Influence of lipids on membrane assembly and stability of the potassium channel KcsA

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Abstract Recently we observed in an in vitro system that newly synthesized KcsA assembles efficiently into a tetramer in lipid vesicles [van Dalen et al. (2002) FEBS Lett. 511, 51–58]. Here we used this system to get insight into the importance of the lipid composition for KcsA membrane association and tetramerization and we compared this to the lipid dependency of the thermo-stability of the KcsA tetramer. It was found that a large amount of phosphatidylethanolamine (>40 mol%) and a lower amount of phosphatidylglycerol (~20–30 mol%) were optimal for efficient KcsA membrane association and tetramerization. Strikingly, vesicles of the abundant and commonly used membrane lipid phosphatidylcholine did not support assembly, further demonstrating the importance of membrane lipid composition for KcsA assembly. The in vitro assembled KcsA tetramer showed similar thermo-stability in biological and pure lipid membranes, demonstrating that both tetramers are alike. In addition, we show that solubilization of the membrane with detergent reduces the thermo-stability of the tetramer. The highest KcsA tetramer stability was observed in intact bilayers in the presence of anionic lipids. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Lipid bilayer; Membrane insertion; Oligomerization; Protein–lipid interaction; Thermo-stability

1. Introduction

Many membrane proteins are present as oligomers or as part of larger protein structures. These complexes are held together by protein–protein interactions and also lipids appear to be involved in the stability of these complexes. The way lipids interact with and influence membrane proteins can be very different. Proteins could bind specific lipids at a particular position, such as observed in the crystal structures of some membrane proteins (e.g. [1,2]); specific lipids can be required in the membrane to provide the right environment for protein function [3,4] or they can influence the process of

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protein integration and folding into a correct conformation [5,6]. Whereas many examples exist of effects of lipids on membrane protein stability and activity, little is known about the role of lipids in the assembly of membrane proteins. A direct effect of lipids on the integration process in the membrane so far has only been studied for small, single membrane spanning proteins [6,7]. Concerning multiple spanning or oligomeric membrane proteins, an important role for lipids has been described only in the trimer formation of the *Escherichia coli* outer membrane porin PhoE [8] and in functional folding of LacY in the inner membrane of *E. coli* [5,9].

To study lipid influences on the complete biogenesis of an oligomeric structure, a suitable model protein is needed. The bacterial potassium channel KcsA is such a protein. It is a homo-tetrameric protein of 160 amino acids in each subunit. Each subunit contributes its C-terminal transmembrane helix to the lining of the pore and its second transmembrane helix faces the lipids. KcsA can be expressed in and functionally isolated from the *E. coli* inner membrane with an N-terminal His-tag [10]. The crystal structure of KcsA revealed a symmetrical tetrameric organization and with site-directed spin-labelling it was found that the N-terminus is localized at the membrane–water interface while the C-terminus is extended into the cytoplasm [11,12]. KcsA is homologues to the eukaryotic voltage-gated K^+ channels, it is well-characterized and its gating and conduction properties have been extensively studied upon reconstition in lipid bilayers [13,14]. Because of these properties and also because the KcsA tetramer is highly stable in a wide range of detergents [15], it is an attractive model for functional and biogenesis studies.

Recently we have shown that tetramer assembly occurs in the *E. coli* inner membrane both in vivo and in vitro [16]. Using the in vitro system it was shown that a functional signal recognition particle (SRP) targeting pathway is required for tetramer formation. Furthermore, KcsA assembly was found not to be highly dependent on the presence of the Sec translocase and YidC in the membrane. In fact, even more efficient tetramerization of newly synthesized KcsA occurred in a membrane only containing purified *E. coli* lipids [17]. These results opened the possibility to directly investigate the lipid specificity required for tetramer formation. The *E. coli* inner membrane is composed of $\sim75\%$ of the zwitterionic phosphatidylethanolamine (PE) and $\sim20\%$ and 5% of the negatively charged lipids phosphatidylglycerol (PG) and cardiolipin, respectively [18]. Here we analyze the relative importance of the two main lipid components, PE and PG, for KcsA assembly and compare it to phosphatidylcholine (PC), which is often present in membranes used to study KcsA functionality. In

addition, we study the lipid dependency of the thermo-stability of the biosynthetically formed and purified KcsA tetramer.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were purchased from Avanti Polar Lipids Inc. Total *E. coli* lipid extract (TLE) was prepared from the wild-type strain W3899 as described [19]. *n*-Dodecyl-β-D-maltoside (DDM) was from Anatrace Inc., Ni²⁺-NTA agarose was obtained from Qiagen and Bio-Beads SM-2 Adsorbent from Bio-Rad Laboratories. *E. coli* inverted inner membrane vesicles (IMVs) and S-135 lysate were prepared form strains MC4100 and MRE600, respectively [20]. Large unilamellar vesicles (LUVs) were obtained by extrusion [21] using 200 nm membrane filters (Anotop 10, Whatman, UK) of a lipid suspension composed of an appropriate mixture of dried lipids in vesicle buffer (20 mM HEPES, pH 8, 100 mM K_2SO_4, 250 mM sucrose).

2.2. In vitro membrane insertion and assembly assay

In vitro transcription, translation of KcsA was performed in the co-translational presence of membranes and KcsA membrane association was assayed as flotation with the membranes in a sucrose gradient as described [17]. Membrane and pellet fractions were analyzed by SDS–PAGE except that the samples were not boiled before electrophoresis unless specifically mentioned. The membrane association is expressed as the relative amount of synthesized KcsA recovered from the membrane fraction. The amount of tetramers is expressed as the relative amount of monomers detected in the tetramer form.

2.3. KcsA purification and reconstitution

KcsA was expressed with an N-terminal His-tag from pT7-KcsA [16] in *E. coli* strain BL21(λDE3). Purification was performed essentially as described [15] except that the purification buffer contained 10 mM HEPES, pH 7.5, 100 mM NaCl and 5 mM KCl. The solubilized membranes in 3 mM DDM were incubated with pre-washed Ni²⁺-NTA agarose beads for 1 h and the bound His-tagged KcsA was eluted in buffer with 300 mM Imidazole and 1 mM DDM. On average, 2 mg tetrameric KcsA per liter culture was purified. C-terminally truncated KcsA (Δ125–160) was obtained by chymotrypsin treatment of wild-type KcsA (250 μg/ml, 60 min room temperature). KcsA or Δ125–160 were reconstituted in different lipid mixtures by the method described to obtain functional reconstitution of protein translocation and insertion [22]. Briefly, LUVs were solubilized with 8 mM Triton X-100 and mixed with KcsA or Δ125–160 in 1 mM DDM in 1:1000 protein:lipid molar ratio. The mixture was 10 times diluted in purification buffer and the detergent was removed using Bio-Beads. The reconstituted vesicles were collected by centrifugation (30 min, TLA 120.2 rotor, 100 000 rpm, 4°C).

2.4. KcsA stability assay

To measure the stability of the KcsA tetramer formed in the in vitro transcription–translation assay, the membrane vesicles were re-isolated from the membrane fraction of the sucrose gradient and resuspended in vesicle buffer. Samples were incubated for 15 min at indicated temperatures, followed by addition of SDS–PAGE sample buffer and chilling on ice. The KcsA or Δ125–160 stability in reconstitution vesicles was measured in purification buffer. The stability in detergent was measured in the presence of 8 mM DDM. After SDS–PAGE and Coomassie brilliant blue staining, the gel was scanned by Densitometer (Bio-Rad Laboratories) and quantified with the program Quantity One. The relative amount of tetramer in each sample was calculated and plotted against the incubation temperature. The melting temperature (T_m) of the tetramer is defined as the mid-point of this curve.

3. Results

3.1. Lipid dependence of KcsA assembly

Formation of the KcsA tetramer occurs in an in vitro transcription–translation assay in the presence of *E. coli* inner

membranes [16]. Recently, we have demonstrated that even more efficient tetramerization occurs in vesicles prepared from a total lipid extract (TLE) of the *E. coli* inner membrane [17]. Fig. 1 shows that efficient KcsA tetramer formation can also take place using LUVs composed of the synthetic lipids DOPE and DOPG in a 7:3 molar ratio, which mimics the *E. coli* inner membrane lipid composition. SDS–PAGE analysis after sucrose gradient flotation reveals most of the synthesized KcsA in the PE/PG membrane fraction, of which 30% is in the tetrameric state. This is comparable to the efficiency in TLE vesicles [17]. The tetramers visible on the gel in the pellet fraction are the result of lipid contamination of the pellet, caused by difficulties in sampling the synthetic lipid vesicles from the sucrose gradient. The efficient membrane assembly of KcsA in the synthetic lipid bilayer excludes the possibility that the previously observed assembly in TLE vesicles is mediated by proteinaceous contamination or requires the presence of a large variation of acyl chains, as is present in the *E. coli* lipids. This efficient tetramerization in the PE/PG membrane with homogeneous acyl chain compo-
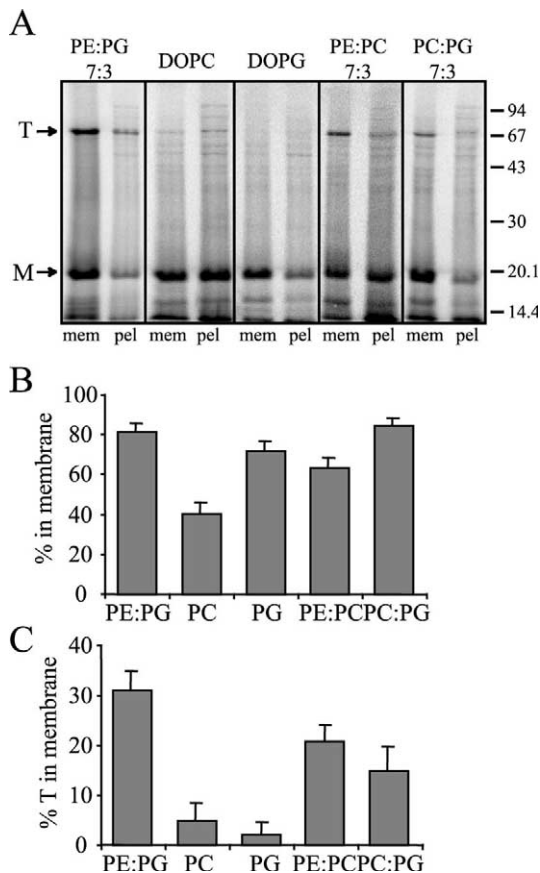

Fig. 1. KcsA assembly with LUVs of different lipid composition. KcsA translation, membrane association and oligomerization were examined in the presence of LUVs composed of different mixtures of DOPE, DOPG or DOPC in a 7:3 molar ratio, or pure DOPC or DOPG. After a sucrose gradient flotation, the samples were analyzed on SDS–PAGE. Monomeric (M) and tetrameric (T) KcsA are indicated and a protein size marker (in kDa) is shown on the right (A). The relative amount of synthesized KcsA recovered from the membrane fraction (B) and the relative amount of tetrameric KcsA formed in the membrane fraction (C) were determined. The average and standard deviations are calculated from at least three different experiments.
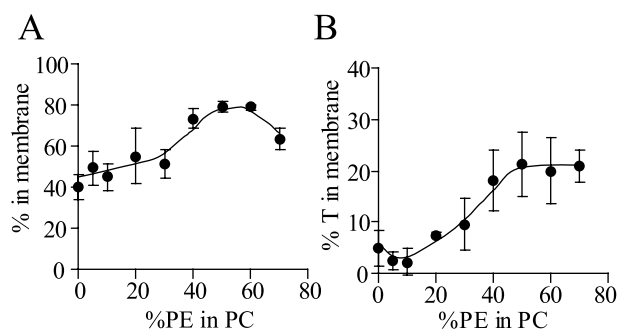
Fig. 2. Influence of DOPE on KcsA assembly. In vitro transcription–translation of KcsA was performed in the presence of LUVs containing varying amounts of DOPE in DOPC. Samples were analyzed as described in the legend of Fig. 1 and the relative amount of membrane-associated KcsA (A) and the relative amount of tetrameric KcsA formed in the membrane fraction (B) were determined. The average and standard deviations are calculated from at least three different experiments.

sition allows systematic analysis of the effect of different lipid headgroups on KcsA assembly.

In most studies on KcsA reported so far PC is present in the bilayer, since this lipid is abundant and forms stable bilayers in which membrane proteins can be functionally hosted. Interestingly, when pure DOPC LUVs were added in the in vitro assay, the extent of membrane association to DOPC was largely reduced compared to the *E. coli* mimicking lipid bilayer of PE/PG (Fig. 1A,B). Moreover, no tetramers were formed in the membrane-associated fraction (Fig. 1C). Because the negatively charged lipid PG is often shown to stimulate protein insertion, pure DOPG bilayers were also tested for KcsA assembly. Although the extent of membrane association was now comparable to the PE/PG lipid bilayer (Fig. 1B), tetramer formation was also abolished in the pure DOPG bilayer (Fig. 1C). These results clearly show that the composition of the lipid membrane has a strong influence on KcsA assembly.

To test to what extent each lipid, PE or PG, is responsible for efficient tetramerization in the PE/PG bilayer, first PG was replaced by PC. From Fig. 1 it is clear that this resulted in a slightly decreased membrane association and also tetramerization was less efficient. On the other hand, replacing PE by PC did not give a change in membrane association, but tetramerization was even more reduced. Similar results were obtained after flotation through a high salt (500 mM KOAc) containing sucrose gradient, excluding the possibility of membrane association only via electrostatic interactions. These results suggest that both PE and PG can affect the extent of KcsA membrane assembly.

To study the effect of PE in more detail, the amount of PE was gradually increased, starting from a pure DOPC bilayer. From quantification of the results it is clear that in the presence of high amounts of PE (>40 mol%) membrane association is almost two-fold increased compared to the pure PC LUVs (Fig. 2A). Strikingly, while tetramerization is absent in pure DOPC bilayers, the presence of only 20 mol% DOPE results already in the formation of tetramers. Up to 20% of the synthesized KcsA is in the tetrameric state in the presence of high amounts of PE (Fig. 2B). Still, the efficiency in tetramer formation is never as high as in the PE/PG membrane (compare Fig. 1).
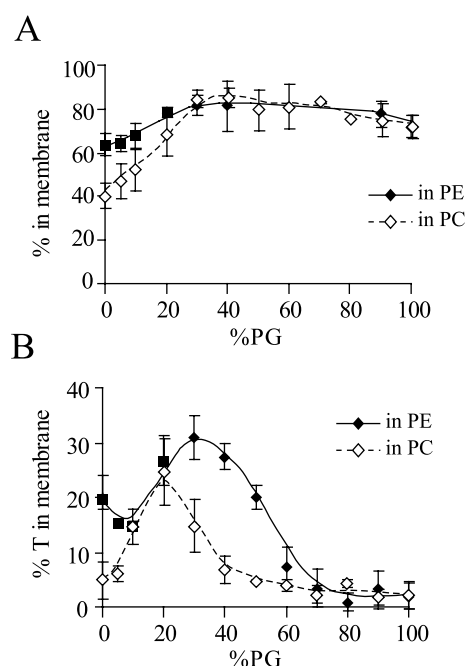

Fig. 3. Influence of DOPG on KcsA assembly. In vitro transcription–translation of KcsA was performed in the presence of LUVs containing varying amounts of DOPG in DOPE ($\blacklozenge$) or in DOPC ($\diamondsuit$). For reasons of bilayer stability, a maximum of 70% DOPE was used; 0–20% PG in 70% PE was supplemented with 30–10% PC ($\blacksquare$). Samples were analyzed as described in the legend of Fig. 1 and the relative amount of membrane-associated KcsA (A) and the relative amount of tetrameric KcsA formed in the membrane fraction (B) were determined. The average and standard deviations are calculated from at least three different experiments.

The effect of PG was studied by gradually increasing the amount of PG in PE and PC membranes. Irrespective of the lipid system, the presence of 30 mol% PG is sufficient to obtain optimal membrane association ($\sim 80\%$), which cannot be increased further (Fig. 3A). In contrast, the ability to form tetramers in the membrane was found to be largely dependent on the amount of PG present (Fig. 3B). In line with the previous results, tetramer formation is more efficient in the PE/PG than in PC/PG bilayers. In both systems, the presence of low amounts of PG increases the amount of tetramers formed. An optimum is reached at 20 mol% PG in PC lipids and 30 mol% PG in PE lipids. Larger amounts of PG in the
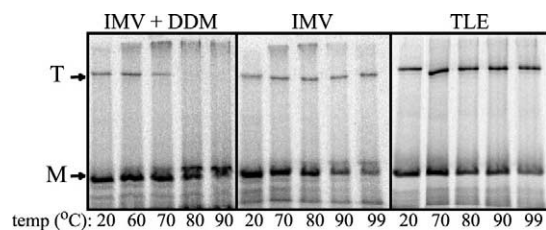

Fig. 4. Stability of KcsA after biogenesis. In vitro transcription–translation of KcsA was performed in the presence of *E. coli* IMVs or TLE LUVs and the samples were separated by a sucrose flotation gradient centrifugation. From the membrane fraction the vesicles were re-isolated and incubated in vesicle buffer in the presence or absence of 8 mM DDM for 15 min at the indicated temperature. After rapid chilling on ice, the samples were analyzed on SDS–PAGE.

lipid membranes reduce and finally even block tetramer formation, whereas membrane association is still optimal. The results demonstrate that a lipid membrane composed of a mixture of PE and PG, in the composition that is similar to the *E. coli* inner membrane, is optimal in supporting efficient membrane assembly of KcsA.

3.2. Stability of the KcsA tetramer

Next we investigated whether lipids influence the stability of the KcsA tetramer. It is known that the purified KcsA tetramer in detergent is highly stable, except upon heat treatment above ∼70°C, which results in disassembly. This can be followed as a tetramer to monomer transition on SDS–PAGE [15]. The in vitro assembled tetramer in the *E. coli* inner membrane was checked for a comparable stability behavior, after its isolation in detergent. As shown in Fig. 4 (left panel), the in vitro formed tetramer indeed starts to disassemble at 70°C and is not stable at higher temperatures. Interestingly, the monomers run at a lower electrophoretic mobility in the gel after heating, possibly due to unfolding. When the IMVs were not solubilized before heat treatment, a remarkable increase in stability of the tetramer was observed (Fig. 4, IMV). Even after incubation above 90°C, KcsA tetramers could be detected. The overall decrease of intensity is caused by increased aggregate formation at higher temperatures. A similar increase of KcsA stability as in intact IMVs was observed for the in vitro formed tetramer in TLE vesicles (Fig. 4, TLE) and in PE/PG synthetic lipid membranes (not shown), indicating that lipids and not proteins are responsible for the increase of the tetramer stability.

To further study the stabilizing effect of lipids, we used a better defined system of reconstituted protein in different lipid bilayers, in which at the start of the experiment only tetramers are present. As shown in Fig. 5A, the purified KcsA tetramer starts to destabilize in detergent after incubation at 70°C and is completely disassembled at higher temperatures. The additional band visible on the gel after incubation at 80°C is probably a KcsA dimer, which is more often observed after heat treatment [15]. KcsA reconstituted in PE/PG lipids displays a comparable tetramer stability as the in vitro assembled KcsA tetramer in the intact lipid bilayer (compare Fig. 5A with Fig. 4). Reconstitution in *E. coli* TLE showed a similar stability behavior (not shown). Interestingly, after reconstitution of purified KcsA in a pure DOPC bilayer, the tetramer stability was decreased as compared to the PE/PG bilayer. As shown on the gel in Fig. 5A, after incubation at 90°C already monomers are clearly visible in the DOPC system and after incubation at 99°C all tetramers are disassembled, while they are still present under these conditions in the PE/PG membrane. Higher protein:lipid ratios (up to 1:100) in the DOPC system did not change the stability behavior (not shown). Quantification of the amount of tetramers remaining after incubation at different temperatures results in melting curves as shown in Fig. 5B (closed symbols). The large stabilizing effect of lipids as compared to the lipid free purified protein is obvious from the shift of tetramer decrease to higher temperatures. The T_m in detergent was determined at 70°C and in DOPC at 93°C. To get a more accurate insight into the difference in tetramer stability between DOPC and PE/PG bilayers, a C-terminal truncated KcsA with a reduced stability [14] was also used (open symbols). The T_m of the Δ125–160 tetramer in PE/PG lipids was determined to be 93°C, which was 6°C high-
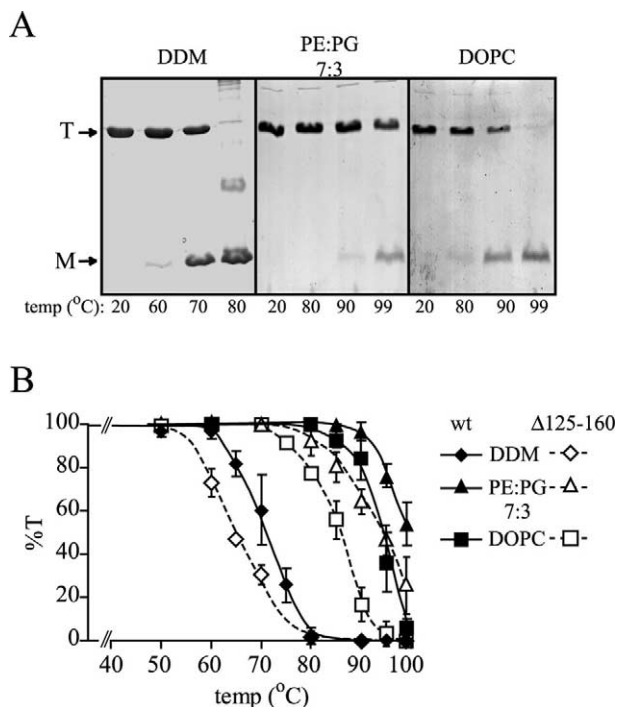

Fig. 5. Stability of purified and reconstituted KcsA. Purified KcsA in 8 mM DDM or reconstituted in a DOPE:DOPG 7:3 molar ratio or in pure DOPC was incubated in buffer for 15 min at the indicated temperature. After rapid chilling on ice, the samples were analyzed on SDS–PAGE and visualized with Coomassie staining (A). The relative amount of KcsA (closed symbols) or Δ125–160 (open symbols) tetramers in DDM (♦/◇), PE:PG 7:3 (▲/△) or DOPC (■/□) after incubation was determined. The average and standard deviations from three different experiments are expressed (B).

er than for this truncate in pure DOPC. Thus, this truncate appears to exhibit a similar lipid dependency as wild-type KcsA.

To what extent are the different lipids responsible for the higher stability in PE/PG versus PC? Replacing PG by PC in the reconstitution assay resulted in a decrease of the thermostability to a comparable level as in pure PC vesicles. This stability was not affected in a large range of PE/PC lipid mixtures, indicating that PE has no specific effect on tetramer stability (data not shown). Replacing PG by the negatively charged DOPA did not affect tetramer stability, suggesting that the negative charge and not the chemical structure of the PG headgroup is responsible for the higher tetramer stability as compared to PC or PE (data not shown).

4. Discussion

4.1. Lipid dependence of membrane assembly of the KcsA tetramer

We first analyzed the effect of lipids on membrane assembly of the in vitro synthesized K^+ channel KcsA. We found that both membrane association and tetramerization of newly synthesized KcsA were most efficient in vesicles containing small amounts of the negatively charged lipid PG together with large amounts of zwitterionic PE. This is remarkably comparable to the lipid composition of the bacterial membrane. In contrast, in vesicles prepared from pure DOPC, membrane

association was relatively low and no tetramer formation was observed.

It is likely that the lipid dependence of the extent of membrane association of KcsA is at least to a large part due to differences in targeting efficiency. Efficient KcsA assembly is dependent on the SRP pathway [17], and FtsY, which is the docking protein in the SRP pathway, has been shown to have less interaction with PC as compared to PE or PG [23,24]. Therefore, docking of the nascent KcsA chain could be less efficient for PC. In addition, since the monomers that did associate with the PC bilayer seemed unable to assemble into tetramers, they may have been not correctly inserted or folded.

The increased membrane association by introducing PG in the PC bilayer can most likely be attributed to an increased targeting to the membrane by FtsY [24]. In addition, PG could contribute directly to targeting, for instance by anchoring the positively charged N-terminus to the *cis*-side of the membrane, as observed in other protein insertion systems [6,7]. The increased tetramerization by the presence of 20–30 mol% PG could be due to a requirement for PG for correct folding of the KcsA monomer, in analogy to the results obtained for the *E. coli* protease, DegP [25]. In this respect, the Arg residue at position 52 is interesting because its side chain sticks out towards the membrane interface [11] and might interact with negatively charged lipids, thereby possibly contributing to a correct positioning of the KcsA monomer in the membrane. Rather surprisingly, higher concentrations of PG in the membrane inhibited tetramerization. A possible explanation for this effect is that a too high negative charge density of the membrane might affect the charged residues in the periplasmic region, either by blocking translocation or by an effect on the positioning of the pore region, which has been suggested to be important for tetramer stability [26,27].

Similar as for PG, the increased membrane association in the presence of PE could be explained by an increased targeting to the membrane via FtsY [24]. Remarkably, PE seems to be the most effective lipid in stimulating tetramerization. This effect could be related to the non-bilayer property of PE. Possibly, the small size of the PE head group facilitates the initial KcsA interfacial insertion, similar as was postulated for the interfacial insertion of the catalytic domain of leader peptidase [28]. After membrane insertion of the monomers, PE then might promote oligomerization as a consequence of the high packing density in the hydrophobic part of the membrane as compared to that of a pure PC bilayer [29]. Alternatively, it is possible that PE influences folding of the KcsA monomer into a tetramerization competent state by a still unknown and lipid specific mechanism, similar to the role of PE suggested in the folding of the membrane protein LacY into its functional conformation after membrane insertion [5,9].

4.2. Lipid dependence of KcsA tetramer stability

Purified KcsA tetramers are highly stable in detergent. We have shown here that when the protein is reconstituted in pure lipid bilayers, the stability of the tetramer is still further increased. The stability of the in vitro assembled KcsA tetramer in either the *E. coli* inner membrane or in pure lipid bilayers was comparable to that of the purified, reconstituted protein. This strongly suggests that the tetrameric state formed in vitro in both membrane systems is similar to that of the purified

tetramer, which has been shown to be fully functional [10,13].

It was found that all lipids tested stabilize the tetramer. The largest stabilizing effect was observed in the presence of negatively charged lipids. Interestingly, these lipids also have been suggested to be needed for KcsA channel activity [13]. Together with the observation in the present study that PG plays a role in assembly, this points to multiple roles of negatively charged lipids. These roles possibly involve specific interactions with KcsA that are needed for a proper conformation of the protein. Also DOPC clearly stabilizes the KcsA tetramer, although we have shown that it does not support tetramer assembly. This supports our notion (see above) that it is incorrect insertion of the monomer in the membrane that causes the inability of the DOPC bilayer to support KcsA assembly, rather than intrinsic properties of the DOPC bilayer itself.

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