBBr is a rapid process, and experiments showed, that after 1 min of labeling, no further increase in specific fluorescence of FNR is observed (Reinhart *et al.*, 2008). Due to the rapid reaction, the method can be used to determine the kinetics of the appearance of accessible Cys residues after shifting anaerobically growing bacteria to oxic conditions. At distinct time points after the shift, samples were withdrawn, and the cells were incubated with mBBr (FIG.R3). Before the shift, about 20% of the Cys residues (corresponding to about one labelled Cys residue / FNR) were accessible to mBBr. With increasing time of O_2 exposure, the amount of label increased. Maximal labeling was achieved 5 to 6 min after the oxygen shift. The degree of Cys labeling after 6 min of O_2 exposure was similar to that of FNR from aerobically grown *E. coli*.

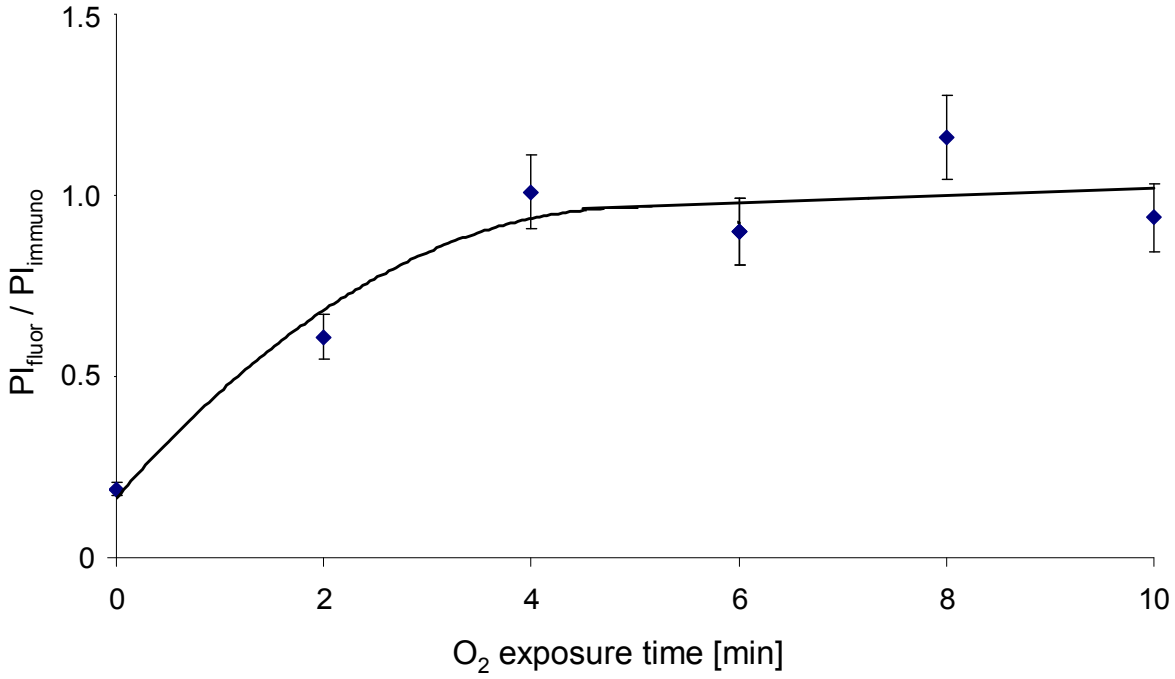


FIG.R3. Kinetics of $[4Fe\text{-}4S]^{2+}$FNR conversion to apoFNR *in vivo*, as determined by labeling of FNR with mBBr. *E. coli* CAG627 (pMW32) was grown anaerobically to an OD_{578} of 1.0 in the presence of IPTG. Aliquots were placed in petri dishes and shaken under air; at specific time points, the samples were labelled with mBBr. After the reaction was stopped by the addition of DTT, the cellular proteins were subjected to SDS-PAGE and Western blotting. FNR fluorescence (integrated pixel intensity of fluorescence [PI_{fluor}]) and immunostaining (integrated pixel intensity of immunostaining [PI_{immuno}]) were quantified using isolated FNR with five accessible Cys residues as a reference as described. Values are shown as means standard deviations from three independent experiments.

4.3 Differentiating FeS-containing FNR and apoFNR by AMS labeling

4.3.1 Accessibility of Cys residues to AMS in aerobically and anaerobically purified FNR

AMS (4-Acetamido-4´-maleimidylstilbene-2,2´-disulfonic acid) has been widely used for labeling of Cys residues in the hydrophilic space of proteins (Taylor *et al*., 2007). When aerobically purified apoFNR (*M*r of 29,000) was reduced by DTT and incubated with AMS, the apparent *M*r increased from 29,000 to 34,000 according to SDS-PAGE (FIG.R4). It can be assumed that this form of FNR is labelled at five Cys residues with AMS, indicating an approximate increase in *M*r of 1,000 per molecule of bound AMS. The increase corresponds to the mass of AMS (536.44 Da), but the negative charge of AMS might contribute to the changed mobility as well.

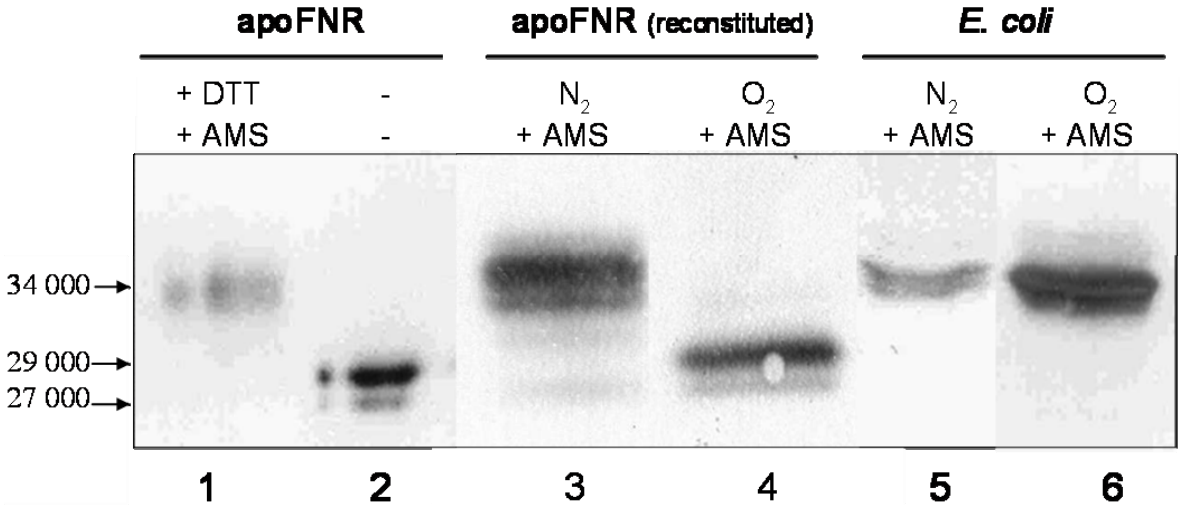


FIG.R4. AMS labeling of purified apoFNR and FNR from aerobically or anaerobically growing *E. coli* CAG627 (pMW32). Purified apoFNR was reduced with excess DTT and subsequently labelled with AMS (8 mM, lane 1). Lane 2 contains purified unlabeled apoFNR for comparison. Reconstituted $[4Fe\text{-}4S]^{2+}$FNR was labelled with AMS either directly (lane 3) or after incubation under air for 20 min (lane 4). For labeling *in vivo*, cells of *E. coli* CAG627 (pMW32) were grown under inducing conditions under anoxic (N_2) (lane 5) or oxic (O_2) (lane 6) conditions to an OD_{578} of 0.8, permeabilized with chloroform, and incubated with 10 mM AMS for 5 min. After the reaction was stopped with the SDS sample buffer, the isolated proteins (2 to 5 µg of purified FNR) and the cellular proteins (30 µg) were separated by SDS-PAGE, blotted onto nitrocellulose, and used for anti-FNR immunoblotting. The positions of M_r 27,000, 29,000, and 34,000 are indicated.

4.3.2 Accessibility of Cys residues to AMS in isolated and reconstituted $[4Fe4S]^{2+}$ FNR

Purified ApoFNR was reconstituted *in vitro* using Fe(II), cysteine, and cysteine desulfurase $NifS_{AV}$ as described previously in Achebach *et al.*, 2005. After exposure of reconstituted FNR to air (20 min) and subsequent labeling with AMS, the 29,000-*M*r form of FNR and a small amount of a 27,000-*M*r form of FNR were left. ApoFNR with a *M*r of 27,000 represents an oxidized form of FNR, which can be converted to FNR of *M*r 29,000 by reduction with DTT (Achebach *et al*., 2005). The experiments indicate that AMS displaces [FeS] clusters from FNR and labels the newly accessible Cys residues. In contrast, air-exposed apoFNR contains large amounts of Cys disulfides, which are not labelled by AMS (Achebach *et al*., 2005). Treatment of the air-oxidized apoFNR with DTT results in reduced Cys residues and AMS-labelled apoFNR of 34 kDa.

4.3.3 *In vivo* Cys accessibility to AMS

Labeling of FNR with the impermeable reagent AMS was performed also *in vivo* after permeabilizing the cells with chloroform. The proteins were analyzed after SDS-PAGE by immunoblotting with anti-FNR serum as described. The immunoblot revealed a form of FNR with a *M*r of 34,000 in both anaerobically and aerobically growing cells (FIG.R4, lanes 5 and 6). FNR proteins with other *M*r values were not found. The 34,000-*M*r form of FNR can be explained by assuming labeling of all Cys residues of FNR.

4.3.4 *In vivo* detection of Cys disulfides in FNR

The labeling pattern of apoFNR from aerobically grown *E. coli* suggested that all Cys residues are accessible to labeling by AMS (FIG.R4, lane 6), indicating that no intra-molecular disulfides are formed. For a more quantitative analysis of the redox state of the cysteine residues, apoFNR from aerobically growing *E. coli* was labelled *in vivo* in non-permeabilized bacteria with NEM. Isolated FNR was then subjected to MALDI-TOF analysis (FIG.R5). The major signal with a mass of 28,732 Da corresponds to FNR (28,111 Da) alkylated at five Cys residues by NEM, each label causing an increment of 124 Da. No significant masses corresponding to smaller FNR species

were detectable. In particular, there were no signals equivalent to FNR labelled at only one or three Cys residues which would be characteristic for the presence of two or one intra-molecular disulfide bonds. Instead, minor bands of higher masses were detected; these bands may represent other proteins or products of alternative thrombin cleavage during FNR preparation.

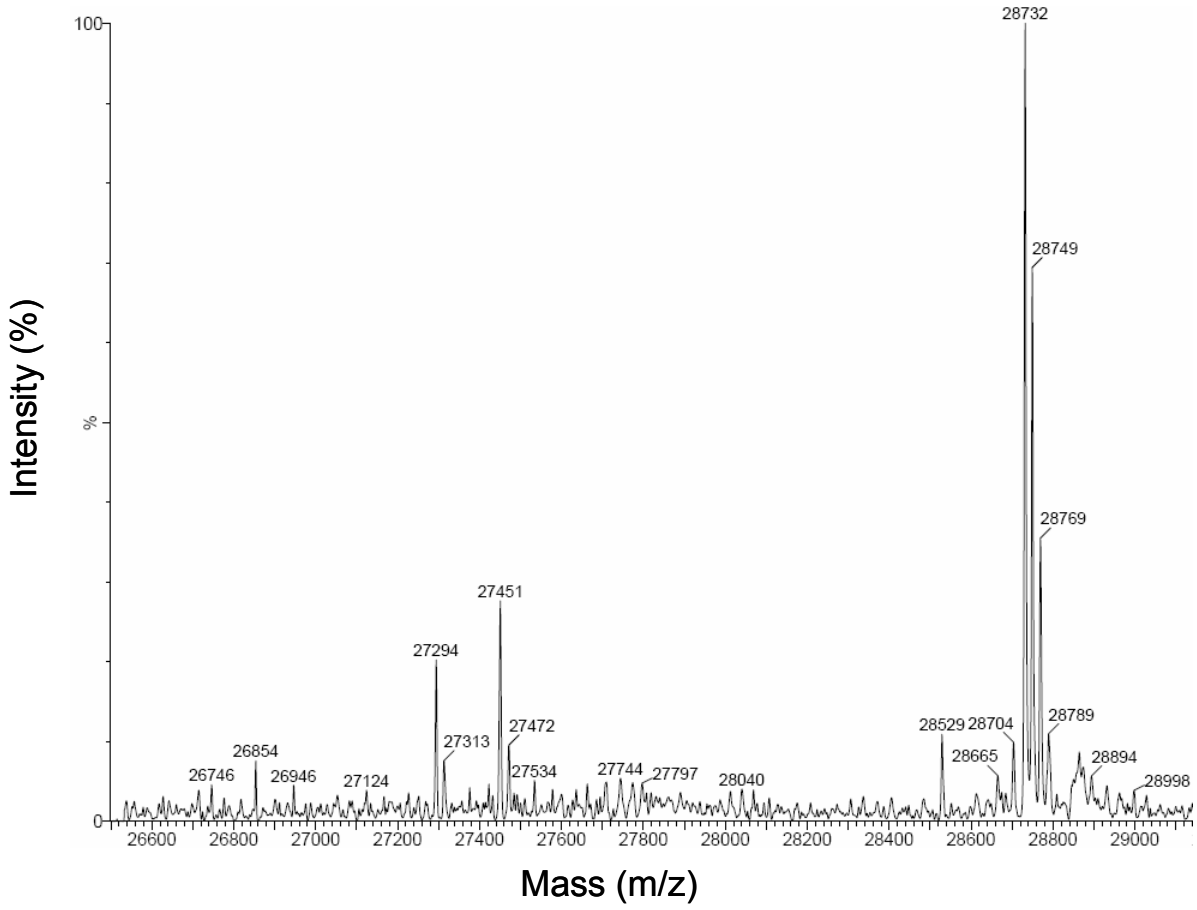


FIG.R5. MS of FNR from aerobically grown bacteria after *in vivo* alkylation by NEM. *E. coli* CAG627 (pMW68) with induced GST-FNR production (OD_{578} of 1.0) was grown under inducing conditions and labelled by adding NEM (10 mM) to the medium for 15 min without permeabilization of the bacteria. FNR was isolated and separated from GST by thrombin. FNR (600 µg/ml) was subjected to reversed-phase HPLC on a C18 column connected online to the MS instrument. Unmodified FNR has a mass of 28,111 Da (cleavage product of FNR-GST); modified forms with one, two, three, four, and five NEM residues have predicted masses of 28,235, 28,359, 28,483, 28,607, and 28,781 Da, respectively.

A similar result was obtained when FNR was labelled in aerobically growing bacteria by iodoacetate. MALDI-TOF analysis revealed a major signal at a mass of 28,398 Da corresponding to apoFNR alkylated at five Cys residues by acetate (increase in molecular mass by 58 Da / acetate) which confirms the presence of apoFNR with five reduced thiol residues. The labeling pattern obtained with NEM and iodoacetate therefore verifies that no significant amount of disulfide is present in apoFNR in aerobically growing *E*. *coli*.

4.4 Labeling Cys residues of NreB from *Staphylococcus carnosus*

4.4.1 Labeling Cys residues of NreB with mBBr

NreB contains four cysteine residues, which are all required for binding of the $[4Fe4S]^{2+}$ or $[2Fe2S]^{2+}$ cluster (Müllner *et al*., 2008). In this study the labeling of accessible Cys residues was used as an approach to differentiate FeS-containing and FeS-free forms of NreB *in vitro* and *in vivo*. For Cys labeling, the fluorescent reagent mBBr, which reacts specifically with Cys thiols, was used. mBBr and IAA are sufficiently membrane permeant for labeling without cell disruption (Mate *et al*., 1994; Sbodio *et al*., 2007). This opens up the opportunity to define the actual state of NreB *in vivo* in aerobically and anaerobically growing cells of *S. carnosus*.

mBBr becomes fluorescent after reaction with thiols. Labeling of the Cys residues of NreB was studied without prior permeabilization of the cytoplasmic membrane to avoid perturbation of the bacteria as previously described for FNR (Reinhart *et al*., 2008). In contrast to *E. coli S. carnosus* is a gram positive bacterium, and the labeling was performed after partial digestion of the cell wall by lysostaphin to allow accession of the reagent to the membrane. After labeling and stopping the reaction by addition of DTT, the bacteria were broken and NreB was sedimented by NreB specific antiserum and protein A Sepharose as described by Qiagen. The proteins of the sediment were subjected to SDS-PAGE, and after blotting of the proteins to a PVDF membrane, the fluorescence of protein bands was visualized (FIG.R6A).

The fluorescent bands at the expected position for NreB showed high intensities after induction of NreB production and were lacking in non-induced cells, suggesting that the bands were derived from NreB (FIG.R6A lanes 1-4). The NreB content of wildtype *S. carnosus* cells was to low for detection, similar to the FNR content in wildtype *E. coli* cells (Reinhart, 2006). Moderate levels of NreB over-expression were sufficient for direct quantisation of NreB from cell extracts by immunoblotting. The detected fluorescence of NreB was much higher for aerobically than for anaerobically grown bacteria (FIG.R6A). In the immunoblot, the protein bands corresponding to the fluorescent bands reacted with anti-NreB serum, and the reaction increased after induction of NreB expression (FIG. R6B). The amount of NreB was comparable for aerobically and anaerobically grown bacteria, suggesting that the specific fluorescence of NreB was increased in aerobic bacteria.

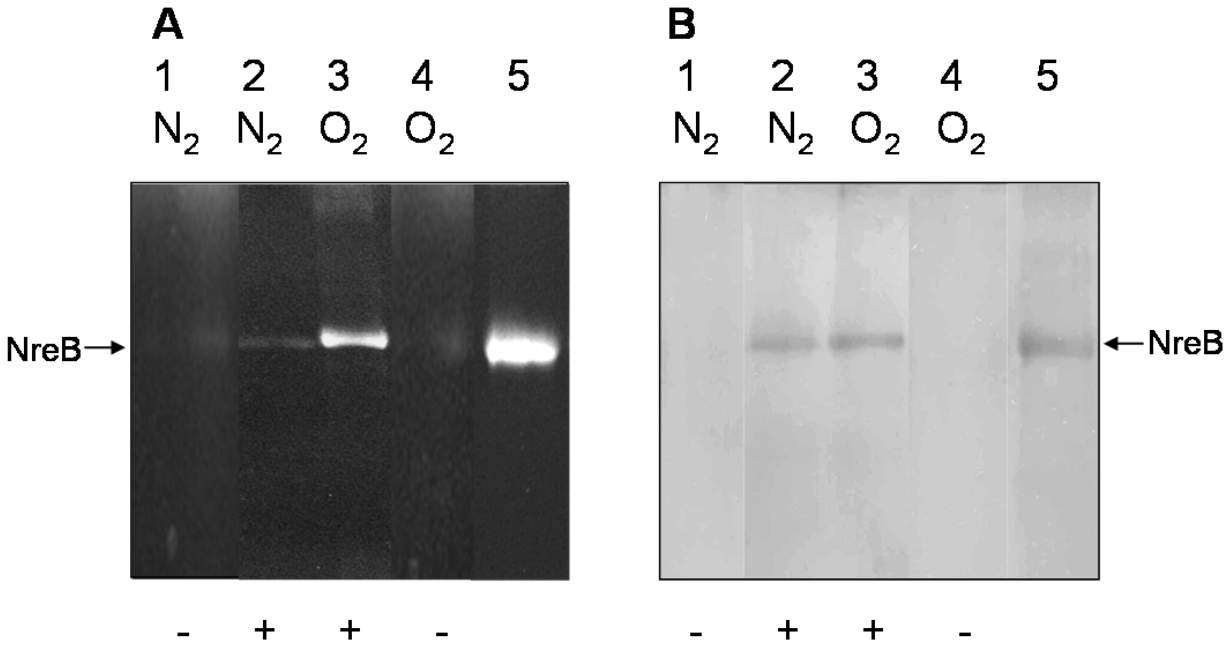


FIG.R6. Direct labeling of native NreB in aerobically and anaerobically growing *S. carnosus* m1 (pCQE1*nreB*). Growing bacteria at an OD_{578} of 1.0 were incubated with lysostaphin and mBBr (1 mM) for 10 min. After the addition of 2 mM DTT and immunoprecipitation of NreB with anti-NreB serum, bacterial proteins (10 µg per lane) were dissolved in SDS sample buffer and subjected to SDS-PAGE. (A) After blotting of proteins to a PVDF membrane, the fluorescence was recorded. (B) NreB was then detected and evaluated by immunostaining with anti-NreB serum. The bacteria were grown under aerobic (O_2; lanes 3 and 4) or anaerobic (N_2; lanes 1 and 2) conditions and under *nreB*-inducing (+) or non-inducing (-) conditions. Lane 5 contains isolated NreB after labeling by mBBr. The position of NreB is indicated on the left and the right.

4.4.2 Quantifying accessible Cys residues of NreB *in vitro*.

To determine the correlation between the number of labelled Cys residues and the fluorescence of mBBr, labeling was performed with aerobically purified NreB protein, similar to the procedure described earlier for FNR (Reinhart *et al*., 2008). To achieve quantitative labeling by mBBr, isolated NreB was denatured by the addition of guanidinium hydrochloride. The sample was then incubated with increasing amounts of mBBr (0, 1, 2, 3, 4, 5, and 10 mol of mBBr per mol of NreB) for 10 min. After SDS-PAGE, the fluorescence and label associated with NreB were determined (FIG.R7). The specific signal for NreB fluorescence increased with increasing amounts of mBBr per NreB in a nearly linear relationship up to levels of 4 mol mBBr per mol NreB. Incubation with larger amounts of mBBr did not result in significantly higher labeling (FIG.R7).

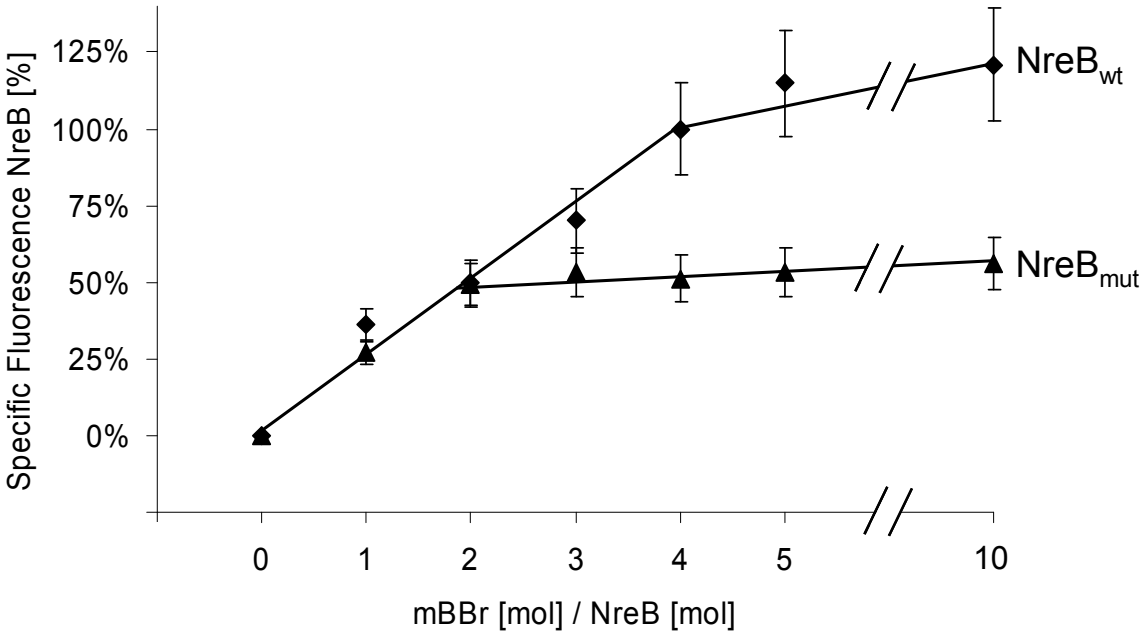


FIG.R7. Labeling of Cys residues of denatured wild type NreB and NreB (C59S C62S) by mBBr. Aerobically prepared wildtype NreB ($NreB_{wt}$, squares) was incubated with guanidinium hydrochloride and then incubated for 10 min with a 0-, 1-, 2-, 3-, 4-, 5-, or 10-fold molar excess of mBBr. Three micrograms of labelled sample was subjected to SDS-PAGE, blotted to a PVDF membrane, and quantified for the fluorescence associated with the protein bands. Afterwards, the amount of NreB was quantified by immunostaining. Each point was derived from three independent labeling experiments. Mutant NreB (C59S C62S) ($NreB_{mut}$, triangles), which contains only 2 Cys residues, was prepared and labelled in the same manner as wild-type NreB. The specific fluorescence of wildtype NreB labelled with fourfold molar excess of mBBr was set 100%.

When the same experiment was performed with mutant NreB (C59S C62S) that retained only two Cys residues, the level of labeling increased only to levels corresponding to about 50 % of the level of wild-type NreB. The labeling remained at this level even in the presence of a large excess of mBBr. The responses of the wild type and the Cys mutant demonstrate that the amount of mBBr incorporated is related to the number of accessible Cys residues. By using fully labelled wildtype NreB and mutant NreB (C62S C59A) as references, labeling of other NreB samples can be evaluated quantitatively and used to determine the number of accessible Cys residues.

4.4.3 Two-step *in vivo* labeling of Cys residues of NreB

For quantitative evaluation of the number of accessible Cys residues, a two-step labeling procedure was established which includes a denaturing step. By this procedure, the lysostaphin-treated bacteria were first incubated with iodoacetate (IAA) to label accessible Cys residues. Iodoacetate is able to diffuse across the cytoplasmic membrane into the cytoplasm at a sufficient rate for the labeling procedure (Quick *et al*., 1999). After stopping the labeling reaction by the addition of DTT, the bacteria and proteins were denatured by the addition of 2 M guanidinium hydrochloride. Subsequently, all Cys residues that had not been labelled in the first reaction with IAA were labelled with mBBr (step two). After separation of the proteins by SDS-PAGE, proteins corresponding to the mass of NreB showed a fluorescent band (FIG.R8A). The band corresponding to NreB was absent in non-induced cells. In induced cells the same band from anaerobically grown bacteria showed a much higher intensity in fluorescence than that from aerobically grown bacteria. The amounts of NreB, however, were comparable for aerobically and anaerobically grown bacteria (FIG.R8B), suggesting that the specific fluorescence of NreB was increased in the anaerobic bacteria.

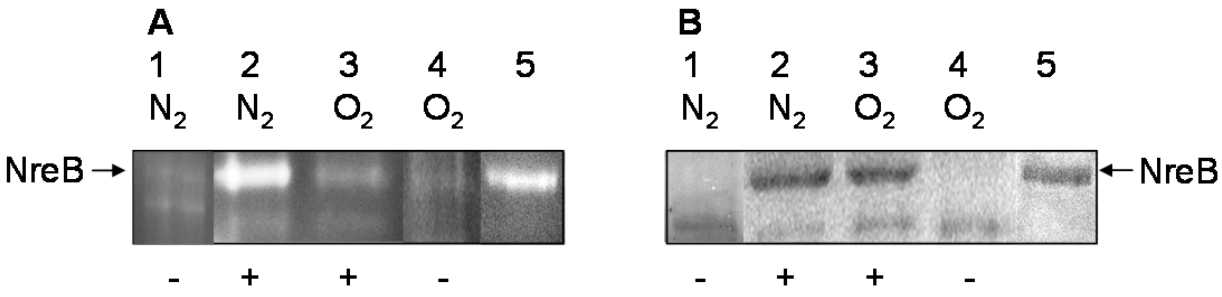


FIG.R8. Two-step labeling of Cys residues of NreB in aerobically and anaerobically growing *S. carnosus* m1 (pCQE1*nreB*). Growing bacteria of OD_{578} 1.0 were incubated with lysostaphin and IAA (1 mM) for 10 min (labeling of native protein). After the addition of DTT (1 mM), 2 M guanidinium hydrochloride and 2 mM mBBr were added (labeling of residual Cys residues in denatured protein). NreB was sedimented with anti-NreB serum, and bacterial proteins (10 µg per lane) were dissolved in SDS sample buffer and subjected to SDS-PAGE. (A) After blotting of proteins to a PVDF membrane, the fluorescence was recorded. (B) NreB was then detected and evaluated by immunostaining with anti-NreB serum. The bacteria were grown under aerobic (O_2; lanes 3 and 4) or anaerobic (N_2; lanes 1 and 2) conditions and under *nreB*-inducing (+) or non-inducing (-) conditions. The position of NreB is indicated on the left and the right.

4.4.4 Two forms of NreB can be differentiated *in vitro* and *in vivo*

For quantitative analysis generally the two-step labeling protocol was used, because it showed higher reproducibility. The accessibility of Cys residues was compared for NreB under aerobic and anaerobic conditions *in vivo* and *in vitro* (FIG.R9). The Cys residues accessible in the native and denatured proteins were labelled differentially with IAA and mBBr. The mBBr fluorescence therefore represents the Cys residues that became accessible only after denaturing of the protein, i.e., the Fe-S-protected residues. The difference in the mBBr label compared to the maximal label, on the other hand, represents the Cys residues that were labelled already by IAA in the native protein.

In aerobically grown bacteria, only a small portion (9%) of the Cys residues were labelled by mBBr, since most of the residues were labelled by IAA in labeling step 1 (FIG.R9A, lane 1, left). In the anaerobic bacteria, most of the Cys residues (89%) were accessible to labeling by mBBr after denaturing of the protein (FIG.R9A, lane 1, right). When in a control experiment the proteins of the bacteria were denatured by guanidinium hydrochloride before the first labeling step, in the second reaction only a small portion was labelled by mBBr for both types of bacteria (FIG.R9A, lane 2). Therefore, the differences in labeling can be explained by different accessibilities,

whereas other factors apparently play no role. Overall, the Cys residues in NreB proteins from aerobic and anaerobic bacteria are largely different in their accessibility to labeling reagents. The difference is observed only when the proteins are in the native state during the labeling in step 1, demonstrating that the differences represent the state of the native protein.

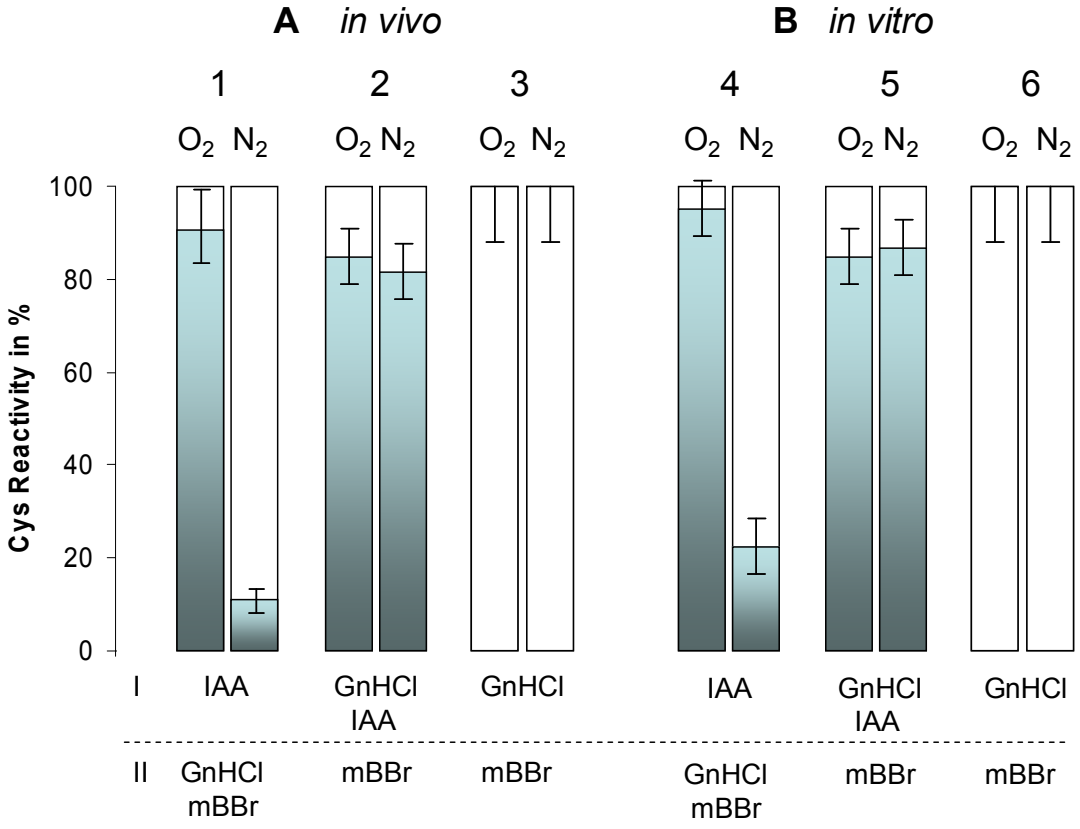


FIG.R9. Labeling of Cys residues in NreB by two-step labeling with IAA (native labeling, blue bars) and mBBr (labeling of denatured protein, white bars). Labeling of NreB in growing bacteria (A) and of isolated NreB (B) is shown. (A) Anaerobically and aerobically growing (OD_{578} = 1.0) *S. carnosus* m1 (pCQE1*nreB*) cells were incubated with iodoacetate (1 mM, 10 min) after finishing *nreB* induction (at an OD_{578} of 0.5; induction for 2 h with 150 mM xylose). After the addition of DTT (1 mM), 2 M guanidinium hydrochloride and 2 mM mBBr were added (experiment 1). In experiment 2, labeling by iodoacetate was performed as in experiment 1, but the guanidinium hydrochloride was added before labeling by IAA. In experiment 3, the bacteria were incubated with 2 M guanidinium hydrochloride (without iodoacetate labeling) and then labelled by 1 mM mBBr. Protein (50 µg per lane) was dissolved in SDS sample buffer and subjected to SDS-PAGE. After blotting of proteins to a PVDF membrane, the mBBr fluorescence and the amount of NreB were determined as described. Each bar gives the labeling by mBBr (white part of the bar) and by IAA (blue part of the bar). Labeling by IAA was calculated as the difference in the mBBr label compared to the maximal label (mBBr label in experiment 3). (B) The same labeling procedure as for panel A was performed with aerobically (O_2) and anaerobically (N_2) prepared NreB.

When the two-step labeling experiment was performed *in vitro* (FIG.R9B) with isolated NreB, the labeling pattern was similar to that in bacteria (FIG.R9B, lanes 4-6). The labeling of aerobic NreB was very high in the first step, i.e., labeling of the native protein by IAA (FIG.R9B, lane 4, left), and low in the second step (labeling by mBBr after denaturing the protein). TAB.R2 summarizes the quantified fluorescence values after the two-step labeling procedure *in vivo* and *in vitro*.

TAB.R2. Two-step labeling of NreB with iodoacetate (IAA) and monobromobimane (mBBr). Labeling was performed in bacteria grown under aerobic or anaerobic conditions (A, B) and with isolated and denatured NreB protein (C, D). Labelling with iodoacetate was performed in native bacteria, and the subsequent labeling with mBBr after denaturing the cells (A, B). Isolated NreB was denatured with 2 M guanidinium hydrochloride and labelled with iodoacetate (step 1) followed by labeling with mBBr (step 2, C). Isolated NreB was denatured with guanidinium hydrochloride and then labelled only with mBBr (D). $PI_{fluor/immuno}$ was detected with Gel Pro imaging software (version 6.0) by Intas.

Growth conditions	Fluorescence label PI_{fluor}	Anti-NreB stain PI_{immuno}	Specific labeling NreB PI_{fluor} / PI_{immuno}	Cys availability to IAA	Cys availability to mBBr
(A) Aerobic *in vivo*	3,488 ± 350	24.2 ± 2.4	144 ± 14	91 ± 9 %	9 ± 1 %
(B) anaerobic *in vivo*	38,510 ± 3,850	26.7 ± 2.7	1,442 ± 140	12 ± 1 %	88 ± 9 %
(C) denat. NreB; IAA & mBBr	60.3 ± 6	7.90 ± 0.8	7.62 ± 0.8	100 %	0 %
(D) denat. NreB; only mBBr	42,122 ± 4,210	25.6 ± 2.6	1,646 ± 165	n.a.	100 %

4.4.5 Numbering of accessible Cys residues by mass spectrometry

Aerobically or anaerobically prepared NreB was incubated with an excess of iodoacetate (IAA) in order to label accessible Cys residues. Prior to labelling, the sample was incubated with small amounts of DTT in order to keep the available Cys residues in the reduced state. After labeling and subsequent inactivation of unreacted iodoacetate by an excess of DTT, the samples were digested with protease Glu-C, which cleaves specifically at the C-terminus of Glu residues. The peptide mixture was then analyzed by mass spectrometry, with particular attention to the presence of the Cys-containing peptides (FIG.R10). Protease Glu-C generates principally three Cys-containing peptides from NreB (PepC1, PepC2 and PepC3) (TAB.R3). One of the

peptides (PepC3) contains two Cys residues. The digest was screened for peptides with masses corresponding to PepC1, PepC2, and PepC3 in the unmodified form and to peptides with masses corresponding to PepC1, PepC2 and PepC3 after alkylation of the Cys residue(s) with iodoacetate. Alkylation results in an increase of mass of 58 Da per Cys residue.

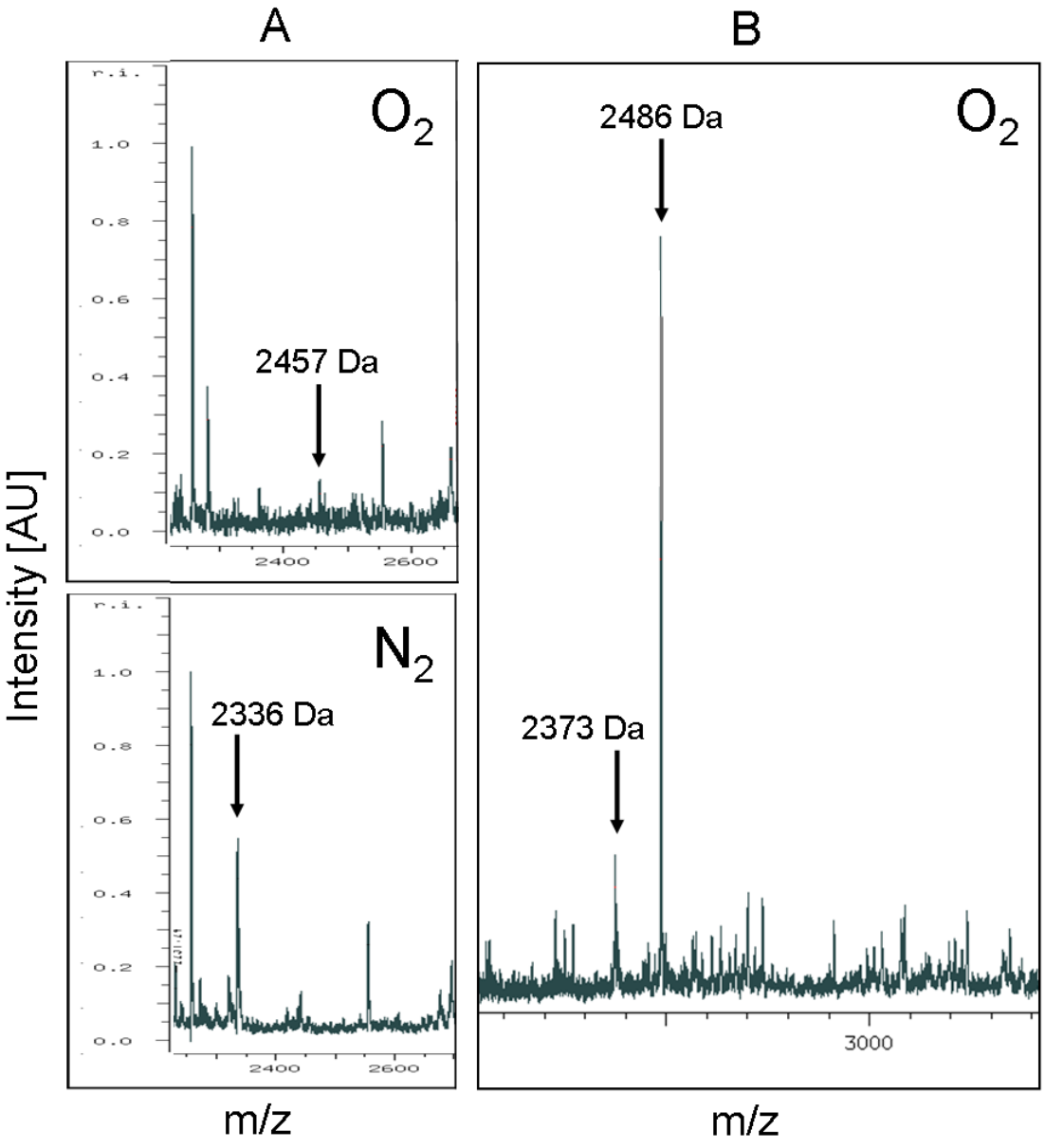


FIG.R10. Mass spectrometry of Glu-C (A)- and Asp-N (B)-derived peptides of NreB from aerobically (O_2) or anaerobically (N_2) grown bacteria after direct labeling by iodoacetate. NreB from aerobically and anaerobically grown bacteria corresponds to apoNreB and $[4Fe4S]^{2+}$NreB, respectively. 100 µg of purified native NreB protein was incubated with 5 mM iodoacetate for 30 min at 4°C and digested with (A) Glu-C (1 µg protease / 100 µg NreB) or (B) Asp-N (1 µg protease / 40 g NreB) for 6 h at 25°C or 37°C, respectively. The fragments were analyzed by MALDI-TOF mass spectrometry. Peptides containing Cys residues are labelled by arrows, and the corresponding masses are indicated.

In the Glu-C digest of aerobic NreB, the Cys-containing peptides were found only as the alkylated forms (PepC1-Ac, PepC2-Ac, and PepC3-Ac_2), whereas the unmodified forms were absent. This is shown in FIG.R10A for PepC3, where the fully alkylated form, PepC3-Ac_2 with two acetyl residues (2,457 Da) was present, whereas unmodified PepC3 and PepC3-Ac_1, with one alkylated Cys residue, were absent. This finding suggests that all of the Cys residues in aerobic NreB are accessible to alkylation.

TAB.R3. Cys-containing peptides of NreB after Glu-C digestion and alkylation with IAA. Aerobically or anaerobically isolated NreB was alkylated with 5 mM iodoacetate (IAA) under aerobic or anaerobic conditions, respectively. After stopping the reaction with DTT, the protein was digested with Glu-C protease as described and analyzed by mass spectrometry. Peptides corresponding to the expected Cys-containing peptides that were present in the mass spectra are indicated. The expected mass shift is + 58 *m/z* for each labelled Cys moiety. The peptides are labelled with the position of the corresponding Glu residue in NreB (E_x). Peptides which were not detected by MALDI-TOF are indicated (N.d.)

Cys containing peptides	Sequence of peptides	Calculated M_r of peptides	Detected M_r of peptides	
			Aerobic	Anaerobic
PepC1	$NCFLE_{80}$	624.7	N.d.	Not detected, but expected to be present
PepC1-Ac	$NC_{Ac}FLE_{80}$	682.7	677	N.d.
PepC2	$YDIMSCE_{75}$	860.0	N.d.	Not detected, but expected to be present
PepC2-Ac	$YDIMSC_{Ac}E_{75}$	918.0	917	N.d.
PepC3	$VFADDNDYSQMTNAVCRRCE_{63}$	2,337.6	N.d.	2,336
PepC3-Ac_{1A}	$VFADDNDYSQMTNAVC_{Ac}RRCE_{63}$	2,395.6	N.d.	N.d.
PepC3-Ac_{1B}	$VFADDNDYSQMTNAVCRRC_{Ac}E_{63}$	2,395.6	N.d.	N.d.
PepC3-Ac_2	$VFADDNDYSQMTNAVC_{Ac}RRC_{Ac}E_{63}$	2,453.6	2,457	N.d.

From anaerobic NreB, on the other hand, PepC3 was present as the unmodified form only (2,336 Da), and none of the alkylated forms of PepC3 was detected. PepC1 and PepC2, which were also expected in the non-modified form, were not detected, suggesting that it is difficult to identify the non-alkylated forms of the latter peptides.

The absence of the alkylated forms of PepC1 and of PepC2 (which were detected in the aerobic forms of NreB) suggests that the corresponding forms are not present in anaerobic NreB. In a preparation of aerobic NreB that was alkylated with iodoacetate and then digested by Asp-N, the peptide PepN1 (2,389 Da) was identified, which might represent the peptide containing Cys59 and Cys62 (FIG.R10B). The Asp-N digest of aerobic NreB contained high levels of PepC3, with two acyl residues (2,505 Da), whereas the unmodified (or protected) form and the form with one acyl residue (2,389 Da and 2,447 Da, respectively) were present at much lower levels. Therefore, the combined data from labeling with iodoacetate and digestion with Glu-C and Asp-N show that the Cys residues of aerobically incubated NreB are accessible to alkylation, whereas those from anaerobically incubated FeS-containing NreB are protected from alkylation by iodoacetate.

4.4.6 Kinetics of $[4Fe4S]^{2+}$ NreB conversion to apoNreB caused by O_2

Earlier, the kinetics for the conversion of $[4Fe4S]^{2+}$NreB to $[2Fe2S]^{2+}$NreB were studied *in vitro* by Mössbauer spectroscopy (Müllner *et al*., 2008). A half-time of about 2.5 min was determined for the conversion. In this study, the kinetics of $[4Fe4S]^{2+}$NreB conversion to apoNreB were studied *in vitro* by measuring the change in Cys accessibility to iodoacetate and mBBr (FIG.R11). Anaerobic $[4Fe4S]^{2+}$NreB was exposed to air, and after various times of exposure, the accessibility of the Cys residues was determined by the two-step labeling procedure.

Labeling of anaerobic NreB by mBBr was high before starting air exposure (about 80 to 90 % of the total Cys residues), which is characteristic for the presence of NreB $[4Fe4S]^{2+}$. The number of mBBr-accessible residues decreased exponentially with increasing time of air exposure. After 15 min of incubation with oxygen, only 11 % of the Cys residues were accessible to mBBr (FIG.R11), demonstrating that most of the NreB was converted to apoNreB. The labeling by iodoacetate was calculated as the difference in mBBr-accessible to total accessible Cys residues. The half-time for the change in accessibility was about 6 min, and after 15 min, approximately 90% of the total Cys residues became accessible.

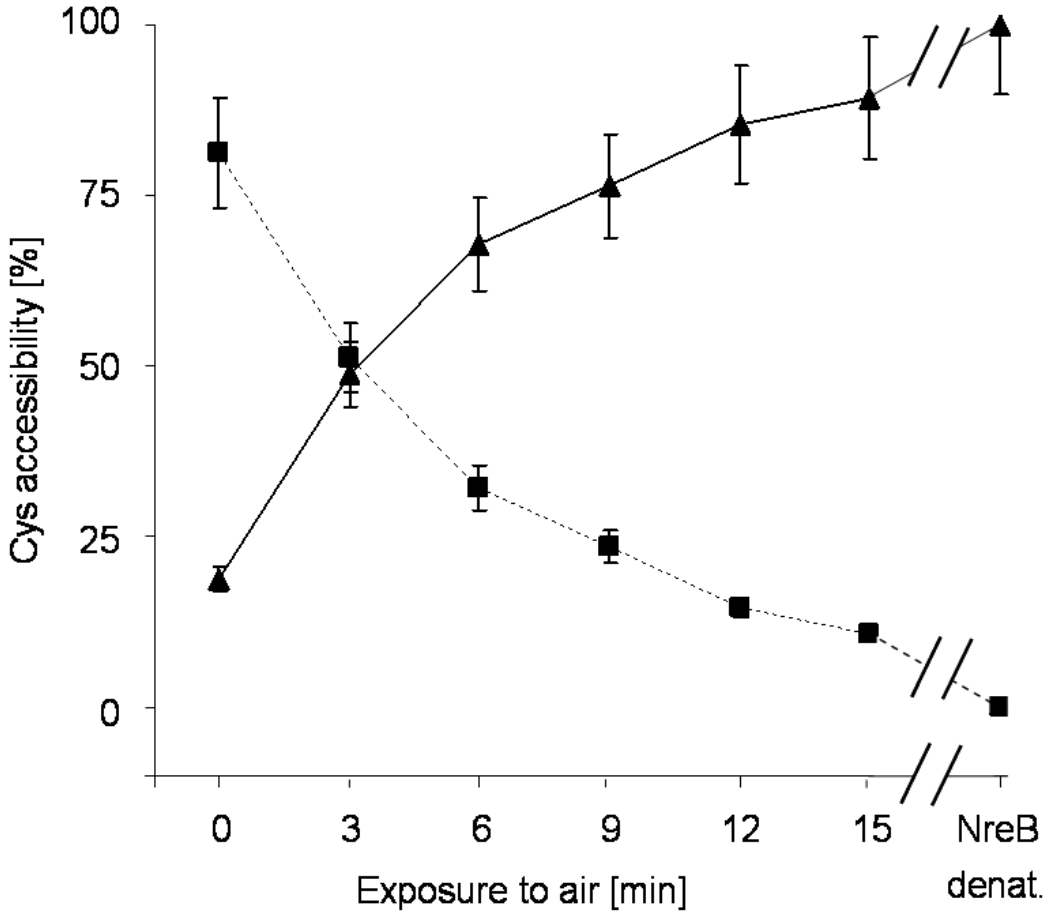


FIG.R11. Kinetics of conversion of purified anaerobic $[4Fe4S]^{2+}$NreB to apoNreB by exposure to air oxygen. Isolated anaerobic $[4Fe4S]^{2+}$NreB (1 mg / ml) was exposed to air and mixed at the indicated time points with iodoacetate. After stopping the iodoacetate reaction with DTT, the sample was denatured by 2 M guanidinium hydrochloride and incubated with excess mBBr. After SDS-PAGE and immunoblotting, the specific label of mBBr fluorescence (■) was determined. The Cys accessibility of NreB protein which was labelled only with mBBr was set to 100%. The amount of Cys labeling by iodoacetate (▲) was calculated as the difference between the 100% label and the mBBr label for the given sample.

4.5 Significance of NreA for the NreBC two-component system

4.5.1 *In silico* analysis of NreA

The function of NreA, encoded by the first gene of the three-cistron operon *nreABC*, is as yet unknown. Preceding experiments showed that NreA deletion mutants of *S. carnosus* constantly consume nitrate and perform nitrate respiration, even under aerobic conditions (personal communication S. Schlag, Tübingen). NreA is composed almost exclusively of a GAF domain (Schlag *et al*., 2008). The GAF domains represent one of the largest families of regulatory domains, which can serve a diverse range of functions, e.g. binding of small molecules including cyclic nucleotides, dimerisation and DNA-binding (Martinez, *et al.,* 2002). Despite of a high number of proteins containing GAF domains, there are only few proteins known consisting exclusively of GAF domains without any additional domains (Aravind *et al*., 1997). NreA is well conserved and found in all NreABC clusters of firmicutes, including *Bacillus* sp., *Staphylococcus* sp. and *Lactobacillus* sp.

The GAF domain is a widespread module in proteins of diverse function, including cell signalling proteins and transcription factors (Levdikov *et al*., 2009). Its structure, typically spans 150 residues and has three tiers: a basal layer of two or more α-helices, a middle layer of β-sheets and a top layer formed by segments of the polypeptide that connect strands of the β-sheets (for a detailed review see Martinez *et al.*, 2002). *In silico* sequence analysis of NreA identified a GAF domain harbouring a putative helix-turn-helix motif (FIG.R12). The ExPASy and PSIPRED algorithms delivered a concurrent structure prediction with relatively high confidence. The transcription factor CodY from *B. subtilis* features a GAF domain for dimerisation and cofactor binding and an additional helix-turn-helix domain for DNA binding, including a conserved serine residue which is important for transcription regulation (Sonenshine *et al*., 2009). The N- and C-terminal helices of CodY GAF domain are involved in dimerisation. The serine residue identified in CodY is also present in the helix-turn-helix motif of NreA (S99, FIG.R12A). The α-helices and S99 are well conserved in NreA, including the helices flanking the N- and C-terminus of NreA.

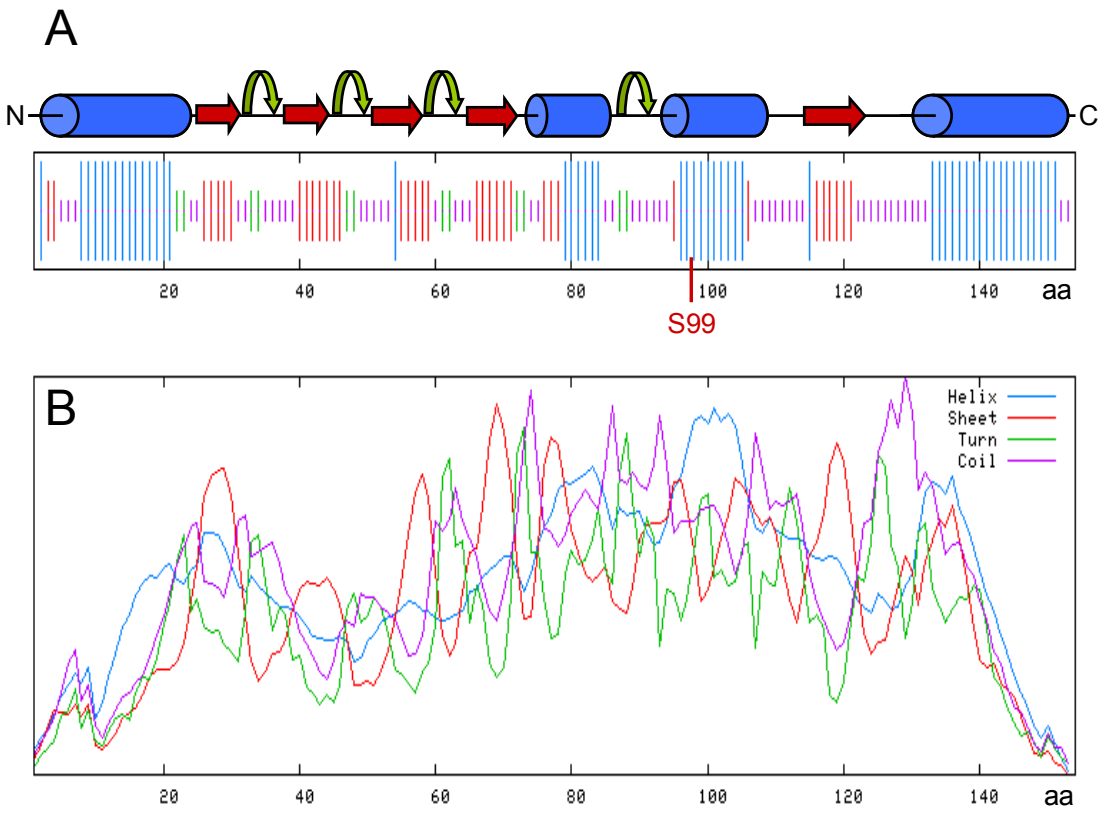


FIG.R12. *In silico* structure predictions for NreA (154 aa) from *S. carnosus* (A): The ExPASy proteomics server predicts two α-helices (blue cylinders) at the C- and N-terminus, respectively. Additionally two central α-helices, separated by a turn (green arrows) are predicted. Between the helices 5-6 β-sheets (red arrows) are predicted. The confidence of prediction is indicated by the height of the graph in (B). The putative helix-turn-helix motif spanning residues 79 to 106 and containing serine residue 99 (indicated as S99) is predicted with a high level of confidence. In contrast, the structure of the N- and C-terminus of NreA is predicted with relatively low confidence. These findings were confirmed by the PSIPRED protein prediction server.

4.5.2 Electro Mobility Shift Assays with NreA (EMSAs)

To investigate putative DNA-binding abilities of the identified helix-turn-helix motif, NreA was purified aerobically or anaerobically from *E. coli* M15 carrying plasmid pQE31*nreA* (Schlag *et al*., 2007) using a Ni-NTA column (Qiagen). Samples of the elution fractions were run on a SDS-PAGE and stained with Coomassie blue to check for impurities. NreA could be purified aerobically up to 3.2 mg protein per ml and anaerobically up to 0.8 mg protein per ml NreA without detectable impurities (FIG.R13). NreA was then incubated with putative promoter DNA fragments of *narG*, *narT* and *nirB* (FIG.R14; A - C), which have been amplified by PCR from genomic wildtype DNA of *S. carnosus* TM300. Samples were run on a native 6 % acryl amide

gel and DNA was stained with SYBR Green I. An additional DNA band appeared when low-salt, anoxic conditions were applied (FIG.R14; A - C; lanes 3 and 6). This was the case for all three DNA fragments (P_{narT}, P_{narG} and P_{nirB}). All other tested conditions showed no mobility shift.

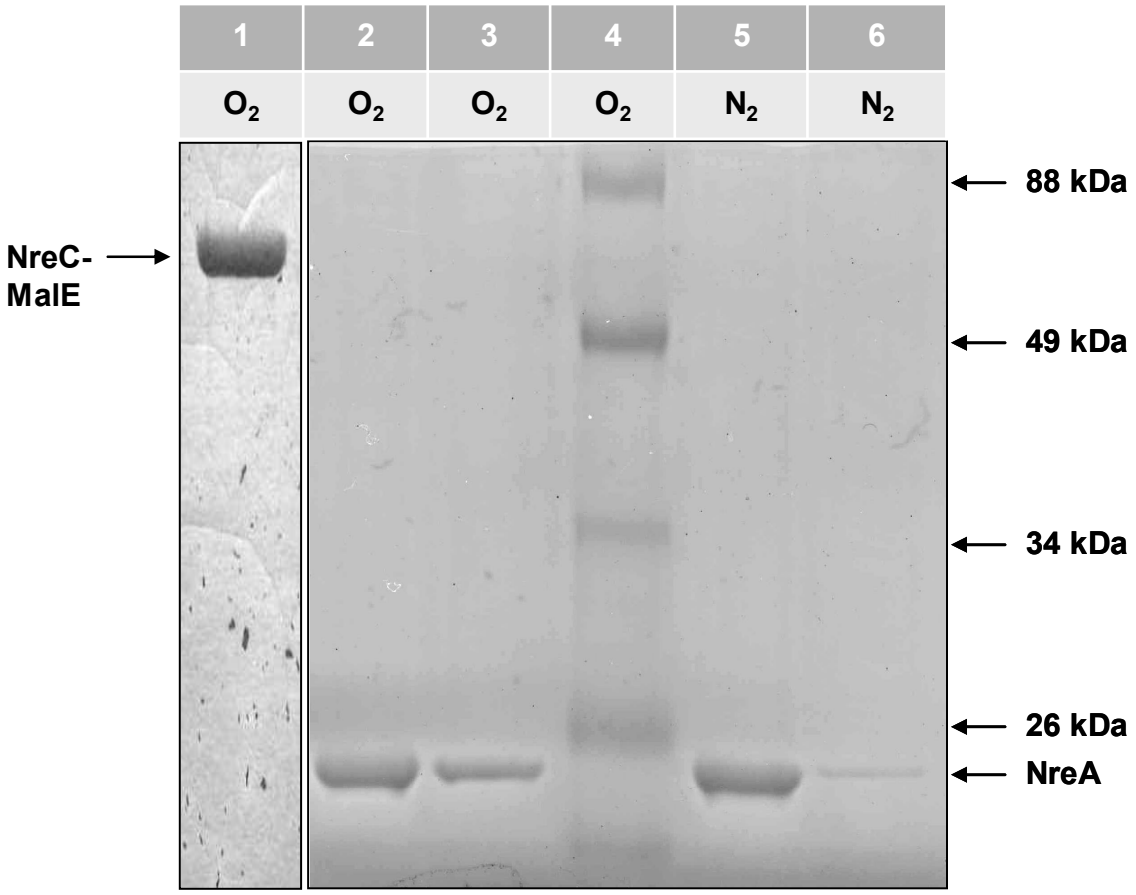


FIG.R13. Aerobic purification of NreC-MalE from *E. coli* EC3 (pMAL*nreC*) (lane 1) and aerobic and anaerobic purification of 6xHis-NreA (18 kDa, SwissProt) from *E. coli* M15 (pQE31*nreA*) (lanes 2-6). Elution fractions 1 and 2 from aerobic preparation (lane 2 and 3) and fractions 1 and 2 from anaerobic preparation (lane 5 and 6) were subjected to SDS-PAGE (2.5 µl each) followed by Coomassie blue protein staining. A premixed protein marker (lane 3) was used to estimate the size of the isolated protein and the positions of marker proteins are indicated on the right.

4.5.3 Formaldehyde crosslinking of NreA to NreB

To investigate a putative protein interaction between NreA and NreB, the two proteins were isolated aerobically or anaerobically from *E. coli* M15 (pQE31*nreA*) and *S. carnosus* m1 (pCQE1*nreB*), respectively. Protein samples were incubated with 0.5% formaldehyde (FA) and subjected to SDS-PAGE and Coomassie staining. In

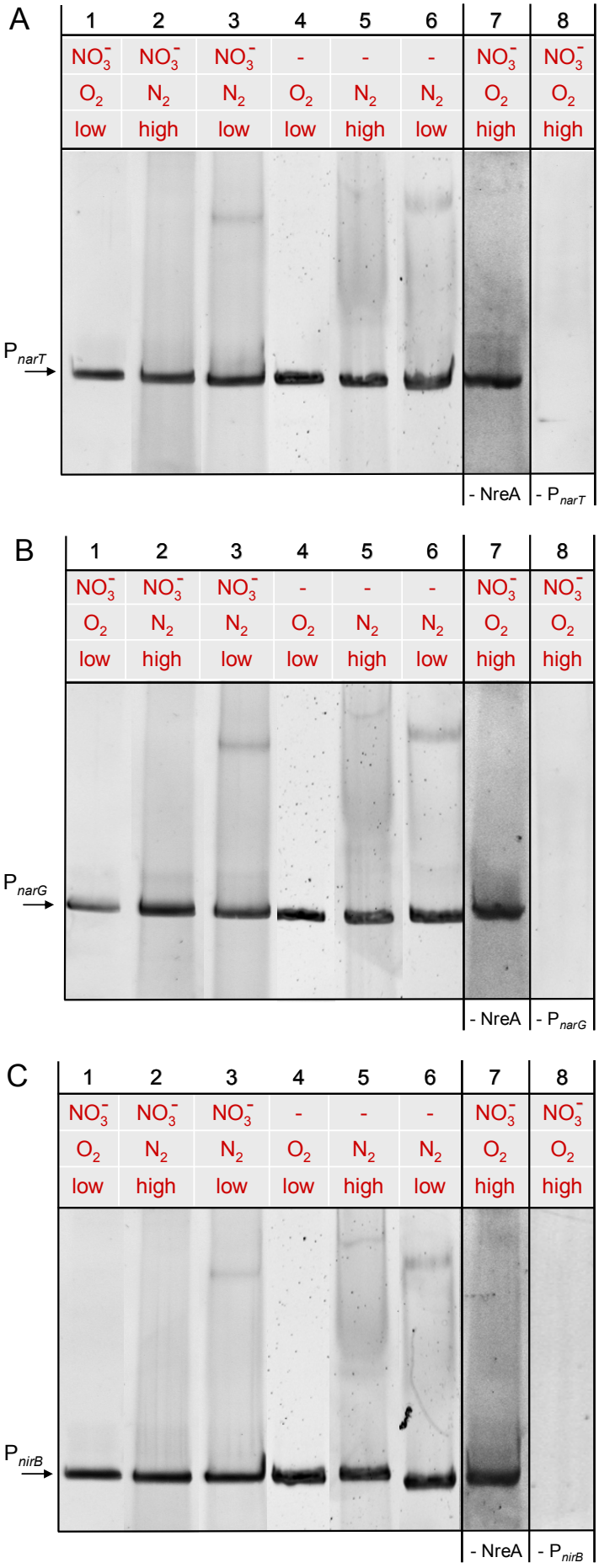


FIG.R14. Binding of isolated NreA protein to the putative promoter regions of *narT* (**A,** 628 bp), *narG* (**B,** 622 bp) and *nirB* (**C,** 483 bp). The upstream regions of *narT* (P_{narT}), *narG* (P_{narG}), and *nirB* (P_{nirB}) were amplified by PCR from genomic wildtype DNA of *S. carnosus* TM300, and incubated with aerobically or anaerobically isolated NreA protein for 30 min on a rocking platform. Anaerobic samples were incubated in an anoxic chamber (lanes 2 & 3; 5 & 6). The DNA binding buffer contained 5 mM NaCl for low salt conditions ("low") and 50 mM NaCl for high salt conditions ("high"). Additionally 10 mM $NaNO_3$ was added where indicated (A-C; lanes 1 -3; 7 & 8). Lane 7 (A-C) contained target DNA in DNA binding buffer with 10 mM $NaNO_3$, but without NreA protein. Lane 8 contained aerobic NreA protein and 10 mM $NaNO_3$, but without target DNA. The samples were separated on a native 6 % acryl amide gel (170 V for 45 min) and subsequently stained with SYBR green I solution (1:10,000). Fluorescence signals were detected on a UV-bench. The positions of the three native DNA fragments P_{narG}, P_{narT} and P_{nirB} are indicated on the left, respectively.

presence of formaldehyde NreA (18 kDa, SwissProt) and NreB (40 kDa, SwissProt) form crosslink products of approx. 35 kDa (FIG.R15, lane 1) and approx. 100 - 120 kDa (FIG.R15, lane 3), respectively. The band intensities corresponding to monomeric NreA and monomeric NreB decrease in presence of formaldehyde (FIG.R15, lanes 1 & 3). Aerobic NreA and NreB together form a crosslink product of approx. 100 - 120 kDa (FIG.R15, lane 7). Under anaerobic conditions the band intensity (and hence the amount) of this crosslink product increases significantly (FIG.R15, lane 6).

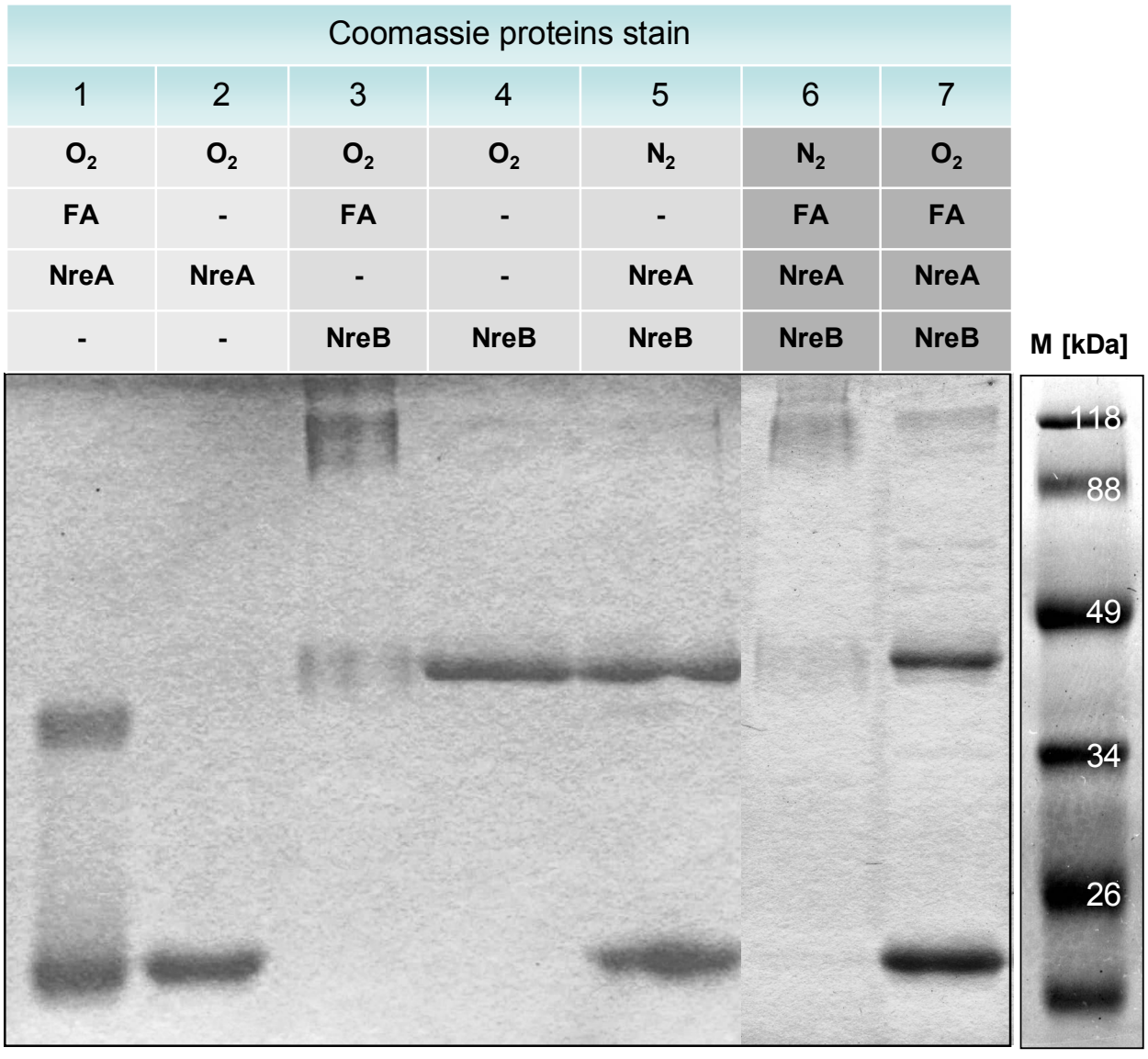


FIG.R15. Coomassie proteins stain of NreA / NreB formaldehyde (FA) crosslinks. NreA and NreB were isolated aerobically (lanes 1-4 and 7) or anaerobically (lanes 5 and 6) from *E. coli* M15 (pQE31*nreA*) and *S. carnosus* m1 (pCQE1*nreB*), respectively. Protein samples were incubated with 0.5 % formaldehyde (lanes 1, 3, 6 and 7) and subjected to SDS-PAGE and Coomassie proteins staining. The anaerobic samples were prepared in an anoxic chamber. For size comparison a marker (M) with indicated molecular sizes in kDa is shown (right).

The putative NreA and NreB crosslink products from lanes 1, 3, 6 and 7 in FIG.R15 reacted with antiserum specific for NreB (FIG.R16). The amount of NreA (or NreB) corresponding to the monomeric form decreases simultaneously with increasing amounts of crosslink product in presence of formaldehyde (FIG.R15 lanes 1, 3, 6 and 7). Glutaraldehyde crosslinks showed similar results, but the resulting protein bands were more blurred than bands resulting from formaldehyde crosslinks.

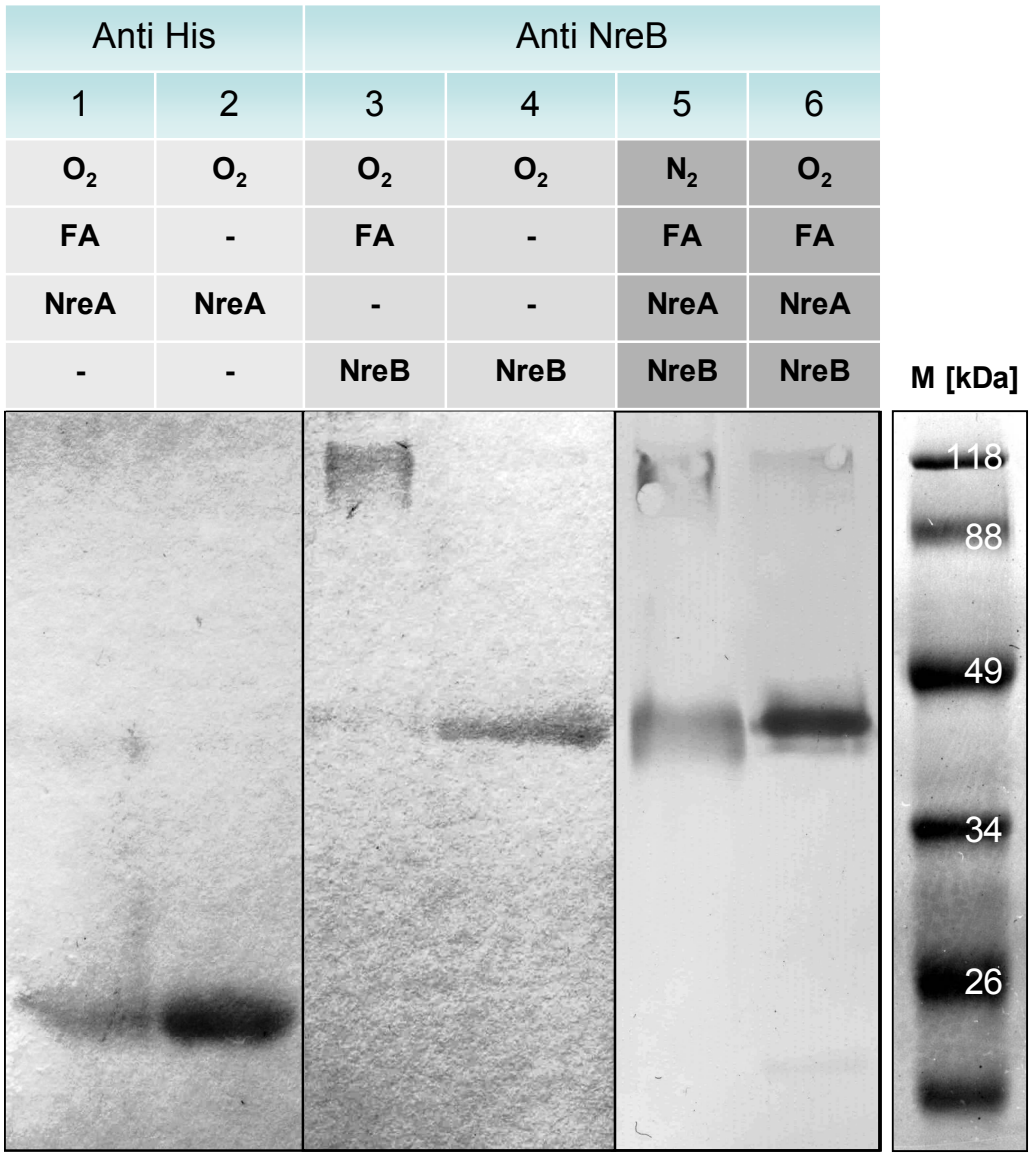


FIG.R16. Western blots of NreA and NreB formaldehyde (FA) crosslinking. NreA and NreB were isolated aerobically (lanes 1-4, 6) or anaerobically (lane 5) from *E. coli* M15 (pQE31*nreA*) and *S. carnosus* m1 (pCQE1*nreB*), respectively. Protein samples were incubated with 0.5 % formaldehyde (lanes 1, 3, 5 and 6) and subjected to SDS-PAGE and Western blotting. Lanes 1 and 2 were incubated with His-Tag specific antiserum. Lanes 3 – 6 were incubated with NreB specific antiserum. In all experiments a secondary antibody coupled to horseradish peroxidase (HRP) and a developing solution containing 4-Chlor-1-naphtol was used. For size comparison a pre-stained protein marker (M) with indicated molecular sizes in kDa is shown on the right.

4.5.4 Formaldehyde crosslinking of NreA to NreC

To investigate a protein interaction between NreA and NreC, the two proteins were isolated aerobically from *E. coli* M15(pQE31*nreA*) and *E. coli* EC3(pMAL*nreC*), respectively. NreC was isolated as NreC-MalE fusion-protein and cleaved with factor *X*a protease to seperate MalE from NreC. Protein samples were incubated with 0.5% formaldehyde (FA) and subjected to SDS-PAGE and Coomassie staining. In presence of formaldehyde NreA and NreC form new bands of approx. 35 kDa (FIG.R17, lane 1) and approx. 120 kDa (FIG.R17, lane 3), respectively. The band intensities of NreA of 18 kDa and of NreC of 35 kDa decrease in presence of formaldehyde (FIG.R17, lanes 1 & 3). In presence of formaldehyde a mixture of NreA and NreC form a crosslink product of approx. 120 kDa (FIG.R17, lane 5), while the band intensities of NreA of 18kDa and NreC of 35 kDa decrease. The band intensity of MalE does not change after addition of formaldehyde.

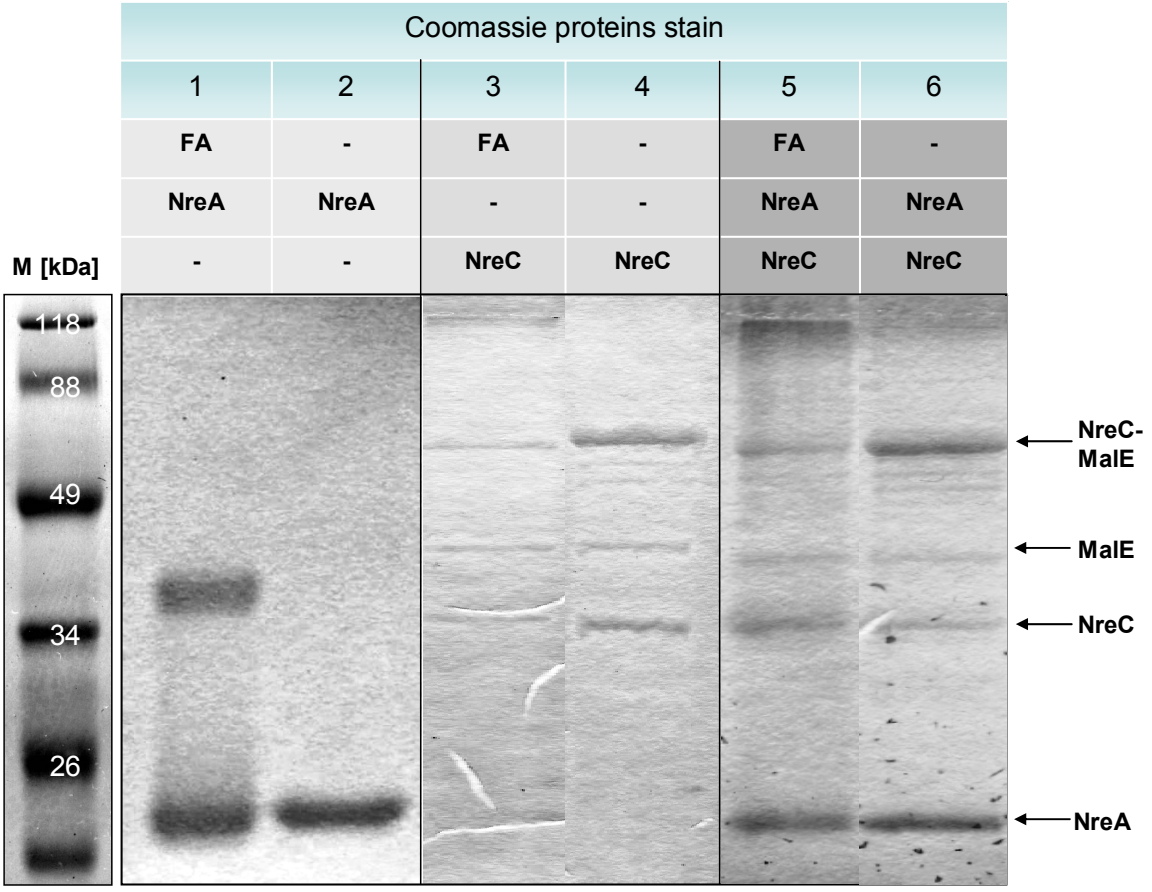


FIG.R17. Formaldehyde crosslinking of a NreA / NreC mixture. NreA and NreC were isolated aerobically from *E. coli* M15 (pQE31*nreA*) and *E. coli* EC3 (pMAL*nreC*), respectively. NreC-MalE fusion protein was cleaved with factor *X*a protease. Protein samples were incubated with 0.5 % formaldehyde (FA, lanes 1, 3, and 5) and subjected to SDS-PAGE and Coomassie staining. For size comparison a marker (M) with indicated molecular sizes in kDa is shown on the left.

Instead of isolating NreC from anaerobically growing cells, 'aerobic' NreC was subjected to *in vitro* phosphorylation. To investigate if activation of NreC by phosphorylation has an effect on protein interaction with NreA, NreC was phosphorylated *in vitro* by incubation with carbamoylphosphate. Phosphorylated NreC together with formaldehyde formed a crosslink product at approx. 120 kDa (FIG.R18, lanes 3 & 4), while the band intensities of NreA of 18kDa and of NreC-P of 35 kDa decrease, similar to the non-phosphorylated form of NreC. The amount of monomeric NreA further decreased in the crosslink after phosphorylation of NreC (FIG.R18, lanes 5 & 6). The amount of MalE slightly decreased after addition of formaldehyde, independently of NreA addition.

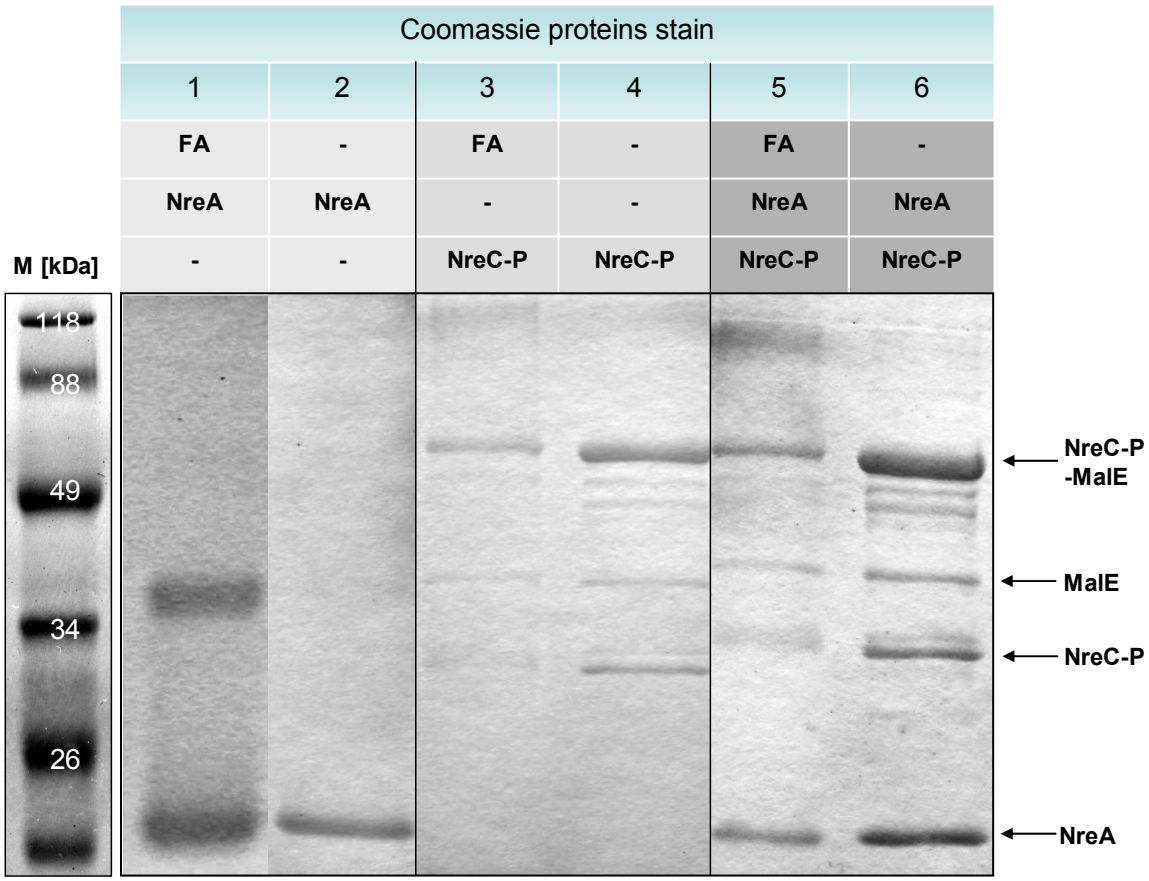


FIG.R18. Formaldehyde crosslinking of a NreA / NreC-P mixture. NreA and NreC were isolated aerobically from *E. coli* M15 (pQE31*nreA*) and *E. coli* EC3 (pMal*nreC*), respectively. NreC-MalE fusion protein was cleaved with factor *X*a protease. NreC was phosphorylated *in vitro* after isolation and after cleavage by incubation with 20 mM carbamoylphosphate. Protein samples were incubated with 0.5 % formaldehyde (FA, lanes 1, 3, and 5) and subjected to SDS-PAGE and Coomassie staining. For size comparison a marker (M) with indicated molecular sizes in kDa is shown on the left.

4.5.5 Cloning of NreC

NreC is the response regulator of the NreBC two-component system. NreC is phosphorylated by active $[4Fe4S]^{2+}$NreB under anaerobic conditions (Kamps *et al*., 2004 and Müllner *et al*., 2008). Phosphorylated NreC binds to the target DNA and acts as a transcription regulator. To test the hypothesis, that NreC interacts at some point with NreA, NreC from *S. carnosus* was cloned in the inducible expression vector pQE30. *nreC* was amplified from genomic wildtype DNA of *S. carnosus* TM300 via PCR using Phusion polymerase (FIG.R19). Using other polymerases (e.g. *pfu*) resulted in no PCR product. The amplified DNA fragments were cloned into the *Kpn*I and *Sal*I sites of pQE30 (FIG.M2) using 1:1, 1:3, 1:5 and 1:10 insert to vector DNA ratios and electro-transformed into *E. coli* JM109 (Farinha *et al*., 1990). The 1:5 ligation (amount of DNA_{vector} to DNA_{insert}) sample showed the highest transformation efficiency (up to $2*10^3$ cfu / µg DNA). In a control experiment circular plasmid pQE30 without *nreC* insert and a sample lacking DNA were transformed. For the transformation with uncut pQE30 efficiencies up to $6*10^5$ colonies per µg DNA were observed. Plasmid pQE30*nreC* was purified from transformed cells and subjected to DNA sequencing using the self designed sequencing primer pQE30nreC_seq (TAB.M4). The DNA sequences were confirmed at least three times. However, in all cases only the 3' and 5' ends of *nreC* were cloned into pQE30, missing the major middle part of the gene and resulting in non-functional NreC protein. For further investigations, NreC purified as MalE-NreC fusion protein from strain *E. coli* EC3 (pMal*nreC)* from Fedtke *et al*. (2002) was used instead.

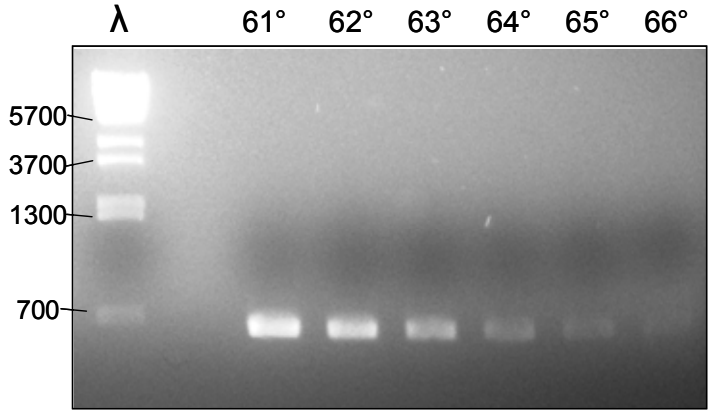


FIG.R19. Amplification of *nreC* (677 bp) from genomic DNA of *S. carnosus* TM300. The gradient PCR was conducted at annealing temperatures of 61 - 66 °C and with primers NreC-KpnI-for and NreC-SalI-rev. After digestion with *KpnI* and *SalI* and three purification steps the DNA was subjected to gel electrophoresis and stained with Ethidiumbromid. For size estimation selected sizes in [bp] are indicated on the left.

5. Discussion

5.1 Function and properties of the oxygen sensor FNR from *E. coli*

5.1.1 The physiological relevant form of FNR in aerobically growing *E. coli* is apoFNR

FNR of aerobically growing *E. coli* is predominately found as apoFNR. Labeling reagents like mBBr or AMS which selectively label accessible Cys residues without removing the labile FeS cluster allowed identification of apoFNR and differentiation from FeS-containing FNR *in vivo*. FNR with non-accessible Cys residues (i.e., FeS-containing FNR) was found under the same conditions as in earlier studies by Mössbauer spectroscopy (Sutton *et al*., 2004a and 2004b). The labeling methods used here allowed direct demonstration of apoFNR, and in addition, of the redox state of the Cys residues. $[2Fe2S]^{2+}$ and $[4Fe4S]^{2+}$-containing FNR showed similar sensitivities to the displacement of the FeS cluster, and both forms were not differentiated in the labeling studies.

ApoFNR in aerobically grown *E. coli* is either newly synthesized or the conversion product of $[4Fe4S]^{2+}$FNR. The relative contribution of both processes to apoFNR formation is not known. The anaerobic / aerobic shift experiments in growing bacteria (Engel *et al*., 1991; Popescu *et al*., 1998) and *in vitro* (Achebach *et al*., 2005) demonstrate that conversion takes place efficiently. The reagent mBBr allowed quantitative *in situ* studies on apoFNR. Up to five Cys residues per FNR become labelled by mBBr, and the number of reactive residues varies between one and two and about four Cys residues *in vivo* per FNR depending on the presence of O_2.

For $[4Fe4S]^{2+}$FNR, $[2Fe2S]^{2+}$FNR and apoFNR one and five accessible and labelled Cys residues are expected, respectively. Since both $[4Fe4S]^{2+}$ and $[2Fe2S]^{2+}$ clusters require four Cys ligands, FNR with more than one accessible Cys residue must be apoFNR. The presence of one or two accessible Cys residues in anaerobic cells of *E. coli* suggests that the bacteria contain some apoFNR. Mössbauer spectroscopy of anaerobic bacteria also showed significant amounts apoFNR without incorporated

FeS cluster (Popescu *et al.*, 1998). The suboptimal labeling by mBBr in aerobic bacteria (labeling of four compared to five Cys labels) could be due either to incomplete labeling and accessibility or to the presence of some residual FNR with bound FeS cluster. Labeling of FNR *in vivo* in aerobically growing bacteria with NEM followed by MALDI-TOF analysis (FIG.D1) showed indeed the theoretical value of five accessible Cys residues as predicted for apoFNR, and a significally lower accessibility to NEM for $[4Fe4S]^{2+}$FNR.

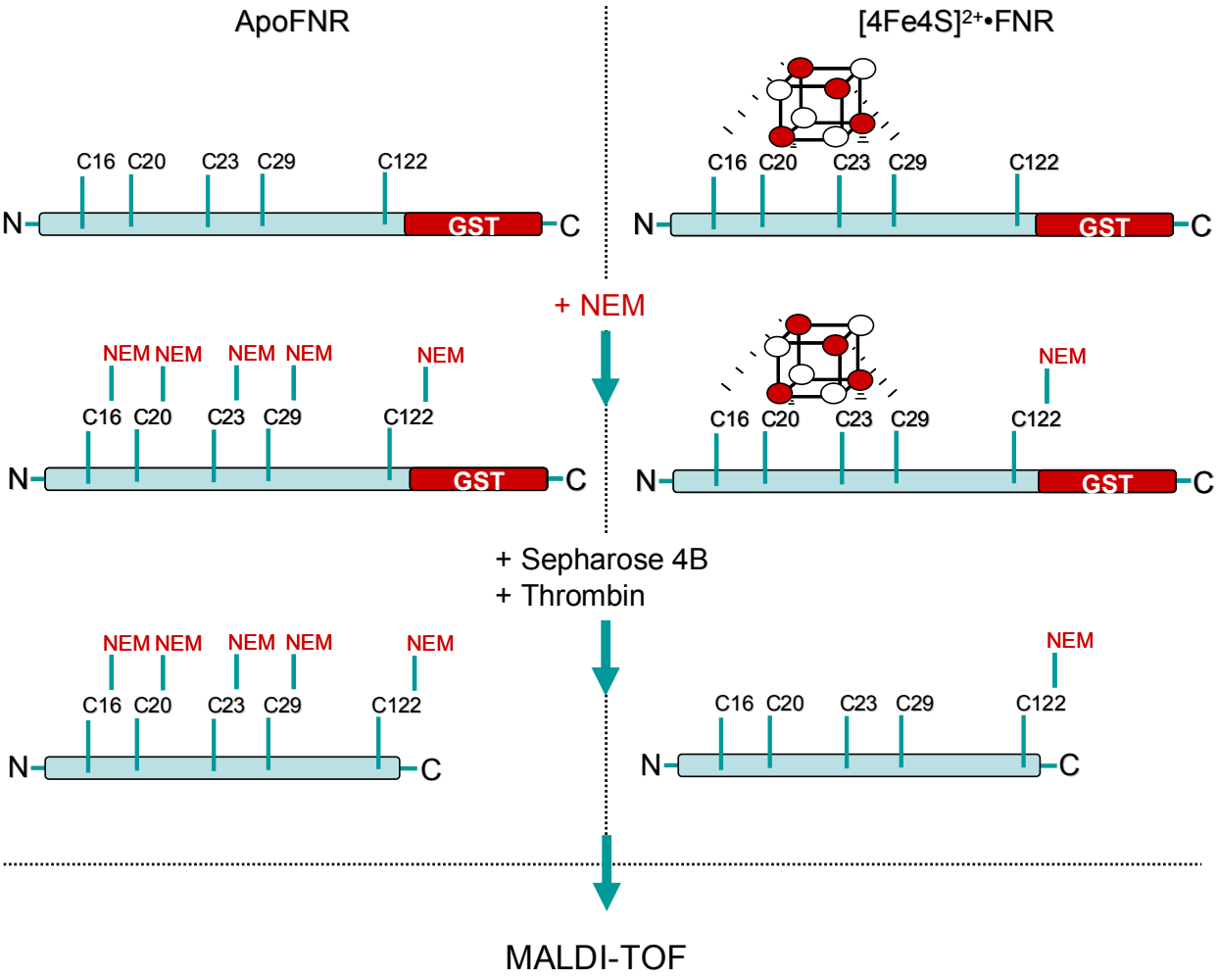


FIG.D1. Flow chart for FNR sample preparation for mass spectroscopy and differentiation of apoFNR and $[4Fe4S]^{2+}$FNR. Aerobic or anaerobic cells of *E. coli* CAG627 (pMW32) over-expressing GST-FNR were incubated with N-ethylmaleimide. The labelling reaction was stopped by adding excess DTT. GST-FNR bound to glutathione-sepharose 4B was digested for 2 h at 20 °C with 20 U thrombin in 50 mm Tris / HCl, pH 7.6 and then eluted from the column in protein isolation buffer C. FNR obtained from GST-FNR in this way contains a Gly-Ser extension in front of the N-terminal Met-Ile-Pro of wild-type FNR, and was subsequently analysed by mass spectroscopy.

5.1.2 ApoFNR forms no disulfide bonds *in vivo*

The quantitative conversion of $[4Fe4S]^{2+}$FNR to apoFNR *in vivo* within a six minutes demonstrates the physiological relevance of the reaction. *In vitro*, air inactivation of $[4Fe4S]^{2+}$FNR yields apoFNR with ~1.5 Cys-disulfides (Achebach *et al*., 2005). The experiments shown here demonstrate that apoFNR in bacterial cells contains no significant amounts of disulfides. Obviously the disulfides are formed *in vitro* only in the absence of the highly reduced thiol / glutathione redox buffer of *E*. *coli*. In the bacterial cells, more than 90% of the free thiols are in the thiol state (De Crouy-Chanel and Richarme, 2001), providing strongly reducing conditions.

Before the presence and function of the $[4Fe4S]^{2+}$ cluster in FNR was known, the Cys residues of aerobically growing *E*. *coli* were shown to be more readily accessible to alkylation than those from anaerobically growing *E*. *coli* (Trageser and Unden, 1989). The change in the labeling behaviour was then attributed to a thiol / disulfide change and to metal ion binding by FNR in response to O_2 availability. After identification of the O_2-sensitive $[4Fe4S]^{2+}$ cluster and the $[4Fe4S]^{2+}$ to $[2Fe2S]^{2+}$ switch of FNR, this model had to be dismissed. It now becomes apparent that the increase in the number of accessible Cys residues in FNR of aerobically growing *E*. *coli* is functionally relevant, but it has to be attributed to the release of the FeS clusters resulting in formation of apoFNR.

5.1.3 Oxygen causes a two-step inactivation of $[4Fe4S]^{2+}$ FNR

Overall, the earlier experiments and the present experiments identify three different functional forms of FNR *in vivo*: $[4Fe4S]^{2+}$FNR, $[2Fe2S]^{2+}$FNR, and apoFNR. The rapid formation of the two inactive forms, $[2Fe2S]^{2+}$FNR and apoFNR, suggests that both play a role in bacterial cells. $[2Fe2S]^{2+}$FNR is formed from $[4Fe4S]^{2+}$FNR and then degraded within a few minutes to apoFNR (Sutton *et al*., 2004a). In the absence of further sufficient oxygen $[2Fe2S]^{2+}$FNR is rather stable (Sutton *et al*., 2004b). Similar kinetics in the minute range for the formation of apoFNR from $[4Fe4S]^{2+}$FNR in aerobically growing cells were found in this study. Thus, under oxic conditions, $[2Fe2S]^{2+}$FNR appears to be an intermediate in the formation of apoFNR, whereas apoFNR represents the major inactive form of FNR in aerobically growing *E*. *coli*.

Switch experiments suggest that the active form of FNR and apoFNR can be interconverted in both directions (Engel *et al.,* 1991; Dibden *et al*., 2005). However, apoFNR exhibits a decreased half-life (Mettert *et al*., 2005).

5.1.4 Possible functions of $[2Fe2S]^{2+}$ FNR and apoFNR *in vivo*

When O_2 is supplied only briefly or at low concentrations, inactivation of $[4Fe4S]^{2+}$ FNR might stop at the level of $[2Fe2S]^{2+}$FNR. The presence of $[2Fe2S]^{2+}$FNR instead of apoFNR could possibly allow a rapid return to the $[4Fe4S]^{2+}$FNR state upon reversal to anoxic conditions and thereby rapid restoration of anaerobic growth. Such a situation could be important for bacteria growing in microaerobic biotopes where oxygen supply is not permanent or close to the regulatory $pO_{0.5}$ value of FNR (~1-5 µM; Becker *et al*., 1996).

For long-term growth under oxic conditions, on the other hand, a stable repression of genes encoding enzymes of anaerobic metabolism is appropriate. The presence of apoFNR instead of $[2Fe2S]^{2+}$FNR might prevent frequent shifts to anaerobic metabolism during short-term lack of oxygen. Such a two-step inactivation of FNR might provide a more stable regulation, which is sensitive to oxygen, and on the other hand buffers small changes in oxygen supply without permanent aerobic / anaerobic shifting of the transcription machinery.

5.1.5 Comparison of FNR_{Ec} and FNR_{Bs}

Signal perception of oxygen tension by FNR_{Bs} from *B. subtilis* involves a labile $[4Fe4S]^{2+}$ cluster and is similar to the mechanism of signal perception of FNR_{Ec} from *E. coli* (Reents *et al*., 2006b). However, the predicted protein structures of *B. subtilis* FNR and *E. coli* FNR reveal significant differences (FIG.D2). The four Cys residues coordinating the $[4Fe4S]^{2+}$ cluster of FNR_{Ec} are located at the N-terminal part of the protein while FNR_{Bs} possesses a C-terminal protein extension containing the three cysteine residues and putatively a fourth, yet unknown ligand needed for $[4Fe4S]^{2+}$ cluster formation (Reents *et al*., 2006a). FNR_{Bs} forms a stable dimer under aerobic and anaerobic conditions independently of FeS cluster formation, whereas

dimerisation of FNR_{Ec} occurs exclusively under anaerobic conditions in the presence of a $[4Fe4S]^{2+}$ cluster.

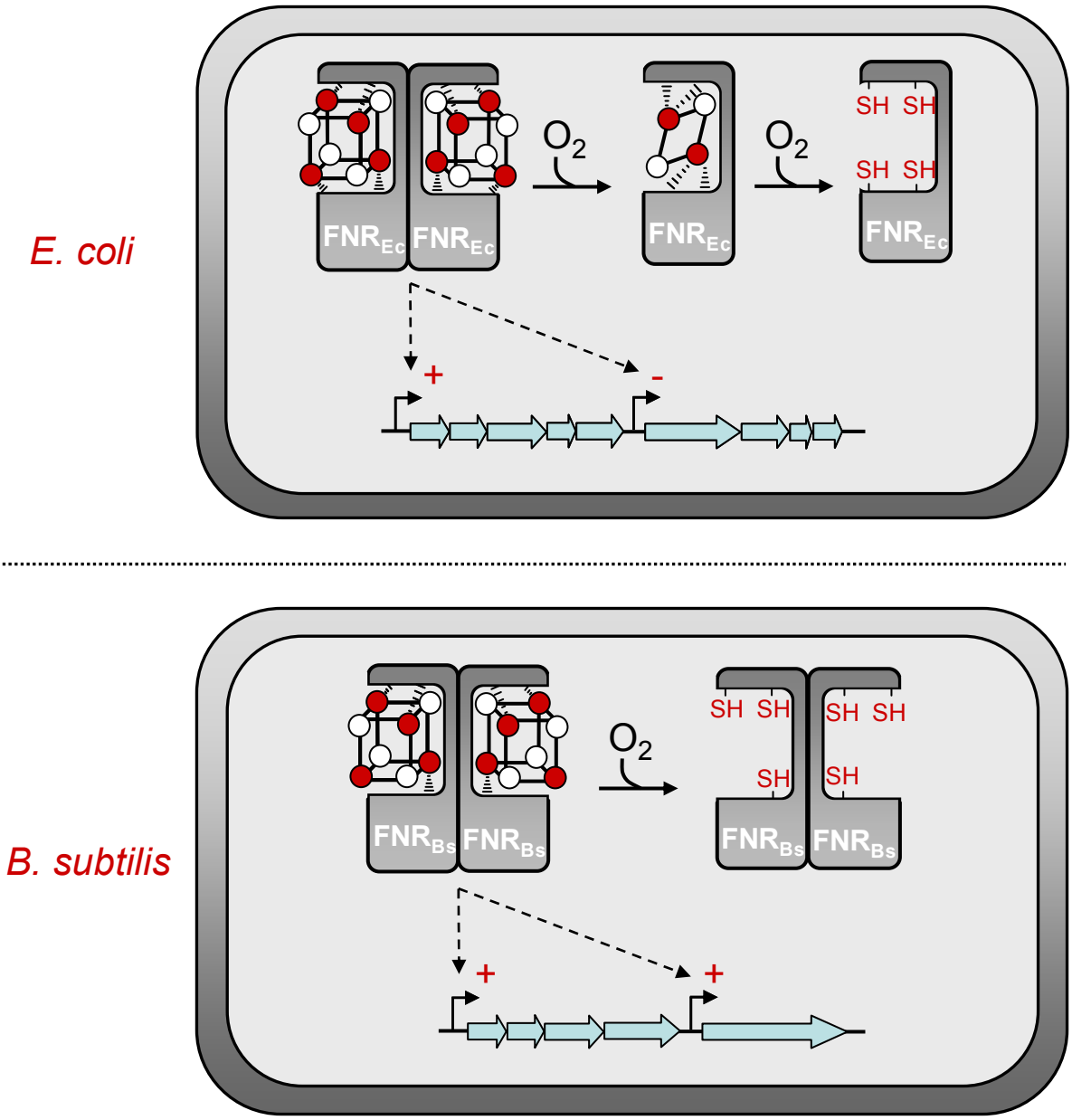


FIG.D2. Comparison of the cytoplasmatic oxygen sensors FNR_{Ec} from *E. coli* (top) and FNR_{Bs} from *B. subtilis* (bottom). The mechanism by which oxygen is sensed is similar in both proteins and involves an oxygen labile $[4Fe4S]^{2+}$ cluster. FNR_{Ec} depends on dimerisation for DNA binding and induces genes of anaerobic respiration while repressing genes of aerobic respiration. The $[4Fe4S]^{2+}$ cluster is required for dimerisation of FNR_{Ec} and bound by four Cys residues. On the other hand, FNR_{Bs} is always a dimer independently from oxygen. In anaerobic FNR_{Bs} the $[4Fe4S]^{2+}$ cluster is bound by only three Cys residues and a yet unknown fourth ligand (Reents *et al.*, 2006b). Active $[4Fe4S]^{2+}$ FNR_{Bs} positively regulates the transcription of the nitrate reductase operon *narGHJI* and the nitrite extrusion protein gene *narK* in *B. subtilis*.

Despite the similar mechanisms of oxygen sensing, differences in anaerobic gene regulation of FNR_{Ec} and FNR_{Bs} are described (Reents *et al*., 2006). Under oxygen limiting conditions the $[4Fe4S]^{2+}$ cluster is coordinated in FNR_{Bs} by only three Cys residues localized at the C-terminus and a yet unknown fourth ligand of the regulator. Cluster formation is supposed to affect DNA-binding, which allows promoter recognition and transcriptional activation of target genes. In contrast FNR_{Ec} forms a dimer only in the presence of the oxygen-sensitive $[4Fe4S]^{2+}$ cluster localized at the N-terminus of the protein and coordinated by four Cys residues. In *E. coli* dimeric $[4Fe4S]^{2+}FNR_{Ec}$ binds to its promoter sequences and mediates transcriptional activation or repression. In contrast, only positively regulated target genes of $[4Fe4S]^{2+}FNR_{Bs}$ are known so far in *B. subtilis*, e.g. the nitrate reductase operon *narGHJI* and the nitrite extrusion protein gene *narK* (Reents *et al*., 2006a).

5.2 The oxygen sensing two-component system NreBC from *Staphylococcus carnosus*

5.2.1 *In vivo* Mössbauer studies on NreB are difficult

Mössbauer studies have been used to differentiate $[4Fe4S]^{2+}$ and $[2Fe2S]^{2+}$-containing forms of FNR_{Ec} *in vivo* (Popescu *et al*., 1998). However, $NreB_{Sc}$ cannot be overproduced and concentrated to sufficient levels (~100 µM; Popescu *et al*., 1998) to allow *in vivo* Mössbauer studies with cells of *S. carnosus*. Cys labeling therefore enables studies on proteins *in vivo* which cannot be overproduced to extents required for Mössbauer studies. More important is that the studies can be performed with moderate levels of NreB overproduction, and physiological conditions can be applied. According to estimations from immunoblotting, wildtype *S. carnosus* contains 0.16 µg of NreB per mg of total cell protein or less (corresponding to an approximate concentration of 0.4 µM NreB in the bacterium). In the expression strain, about 4 µg of NreB per mg of total cell protein was found under the conditions of the labeling experiments. This suggests an about 30-fold increase compared to the wild type, which is, however, still a factor of 10 below the levels required for *in vivo* Mössbauer spectroscopy (Popescu *et al*., 1998).

Even more important is that the labeling experiments can be performed at low cell densities and under aerobic and anaerobic conditions, whereas for *in vivo* Mössbauer spectroscopy, very high cell densities are required, which makes maintenance of aerobic conditions and studies on the aerobic-anaerobic switch very difficult. Therefore, labeling of the Cys residues of labile FeS clusters is a useful method for studies on cluster degradation *in vivo* and *in vitro* and complementing Mössbauer studies. Similar arguments apply to *in vivo* studies on FNR_{Ec} by Cys labeling versus Mössbauer studies.

5.2.2 Functional Cys labeling studies of NreB

Mössbauer studies have shown that anaerobic $[4Fe4S]^{2+}$NreB is converted *in vitro* in the presence of O_2 to $[2Fe2S]^{2+}$NreB and further to apoNreB (Müllner *et al*., 2008). On the basis of these results, the Cys labeling experiments described here demonstrate that the same reactions take place *in vivo* in cells of *S. carnosus* and that apoNreB is the physiological relevant form of NreB in aerobically growing bacteria. Overall, oxygen causes similar reactions and conversions of NreB *in vitro* and *in vivo*. $[4Fe4S]^{2+}$NreB and apoNreB are the physiologically relevant forms under anaerobic and aerobic conditions, respectively. The experiments also show that apoNreB is formed *in vitro* from $[4Fe4S]^{2+}$NreB, with a half-time of 6 min, which fits the kinetics of $[4Fe4S]^{2+}$ to $[2Fe2S]^{2+}$ cluster conversion, with a half-time of ~2.5 min, that was obtained by Mössbauer studies before (Müllner *et al*., 2008).

5.2.3 A two-step Cys labeling procedure allows quantitative labeling studies on NreB

Quantitative evaluation of the accessibility of the Cys residues of NreB and the clear-cut differentiation between apoNreB and FeS-containing NreB required a two-step labeling procedure. In this procedure, the first labeling step is performed with the native protein, using the thiol reagent iodoacetate (IAA), and the second labeling step is performed with the denatured protein, using monobromobimane (mBBr) (FIG.D3). The IAA (or NEM) / mBBr two step labeling procedure is basically similar to the AMS (or NEM) / PEG-mal labeling procedure that relies on detection of labelled samples by mass shift. The quantitative labeling by IAA was confirmed by alkylation and mass

spectrometry (FIG.D4). The two-step procedure using IAA and mBBr allowed work *in vitro* and *in situ* in growing bacteria when the NreB concentrations were relatively low.

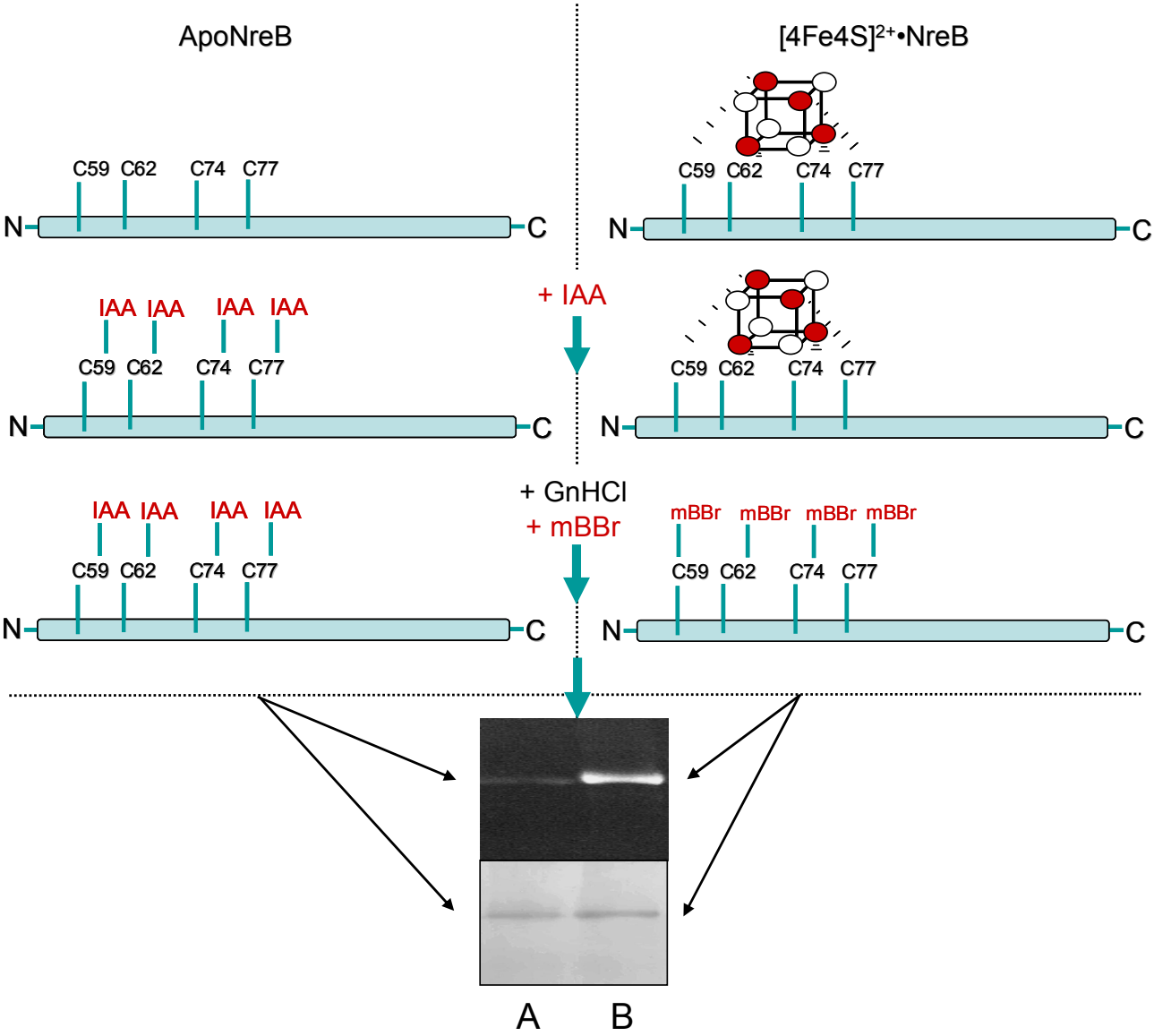


FIG.D3. Flow-chart for differentiation between ApoNreB and $[4Fe4S]^{2+}$NreB by two-step Cys labeling. In the first labeling step, cells of *S. carnosus* were incubated with iodoacetate (IAA), which is membrane permeable and highly reactive to native, surface-exposed Cys residues. The presence of a $[4Fe4S]^{2+}$ cluster in anaerobic NreB prevents Cys labeling. After denaturing with GnHCl, the formerly protected residues are labelled with monobromobimane (mBBr). The cell walls are disrupted and the lysate is subjected to SDS-PAGE and western blotting. The specific fluorescence of NreB is calculated from the fluorescence intensity and the total NreB content of the sample. Low fluorescence (A) means Cys residues were accessible to IAA, high fluorescence (B) means Cys residues were accessible to mBBr. By this procedure, *in vivo* differentiation of apoNreB and FeS-NreB is possible.

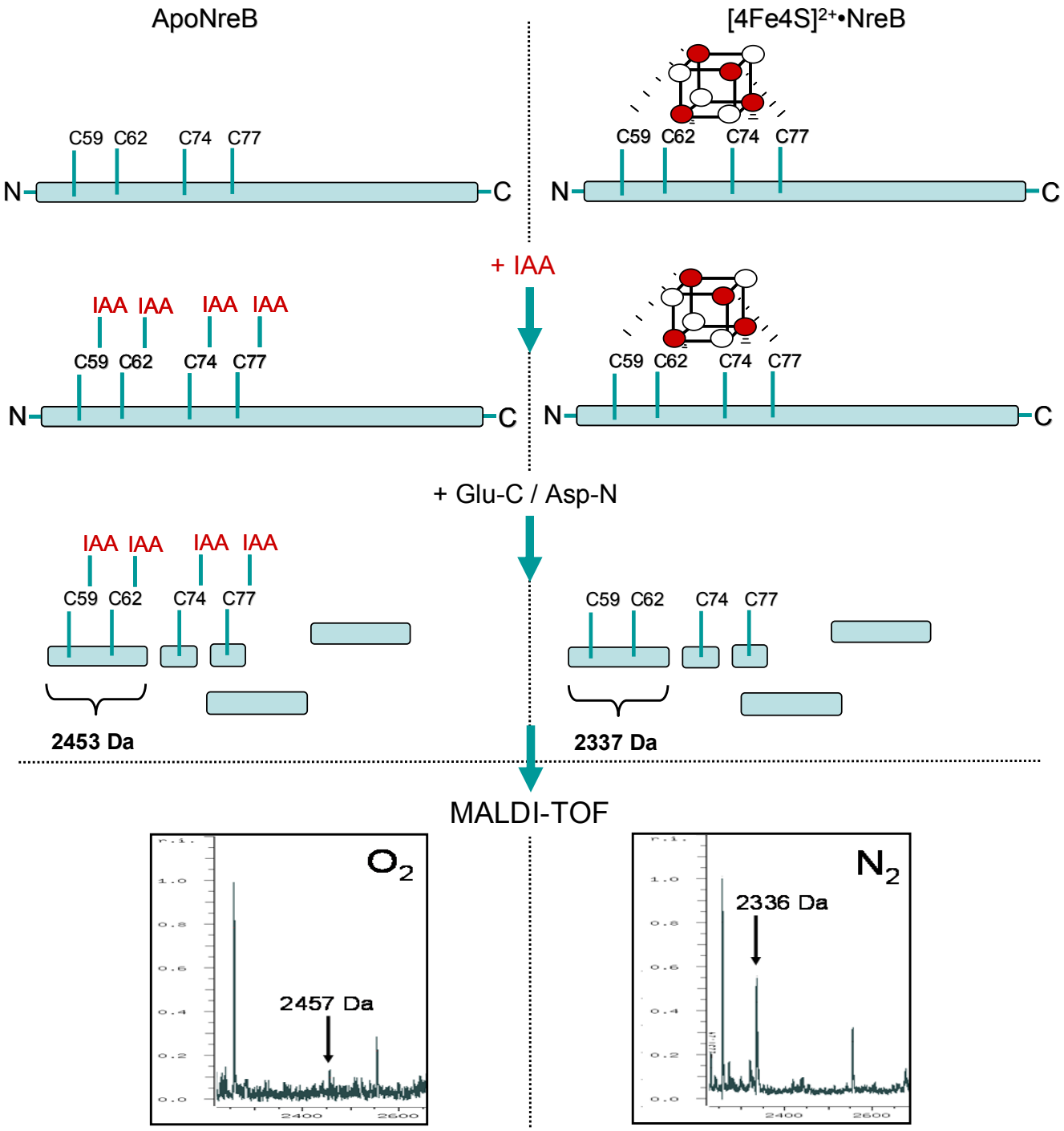


FIG.D4. Flow chart of NreB sample preparation for mass spectroscopy and differentiation of apoNreB and $[4Fe4S]^{2+}$NreB. Cells moderately over-expressing NreB (30-fold increase compared to wildtype) were incubated with Iodoacetate and NreB was isolated and digested with one of the two strongly sequence specific proteases GluC and AspN (both sequencing grade). The masses of the resulting protein fragments can be predicted. For the unlabelled double Cys digestion fragment a mass of 2337 Da and for the 2x labelled fragment a mass of 2453 Da is predicted (58 Da increase per IAA-label). In aerobic MALDI-TOF samples a peak corresponding to the 2x labelled NreB fragment was detected (2457 Da). In anaerobic samples a peak corresponding to the non-alkylated species of this protein fragment was detected (2336 Da). This confirms that in aerobic cells of *S. carnosus* apoNreB is the major form of NreB.

The difference in labeling cannot be explained by simple changes in the redox state of the Cys residues in NreB, since high levels of labeling for the native protein (i.e., labeling by IAA) were found for aerobically grown bacteria and low levels of NreB were found for anaerobically grown bacteria. The labeling can be explained by assuming protection of the Cys residues from labeling by bound FeS clusters in anaerobic bacteria. Moreover, the finding is in agreement with the *in vitro* studies on bound FeS clusters and accessible Cys residues. Therefore, two-step labeling of the Cys residues of NreB can be used to differentiate FeS-containing NreB from apoNreB.

In anaerobic $[4Fe4S]^{2+}$ NreB, most of the Cys residues (77 %) became accessible to labeling only in the second step with mBBr, that is, after denaturing the protein. This was similar to the reaction of the total protein in bacteria, but the amount of mBBr-labelled residues was smaller (77 % compared to 89 %). Since the mBBr-reacting residues corresponded to the FeS-protected residues, it can be assumed that a measurable part of the anaerobic NreB is no longer in the $[4Fe4S]^{2+}$ NreB form. In agreement with this observation, it was suggested earlier (Müllner *et al*., 2008) that part of the isolated anaerobic NreB had lost the FeS cluster. Again, when NreB was denatured in a control experiment by guanidinium hydrochloride before the first labeling step, in the second labeling reaction only a small portion was labelled by mBBr. This applies in the same way to aerobic and anaerobic NreB

5.2.4 apoNreB is the physiological relevant form of NreB in aerobically growing *S. carnosus*

Oxygen sensing is generally complicated by the fact that the direct stimulus of the sensor is often not well defined. Other oxidants might have similar effects and are interchangeable with molecular oxygen. Therefore, it is important to study oxygen sensors *in vivo* in bacteria and to verify the *in vitro* reactions. This approach is of particular significance for NreB, for which two different air-inactivated forms ($[2Fe2S]^{2+}$NreB and apoNreB) have been shown *in vitro*. Anaerobically growing *S. carnosus* contained only NreB with bound FeS-cluster, no significant levels of apoNreB were detected. Since $[4Fe4S]^{2+}$NreB represents the only active form of NreB and was isolated as the major form from anaerobic bacteria (Müllner et al.,

2008), the FeS-containing form of NreB in the anaerobic bacteria has to be $[4Fe4S]^{2+}$NreB. The aerobically growing bacteria, on the other hand, contained only apoNreB; $[2Fe2S]^{2+}$NreB was not present in significant amounts. Thus, apoNreB is the physiologically relevant form in aerobically growing *S. carnosus*. Many Gram-positive bacteria, such as *Staphylococcus* and *Bacillus*, lack glutathione, which is replaced by cysteine and other low-molecular-weight thiols (Leichert *et al*., 2003; Newton *et al*., 1996). Glutathione and cysteine equilibrate only slowly with molecular oxygen and are present in the cytoplasm essentially in the reduced state, even under aerobic conditions, when O_2 diffuses into the cytoplasm.

Overall, it appears that labeling by iodoacetate and mBBr in the two-step procedure detects the number of accessible Cys residues *in vivo* and *in vitro* in a very similar way and that the labeling method allows differentiation of Cys residues that bind an FeS cluster and those not involved in binding. Accordingly, the differences in Cys residues that are accessible in the native state under aerobic and anaerobic conditions represent FeS-protected Cys residues. Iodoacetate obviously does not displace the FeS cluster, which is demonstrated by the high level of protected Cys residues under anaerobic conditions.

5.2.5 Possible functions of the intermediate product $[2Fe2S]^{2+}$ NreB

It is not clear whether $[2Fe2S]^{2+}$NreB is also of physiological significance to *S. carnosus*, similar to the situation found in *E. coli* and FNR. $[2Fe2S]^{2+}$NreB might be important for transitional stages when the oxygen supply is limited. The presence of $[2Fe2S]^{2+}$NreB as an intermediate might facilitate and accelerate switching between the active and inactive forms of the sensor kinase. It is, however, also conceivable that the $[2Fe2S]^{2+}$ form is a degradation intermediate which is formed only for chemical reasons, without physiological significance, indicating that $[2Fe2S]^{2+}$ intermediates are generally formed during degradation of O_2-labile $[4Fe4S]^{2+}$ clusters.

5.2.6 The $[4Fe4S]^{2+}$ cluster is a universal cofactor for oxygen sensing in bacteria

The mechanism for oxygen sensing, in particular the use and reaction of the $[4Fe4S]^{2+}$ cluster by FNR_{Ec} and $NreB_{Sc}$ is very similar, although FNR and NreB are completely different proteins (FIG.D5). In NreB the FeS-cluster is coordinated by a PAS domain, which shows no sequence homology to the FeS-cluster binding domain of FNR. It can be assumed, that during evolution FNR and NreB independently acquired a $[4Fe4S]^{2+}$ cofactor for oxygen sensing. The signal transduction pathway differs greatly in both oxygen sensor proteins (FIG.D5).

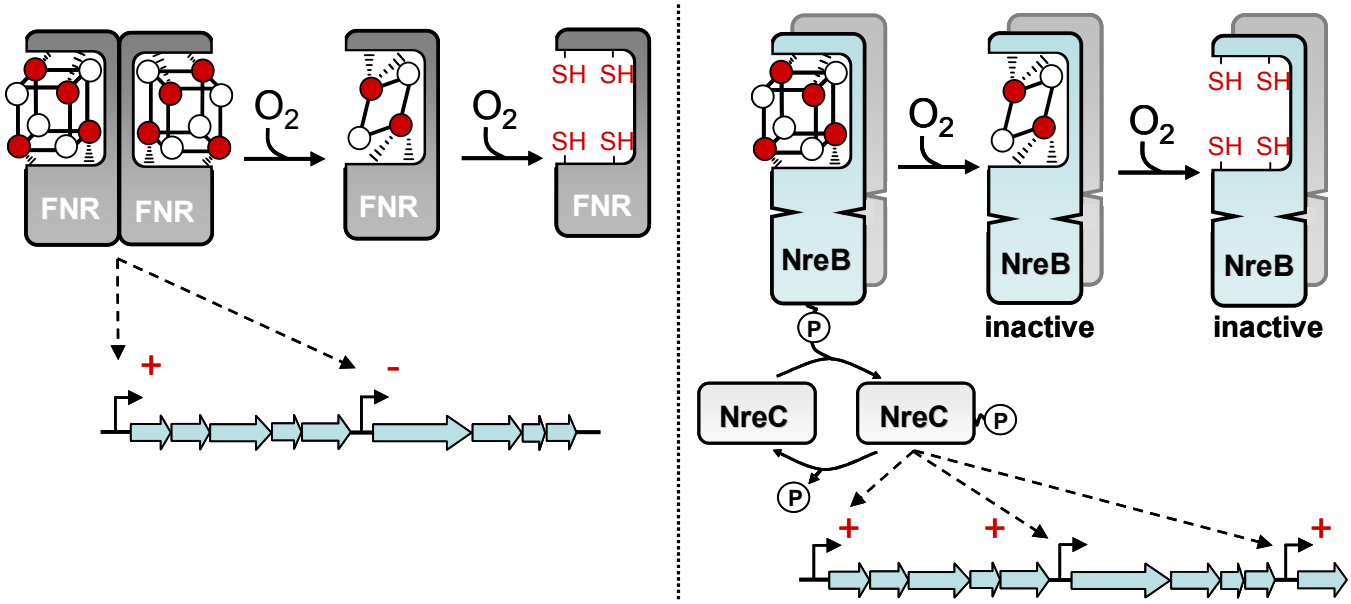


FIG.D5. Comparison of the one-component system FNR from *E. coli* (left) and the two-component system NreBC from *S. carnosus* (right). FNR is a one-component system with a Crp domain and depends on dimerisation for DNA binding and gene regulation. FNR induces genes of anaerobic respiration and represses genes of aerobic respiration. In contrast NreB from *S. carnosus* features a PAS domain, is always a dimer, independently of oxygen, and phosphorylates the response regulator NreC for gene regulation. Phosphorylated NreC induces genes for anaerobic respiration. But like FNR, in anaerobic bacteria most of the NreB exists as $[4Fe4S]^{2+}$ NreB, whereas in aerobic bacteria the FeS-less apo form prevails. It can be assumed, that the $[2Fe2S]^{2+}$ intermediate form has no physiological relevance in both oxygen sensors.

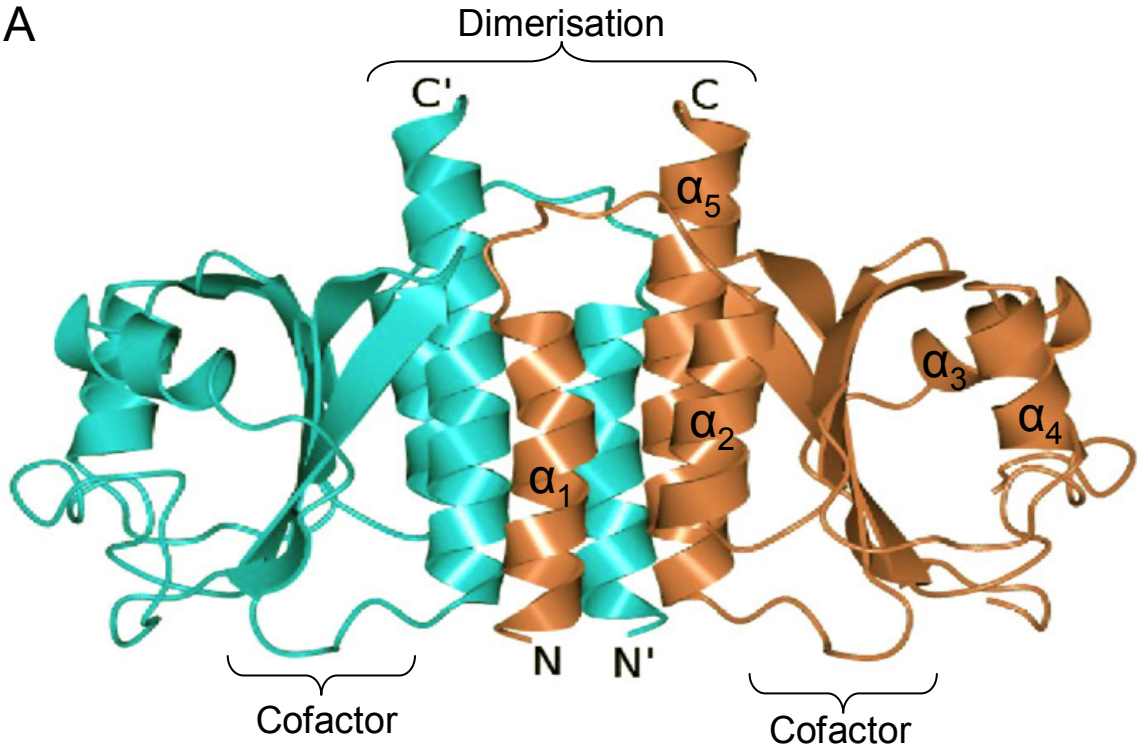
5.3 Significance of NreA for the NreBC two-component system

5.3.1 Structure comparison of NreA and CodY

The GAF domain of NreA from *S. carnosus* shows 22.8% amino acid identity and 36.1% similarity to the GAF domain of CodY from *B. subtilis* (FIG.D6B). CodY controls the expression of stationary phase genes and is one of the factors initializing sporulation (Majerczyk *et al*., 2008; Sonenshine *et al*., 2005). GTP activates the repressor function of CodY by interacting with the GTP-binding motif of the CodY GAF domain (residues 1-155, FIG.D6A). The affinity of CodY for GTP is sufficiently low, so that CodY can distinguish between 2 mM GTP in rapidly growing cells and 300 mM in stationary phase cells. CodY interacts with target DNA through a helix–turn–helix (HTH) motif (residues 202-221) and X-ray crystallography has revealed that the two helices are placed at an angle of about 120°. The helices are linked by a flexible region of 3 to 4 amino acids. The stabilizing helix sits above the major groove, near the DNA backbone, and the flexible turn region allows the recognition helix to form a sequence-specific interaction with DNA and regulate more than 100 target genes in *B. subtilis* (Joseph *et al*., 2005).

The mechanism in CodY by which the signal of a bound cofactor is communicated to the C-terminal helix-turn-helix motif is not fully understood. DNA-binding of CodY depends on serine residue 215, which is located in the C-terminal helix-turn-helix motif and might be a target for phosphorylation (Joseph *et al*., 2005). Serine / Threonine kinases are common in gram positive bacteria (Macek *et al*., 2007) and have also been described in *Staphylococcus aureus* (Debarbouille *et al*., 2009). Many gram positive bacteria, including *S. carnosus*, encode a homolog of *B. subtilis* CodY. In *B. subtilis*, the repressor function of CodY is activated by interaction with two different effectors, GTP and isoleucine, which independently and additively increase the affinity of CodY for its target DNA sites (FIG.D7). Depletion of either GTP or isoleucine is sufficient to induce CodY-regulated genes. CodY is activated by isoleucine at 10–15 mM, the concentration range found in rapidly growing cells. The GTP signal presumably reflects the energy status of the cell.

A



B

```
CODY_BACSU        1 MALLQKTRIINSMLQAAAGKPVN-F--KEMA--ETLR-----D---V-I-     35
                          :||:: |:     : |  :: |  | :|     |   : :
NREA_STACA        1 ---------MNSVI-AS-----DYFDYQD-ALDE-IRETEKFDFAAIALP     33

CODY_BACSU       36 -D---SNIF-VVSRRGKLLGY-SI-NQQIEND-RMKKM--LEDRQFPEEY     75
                     |   |     |:  :.|   | |  |  | |  |. :|  |  |  |
NREA_STACA       34 EDGLHS---AVI--KWK---YAS-GN--I-N-YRY-RMIVL--R--P---     62

CODY_BACSU       76 T-KNL--FNVP-ETSS-NL--DINSEYTAFPVENRD-L-F-----QAGLT    111
                      |.|  . |  .|.| .:  |:::|.:    :| | | :     :| ||
NREA_STACA       63 -GKGLAGL-V-IRTGSRKIVEDVDAELS----QN-DKLGYPIVLSEA-LT    103

CODY_BACSU      112 T-I-VPI-----I-GG---G--E-R-L--G-TLILS-RLQDQFNDDDLIL    142
                    . : :|:     : |.   |  | | |  | |   : |: :| .   |
NREA_STACA      104 AMVAIPLWKQNRVYGALLLGQREGRPLPEGST---TFRI-NQ-R---L--    143

CODY_BACSU      143 AEYGA-TVVGM-EIL----    155
                       |: |     ||
NREA_STACA      144 ---GSFT----DEI-NKQP    154
```

FIG.D6. (A) Structure of the GAF-domain of CodY (residues 1-167) from *B. subtilis.* The picture shows a homodimer of two GAF domains. The C- and N-terminal helices for dimerisation and the β-sheets for cofactor binding are indicated. Numbering of the α-helices corresponds to numbering in FIG.D7. This structure of CodY GAF-domain was calculated after protein crystalisation (from Levdikov, 2009). (B) Alignment of the CodY GAF-domain (residues 1-155) from *B. subtilis* and NreA from *S. carnosus* shows 22.8% amino acid identity and 36.1% similarity.

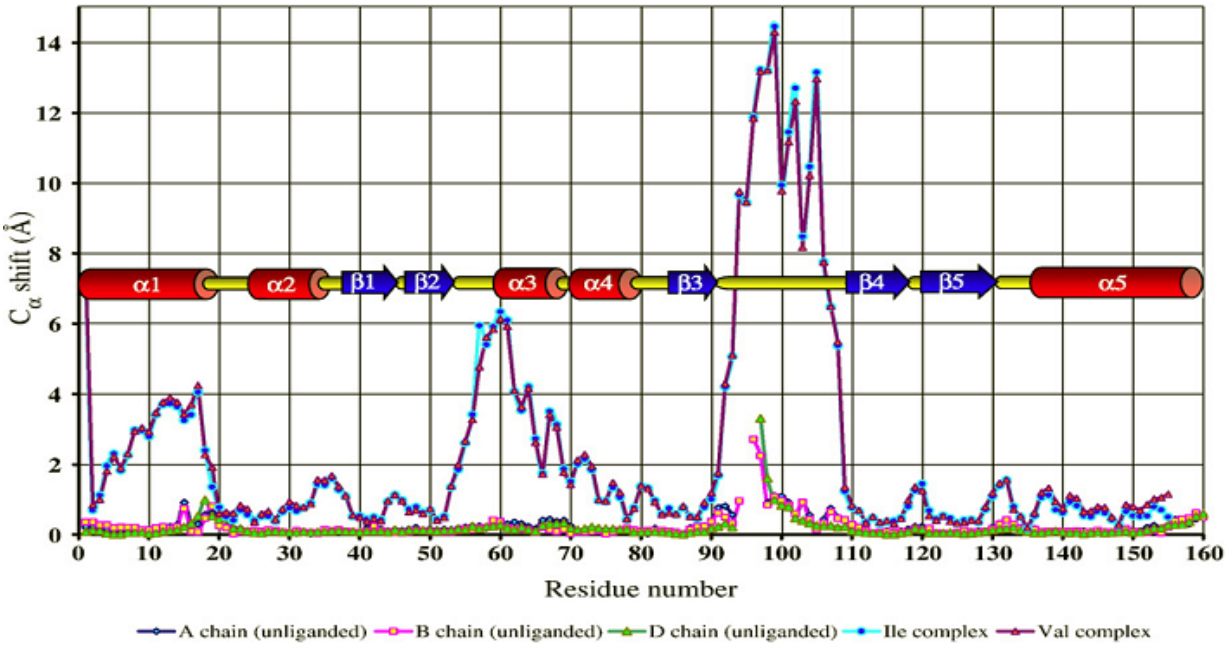


FIG.D7. Changes in secondary structure of CodY GAF domain from *B. subtilis.* Changes in secondary structure are shown after ligand binding as C_α-shift (in Å) in relation to topology. A comparison of the crystallised chains of the unliganded N-CodY structure with N-CodY-ILE and N-CodY-VAL structures. The C_α shift is plotted against residue number. Secondary structure elements as a function of residue number are indicated Most of the rearrangements take place in the β-sheets, which bind the cofactor GTP. The dimerisation helices α_1, α_2 and α_5 show no rearrangement, this is in agreement with the finding that dimerisation of CodY is independent of cofactor binding. (from Levdikov *et al.*, 2009).

5.3.2 Direct DNA binding of NreA

Electrophoretic mobility shift assays (EMSAs) with NreA and putative target DNA fragments (*narG*, *narT* and *nirB*) showed no direct binding of NreA to DNA. The experiments were conducted under aerobic or anaerobic conditions, at variable salt concentrations, and in presence or absence of nitrate. However, it is difficult to reproduce the *in vivo* conditions *in vitro* due to the complex redox situation in the cell and the wide array of potential cofactors. Therefore a possible DNA interaction under certain conditions *in vivo* cannot be ruled out.

Sequence alignments show that the DNA binding domain of CodY, including the serine residue which is important for regulation of DNA binding, is well conserved in NreA (FIG.D8). Residues located on one side of the putative helix are remarkable well conserved; the residues are spaced 3 to 4 amino acids apart from each other in

the primary amino acid sequence and located directly on top of each other in the secondary helix structure.

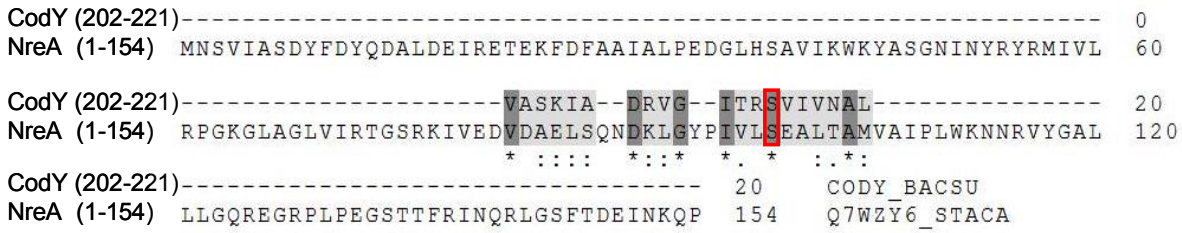


FIG.D8. Alignment of NreA from *S. carnosus* and the helix-turn-helix motif of CodY from *B. subtilis* (residues 202-221). Residues with a distance of 3.6 amino acids apart are positioned above each other in the helix. The position of the conserved serine residue 215, which is important for DNA-binding of CodY, is indicated.

5.3.3 NreA, a novel nitrate sensor in *S. carnosus*?

Protein crosslink studies with formaldehyde as chemical crosslinker showed that NreA and NreC each form crosslink products with the molecular mass of homodimers. This was expected for NreC, as DNA-binding proteins usually act as dimers or oligomers. Secondary structure predictions for NreA show one N-terminal helix and one C-terminal helix. It has been described previously that terminal helices of GAF domains play a role in protein dimerisation, e.g. in case of CodY (Sonenshine *et al*., 2009). Studies on NreA mutants, which do not form a homodimer in crosslink experiments, will be a useful addition to investigate if the physiological function of NreA depends on protein dimerisation.

In *E. coli* the expression of the *narGHJI* promoter is under control of a complex regulatory network, involving the oxygen sensor FNR and the regulator proteins NarL, NarP and IHF (Stewart, 1994). It can be assumed that the oxygen sensing NreBC system is not the only regulation system for *narGHJI* expression in *S. carnosus*. It is still unknown how the presence of nitrate is sensed and how nitrate influences the expression of genes involved in nitrate respiration in *S. carnosus*. GAF domains are known for binding small molecules, like GTP or amino acids (Levdikov *et al*., 2009), underlining the possible function of NreA as a nitrate or nitrite sensor. Crosslink experiments with formaldehyde as chemical crosslinker show that NreA

interacts with purified NreB and NreC. NreA together with NreB or NreC forms a crosslink product corresponding to a molecular mass of approx. 120 kDa, respectively, while the amount of NreA homodimer decreases significantly in presence of NreB or NreC. This shows that NreA is a third component interacting with the NreBC two-component system.

5.3.4 Sensing nitrate in *E. coli*: The NarXL system

In *E. coli* nitrate stimulates the autophosphorylation rate of NarX, which then phosphorylates the response regulator NarL to activate and repress target gene expression (Noriega *et al.*, 2008). NarX is a membrane bound histidine kinase that detects the presence of nitrate in the periplasm. The periplasmic domain of NarX contains 17 conserved residues, which are involved in nitrate sensing (Cavicchioli *et al*., 1996). NarX transfers this signal to the response regulator NarL, which then regulates gene expression in response to nitrate availability. Phosphorylated NarL activates the expression of the nitrate reductase *narGHJI* and the nitrate antiporter *narK*, while it represses the fumarate reductase genes *frdABCD* (Schröder *et al*., 1994).

In silico sequence analysis indicate that the NarXL system is also present in *P. aeruginosa* (lacking experimental proof), but it is missing in *P. denitrificans* and *S. carnosus*. It can be assumed that nitrate, which plays a major role in natural habitats of *S. carnosus* or *P. denitrificans*, has an impact on gene regulation. However, until now a nitrate sensor in those species and many gram-positive bacteria has not been identified.

5.3.5 The tri-component system NreABC

There is increasing evidence that histidine kinase sensors from traditional two-component systems employ accessory proteins as co-sensors (Tetsch *et al*., 2009). In *E. coli* the C_4-dicarboxylate transporter DcuB influences the histidine kinase DcuS under anaerobic conditions. In the absence of C_4-dicarboxylates, DcuB interacts with DcuS and prevents its autophosphorylation. In the presence of C_4-dicarboxylates, DcuB presumably acts as a transporter and releases DcuS which can now

phosphorylate the response regulator DcuR, inducing genes for fumarate reduction (Janausch *et al*., 2002).

In *S. carnosus* NreB phosphorylates the response regulator NreC under anaerobic conditions and NreC then induces expression of target genes for nitrate respiration. It can be speculated that in absence of nitrate, NreA interacts with NreB and / or NreC and prevents signal transduction (FIG.D9). This could be achieved by: (i) preventing the phosphate transfer from NreB to NreC, (ii) inhibiting DNA-binding of phosphorylated NreC, or (iii) dephosphorylation of activated NreC. It remains to be determined if there is a direct or indirect interaction between NreA and nitrate. Alternatively NreA with bound nitrate might stimulate the NreBC system to full activity.

In vitro phosphorylation of NreC resulted in decreased amount of monomeric NreA in crosslink assays. This could mean that NreA-NreC protein interaction is promoted by phosphorylation of NreC. However, confirmation of these findings is required.

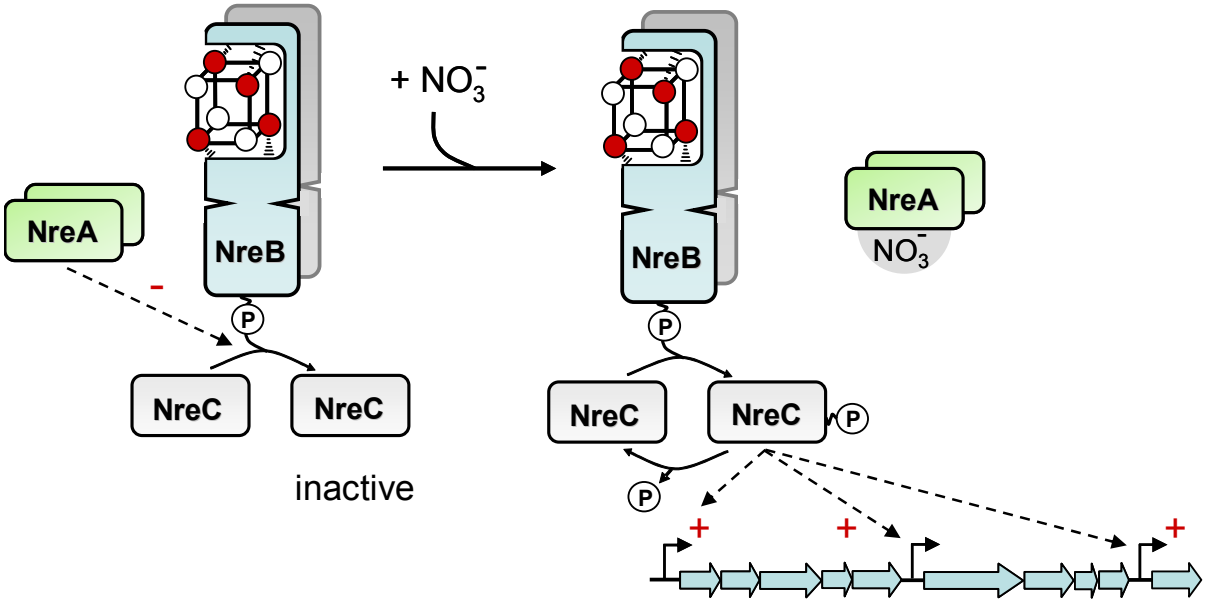


FIG.D9. Proposed scheme of NreA function in anaerobic cells of *S. carnosus*. In absence of nitrate NreA interacts with NreB and / or NreC and inhibits signal transduction of NreC. The molecular mechanism of NreA function remains to be determined. NreA could prevent the phosphate transfer from NreB to NreC by interacting with both proteins. In presence of nitrate NreA binds to nitrate and does not interact with NreB and / or NreC anymore, allowing induction of genes for nitrate respiration by phosphorylated NreC.

The NreA working model in FIG.D9 can explain why NreA deficient mutants of *S. carnosus* induce genes for nitrate respiration even in the absence of nitrate. In wildtype cells NreA interacts with nitrate directly or indirectly, preventing interaction with NreB or NreC and allowing induction of the genes for nitrate respiration under anaerobic conditions by phosphorylated NreC. This implies that in *S. carnosus* two different signal inputs are considered for complete induction of nitrate respiration genes. First, the absence of oxygen has to be detected by NreB, and secondly, the presence of nitrate has to be detected by NreA.

Recent experiments by S. Nilkens (2010) showed that deletion of the complete NreABC operon or mutation of one of the four Cys residues in NreB results in significantly decreased *narG* promoter activity, independent of oxygen and nitrate. However, the presence of an additional nitrate sensing system in *S. carnosus* cannot be ruled out and it is possible that a yet unknown nitrate sensor depends on the NreABC system for signal transduction.

List of abbreviations

aa	Amino acid(s)
Amp	Ampicillin
AMS	4-Acetamido-4´-maleimidylstilbene-2,2´-disulfonic acid
APS	Ammoniumpersulfat
ATP	Adenosin-triphosphat
BM	Basic medium
B. s.	*Bacillus subtilis*
BSA	Bovine serum albumin
Cam	Chloramphenicol
crp	cAMP receptor protein
Cys	Cysteine
DNA	Desoxyribonucleinacid
DTT	Dithiothreitol
E. c.	*Escherichia coli*
EDTA	Ethylendiamintetraacetat
EPR	Electron Paramagnetic Resonance
Ery	Erythromycin
Fig.	Figure
FNR	Fumarat-Nitrat-Reduktase-Regulator
h	Hours
HEPES	2-(4-(2-Hydroxyethyl)-1-piperazinyl)-Ethansulfonic acid
IAA	Iodoacetate
IPTG	Isopropyl-β-D-thiogalactopyranosid
kb	Kilo bases
kDa	Kilo Dalton
LB	Luria-Bertani
mBBr	Monobromobimane
min	Minutes
MOPS	N-morpholino propansulfonic acid
Mr	Standard atomic weight
NaPi	Natriumphosphat
NEM	N-Ethylmaleinimid
Nre	Nitrogen regulatior

OD	Optical density
PAB	Penassy broth
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
qBBr	Monobromo-trimethylammonio-bimane
RM	Rich medium
RT	Room temperature
S. c.	*Staphylococcus carnosus*
SDS-PAGE	Sodium-dodecyl-sulfate-polyacrylamid-gel-electrophoresis
SMM	Saccharose maleic acid $MgCl_2$
SMMP-B	Saccharose maleic acid $MgCl_2$ PAB - BSA
Tab.	Table
TAE	Tris acetat EDTA
TEMED	N,N,N',N'-Tetramethylethylendiamin
rpm	Rounds per minute
v/v	Volume per volume
w/v	Weight per volume
wt	Wildtype

7. Publications

Reinhart, F., Huber, A., Thiele, R., and Unden, G. 2010. Response of the oxygen sensor NreB to air *in vivo*: Fe-S-containing NreB and apoNreB in aerobically and anaerobically growing *Staphylococcus carnosus*. J. Bacteriol. **192**(1); 86-93

Reinhart, F., Achebach, S., Koch, T., and Unden, G. 2008. Reduced apo-fumarate nitrate reductase regulator (apoFNR) as the major form of FNR in aerobically growing *Escherichia coli.* J. Bacteriol. **190**(3); 879-886

Unden G., Müllner M., and Reinhart, F. 2010. Sensing of oxygen by bacteria. p.289-306 *In* Reinhard Krämer and Kirsten Jung (ed.) Bacterial Signaling, WILEY-VCH Verlag GmbH & Co., Weinheim, Germany, ISBN 978-3-527-32365-4

Unden, G., Müllner, M., and Reinhart, F. 2007. Eisen-Schwefel-haltige Sensoren aus Bakterien. Biospektrum. **13** 03/07, Spektrum Akademischer Verlag; 2-5

Oral presentations

Reinhart, F., Huber, A., Thiele, R., and Unden, G. 2010. Response of the oxygen sensor NreB to air *in vivo* in aerobically and anaerobically growing *Staphylococcus carnosus*. VAAM general convention 2010, Hannover

Poster presentations

Nilkens, S., Singenstreu, M., Reinhart, F., and Unden, G. 2010. Response of the tri-component system NreABC of *Staphylococcus carnosus* to oxygen and nitrate. Symposium on Gene Regulation in Procaryotes 2010, Neustadt a. d. Weinstraße

Reinhart, F., Müllner, M., Bill, E., and Unden, G. 2009. ApoNreB as the major form of the O_2-Sensor NreB in aerobically growing *Staphylococcus carnosus*. VAAM study group meeting on Gene Regulation 2009, Göttingen

Reinhart, F., Müllner, M., Bill, E., and Unden, G. 2009. The O_2-Sensor NreB of *Staphylococcus carnosus* contains a PAS domain with an oxygen sensitive $[4Fe4S]^{2+}$ cluster. VAAM general convention 2009, Bochum

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