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Membrane properties control the ATPase activity of the ABC transporter BmrA

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ABSTRACT

The structure and the function of membrane proteins can be affected by the lipid bilayer environment, yet its impact is often neglected in *in vitro* studies where proteins are typically analyzed in membrane mimetics, mostly liposomal systems. It has been observed that the activity of the bacterial ATP-binding cassette (ABC) transporter BmrA (*Bacillus* multidrug resistance ATP) differs when measured in detergent *vs.* a model membrane environment, indicating that the physico-chemical properties of the membrane environment crucially affect the protein's activity. We now performed a systematic analysis to elucidate the impact of individual lipid/membrane properties on the activity of BmrA and identified three parameters controlling the BmrA activity in lipid bilayers: (i) the hydrophobic thickness of the membrane, (ii) a negative surface charge, and (iii) the packing of lipids in the acyl-chain and head group regions. Our study provides valuable insights into how a specific lipid composition can influence the basal ATPase activity of BmrA and emphasizes that the lipid composition should be carefully selected in *in vitro* studies of membrane proteins.

1. Introduction

Integral membrane proteins are embedded in a dynamic and highly regulated lipid bilayer whose physico-chemical properties vary depending on the exact lipid composition. In eukaryotes, the exact lipid composition differs between the different cellular membrane systems, and in prokaryotes the spatio-temporal lipid composition is strongly regulated by the environment and various stressors [1,2]. Several studies have emphasized that the structure and activity of certain membrane proteins are affected by membrane lipids, and it is believed that membrane properties influence/regulate membrane proteins in general [3–6]. Individual lipid species can interact with the membrane protein either specifically (*e.g.* through hydrogen bonding or charge interactions) or non-specifically, due to combined physical bulk properties (membrane thickness, curvature, lateral pressure/stress and fluidity) if the bilayer is considered as a macrostructure [7–10]. For example, in the case of the human p24 transmembrane helix dimer, sphingomyelin binds to a specific binding site, which modulates the membrane protein's structure and activity [11,12]. The structure of the human glycophorin A transmembrane helix dimer also is dramatically affected by the physico-chemical properties of the lipid environment, including the hydrophobic thickness, fluidity and membrane curvature [13,14].

Depending on the lipid composition, the hydrogen bonding patterns between residues of the membrane protein and the lipid head groups can vary and influence the activity of more complex membrane proteins, as has *e.g.* been described for the rhomboid protease [15]. Also the activity of the Ca^{2+}-ATPase from the sarcoplasmic reticulum is regulated by different physical properties of the surrounding membrane, such as the hydrophobic thickness, fluidity and curvature [7]. Despite these (and many other) well-known examples, the native membrane environment is often neglected when the structure and/or activity of membrane proteins is studied. Even though studies in simple systems, such as detergent micelles, can provide important insights into the structure and function of membrane proteins, they typically obscure reality to some extent.

Members of the large superfamily of ATP-binding cassette (ABC) transporters catalyze the import or export of various substrates into cells, *e.g.* smaller ions, sugars, lipids and even larger biomolecules, such as antibiotics and anti-cancer drugs [16,17]. Driven by ATP hydrolysis, they enable substrate transport against a chemical gradient across a membrane [18]. ABC transporters are present in all kingdoms of life,

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and, although diverse in their respective substrate spectrum, they all share a common architecture [18,19]. A functional transporter consists of four entities: two transmembrane domains (TMDs) and two cytosolic nucleotide-binding domains (NBDs), whereby all four domains can be expressed either on one, two (one NBD is fused to one TMD) or individual polypeptide chains. While a standardized model of the transport mechanism for all ABC transporters remains elusive, and the existence of such a unifying model is highly controversial, the proposed models (ATP-switch, alternating site and constant contact) share common features [18]. These are that ATP binding occurs at the structurally conserved NBDs followed by their dimerization [18,20–22] and that the protein structure undergoes a transition from an "open" state - also called the inward-facing (IF) state, where the NBDs are separated and the protein has an overall V-shaped structure [23], to a "closed" (outward-facing, OF) state, where the NBDs are in close contact [18]. This structural rearrangement is coupled with the release of a bound substrate.

As the structure of the TMDs within the membrane significantly changes during a transport cycle and with that the overall conformation of the transporter, the activity of ABC transporters is likely to be influenced by (non-)specific interactions with membranes. In fact, it has been reported that lipid/membrane properties influence the activity and function of different ABC transporter. For example the basal ATPase activity of the maltose ABC transporter $MalFGK_2$ is decreased drastically in the presence of lipids with long acyl-chains (>C14), possibly due to the lipids affecting the motion of the TMDs [24]. Furthermore, the activity of the "transporter associated with antigen processing" (TAP) is stimulated by the lipid head groups of phosphatidylinositol (PI) and phosphatidylethanolamine (PE) [25]. However, it is difficult to distinguish whether lipids allosterically modulate a transporter function by binding directly to a specific binding site, act as a structural stabilizer of different conformations, chang the bulk membrane properties, or are "simply" a substrate of the transporter, as known for P-glycoprotein (P-gp) [26,27]. Yet, the impact of a membrane (system) on ABC transporters cannot be neglected, as some ABC transporter (*e.g.* P-gp or human ABCG1) do not exhibit a basal ATPase activity in detergents but in liposomal systems [28,29]. Typically, the ATPase activity of ABC transporters is further stimulated by substrate binding [30–32]. The detergent-solubilized *Bacillus* multidrug resistance ATP (BmrA) protein has a high basal ATPase activity already in the absence of substrates, yet, this activity appears to further increase when the protein is embedded in a lipid environment [33–36]. Due to this observation, we aimed at elucidating the effect of lipid and membrane properties on BmrA's function. We observed an increased basal ATPase activity of BmrA in membranes with higher hydrophobic thicknesses. Furthermore, high lateral pressure in the acyl-chain region and loose packing of the lipid head groups further stimulate BmrA. Finally, lipids with a negative head group charge significantly stimulate the basal ATPase activity of BmrA. Our observations nicely illustrate that membrane and lipid properties can significantly modulate the BmrA activity. As BmrA has a high, futile ATPase activity even in absence of substrates, we suggest that its activity is spatio-temporally controlled *in vivo* by its intimate membrane environment.

2. Experimental procedures

2.1. Cloning

The *bmrA* gene, encoding the full-length protein BmrA of *B. subtilis* strain 168, was cloned into the expression plasmid pET303-CT/His (Invitrogen, Carlsbad, USA) as recently described in [37]. The plasmid carrying the gene with the BmrA-SNAP-tag fusion protein was constructed *via* Gibson assembly [38]. The sequence for the SNAP-tag protein was amplified with appropriate overhangs to the target plasmid by PCR using the pSNAP-tag (T7)-2 vector (New England Biolabs GmbH, Ipswich, MA, USA) as a template. Additionally, a short $(GGS)_2$ linker was inserted between the BmrA and SNAP-tag sequences. Matching primers were used to amplify the target plasmid (pET303-CT/His-BmrA). The gene coding for a variant carrying eight amino acid replacements (BmrA 8xM) was cloned by eight consecutive site-directed mutagenesis affecting residues in the following order: R20A, R21A, K13A, K11A, K9A, K7A, K5A and K4A. All oligonucleotides used in this study are listed in Table 1. The corresponding plasmids, which contain a C-terminal His_6-tag, were used for heterologous expression of BmrA wt, BmrA-SNAP-tag and BmrA-8xM in *Escherichia coli* (*E. coli*).

2.2. Expression and purification

Full-length BmrA wt, BmrA-SNAP-tag and BmrA 8xM were heterologously expressed in *E. coli* C41(DE3) cells and solubilized from *E. coli* membranes with the detergent DDM (n-dodecyl β-D-maltoside) as described in detail in [37,39]. For proteoliposome formation, the buffer was exchanged to reconstitution buffer (50 mM Tris-HCl, 300 mM NaCl, 0.5 mM DDM, pH = 8.0) using a PD-10 column (Cytiva, Munich, GER). After determining the protein concentration *via* measuring the absorption at 280 nm, the protein concentration was adjusted to 15.5 μM ($\varepsilon_{BmrA\ wt}$ = 38,850 M^{-1} cm^{-1}, $\varepsilon_{BmrA\text{-}SNAP\text{-}tag}$ = 60,070 M^{-1} cm^{-1}, $\varepsilon_{BmrA\ 8xM}$ = 38,850 M^{-1} cm^{-1}; ExPASy ProtParam [40]). Aliquots of the proteins were stored at −20 °C until use.

2.3. Proteoliposome formation

All phospholipids used in this study (Table 2) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). The organic solvent ($CHCl_3$) of a total of 1.25 μmol pure lipids or lipid mixtures was dried under a gentle nitrogen flow. The lipid film was kept under vacuum overnight to ensure complete solvent evaporation. Dried lipids were solubilized in rehydration buffer (50 mM Tris-HCl, pH = 8.0) at 37 °C for 30 min to a final concentration of 5 mM. Subsequently, large unilamellar vesicles (LUVs) were formed by four freeze-thaw cycles (in liquid nitrogen) of the lipid suspension. The liposomes were destabilized by addition of (amount as indicated in Table S1) Triton™ X-100 (Sigma-Aldrich Chemie GmbH, Taufkirchen, GER). Subsequently, destabilized liposomes were incubated with 1 mg/mL purified protein in reconstitution buffer (ratio 333:1 lipid/protein) at 37 °C for 30 min. Proteoliposome formation was achieved *via* slow detergent removal. Therefore, the protein samples were incubated consecutively four times with 25 mg (for 500 μL proteoliposome dispersion) fresh Bio-Beads SM-2 (Bio-Rad, Munich, GER) at 4 °C for 30 min, 1 h, overnight and again 1 h. Potentially aggregated proteins were removed from the formed proteoliposomes by centrifugation at 13,000*g* for 10 min. Finally, proteoliposomes were collected by ultra-centrifugation at 285,000 *g* for 1 h and resuspended in reaction buffer (50 mM Hepes-KOH, pH = 8.0). To verify proteoliposome formation, the supernatant and the same volume of resuspended pellet after ultra-centrifugation were analyzed on 10% SDS-PAGE gels and gels were stained after electrophoresis with Coomassie brilliant blue R520. The total protein concentration of proteoliposomes was determined using the commercially available Pierce™ bicinchoninic acid (BCA) protein assay kit (Thermo Scientific™, Schwerte, GER). As recommended by the manufacturer, a 2% SDS solution was added to the proteoliposomes beforehand. ATPase activity measurements or SNAP-tag labeling was carried out at the same day or at the latest after 2 days while the proteoliposomes were stored at 4 °C.

2.4. ATPase activity assay

The ATPase activity of detergent-purified BmrA wt was measured *in vitro* using a ATP regenerative system in buffer containing 5 mM DDM as described [37,39]. The ATPase activity of BmrA wt and BmrA 8xM in proteoliposomes was measured in the absence of detergent. Where indicated, purified or membrane-reconstituted protein was pre-incubated with 0, 1, 2, 4, 8 or 12 mM 2-phenylethanol (PhEtOH) for

Table 1
Oligonucleotides used in this study. Bases in classic font represent the bases from the original plasmid pET303-CT/His-BmrA and bases highlighted in bold belong to the pSNAP-tag (T7)-2 vector. Sequences coding for the short $(GGS)_2$-linker are underlined.

Primer	5′-Sequence-3′
pET303-CT/His-BmrA-fw	**GCAGGCGGATCCCCCGGGCTCGAGGTTAAT**GAATTCCACCACCACCAC
pET303-CT/His-BmrA-re	CTTAGAAAACAAAGCCGGGGGTGGAAGTGGAGGATCA**GACAAAGATTGCGAAATG**
pSNAP-tag (T7)-2-fw	AACAAAGCCGGGGGTGGAAGTGGAGGATCA**GACAAAGATTGCGAAATGAAAC**
pSNAP-tag (T7)-2-re	ATCTCAGTGGTGGTGGTGGTGGTGGAATTC**ATTAACCTCGAGCCCGGGGGATCC**
BmrA-R20A-fw	CTTTTTTGCCTTAGTAGCCCGGACCAATCCTTC
BmrA-R20A-re	GAAGGATTGGTCCGGGCTACTAAGGCAAAAAAG
BmrA-R21A-fw	CTTTTTTGCCTTAGTAGCCGCCACCAATCCTTCTTATG
BmrA-R21A-re	CATAAGAAGGATTGGTGGCGGCTACTAAGGCAAAAAAG
BmrA-K13A-fw	CTAAAAGTAAATTGGCCCCCTTTTTTGCCTTAG
BmrA-K13A-re	CTAAGGCAAAAAAGGGGGCCAATTTACTTTTAG
BmrA-K11A-fw	CAAAAATCTAAAAGTGCCTTGGCCCCCTTTTTTG
BmrA-K11A-re	CAAAAAAGGGGGCCAAGGCACTTTTAGATTTTTG
BmrA-K9A-fw	CAAGAAACAAAAATCTGCCAGTGCCTTGGCCC
BmrA-K9A-re	GGGCCAAGGCACTGGCAGATTTTTGTTTCTTG
BmrA-K7A-fw	CAACCAAGAAACAAGCCTCTGCCAGTGCC
BmrA-K7A-re	GGCACTGGCAGAGGCTTGTTTCTTGGTTG
BmrA-K5A-fw	GCCAACCAAGGCCCAAGCCTCTGCC
BmrA-K5A-re	GGCAGAGGCTTGGGCCTTGGTTGGC
BmrA-K4A-fw	CTAGAATGCCAACCGCCGCCCAAGCCTCTGC
BmrA-K4A-re	GCAGAGGCTTGGGCGGCGGTTGGCATTCTAG

Table 2
Lipids and lipid compositions investigated in this study.

Lipids/extract		Lipid composition	
IUPAC nomenclature	Abbreviation	% studied	% of background lipid
1,2-dioleoyl-sn-glycero-3-phosphocholine	DOPC (18:1PC)	–	Background lipid
1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol)	DOPG (18:1PG)	10–100 %	0–90 % DOPC
1,2-dioleoyl-sn-glycero-3-phospho-L-serine	DOPS (18:1PS)	10–100 %	0–90 % DOPC
1,2-dioleoyl-sn-glycero-3-phosphoethanolamine	DOPE (18:1PE)	10–70 %	30–90 % DOPC **or** const. 30 % DOPG + 0–70 % DOPC
1′,3′-bis[1,2-dioleoyl-sn-glycero-3-phospho]-glycerol	CL	10–70 %	30–90 % DOPC
1,2-dimyristoleoyl-sn-glycero-3-phosphocholine	14:1PC	100 %	–
1,2-dipalmitoleoyl-sn-glycero-3-phosphocholine	16:1PC	100 %	–
1,2-dieicosenoyl-sn-glycero-3-phosphocholine	20:1PC	100 %	–
1,2-dierucoyl-sn-glycero-3-phosphocholine	22:1PC	100 %	–
1-palmitoyl-2-oleoyl-glycero-3-phosphocholine	POPC (16:0–18:1PC)	10–100 %	0–90 % DOPC
E. coli polar lipid extract	EPL	~70 % PE, ~20 % PG, ~10 % CL	–

30 min at 25 °C. Measurements were carried out with 0.05 mg/mL protein (in DDM micelles or proteoliposomes) at 25 °C in reaction buffer with 3.5 mM Na_2ATP, 10 mM $MgCl_2$, 0.28 mM NADH, 2 mM phosphoenolpyruvate, 6–10 units/mL pyruvate kinase and 9–14 units/mL lactate dehydrogenase (Sigma-Aldrich, Merck KGaA (Darmstadt, GER). NADH consumption was followed *via* measuring the absorbance at 340 nm over 5 min and finally converted to the ATPase activity in [μmol/min·mg] *via* Eq. (1) using the extinction coefficient of NADH (ε_{NADH} = 6220 M^{-1} cm^{-1}) and a pathlength of d = 1 cm.

$$ATPase\ activity = -\frac{\Delta A_{340\ nm}}{\Delta t}\cdot\frac{1}{d \bullet \varepsilon_{NADH} \bullet c_{Protein}} \quad (1)$$

2.5. Analysis of protein orientation in proteoliposomes

BmrA-SNAP-tag containing proteoliposomes were prepared as described and split into three samples. The samples (each 25 μg proteoliposomes) were either labeled with a 2-times molar excess of SNAP-Surface® Alexa Fluor® 488 (New England Biolabs GmbH, Ipswich, MA, USA), or of SNAP-Cell® 647-SiR (New England Biolabs GmbH, Ipswich, MA, USA) or with both sequentially. The labeling was carried out in reaction buffer with 1 mM DTT for 1 h at room temperature in the dark. To each sample, SDS-PAGE sample buffer was added to a final concentration of 50 mM Tris-HCl (pH 6.8), 10% (*v*/*v*) glycerol, 2% SDS (*w*/*v*) and 0.04% (w/v) bromophenol blue and the samples were afterwards stored at −20 °C. 10 μL of the labeled proteoliposome samples were separated on a 10% SDS-PAGE gel and immediately after electrophoresis the in-gel fluorescence of SNAP-Surface® Alexa Fluor® 488 (blue excitation, 526 nm-short pass emission filter) and afterwards of SNAP-Cell® 647-SiR (green excitation, 670 nm-bandpass emission filter) was analyzed with the Typhoon Trio + (Amersham Biosciences, Amersham, GBR). Fluorescence signals were quantified using ImageJ [41]. Gels were afterwards stained with Coomassie brilliant blue R520. The protein orientation was estimated by setting the signal of all labeled proteins with SNAP-Cell® 647-SiR to 100% and subtracting the signal of only inside-oriented protein and the background signal.

2.6. Laurdan fluorescence spectroscopy

DOPC/DOPG (50/50) LUVs containing Laurdan in a molar ratio of 1:500 (Laurdan: lipid) were prepared as described above and used for proteoliposomes preparation. Laurdan-containing proteoliposomes were incubated for 30 min with 0, 1, 2, 4, 8 or 12 mM PhEtOH at 25 °C. Subsequently, Laurdan fluorescence emission spectra of 0.25 mM proteoliposomes (lipid concentration) were measured in triplicate using a FluoroMax-4 fluorescence spectrometer (Horiba Scientific, Kyoto, JPN) at 25 °C. Spectra were recorded between 400 and 550 nm upon excitation at 350 nm. The excitation and emission slits were set to 1 nm and 5 nm, respectively. Changes in the Laurdan fluorescence emission spectra were quantified by calculating the Generalized Polarization (GP) value *via* Eq. (2) using Laurdan's fluorescence emission at 440 and 490 nm [42].

$$GP\ value = \frac{I_{440} - I_{490}}{I_{440} + I_{490}} \quad (2)$$

3. Results

3.1. The membrane environment affects the BmrA ATPase activity

While it has been observed that the activity of the bacterial ABC transporter BmrA differs when measured in detergent *vs.* a model membrane environment [33–36], indicating that the physico-chemical properties of the membrane environment crucially affect the protein's activity, it still remains unclear which lipid and/or membrane properties exactly control the BmrA activity. Hence, we reconstituted BmrA purified in DDM micelles into liposomes with defined lipid compositions to elucidate in detail the impact of specific membrane lipid and bilayer properties on the (ATPase) activity of BmrA. Since BmrA was heterologously expressed and is highly active in *E. coli* membranes [33,36], we first prepared BmrA-containing proteoliposomes using *E. coli* polar lipid (EPL) extract (Fig. 1A, B) and determined the ATPase activity of BmrA in DDM-micelles and BmrA reconstituted into EPL liposomes (Fig. 1C, D). When dissolved in DDM micelles, BmrA had an ATPase activity of 0.14 ± 0.02 μmol/min*mg (k_{cat} = 0.16 ± 0.02 1/s). In contrast, when BmrA was embedded into an EPL membrane, the ATPase activity increased >3-fold to 0.50 ± 0.05 μmol/min*mg (k_{cat} = 0.55 ± 0.06 1/s). Based on these observations it is obvious that membrane and/or lipid properties control the BmrA activity. Depending on the orientation of the protein

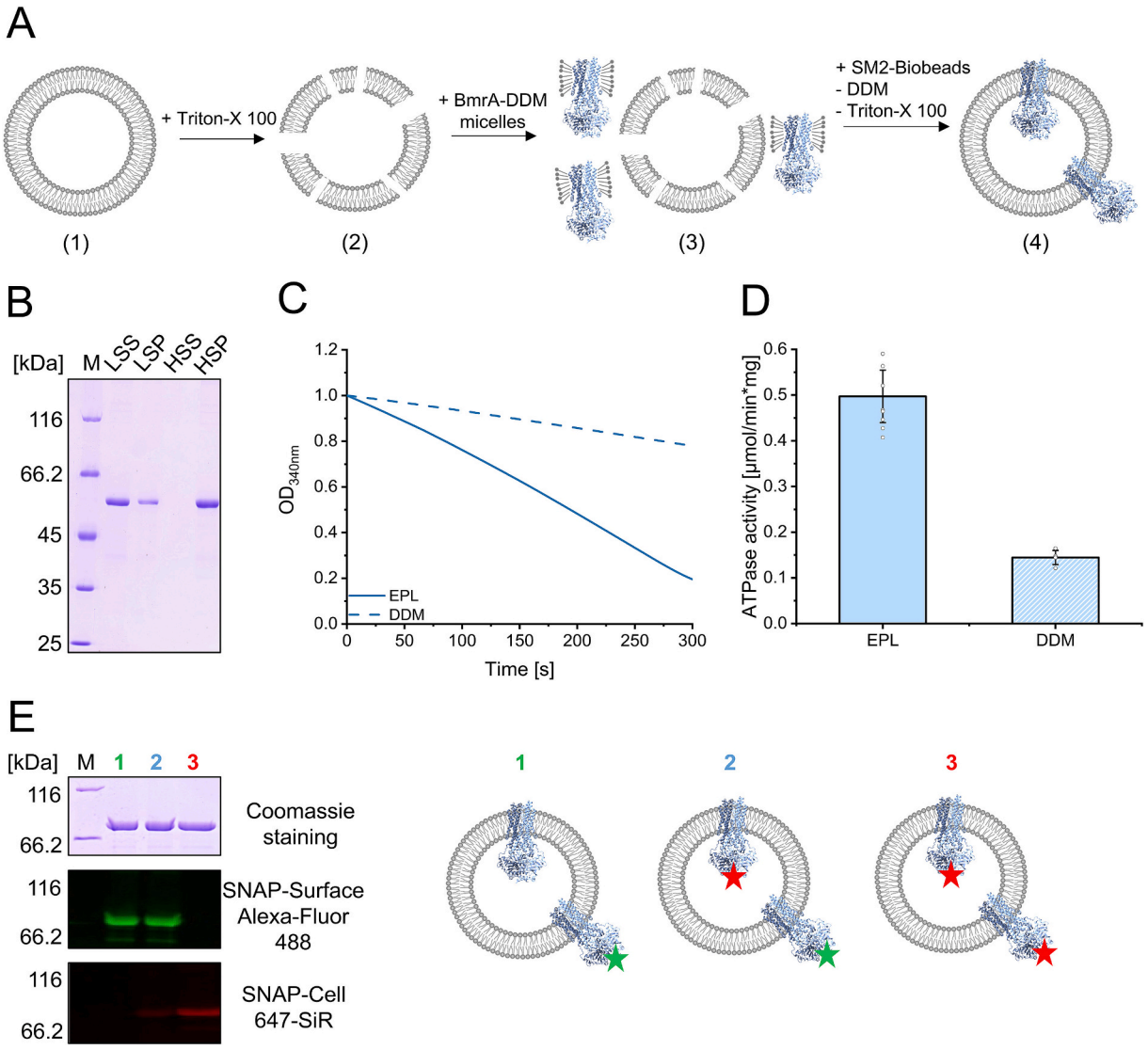

Fig. 1. Preparation, ATPase activity and topology of BmrA in EPL proteoliposomes.
(A) Schematic overview of proteoliposome preparation. Preformed large unilamellar vesicles (LUVs) (1) were destabilized by addition of Triton-X 100 (2) and afterwards incubated with BmrA in DDM-micelles (3). Slow removal of detergent with SM2-Biobeads led to proteoliposome formation (4). (B) SDS-PAGE analysis of the protein during reconstitution. The proteoliposome fraction (LSS = low speed supernatant) was separated from potentially aggregated protein and multilamellar vesicles (MLVs) by centrifugation (13,000 *g*, 10 min) (LSP = low speed pellet). Finally, reconstitution was verified by ultra-centrifugation (285,000 *g*, 1 h), where proteoliposomes (HSP = high speed pellet) were separated from non-reconstituted protein (HSS = high speed supernatant). M = Marker. (C) ATPase activity of BmrA measured in DDM-micelles (dashed line) and reconstituted in EPL liposomes (solid line) *via* following the NADH consumption at 340 nm. (D) Mean ATPase activity of BmrA in DDM-micelles (n = 4, ± SD) and reconstituted in EPL liposomes (n = 7, ± SD). Single datapoints of independent proteoliposome preparations are shown. (E) Orientation of BmrA-SNAP-tag in EPL proteoliposomes. BmrA-SNAP-tag reconstituted into EPL liposomes was labeled either (1) with the membrane impermeable SNAP-tag dye (SNAP-Surface® Alexa Fluor® 488), (2) subsequently also with the membrane permeable SNAP-tag dye (SNAP-Cell® 647-SIR) or (3) only with the membrane permeable dye. Afterwards, labeled proteins were separated on a 10% SDS-PAGE gel and the in-gel fluorescence was determined, followed by Coomassie-staining. M = Marker.

within the liposomal membrane, the measured activities might even be underestimated, as proteins with the NBDs localized inside the liposomes would not be able to hydrolyze any ATP added from the outside (compare Fig. 1A). Nevertheless, as the NBD is the sole large soluble protein domain, a preferred orientation with the NBDs orientated outwardly was expected. To experimentally determine the orientation of BmrA, we reconstituted a purified chimeric BmrA protein with a SNAP-tag fused to the C-terminal end of the BmrA NBD into EPL liposomes. A membrane permeable plus an impermeable SNAP-tag substrate was used for labeling the different possible orientations of the reconstituted protein, with the membrane-permeable substrate labeling all proteins whereas the membrane-impermeable substrate exclusively labels BmrA with an NBD_{out} orientation. Based on the fluorescence signals we calculated that around 93% of all proteins are oriented in an NBD_{out} orientation (Fig. 1E), indicating that the measured ATPase activity in EPL proteoliposomes is only slightly underestimated. For each lipid composition tested in this study, the majority of all proteins (93–96 %) were oriented NBD_{out} (Fig. S1).

3.2. BmrA is more active in thicker membranes

In contrast to biological membranes, the hydrophobic core of micellar systems is more adjustable to the hydrophobic thickness of a detergent-dissolved membrane protein. The hydrophobic thickness of a membrane depends on the lipid acyl chain lengths, and often a membrane protein's activity is highest when the thickness of the hydrophobic membrane core matches the hydrophobic thickness of the protein [43]. Because of this and as a basis for subsequent analyzes in model membrane systems, we next tested whether the membrane thickness impacts the BmrA activity. For this purpose, BmrA-containing proteoliposomes were prepared using the following PC lipids: 14:1, 16:1, 18:1 (DOPC), 20:1 and 22:1 (Fig. S2). Although *B. subtilis* and *E. coli* membranes do not naturally contain DOPC, we still used lipids with a PC head group, since these lipids were available with different carbon chain lengths (14–22). Importantly, all lipids have transition temperatures far below 25 °C, where the ATPase activity was measured, and *i.e.* all membranes were in a fluid state. In thinner membranes (14:1PC/16:1PC), BmrA had a low ATPase activity (Fig. 2), which was even below the activity measured in DDM micelles (Fig. 1C/D). However, when reconstituted into membranes composed of PC lipids with longer acyl chains (18:1–22:1), the ATPase activities significantly increased, reaching the activity measured in DDM micelles (Fig. 2). As no differences in the determined ATPase activities were observed between lipids with longer acyl chains (18:1–22:1), BmrA appears to tolerate a certain thickness range. However, when comparing the BmrA ATPase activities determined in C18:1–C22:1 PC proteoliposomes with the activity determined in EPL liposomes (Fig. 1C/D), it becomes evident that in addition to the hydrophobic thickness other membrane properties appear to further stimulate the BmrA activity.

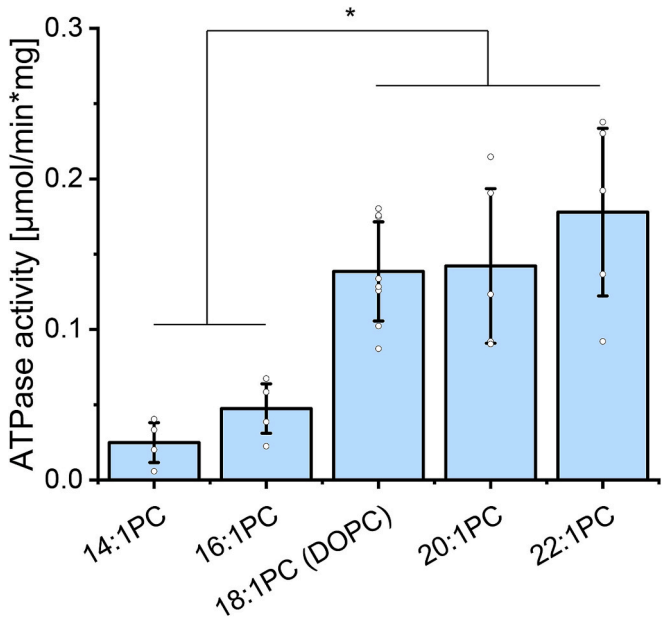

Fig. 2. ATPase activity of BmrA proteoliposomes in membranes with increasing thickness.
BmrA was reconstituted into liposomes containing PC lipids with increasing acyl chain length (14:1PC – 22:1PC). Shown are the mean ATPase activities and the single datapoints of independent proteoliposomes preparations ($n > 4$, ± SD, two-sample *t*-test, $p < 0.05$).

3.3. Negatively charged lipids modulate the BmrA ATPase activity

Since the lipid species in natural membranes do not only differ in their acyl chain length but also in their head group chemistry, we next analyzed the impact of different lipid species on the BmrA activity. EPL, where BmrA is highly active, contains about 70% PE, as well as 20% and 10% of the negatively charged lipids PG and CL, respectively.

Due to its unfavorable head group-to-acyl chain volume ratio, the major lipid species PE is a non-bilayer forming lipid causing negative curvature stress in biomembranes. As curvature stress can affect a membrane protein's activity [7,44], we first mixed the non-bilayer forming lipid PE with the bilayer-forming lipid PC in different ratios and determined BmrA's ATPase activity. Based on our previous results (Fig. 2), we chose 18:1PC (DOPC), a typical zwitterionic and bilayer-forming lipid, as a background lipid and varied the relative amount of DOPE.

Up to a PE content of 70%, where the lipid bilayers were still stable, no statistically significant changes in the BmrA ATPase activity were detected (Fig. 3A), indicating that stored curvature elastic stress does not affect the BmrA activity, at least not in pure PC/PE membranes (compare 3.4).

Next, we investigated a possible dependence of the BmrA ATPase activity on the negative lipid head group charge, again using C18:1-based lipids with different head groups. In pure DOPC liposomes, BmrA has an ATPase activity of 0.14 ± 0.03 µmol/min*mg (Fig. 3B). Yet, when the DOPG content was increased from 0 to 50%, the ATPase activities constantly increased up to 0.49 ± 0.10 µmol/min*mg, a value similar to the activity determined in EPL proteoliposomes, whereas further addition of DOPG (up to 100%) led to a subsequent decrease in the ATPase activity (Fig. 3B). To analyze whether the observed initial activity increase was specific for the DOPG head group or due to the general increase in negative membrane surface charge, we next replaced DOPG by DOPS, a negatively charged lipid not naturally present in *E. coli* or *B. subtilis* membranes in significant amounts. Similar to the previous experiment, the ATPase activities increased when the DOPS content was raised from 0 to 50%, reaching an ATPase activity of 0.39 ± 0.05 µmol/min*mg at 50% DOPS, and the activity was again reduced upon further DOPS addition (Fig. 3C). These observations strongly suggest that the ATPase activity is modulated by the negative membrane surface charge. In line with this, when introducing CL, which carries two negative charges, into DOPC liposomes, the highest ATPase activity (0.39 ± 0.09 µmol/min*mg) was already reached when about 30% CL was present and thereafter the activity decreased again (Fig. 3D). Noteworthy, as pure CL membranes are not stable, we determined the BmrA ATPase activities only until a CL content of 70%. Overall, our results clearly suggest that a negative lipid head group charge modulates the ATPase activity of BmrA and a maximal ATPase activity is reached when BmrA is reconstituted into liposomes containing about 50% lipids with a single negative charge.

When embedded into a membrane, the first N-terminal helix (often referred to as the elbow helix) of BmrA is in direct proximity to the lipid head groups (Fig. 4A). The 21 amino acid long elbow helix contains

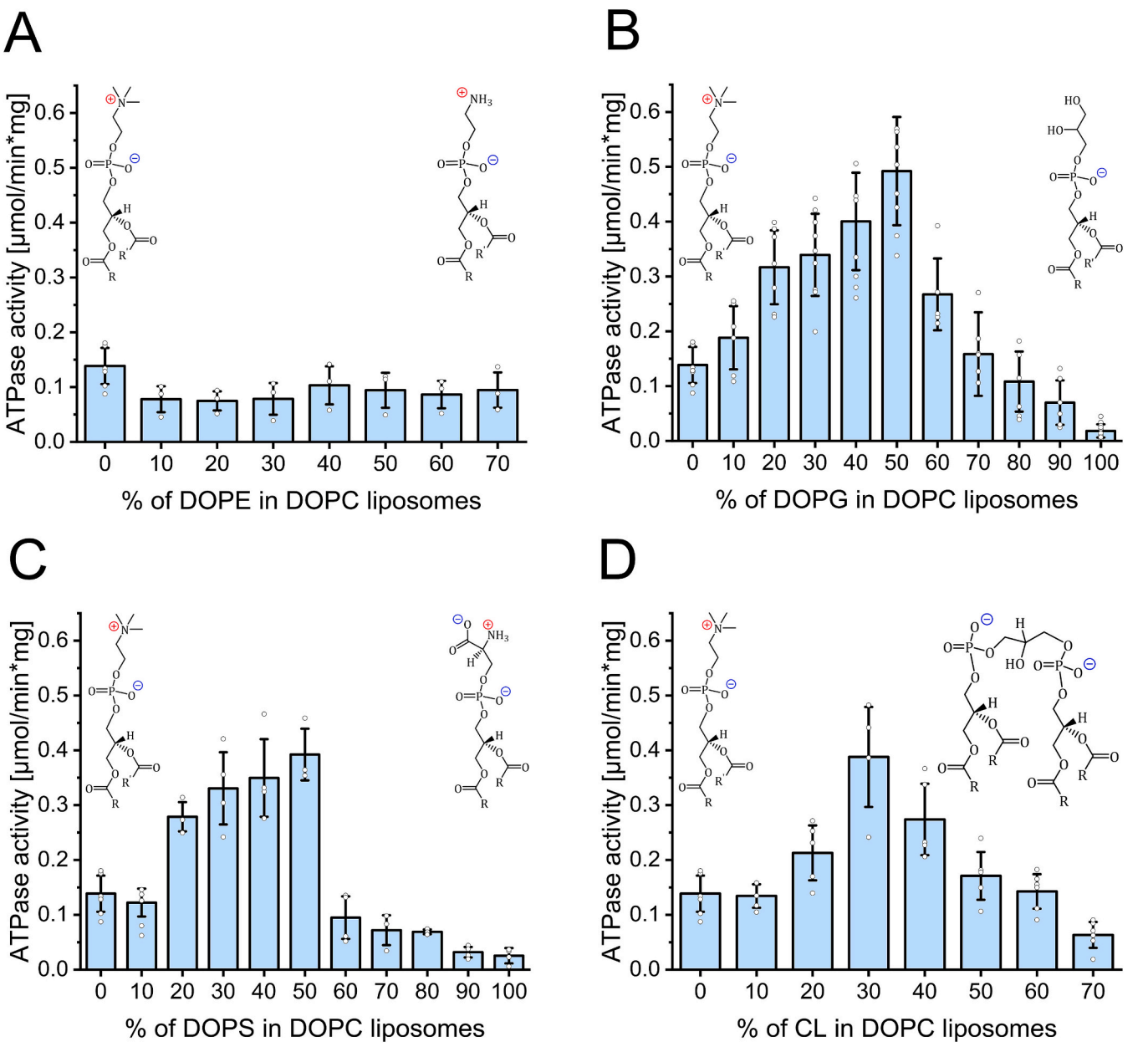

Fig. 3. ATPase activity of BmrA in proteoliposomes containing negatively charged lipids.
BmrA was reconstituted into DOPC liposomes with increasing amount of (A) DOPE (0–70%), (B) DOPG (0–100%), (C) DOPS (0–100%) or (D) CL (0–70%). Shown are the mean ATPase activities and the single datapoints of independent proteoliposome preparations (DOPE/DOPC and DOPS/DOPC: $n > 3$, DOPG/DOPC: $n > 5$ and CL/DOPC: $n > 4$, ± SD). Head group structures of DOPC/DOPE in (A), DOPC/DOPG in (B), DOPC/DOPS in (C) and DOPC/CL in (D) are also shown.

eight positively charged amino acids, which could potentially interact electrostatically with a negatively charged membrane. To test this assumption, we created a BmrA variant lacking all eight positive charges by exchanging them to Ala. Yet, when reconstituted into EPL proteoliposomes, BmrA 8xM displayed a wt-like ATPase activity of 0.44 ± 0.10 µmol/min*mg (Fig. 4B). This indicates that the positively charged amino acids of this helix do not modulate the BmrA activity by electrostatic interactions with the membrane.

3.4. High lateral pressure in the acyl chain region and loosely packed lipid head groups stimulate the BmrA ATPase activity

Not only the lipid head group chemistry, but also more global membrane properties, such as the lateral pressure, curvature elastic stress and/or the membrane fluidity can significantly affect a membrane protein's function. As PE, the major lipid in *E. coli* membranes, has a conical shape with a small head group, it imposes negative curvature stress to membranes [7,44]. Yet, in the absence of negatively charged lipids no changes in the BmrA ATPase activities were observed when the DOPE content was increased (Fig. 3A).

To further study the potential influence of curvature elastic stress on the BmrA activity, we next selected the bilayer-forming, net-uncharged lipid DOPC together with a constant amount of 30% DOPG as a background lipid to stimulate the ATPase activity of BmrA (Fig. 3B). An amount of only 30% of bilayer-forming DOPG ensures that an addition of up to 70% of DOPE, *i.e.* replacing DOPC by DOPE, can be achieved. Due to the DOPE structure with a small volume of the head group region relative to the volume of the acyl-chain region, the molecule influences the lateral lipid packing, and lipids are tighter packed in the acyl chain region whereas the relatively small head group causes weak packing in

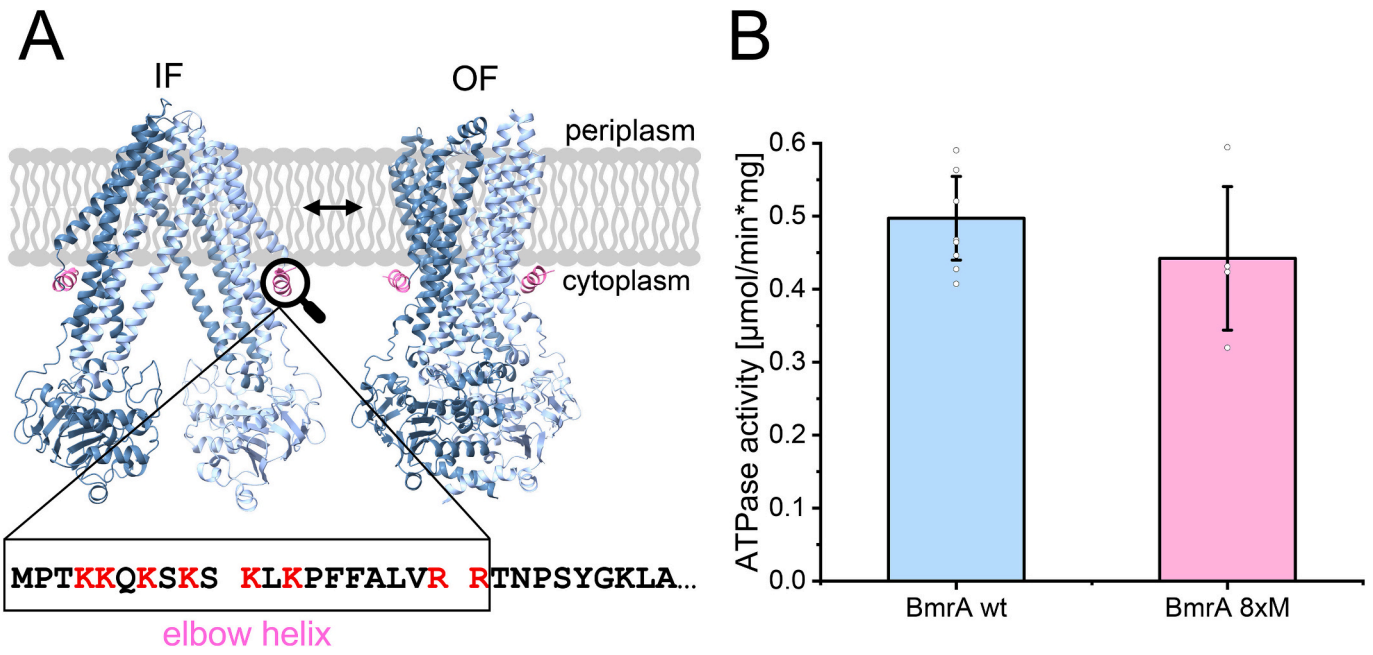

Fig. 4. Positively charged amino acids in the elbow helix of BmrA do not interact crucially with the negative charged membrane surface.
(A) IF [8QOE, [1]] and OF [7OW8] conformation of a BmrA dimer (single monomers in light and dark blue) in a lipid bilayer. The N-terminal elbow helix (residues 1–21) lies perpendicular below the membrane surface and is highlighted in pink. An enlarged view onto the amino acid sequence of the elbow helix is given and the positively charged amino acids, that were exchanged to Ala in BmrA 8xM, are highlighted in red. (B) Mean ATPase activities of reconstituted BmrA wt (blue, n = 7, ± SD) and BmrA 8xM (K4A/K5A/K7A/K9A/K11A/K13A/R20A/R21A, pink, n = 4, ± SD) determined in EPL liposomes. Single datapoints of independent proteoliposome preparations are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the head group region [44].

When increasing the DOPE content in DOPC/DOPG liposomes from 0 to 40%, the ATPase activity did not significantly change (Fig. 5A). Yet, above 50% DOPE, the ATPase activity increased significantly, reaching the value observed with 50/50 DOPC/DOPG in the previous experiment (Fig. 3B), indicating a stimulating effect of DOPE on the (pre-stimulated) ATPase activity of BmrA, caused by the increased lateral membrane pressure in the acyl chain region or the reduced packing in the lipid head group region (or both).

To separate these packing effects, we next analyzed the impact of acyl-chain packing using BmrA proteoliposomes containing DOPC plus increasing amounts of POPC. The latter contains a saturated and an unsaturated acyl-chain, *i.e.* increasing amounts of POPC led to a reduced lateral pressure in the acyl-chain region whereby the head group regions remained unaffected. With increasing amounts of POPC the BmrA ATPase activity steadily decreased (Fig. 5B), again suggesting that a more tightly packed membrane inhibits the ATPase activity of BmrA. Note that one acyl-chain of POPC is shorter (C16) compared to the other acyl-chain (C18), which might also influence the activity of BmrA, as we showed in Fig. 2.

Next, we focused on the lipid head group packing and examined the (pre)stimulated ATPase activity of BmrA proteoliposomes (DOPC/DOPG: 50/50) upon addition of increasing concentrations of the alcohol PhEtOH (0–12 mM). PhEtOH is known to incorporate into the lipid bilayer at the interface between the polar and the unpolar membrane regions, which disturbs the membrane organization in the lipid head group region, yet not in the hydrophobic membrane core, resulting in an increased membrane fluidity [45]. At first, we investigated changes in the membrane fluidity upon addition of 0–12 mM PhEtOH to BmrA-containing DOPC/DOPG (50:50) proteoliposomes using the fluorescent probe Laurdan. While the membrane was already quite fluid in the absence of PhEtOH (Fig. 5C), further increasing the PhEtOH concentration led to an increased fluidity, as indicated by the decreasing GP value. Also, upon PhEtOH addition BmrA's ATPase activity in proteoliposomes increased by a factor of 1.5 from zero to the highest PhEtOH concentration studied (Fig. 5D, black). Importantly, PhEtOH had no significant impact on the ATPase activity of BmrA in DDM micelles (Fig. 5D, blue), indicating that PhEtOH does not directly stimulate the BmrA activity, at least when assuming that PhEtOH does not differently interact with BmrA in detergent *vs.* liposomes. Thus, the observed effect appears to be membrane-mediated. Taken together, our results emphasize that both high lateral pressure in the acyl-chain region and weaker lipid head group packing stimulates the ATPase activity of BmrA.

4. Discussion

The activity of membrane proteins might be controlled by three key features; its (proper) structure, structural dynamics and the natural environment [5]. Yet, especially the natural membrane environment is often neglected in biochemical and biophysical *in vitro* analyzes of membrane protein's structure and activity [46], albeit several recent observations have emphasized the importance of membranes and lipids for proper functioning of some membrane proteins [4–8,43,44,46]. However, in most cases the exact impact of a defined lipid environment on the activity of a membrane protein cannot be simply predicted, except when lipids are tightly bound to an integral part of a protein (complex) structure [26,47,48]. Typically, the optimal lipid environment has to be experimentally tested, and the lipid composition of the membrane the protein naturally resides in, might be a good starting point. Nevertheless, as the membrane lipid composition might depend on the exact growth conditions, especially in bacteria, there might not be "the one" typical lipid environment, rather membrane proteins have to function in a certain range of lipid compositions and/or membrane properties. Furthermore, changing the intimate lipid environment might be used by organisms to control the activity of environment-sensitive membrane proteins.

Here, we analyzed in proteoliposomes with defined lipid compositions the impact specific lipids and membrane properties have on the activity of the bacterial ABC transporter BmrA. Of note, additionally to

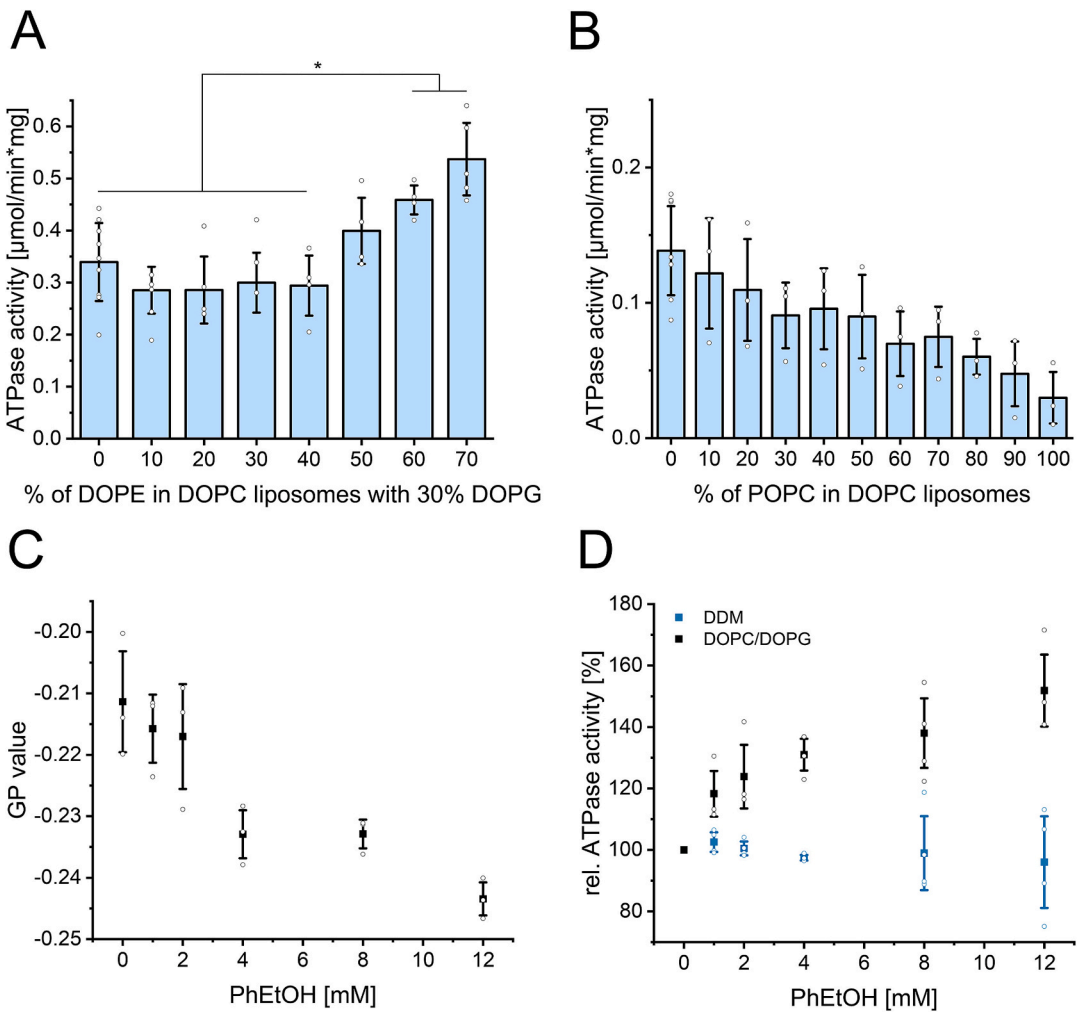

Fig. 5. ATPase activity of BmrA proteoliposomes in membranes with varying fluidity.
BmrA was reconstituted into DOPC liposomes containing (A) increasing amounts of DOPE (0–70%) and a constant content of 30% DOPG and (B) increasing amounts of POPC (0–100%). Shown are the mean ATPase activities and the single datapoints of independent proteoliposome preparations (DOPE/DOPG/DOPC: $n > 4 \pm$ SD, two-sample *t*-test, $p < 0.05$, POPC/DOPC: $n > 3 \pm$ SD). (C) Fluidity of DOPC/DOPG (50/50) proteoliposomes containing reconstituted BmrA and the fluorescence probe Laurdan in presence of 0–12 mM PhEtOH. Mean GP values were calculated from the Laurdan fluorescence emission spectra of independent proteoliposome preparations ($n = 3$, ± SD) and shown with single datapoints. (D) Mean relative ATPase activities of BmrA in DDM-micelles ($n > 3$, ± SD, blue) and reconstituted in DOPC/DOPG (50/50) liposomes ($n > 3$, ± SD, black) in the presence of 0–12 mM PhEtOH. Additionally, single datapoints of independent proteoliposome preparations or protein purifications are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the lipid composition the chosen membrane model system (liposomes, nanodiscs *etc.*) appears also to be important for a transporter function, as the size of nanodiscs was reported to significantly modulate the activity of an ABC transporter by narrowing down the transporter's conformational spectrum [6,49]. When incorporated into EPL liposomes, the BmrA ATPase activity was significantly higher compared to a micellar environment, in line with previous reports [33,35,50]. Yet, the influence of specific lipid and/or membrane properties on the BmrA activity is largely unknown.

A biological membrane provides a defined environment with specific physical properties, including the hydrophobic membrane thickness, the lateral pressure and the membrane surface charge, which all potentially modulate the structure and/or activity of membrane proteins [4,7,43]. Ideally, the hydrophobic thickness of membranes and the hydrophobic thickness of protein transmembrane regions roughly match, preventing high energy costs for exposing fatty acids and/or hydrophobic protein regions, which either distorts the membrane or the protein, both potentially affecting the protein's structure and activity [7]. A dramatic impact of a membrane's hydrophobic thickness has been nicely illustrated using simple model systems, such as the human glycophorin A transmembrane helix dimer [13].

The hydrophobic thickness of BmrA is predicted to be around 31 Å (OPM, as of March 2025, [51]). As observed in the present study, BmrA clearly prefers membranes thicker than 16:1 PC (Fig. 2). Yet, BmrA was similarly active in DOPC and 22:1 PC membranes with hydrophobic carbon core thicknesses of 27 Å and 34 Å, respectively [52], which indicates a certain flexibility of BmrA in maintaining its activity at different membrane thicknesses.

For several membrane proteins it has been reported that negatively charged lipids modulate the protein structure and/or function [3,53,54]. Our results suggest that also in case of BmrA negatively charged lipids stimulate the ATPase activity up to a content of 50% (Fig. 3A–D). In fact, the exact chemical nature of the lipid head group appears to play only a minor role, as PS stimulates the BmrA activity essentially as well as PG. Based on the here presented observations, a membrane surface where approximately 50% of the lipids contain a single negative charge, is optimal for the BmrA activity. In perfect agreement, in case of CL, a lipid containing two negative charges, we observed the highest activity at around 30% of CL (Fig. 3D). It is reasonable to assume that the negative surface charge is crucial for the

BmrA activity due to interactions and (potentially) stabilization of protein regions containing positively charged residues. Thus, membrane interactions of these regions might be involved in stabilization of an active BmrA conformation. In case of BmrA, a small N-terminal α-helix, often referred to as the elbow helix (residues 1–21), lies perpendicular to the membrane surface and in fact this helix contains a high amount of the positively charged residues Lys and Arg. Yet, replacing these residues by Ala did not result in a reduced BmrA activity in EPL proteoliposomes (Fig. 4B), and thus interaction of this helix with negatively charged membrane surfaces is not crucial for the BmrA activity, at least not in lipid bilayers. The protein domains critically involved in establishing membrane-protein contacts *via* ionic interactions thus remain currently elusive.

Furthermore, not only a negative membrane surface, yet additionally changes in lipid packing (Fig. 5) appear to stimulate the BmrA ATPase activity, and both the acyl-chain region (Fig. 5A/B) as well as the head group region (Fig. 5C/D) contribute. Non-bilayer forming lipids, such as PE, exert a lateral pressure that leads to negative curvature of a monolayer. This intrinsic tendency to bend becomes frustrated in a lipid bilayer resulting in curvature stress [7,55]. Since PE is the most abundant phospholipid in *E. coli* membranes, it is obvious that the role of this lipid and the lateral pressure on membrane proteins is of major importance [43,56,57]. However, high lateral pressure in the acyl chain region goes hand in hand with loose packing of lipid head groups. The latter result in local membrane defects, *i.e.* local exposure of the hydrophobic tails of membrane lipids, and probably enables amphipathic helices to bind better to the membrane surface, which might stabilize defined BmrA structures. The transport cycle of BmrA involves a structural transition from a V-shaped, wide open IF *via* an occluded to a OF state, and the thermodynamic equilibrium between these states might control the protein's ATPase and transport activity [58,59]. A membrane with high positive curvature or high positive curvature elastic stress might (de)stabilize a defined protein structure/state, thereby shifting the equilibrium, resulting in the (here observed) activity changes.

In summary, we here identified three parameters controlling the BmrA activity in lipid bilayers: (i) the hydrophobic thickness of the membrane, being optimal at around 27–34 Å, (ii) a negative surface charge with an optimum at around 50% and (iii) changes in lipid packing, especially high lateral pressure in the acyl-chain region and loosely packed head groups stimulates the BmrA activity.

Interestingly, the *E. coli* membrane contains about 70% of the non-bilayer forming lipid PE, plus 20% and 10% of the negatively charged lipids PG and CL, respectively [60], *i.e.* about a negative surface charge of 30% (note that cardiolipin carried two negative charges which corresponds to a net charge of 40%). Also the thickness of *E. coli* membranes (37.5 ± 0.5 Å [61]) is close to the optimal conditions identified in the present study. This explains the observed high background ATPase activity BmrA has in *E. coli* membranes or when incorporated into EPL liposomes (Fig. 1C/D).

Similarly, also the composition and properties of *B. subtilis* membranes (natural environment) can be expected to support the BmrA activity well. The membrane lipid composition appears to highly depend on the exact growth conditions, yet a high amount of negatively charged lipids (up to ~12% CL and ~36% PG) can be naturally present in *B. subtilis* inner membranes [62]. While not further analyzed here, the charges of PG and CL likely cannot simply be added up, and thus probably both *E. coli* and *B. subtilis* offer a membrane surface charge that highly supports the BmrA activity. Furthermore, *B. subtilis* membranes might contain 20–40% of the non-bilayer forming phospholipid PE, *i.e.* less than *E. coli* membranes, yet can additionally contain up to 30% of the neutral, non-bilayer forming lipid diacylglycerol [63]. Thus also *B. subtilis* membranes can have high negative curvature stress, which, as we show here, affects the BmrA activity. On the other hand, the *B. subtilis* membrane appears to be thinner than *E. coli* membranes as the average lipid acyl chain length of membrane lipids is C16 [63,64], which corresponds to a hydrophobic membrane thickness of around 26 Å [52,65], suggesting that BmrA is not optimized for the *B. subtilis* membrane. Yet, as shown for other membranes, the average *in vivo* membrane thickness is not exclusively determined by the lipid acyl chain length but also by membrane incorporated proteins [61]. Furthermore, other membrane constituents besides the here studies phospholipids might additionally affect the structure and thickness of the *B. subtilis* membrane. Thus, the thickness of *B. subtilis* membrane likely differs from the value anticipated solely based on the average acyl chain length, again suggesting that the *B. subtilis* membrane environment stimulates well the BmrA activity.

It is still unclear why the ATPase activity of BmrA is so high in absence of substrates, resulting in constant futile ATP hydrolysis that is not typical for all ABC transporter. The here presented observations now show that the intimate lipid environment can control the activity of BmrA. As *B. subtilis* membranes are structured and defined lipid domains with specific lipid compositions appear to exist [66,67], it is feasible to assume that the activity of BmrA is spatio-temporally controlled in living *B. subtilis* cells by the intimate lipid environment. Activation of the protein by sorting it into or out of membrane regions with an activity-stimulating lipid composition would allow cells to regulate the activity of the protein and prevent futile ATP hydrolysis *in vivo*. Thus, we suggest that the activity of BmrA might be controlled *in vivo via* a dynamic (re) localization of the protein into or out of membrane regions with defined lipid compositions and (potentially) membrane thicknesses.

CRediT authorship contribution statement

Veronika Osten: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Dirk Schneider:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dirk Schneider reports financial support was provided by the Deutsche Forschungsgemeinschaft. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamem.2025.184430.

Data availability

All discussed data are presented within the article and the supporting information and will be made available on request.

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