Lipids in the Structure, Folding, and Function of the KcsA K^+ Channel†

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Received May 30, 2002; Revised Manuscript Received July 14, 2002

ABSTRACT: Lipid molecules surround an ion channel in its native environment of cellular membranes. The importance of the lipid bilayer and the role of lipid protein interactions in ion channel structure and function are not well understood. Here we demonstrate that the bacterial potassium channel KcsA binds a negatively charged lipid molecule. We have defined the potential binding site of the lipid molecule on KcsA by X-ray crystallographic analysis of a complex of KcsA with a monoclonal antibody Fab fragment. We also demonstrate that lipids are required for the in vitro refolding of the KcsA tetramer from the unfolded monomeric state. The correct refolding of the KcsA tetramer requires lipids, but it is not dependent on negatively charged lipids as refolding takes place in the absence of such lipids. We confirm that the presence of negatively charged lipids is required for ion conduction through the KcsA potassium channel, suggesting that the lipid bound to KcsA is important for ion channel function.

Integral membrane proteins interact with lipid molecules in cellular membranes through their hydrophobic transmembrane regions (*1*). The interactions between membrane proteins and lipids can be categorized as either general or specific (*2*). By general interactions, we mean those resulting from the multiphase (membrane-aqueous) environment created by lipids in water, necessary for the stability of most integral membrane proteins. Specific interactions refer to the close association of certain lipids that may bind to the membrane protein, akin to a cofactor, to confer structural stability or to affect the protein's function. Biochemical studies have demonstrated specific lipid binding to certain integral membrane proteins (*3*, *4*), and high-resolution crystal structures, now available for a few membrane proteins, reveal the presence of associated lipid molecules (*5*−*8*). The precise role of these specific lipid interactions is yet to be defined, but their importance is revealed by functional assays demonstrating that a number of membrane proteins such as electron transfer complexes I and III, cytochrome *c* oxidase, and a number of transporter proteins require specific lipids for optimal activity (*9*−*12*).

Here we address the issue of protein−lipid interactions in the context of ion channels, the integral membrane proteins that catalyze the selective conduction of ions across biological membranes. We have investigated the interactions between the bacterial potassium channel, KcsA, and lipid molecules through three different lines of investigation. First, the presence of specific lipid interactions was investigated by high-resolution X-ray crystallographic analysis of a complex of KcsA with a monoclonal antibody Fab fragment. A biochemical analysis was then used to identify a lipid bound to KcsA. Second, an in vitro refolding assay was developed for refolding KcsA from the unfolded state and was used to investigate the role of lipids in the assembly of native KcsA. The in vitro refolding assay also allows us to dissect the properties of lipids that may be important for the assembly of the native KcsA molecule. Third, the presence of specific protein lipid interactions was investigated by analyzing the dependence of KcsA function on the composition of the lipid bilayer.

MATERIALS AND METHODS

Crystallographic Analysis. KcsA was purified and crystallized in complex with an antibody Fab fragment as described previously (*13*). Frozen crystals diffracted X-rays to 2.0 Å Bragg spacing at the A1 station of the Cornell High Energy Synchrotron Source. Phases were determined by molecular replacement using the KcsA structure (PDB entry 1BL8) (*14*) and an antibody Fab structure (PDB entry 1MLC) (*15*). Structure refinement was carried out as described previously (*13*). The lipid omit difference electron density shown in Figure 1 was calculated using $F_o - F_c$ coefficients in CNS (*16*) and contoured at 3.0σ.

Coordinates. Coordinates for the KcsA−antibody Fab complex have been deposited in the Protein Data Bank (entry 1K4C).

Phosphate Assays. KcsA [80 μg, calculated using an extinction coefficient of 33 750 M^{-1} cm^{-1} (*17*)] in 0.1 mL of protein buffer was hydrolyzed by addition of 0.3 mL of 0.6 M H_2SO_4 and 0.21 M $HClO_4$ and incubation at 120 °C for 12 h. Following hydrolysis, the sample was clarified by centrifugation and assayed for phosphate as described previously (*18*). Briefly, a small aliquot of the hydrolyzed sample was diluted to 0.5 mL using H_2O and then mixed with 0.3 mL of assay solution containing 0.32 mM malachite green oxalate, 16 mM ammonium heptamolybdate, and 1.5

† This work was supported by a grant from the National Institutes of Health (GM43949). R.M. is an investigator in the Howard Hughes Medical Institute.

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10.1021/bi026215y CCC: $22.00
Published on Web 08/10/2002

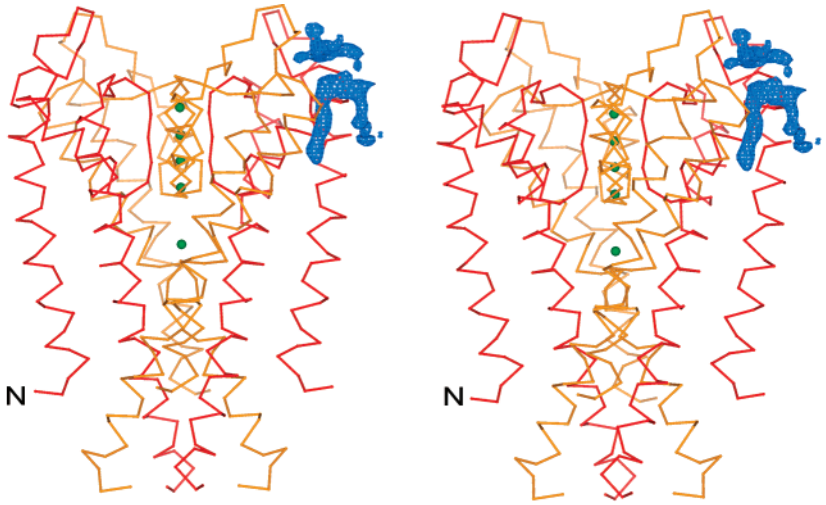

FIGURE 1: Lipid molecules in KcsA crystals. A stereoview of the KcsA structure with electron density corresponding to the lipid molecule. The backbone of KcsA is shown as a red and yellow trace. Green spheres represent potassium ion binding sites. The $F_o - F_c$ map (contoured at 3σ) was calculated using a model that does not contain lipid molecules. For clarity, density corresponding to only one of the lipid molecules is shown. The KcsA monomer consists of an N-terminal outer helix, a central pore helix, and a C-terminal inner helix. This figure was prepared with MOLSCRIPT (*31*) and Raster3D (*32*).

M H_2SO_4. After incubation for 20 min at 22 °C, absorbance was measured at 650 nm and the amount of phosphate present was determined by comparison to a standard curve generated using NaH_2PO_4.

Thin-Layer Chromatographic Analysis. The phospholipid bound to KcsA was identified by TLC on silica gel plates (Whatman K6F) using two different solvent systems. Initially, the lipid present in the KcsA sample was separated from the protein and the detergent by TLC using a $CHCl_3$/MeOH/NH_4OH mixture (65:25:4, v/v) (*19*). KcsA (80 μg) in buffer [50 mM Tris-HCl (pH 7.5), 150 mM KCl, and 100 mM DM] was spotted on the TLC plate along with 7.5 μg of the lipid standards (Avanti Polar Lipids Inc.) dissolved in the same buffer. Lipid spots were visualized by iodine staining. The lipid spot corresponding to the KcsA sample was scraped off and the lipid extracted with a $CHCl_3$/MeOH mixture (1:1, v/v). Following removal of the silica particles by centrifugation, the organic solvent was evaporated under a stream of argon and the residual lipid dissolved in a minimal volume of $CHCl_3$. The lipid was then identified by TLC using a $CHCl_3$/MeOH/H_2O mixture (65:25:4, v/v) (*19*) by comparison to lipid standards (5 μg in $CHCl_3$) that were also spotted on the same TLC plate.

Protein Unfolding and Refolding. KcsA was expressed and purified using the C-terminal hexahistidine affinity tag as described previously (*14*). For TFE/acid unfolding of KcsA, an equal volume of TFE containing 1% TFA acid was added to the KcsA solution and incubated at room temperature for 30 min. The TFE and acid present were removed by dialysis against 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 15 mM KCl, and 0.2% SDS. For refolding, an equal volume of lipid vesicles at 30 mg/mL in 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 15 mM KCl, and 10% glycerol was added. The protein/lipid mixture was sonicated in a water bath sonicator for 5 min and then incubated at room temperature. The extent of refolding was monitored by SDS−PAGE.

Single-Channel Analysis of KcsA. For single-channel analysis of the refolded KcsA, the protein was initially extracted from lipid vesicles using 40 mM DM.[1] The refolded tetramer was separated from most of the unfolded monomer on the basis of the differing affinities for the Co^{2+} resin (Clontech, Palo Alto, CA). The purified protein (10 μg) was mixed with 0.4 mL of a lipid solution containing POPE (7.5 mg/mL) and POPG (2.5 mg/mL) in 10 mM HEPES, 450 mM KCl, and 4 mM NMG solubilized by 34 mM CHAPS. Lipid vesicles were formed by removal of the detergent by dialysis.

For single-channel measurements, planar lipid bilayer membranes of POPE (15 mg/mL) and POPG (5 mg/mL) in decane were painted over a 300 μm hole in a polystyrene partition separating chambers containing the internal and external solutions. To induce fusion of channel-containing vesicles, the external solution contained 150 mM KCl and 10 mM HEPES (pH 7.0) and the internal solution contained 20 mM KCl and 10 mM succinate (pH 4.0). Following channel fusion, the KCl concentration of the internal solution was adjusted to 150 mM by addition of a requisite amount of a 3 M KCl solution. The membrane voltage was controlled and the current recorded by an Axopatch 200B amplifier with a Digidata 1322A analogue-to-digital converter and Axoclamp software (Axon Instruments, Union City, CA) (*20*, *21*).

Immunoblotting Analysis of KcsA. For immunoblotting analysis, the refolding mixtures were initially solubilized by addition of SDS to a concentration of 2% followed by incubation at 37 °C for 30 min. Folded KcsA and unfolded KcsA present in the refolding mixtures were separated by SDS−PAGE. After electrophoresis, proteins in the gel were electrophoretically transferred onto a Hybond-P membrane (Amersham Biosciences, Buckinghamshire, England). Conditioned medium from cultures of mouse monoclonal hybridoma cells (cell line KcsA-1) was used as a source of the

[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CL, cardiolipin; DM, *n*-decyl β-D-maltoside; MES, 2-morpholinoethanesulfonic acid; NMG, *N*-methyl-D-glucamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol.

monoclonal antibody (*22*) and diluted 1:1000 prior to use as the primary in immunoblots. Immunoblots were developed using the "ECL Plus System" (Amersham Biosciences).

$^{86}Rb^+$ Flux Assay. For $^{86}Rb^+$ flux assays (*23*), KcsA was reconstituted into POPE/POPG (3:1) or POPE/POPC (3:1) lipid vesicles at 2.5−10 μg of protein/mg of lipid as previously described. Prior to the $^{86}Rb^+$ flux assays, the extravesicular solution was exchanged for uptake buffer [400 mM sorbitol, 10 mM MES (pH 6.5), 4 mM NMG, and 40 μM KCl]. $^{86}Rb^+$ uptake was initiated by mixing the lipid vesicles with 4 volumes of uptake buffer containing $^{86}Rb^+$ at a final concentration of ~0.5 μCi/mL. At each time point of the flux assay, extra liposomal $^{86}Rb^+$ was removed by passing a 0.1 mL aliquot of the reaction through a 1.5 mL Dowex cation exchange column in the NMG^+ form. The sample loaded on the column was eluted with 1.5 mL of 400 mM sorbitol into a scintillation vial, and the amount of $^{86}Rb^+$ present within the vesicles was determined by liquid scintillation counting. Nonspecific binding of lipid vesicles to the Dowex column was minimized by prewashing the column with 2 mL of 5 mg/mL BSA in 400 mM sorbitol followed by 2 mL of 400 mM sorbitol. At the end of the time course of the uptake reaction, valinomycin was used to determine the level of equilibrium $^{86}Rb^+$ uptake into all the lipid vesicles present. For this purpose, valinomycin was added to the reaction mixture at a final concentration of 1 μg/mL and an aliquot measured for $^{86}Rb^+$ uptake after incubation for 1−2 min.

RESULTS

Crystallographic Observation of Lipid Molecules in KcsA Crystals. A complex of KcsA and the Fab fragment of the anti-KcsA monoclonal antibody was crystallized, and the structure was refined to 2.0 Å resolution as described previously (*13*). An $F_o - F_c$ electron density map calculated in the absence of modeled lipids showed the presence of a hairpin-shaped structure at the interface between two KcsA monomers. The protein, the detergent, or any of the components of the protein buffer or crystallization solutions could not account for this excess electron density. The shape of the electron density seemed to be indicative of a lipid molecule, raising the possibility that KcsA has a tightly bound lipid molecule (Figure 1).

Biochemical Identification of the Lipid in KcsA. As a first step toward confirming the presence of a phospholipid in the KcsA structure, we looked for the presence of phosphate in purified preparations of KcsA. Phosphate assays indicated that after standard biochemical purification, KcsA contained approximately 0.7 mol of phosphate per mole of the KcsA monomer (Figure 2a). This would be consistent with the presence of approximately one lipid molecule per subunit. The phosphate in the KcsA sample could be detected only after acid hydrolysis of the sample, which is consistent with the phosphate originating from phospholipids (Figure 2a).

Further confirmation and identification of the phospholipid that was present was performed using TLC. KcsA after purification in DM was first analyzed by TLC using a $CHCl_3$/MeOH/NH_4OH solvent system. This solvent system was used to separate lipids that might be present in the KcsA sample from the detergent used in the purification. TLC analysis using this solvent system indicated the presence of a lipid

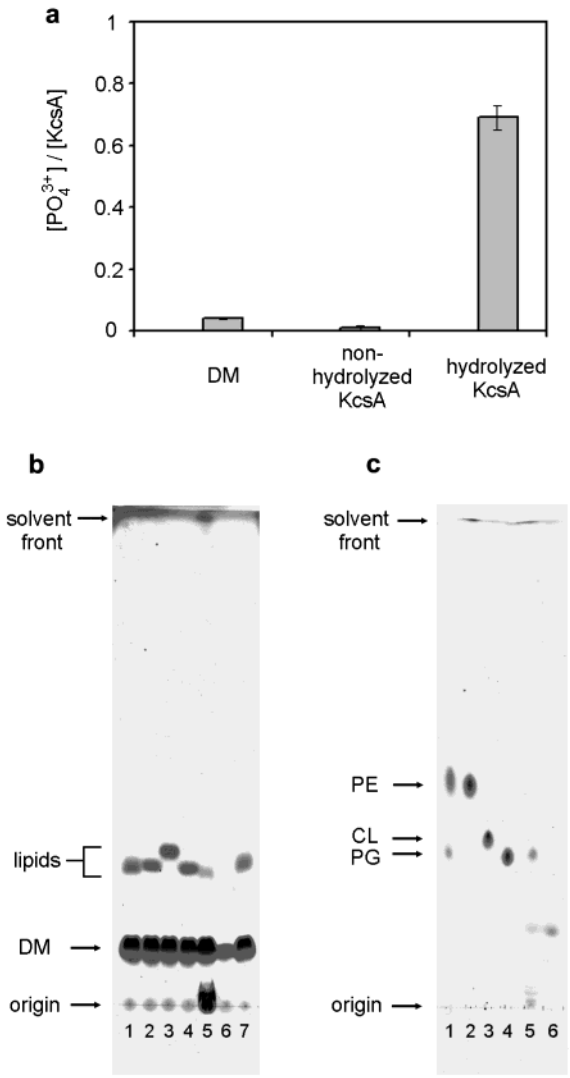

FIGURE 2: Identification of lipids associated with KcsA. (a) Phosphate assays. KcsA was purified in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM KCl, and 5 mM DM. Phosphate assays were performed on KcsA samples after hydrolysis as described in Materials and Methods. Data represent the average of three different preparations of purified protein. KcsA without hydrolysis and buffer with hydrolysis treatment were assayed as controls. (b) Thin-layer chromatography analysis. All samples were dissolved in the buffer described in Materials and Methods: *E. coli* polar lipid extract (lane 1), PE (lane 2), CL (lane 3), PG (lane 4), KcsA (lane 5), DM (lane 6), and *E. coli* total lipid extract (lane 7). TLC was carried out using a $CHCl_3$/MeOH/NH_4OH system (65:25:4, v/v). (c) All samples were dissolved in $CHCl_3$ or a $CHCl_3$/MeOH system (1:1): *E. coli* polar lipid extract (lane 1), PE (lane 2), CL (lane 3), PG (lane 4), lipids extracted from lane 5 of the TLC plate shown in panel b (lane 5), and control extraction on a blank area of the TLC plate (lane 6). TLC was carried out using a $CHCl_3$/MeOH/H_2O system (65:25:4, v/v).

in the KcsA sample (Figure 2b). The identity of the lipid could not be unambiguously assigned using this solvent system. The lipid was therefore extracted with $CHCl_3$/MeOH and rerun using a $CHCl_3$/MeOH/H_2O solvent system along with lipid standards. The second TLC step showed that the lipid present in KcsA comigrated with PG, indicating that the lipid bound to KcsA is a PG molecule (Figure 2c).

Lipid-Binding Site of KcsA. Following biochemical confirmation of a tightly associated lipid with KcsA, a lipid molecule was modeled into the electron density. Due to

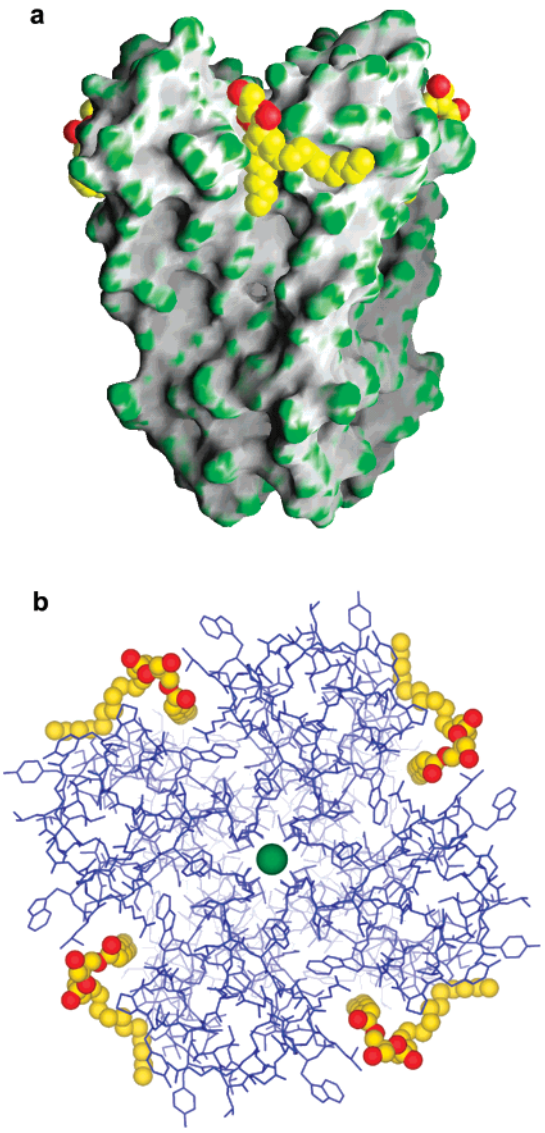

FIGURE 3: Structural analysis of lipid binding to KcsA. (a) Binding surface of the lipid molecule. The surface of KcsA is colored according to curvature (green, convex; gray, concave). The lipid molecule, built as 1,2-diacylglycerol, is shown in CPK representation with oxygen atoms colored red and carbon atoms colored yellow. (b) Lipid-binding site viewed from the extracellular side along the 4-fold axis of KcsA. The channel is colored blue. The green sphere represents the potassium ion. The lipid molecule is in CPK representation colored as in panel a. Panel a was prepared with GRASP (*33*). Panel b was prepared with MOLSCRIPT (*31*) and Raster3D (*32*).

partial disorder, the lipid headgroup was not well resolved; as a result, only the 1,2-*sn*-diacylglycerol portion of the lipid molecule was built into the model. The fatty acyl chains also had to be truncated at nine carbon atoms at the *sn*-1 position and at 14 carbon atoms at the *sn*-2 position. The lipid is bound at the interface between two KcsA monomers with its headgroup near the extracellular surface and its tail projecting into the outer membrane leaflet (Figure 3a,b). The C9 lipid tail lies in a groove between the inner and pore helices of adjacent monomers, while the C14 lipid tail appears to interact less well with the protein. The location of the lipid molecule suggests that its negatively charged phosphatidylglycerol headgroup lies relatively near two Arg side chains, Arg64 and Arg89, which would provide favorable electrostatic interactions between the negatively charged lipid headgroup and the channel.

Unfolding and Refolding of KcsA. The crystallographic and biochemical analyses indicate that there are four lipid molecules bound to the KcsA tetramer. A possible role for these lipid molecules is that they are required for the assembly of the KcsA tetramer. To test a lipid requirement for assembly, we investigated the requirement of lipids for the in vitro assembly of the tetramer from the unfolded, monomeric state. Before determining whether KcsA can be refolded in vitro, we first had to study conditions under which the protein could be unfolded. KcsA is remarkably stable and migrates as a tetramer on SDS−PAGE (Figure 4a, lane 1). The tetramer also persists under commonly used denaturing conditions such as 8 M urea, 6 M guanidinium chloride, and 2% SDS (*24*). For this reason, harsh conditions such as heating at 100 °C for 30 min in the presence of SDS are required to convert KcsA into the monomeric form. The KcsA tetramer is also unstable in the presence of organic solvents such as trifluoroethanol (TFE); for example, the tetramer can readily be disrupted in 50% TFE acidified with trifluoroacetic acid (TFA) (Figure 4a, lane 2). Following the establishment of these protocols for obtaining monomeric KcsA, we next asked if the channel could be reassembled back into its tetrameric state. A survey of refolding conditions revealed that the tetramer could be reassembled from the monomeric form only upon incorporation of the monomeric form into lipid vesicles (Figure 4a, lane 3). The extent of refolding, or formation of the tetramer, was in the range of 20−30%. To test for the correctness of the refolded structure, the refolded material was solubilized using DM and partially purified from the residual unfolded protein using the C-terminal His tag. Purification of the folded tetrameric form from the monomeric form is achieved due to the higher-affinity binding of the refolded tetrameric KcsA to the cobalt resin compared to that of the monomeric form. The purified, refolded protein was reconstituted into lipid vesicles, and channel activity was assayed in planar lipid bilayers. The single-channel activity observed with the refolded protein was similar to that observed with the recombinant protein (Figure 4b). These results indicate that the refolded form of the protein is similar to the native form. These results also clearly indicate that lipids are required for the refolding reaction.

Lipid Requirement for the Refolding Reaction. The lipid requirement for the refolding reaction could arise because the refolding reaction takes place only in a lipid bilayer or alternatively because the tightly bound lipid might be necessary for tetramer formation. To distinguish between these possibilities, we carried out the refolding reaction in the presence of detergents. Addition of DM (to 10 mM) or SDS (to 1%) to the refolding mixture inhibited refolding, indicating that the mere presence of the lipid molecules is insufficient, and that bilayer formation seems to be essential (data not shown). The refolding reaction was quite independent of the nature of the lipids that constitute the bilayer. The refolding reaction was observed using either soybean lipid vesicles or *Escherichia coli* lipid vesicles, which have very different lipid compositions (data not shown). A specific requirement for PG, which was identified in the KcsA structure, was tested by assessing refolding under different mixtures of purified lipids. A mixture of PE and PG (3:1)

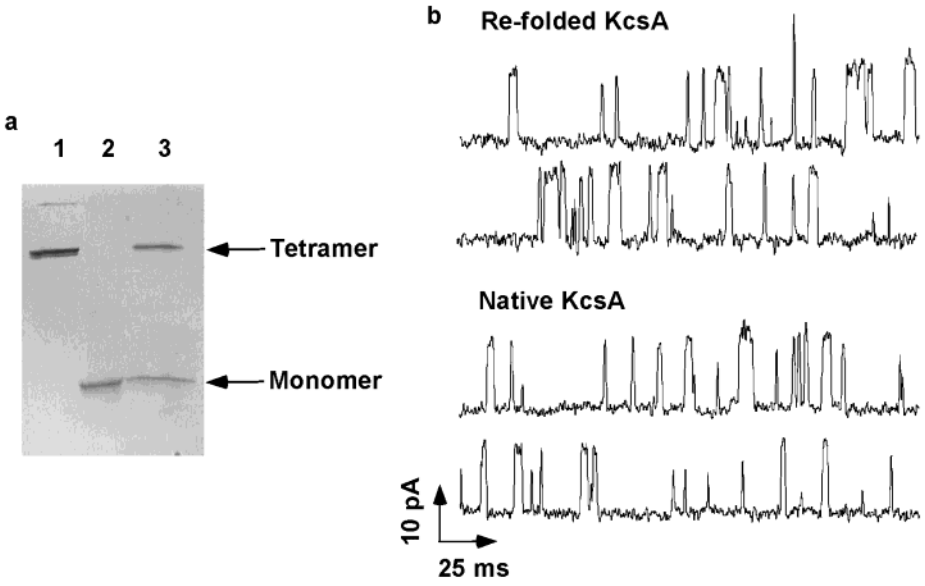

FIGURE 4: Unfolding and refolding of KcsA. (a) SDS−PAGE gel showing recombinant tetrameric KcsA (lane 1), TFE and acid-unfolded KcsA (lane 2), and refolded KcsA (lane 3). (b) Representative single-channel traces for the refolded KcsA are shown along with single-channel traces for native recombinant KcsA. Currents were recorded at 180 mV in symmetrical 150 mM K^+ solutions as described previously (*20*, *21*).

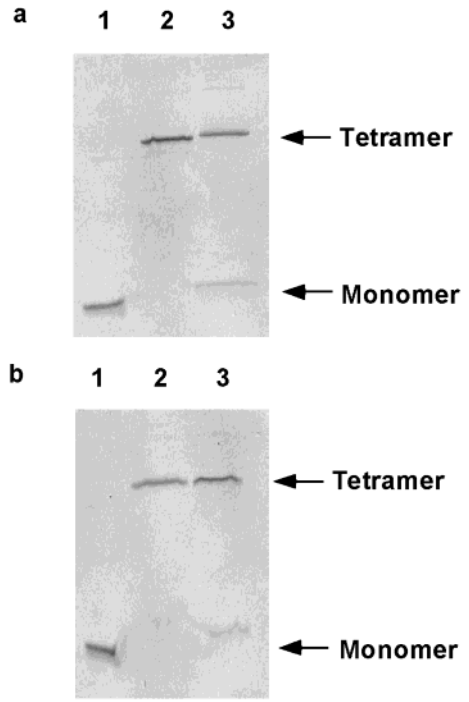

FIGURE 5: Refolding of KcsA by various lipids. (a) SDS−PAGE gel showing TFE and acid-unfolded KcsA (lane 1) and refolding of KcsA by POPE/POPG (3:1, w/w) vesicles (lane 2) and POPE/CL (3:1) vesicles (lane 3). (b) SDS−PAGE gel showing TFE and acid-unfolded KcsA (lane 1) and refolding of KcsA by egg lipid vesicles (lane 2) and POPC lipid vesicles (lane 3).

or PE and CL (3:1) supported refolding, ruling out an absolute requirement for PG (Figure 5a). A requirement for a negatively charged lipid was also ruled out as refolding could take place in either egg lipid vesicles that predominantly consist of phosphatidylcholine or POPC lipid vesicles (Figure 5b). On the basis of these observations, we can conclude that a negatively charged lipid is not required for reassembly of the tetramer from unfolded KcsA.

Analysis of the Folded State Using the Anti-KcsA Monoclonal Antibody. The anti-KcsA monoclonal antibody binds strongly to the tetrameric form of KcsA. The crystal structure of the KcsA−Fab complex indicates that the antibody interacts with regions corresponding to both the N-terminal helix and the pore helix of the KcsA subunit. Key interactions between Tyr45, Arg52, Tyr62, and Arg64 residues of KcsA and the antibody molecule can be identified (Figure 6a). On the basis of these interactions, it is easy to understand why the antibody binds only to the folded state: several amino acids separated in sequence must be brought together in the native conformation. As the antibody binds only to the folded state, antibody binding can be used as a test for the correct folding of the KcsA molecule. Western blots of the refolding mixtures indicated strong antibody binding to the refolded tetramer obtained on refolding with either *E. coli* lipid vesicles or POPC lipid vesicles (Figure 6b). On the basis of these results, we can conclude that the structure of the refolded form, at least in the antibody binding regions, is similar on refolding in the presence or absence of negatively charged lipids.

We note that the absence of antibody binding to the monomeric state suggests that the monomer does not contain the tertiary structure of the folded tetramer. We have also recently completed the semisynthesis and refolding of KcsA (*25*). The refolding of the chemically synthesized peptide enables us to set aside any concerns that we have failed to refold KcsA from the completely unfolded state.

Requirement of Negatively Charged Lipids for KcsA Function. KcsA activity in lipid vesicles can be assayed by a concentrative $^{86}Rb^+$ flux assay (*23*, *26*). The activity of KcsA was tested after reconstitution into lipid vesicles containing PE and PG (3:1) or PE and PC (3:1). $^{86}Rb^+$ uptake was observed with KcsA reconstituted into PE/PG lipid vesicles, whereas uptake was not observed with PE/PC vesicles (Figure 7a). Similar experiments, previously reported, have also indicated a requirement of a negatively charged lipid for KcsA activity (*23*). These results might arise simply because KcsA can only be incorporated into vesicles containing negatively charged lipids. To rule out

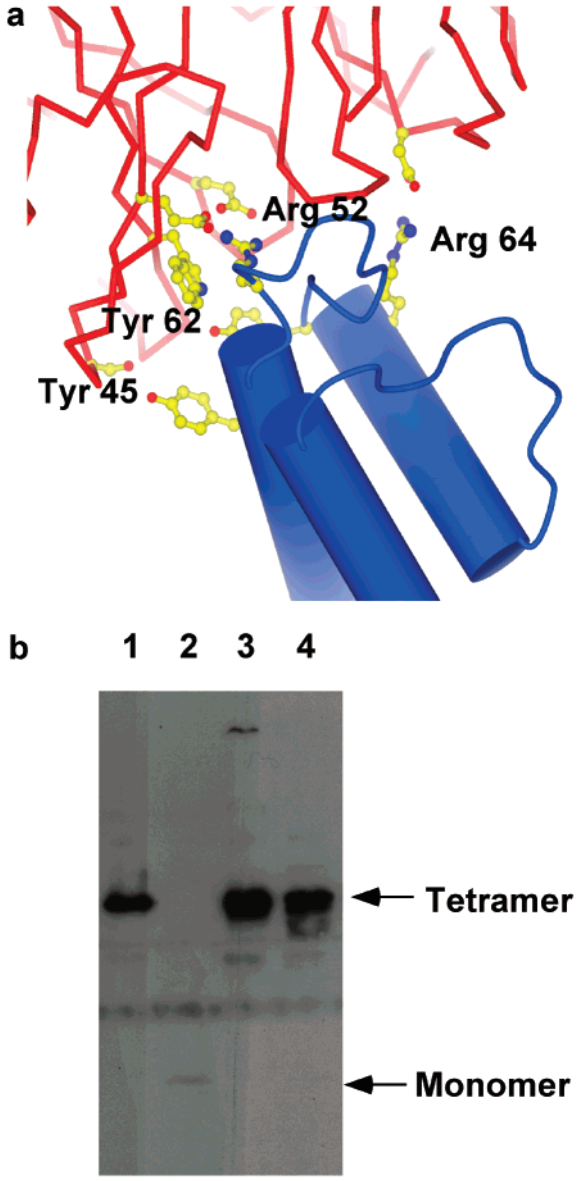

FIGURE 6: Binding of the anti-KcsA monoclonal antibody. (a) Structural details of the binding of the anti-KcsA monoclonal antibody to KcsA. Portions of the antibody molecule (red, represented as a C_α trace) and the KcsA monomer (blue, represented as cylinders) that interact are shown. The residues that form key interactions are depicted in ball-and-stick representation. This panel was prepared with MOLSCRIPT (*31*) and Raster3D (*32*). (b) Immunoblot using the anti-KcsA monoclonal antibody showing recombinant KcsA (lane 1), TFE and acid-unfolded KcsA (lane 2), and KcsA after refolding using *E. coli* lipid vesicles (lane 3) or POPC lipid vesicles (lane 4).

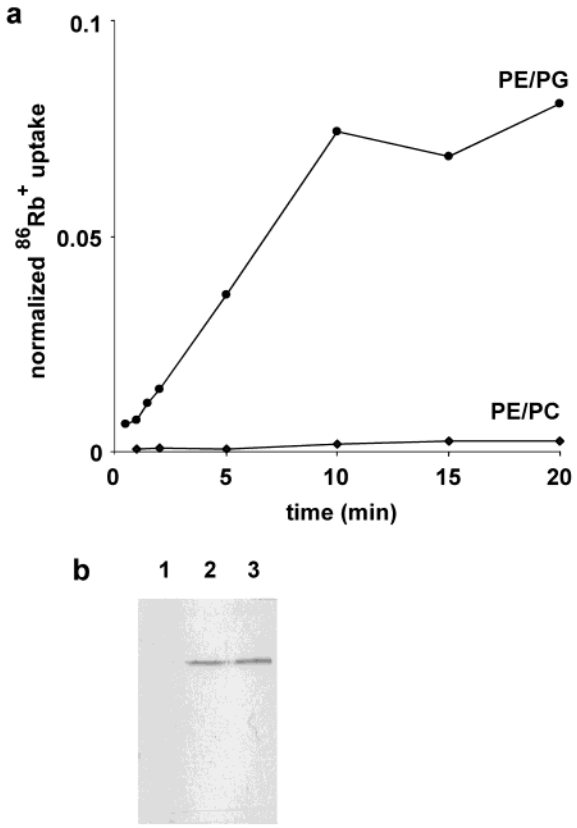

FIGURE 7: Lipid dependence of KcsA function. (a) Time course of $^{86}Rb^+$ uptake into lipid vesicles containing KcsA. KcsA was reconstituted into lipid vesicles at 2.5 μg/mg of lipid. The observed extent of $^{86}Rb^+$ uptake was normalized to the extent of valinomycin-induced uptake in the same vesicles. A similar extent of valinomycin-induced uptake was observed in the case of PE/PG and PE/PC vesicles. (b) Effect of lipid charge on KcsA incorporation into lipid vesicles. SDS−PAGE gel showing the absence of the KcsA tetramer on removal of detergent by dialysis (lane 1) compared to the approximately equal amounts of incorporation of the KcsA tetramer into PE/PG vesicles (lane 2) and PE/PC vesicles (lane 3) by detergent dialysis.

this possibility, the requirement of negatively charged lipids for KcsA reconstitution was tested. For this purpose, KcsA was added to a mixture of PE and PG (3:1) or PE and PC (3:1) lipids solubilized in CHAPS. Lipid vesicles were formed by removal of the detergent by dialysis. The presence of tetrameric KcsA after detergent removal depends on incorporation of KcsA into lipid vesicles. In the absence of lipids, removal of the detergent causes protein denaturation as expected and therefore disappearance of the tetramer band as determined by SDS−PAGE analysis (Figure 7b, lane 1). An approximately similar amount of KcsA tetramer was present in the PE/PG or PE/PC lipid vesicles that formed (Figure 7b, lanes 2 and 3). This result argues that KcsA can be incorporated into lipid vesicles that do not contain negatively charged lipids. However, our results and the results previously reported indicate that ion channel activity is only seen on KcsA reconstitution into vesicles containing negatively charged lipids. On the basis of these results, we can conclude that ion channel activity of KcsA is dependent upon the presence of negatively charged lipids.

DISCUSSION

In this study, we have demonstrated that PG is copurified with KcsA after extraction from *E. coli* membranes and that lipid molecules, presumably PG, are present in the crystal structure of KcsA. Further, we have demonstrated that lipids are required to refold KcsA from the unfolded state to a functional tetramer. Proper refolding requires the presence of lipid bilayers and not the mere presence of individual lipid molecules. Further, the lipid requirement for tetramer assembly is not very specific.

The fact that lipid membranes are required for refolding is somewhat surprising because KcsA is an extremely stable

[2] The presence of PG or other negatively charged lipids might affect the kinetics of the refolding reaction.

protein in detergent micelles; it persists as a tetramer for months at room temperature (*24*, *27*). To the extent that function can be measured in an ion channel extracted from the membrane, a difficult task since the true function of ion channels is to conduct ions across a membrane, KcsA appears to be normal in detergent micelles. Scorpion toxins that require an intact, tetrameric channel to bind do so normally in detergent-solubilized KcsA (*28*). Further, structural studies show that ions bind selectively in the pore of crystallized KcsA (*13*, *14*, *21*), and ionic blockers such as barium also bind inside the pore (*29*). Indeed, the extensive structural analyses of KcsA, which by necessity have been carried out in the membrane-extracted, crystallized form, agree remarkably well with functional analyses carried out in the membrane-embedded form. Therefore, given its stability and apparent functional integrity in the detergent micelle state, it is not apparent why KcsA requires the presence of membranes to fold and assemble into a tetramer, but the requirement is very clear.

In addition to the general lipid interactions that are required to promote KcsA refolding, there appear to be specific lipid interactions as well. PE is more abundant than PG in *E. coli* membranes (*1*), yet PG is isolated with the channel. In the crystal structure, a lipid molecule fills the groove between adjacent subunits. The lipid headgroup lies near two basic amino acids, Arg64 and Arg89, possibly explaining the copurification of PG over PE. The results of refolding experiments presented here have ruled out a requirement for PG or the requirement for a negatively charged lipid for the assembly of the folded tetrameric channel.[2] Monoclonal antibody binding to the refolded tetramer implies that it is in a native conformation.

While negatively charged lipids (and therefore the lipid cofactor) are not essential for the assembly of the KcsA tetramer, the presence of negatively charged lipids is required for ion conduction. While the results of the experiments presented here do not conclusively ascribe a functional role for the lipid in the structure, it is however tempting to speculate that the negative lipid requirement for KcsA function is directly related to the lipid that we see. Given that the transmembrane helices must undergo a conformational change when the channel opens and closes (gates) (*30*), we should expect the lipid cofactor to influence the gating properties of the channel.

ACKNOWLEDGMENT

We thank the staff at the Cornell High Energy Synchrotron Source A1 for help in data collection and Dr. R. Dutzler for assistance in modeling lipid molecules. We thank Dr. C. Miller for help with the flux assays and for helpful discussions.

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BI026215Y