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In Vitro Folding of K_vAP, a Voltage-Gated K^+ Channel

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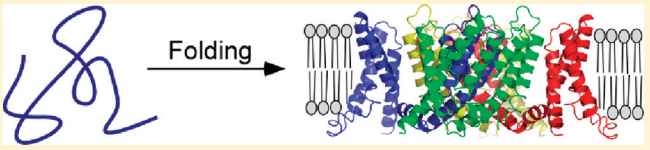
ABSTRACT: In this contribution, we report in vitro folding of the archaebacterial voltage-gated K^+ channel, K_vAP. We show that in vitro folding of the K_vAP channel from the extensively unfolded state requires lipid vesicles and that the refolded channel is biochemically and functionally similar to the native channel. The in vitro folding process is slow at room temperature, and the folding yield depends on the composition of the lipid bilayer. The major factor influencing refolding is temperature, and almost quantitative refolding of the K_vAP channel is observed at 80 °C. To differentiate between insertion into the bilayer and folding within the bilayer, we developed a cysteine protection assay. Using this assay, we demonstrate that insertion of the unfolded protein into the bilayer is relatively fast at room temperature and independent of lipid composition, suggesting that temperature and bilayer composition influence folding within the bilayer. Further, we demonstrate that in vitro folding provides an effective method for obtaining high yields of the native channel. Our studies suggest that the K_vAP channel provides a good model system for investigating the folding of a multidomain integral membrane protein.



Voltage-gated K^+ (K_v) channels are integral membrane proteins (IMPs) that are critical for the generation of electrical impulses by excitable cells.[1] K_v channels are tetrameric proteins with each subunit consisting of six transmembrane segments that are arranged in two distinct domains[2,3] (Figure 1A). The first four transmembrane segments form the voltage sensor domain (VSD), while the last two transmembrane segments form the pore domain (Figure 1B). The pore domain contains the K^+ translocation pathway, while the VSD couples the opening and closing of the pore domain to changes in electrical potential across the membrane. Extensive investigations of K_v channels have been conducted, resulting in a wealth of information about the structure and functional mechanisms of these channels. One aspect of K_v channels that is not well understood is the process of folding to the native state. Understanding the folding of K_v channels is important as folding defects, mainly due to mutations, lead to diseases.[4,5] Studies of the folding of K_v channels reported previously have focused on the integration of individual transmembrane helices into the lipid bilayer, the association of channel subunits, and the trafficking of K_v channels to the cell surface.[6−10] These studies were conducted using in vitro translation or expression in *Xenopus* oocytes or cultured cells. However, there have been no reports of the in vitro folding of a K_v channel from the unfolded state. The advantage of in vitro folding is that it allows us to precisely manipulate the reaction conditions and to thereby identify the factors important for folding.

There are a number of technical challenges encountered during the in vitro folding of IMPs. IMPs require the anisotropic environment provided by the cell membrane for their structural and functional integrity.[11] Therefore, a major challenge during in vitro folding is defining the appropriate detergents or lipids to serve as a mimic for the cell membrane.[12] Another challenge is the lack of a suitable denaturant as those commonly used for soluble proteins, such as urea or guanidine hydrochloride, have limited ability to unfold IMPs.[13,14] Yet another challenge, particularly in the case of ion channels, is the lack of a suitable assay for monitoring the folding process. Because of these challenges, there are only a few reports of the in vitro folding of IMPs.[15] In the family of ion channels, in vitro folding has been extensively investigated only for the KcsA channel.[16−18] In addition, in vitro folding has been reported for the NaK and MscL channels as a part of the chemical synthesis of these channels, and refolding has been demonstrated for a thermally denatured (but not chemically denatured) sodium channel.[19−21]

Here, we investigate the in vitro folding of K_v channel K_vAP from the archaebacterium *Aeropyrum pernix*. The K_vAP channel, though archaebacterial in origin, is functionally very similar to a eukaryotic K_v channel.[22] The K_vAP channel was the first K_v channel for which a crystal structure was reported.[2] Subsequently, crystal structures were reported for the eukaryotic $K_v1.2$ and $K_v1.2$−2.1 chimera channels.[3,23] In the crystal structures of the K_vAP channel, the VSD is distorted, which was shown to be caused by the absence of a lipid bilayer.[24] A structural model for the native state of the K_vAP channel has been proposed on the basis of the structure of the isolated VSD, structural homology to the $K_v1.2$ channel, and disulfide cross-linking and EPR spectroscopic data.[24,25] K_vAP has been used as a model K_v channel for biophysical and spectroscopic investigations because of the structural and functional similarity to eukaryotic K_v channels and the relative ease of overexpression and purification of K_vAP compared to eukaryotic K_v channels.[26,27]

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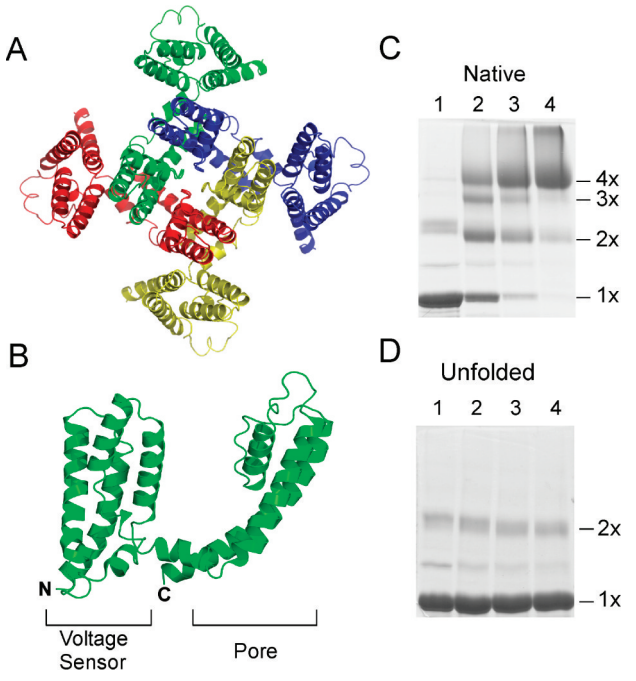

Figure 1. Structure of the K_vAP channel. (A) Top view of the tetrameric K_vAP channel. The structural model of the K_vAP channel as described in ref 24 is shown. (B) Structure of a single subunit of the K_vAP channel. Each subunit consists of two domains, the sensor domain and the pore domain. (C and D) SDS–PAGE gel showing glutaraldehdye cross-linking of the native K_vAP channel (C) and the unfolded protein (D): lane 1, without cross-linker; lanes 2–4, 0.025, 0.05, and 0.1% glutaraldehyde, respectively. The oligomeric nature (1×, 2×, 3×, and 4×) of the cross-linked bands is indicated.

In this study, we demonstrate that the K_vAP channel can be folded in vitro from the extensively unfolded state. We show that lipid bilayers are required for refolding and that the refolded K_vAP channel is biochemically and functionally similar to the native channel. We investigate the factors that are important and determine that refolding depends upon the composition of the lipid bilayer. Interestingly, we observe that refolding of the K_vAP channel is strongly dependent on temperature, with very efficient refolding at elevated temperatures. Using a Cys protection assay, we demonstrate that lipid bilayer composition and temperature do not affect the insertion of the unfolded polypeptide into the bilayer, suggesting that these factors influence the folding of K_vAP within the bilayer. We demonstrate that the efficient in vitro folding coupled with overexpression as inclusion bodies provides a facile approach for obtaining high yields of the K_vAP channel.

EXPERIMENTAL PROCEDURES

Native Expression and Purification of the K_vAP Channel. A His_6-tagged K_vAP channel gene subcloned into the pQE60 plasmid (Qiagen) was kindly provided by R. MacKinnon (The Rockefeller University, New York, NY).[22] The native K_vAP channel was expressed in *Escherichia coli* XL10 (Stratagene) cells as previously described.[22] Following expression, cells were pelleted, resuspended in 50 mM Tris-HCl (pH 7.5), 150 mM KCl, 0.25 M sucrose, 1 mM $MgCl_2$, and DNase (5 μg/mL), and lysed by sonication. Unlysed cells and cell debris were removed by centrifugation at 7500*g*, after which the cell membranes were pelleted by centrifugation at 100000*g*. The membranes were solubilized with decyl β-D-maltoside (DM, 2%, w/v), and the His_6-tagged K_vAP channel was purified by metal affinity chromatography (Talon, Clontech) and size exclusion chromatography.[22] Size exclusion chromatography was conducted on a Superdex S200 column (GE Healthcare) using a running buffer composed of 50 mM HEPES-KOH (pH 7.5), 150 mM KCl, and 0.25% DM.

Expression of K_vAP in Inclusion Bodies. The glutathione *S*-transferase (GST)–K_vAP fusion used for expression as inclusion bodies consists of the K_vAP channel linked to the C-terminus of GST with a linker that consists of a thrombin site, His_6, and a Factor X_a site. Initial expression studies showed premature translation terminations in the N-terminal region of the K_vAP channel. The K_vAP gene consists of a number of rare amino acid codons in the N-terminal region, which could account for the premature terminations. To ensure translation of the complete gene, the rare codons present within the 20 N-terminal amino acids were substituted with the most frequently used codons for the corresponding amino acids. The single native Cys at position 247 was substituted with Ser in the fusion construct to prevent disulfide cross-linking.

The GST–K_vAP fusion was expressed in *E. coli* Rosetta2-(DE3) cells (EMD Biosciences) using the autoinduction procedure.[28] For isolation of the inclusion bodies, cells were pelleted and resuspended in 50 mM Tris-HCl (pH 7.5), 0.2 M NaCl, 1 mM $MgCl_2$, and DNase (5 μg/mL). Lysozyme (0.1 mg/mL) was added, and the cells were incubated at room temperature with gentle stirring. After 30 min, phenylmethanesulfonyl fluoride was added to a final concentration of 1 mM, and the cells were lysed by sonication. Triton X-100 (Tx-100) was added (1%, v/v), and the cell lysate was stirred at room temperature for 30 min. The soluble and insoluble fractions were separated by centrifugation at 12000*g* for 10 min. The insoluble fraction, which contains the inclusion bodies, was washed three times with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1% Tx-100. The inclusion bodies were resuspended in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1% *N*-laurylsarcosine (NLS) and digested with thrombin (Roche, 1 unit/L of culture) overnight, to cleave the K_vAP polypeptide from GST.

For purification of the K_vAP polypeptide, 1 volume of 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl was added to the thrombin cleavage mixture. Tx-100 was added to a final concentration of 2% (v/v), and the K_vAP polypeptide was purified using metal affinity chromatography. The K_vAP polypeptide obtained was unfolded using the protocol described, prior to being used in the refolding studies.

Unfolding of the K_vAP Channel. A multistep protocol was used for unfolding the K_vAP channel. The protocol is based on the unfolding procedure described for bacteriorhodopsin.[13] In the first step, Tx-100 was added to a final concentration of 2% (v/v) and the protein was precipitated by 15% (w/v) trichloroacetic acid (TCA) for 15 min at 4 °C. The protein precipitate was collected by centrifugation at 3000*g*, washed twice with acetone and 0.1% trifluoroacetic acid (TFA), solubilized in 50% trifluoroethanol (TFE) and 0.1% TFA, and lyophilized to provide the unfolded K_vAP channel used for the refolding studies.

Refolding of the K_vAP Channel. The lyophilized K_vAP polypeptide was dissolved in 100 mM sodium phosphate (pH 7.5), 1% sodium dodecyl sulfate (SDS), and 10 mM β-mercaptoethanol. The concentration of the unfolded protein was determined by comparing the band intensities on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–

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PAGE) of the unfolded protein to known concentrations of the native K_vAP channel. The following lipids were used: asolectin (soy total lipid extract, Aso), 1-palmitoyl-2-oleoylglycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoylglycero-3-phosphoglycerol (POPG), 1-palmitoyl-2-oleoylglycero-3-phosphoethanolamine (POPE), and 1,2-diphytanoylglycero-3-phosphocholine (DPhPC) (from Avanti Polar Lipids). Lipids solutions in chloroform were dried under argon, redissolved in cyclohexane, and lyophilized. Lyophilized lipids were hydrated for 1 h at a concentration of 5 mg/mL using 50 mM HEPES-KOH (pH 7.5), 150 mM KCl, and 10 mM β-mercaptoethanol and then sonicated for 5 × 30 s pulses in a water bath sonicator, yielding lipid vesicles. For refolding experiments at room temperature, the unfolded K_vAP polypeptide was diluted 10-fold into lipids or detergents and the mixture sonicated briefly (~5 s pulse). For refolding at elevated temperatures, dilution without sonication was used. A protein concentration of 0.8–1.0 mg/mL was used for the refolding experiments. Refolding of the K_vAP channel was assayed by glutaraldehyde cross-linking for 15 min at room temperature, and the reaction was quenched by the addition of SDS sample buffer that contained 100 mM Tris.

The unfolded K_vAP polypeptide has a strong tendency to aggregate, which complicated analysis of the refolding reactions using glutaraldehyde cross-linking. We observed that in dodecylphosphocholine [2% Fos-12 (w/v)], the native K_vAP channel is stable while nonspecific aggregates dissociate into monomers. The glutaraldehyde cross-linking reaction was therefore conducted after the addition of 2% Fos-12, to distinguish the folded tetrameric species from the nonspecific aggregates. This step was also beneficial as the addition of Fos-12 served to quench the refolding reaction. The refolding samples (15–20 μg of protein) were separated on a 10% SDS–PAGE gel and stained with Coomassie blue. After being destained, the gels were dried and scanned using a flat-bed scanner, and the protein bands were quantified using Scion Image (http://www.scioncorp.com). Protein bands corresponding to the monomer, dimer, trimer, and tetramer in each lane were quantified, and background was subtracted. The refolding yield was calculated as the fraction of the K_vAP polypeptide in each lane that was present as the tetrameric species.

To determine the time course of the refolding of the K_vAP channel, we quenched the folding reaction with Fos-12, followed by GA cross-linking and SDS–PAGE to determine the refolding yield at the various time points. The time course of the refolding showed monoexponential behavior, and a single-exponential fit was used to obtain the half-time ($t_{1/2}$) to maximal yield under the different refolding conditions.

For purification of the refolded K_vAP channel, lipid vesicles used for refolding were solubilized with DM and the refolded channel was purified using metal affinity chromatography followed by size exclusion chromatography, as described for the native channel.

Electrophysiological Measurements of the Refolded K_vAP Channel. Prior to electrophysiological measurements, the native and refolded K_vAP channels were reconstituted into lipid vesicles composed of DPhPC (10 mg/mL) as previously described.[22,29] Functional measurements of the K_vAP channel were conducted using planar lipid bilayers composed of DPhPC. The recording solutions used for channel measurements consisted of 10 mM HEPES-KOH (pH 7.5) and 150 mM KCl on both the cis and trans sides. The voltage dependence of the native and refolded K_vAP channel was determined from a holding potential of −120 mV. Tail currents were measured at −100 mV after a test potential with a duration of 200 ms. The normalized tail current amplitude (I/I_{max}) was plotted as a function of the test potential and fitted with the Boltzmann function $I/I_{max} = 1/\{1 + \exp[-zF(V - V_{0.5})/(RT)]\}$ to obtain values of $V_{0.5}$ and z. Slow inactivation was recorded by a 5 s depolarization to 100 mV from a holding potential of −150 mV. The inactivation time constant (τ_{inact}) was determined by fitting the decay in current after peak activation to the single-exponential equation $i = A \exp(-t/\tau_{inact}) + C$, where i is the current at time t.

Cys Protection Assay Using PEG Maleimide. Single-Cys substitutions of residues 101, 127, 139, 161, 175, and 214 were generated using QuickChange (Stratagene) in a K_vAP channel gene in which the native Cys (Cys247) was substituted with Ser. The Cys mutants were expressed and purified as described for the native protein and unfolded using the protocol previously described.

Refolding of the unfolded Cys mutant proteins was conducted at room temperature as described previously, except that 1 mM DTT was used instead of 10 mM β-mercaptoethanol. The unfolded Cys mutant proteins were diluted into lipids or into 2% Fos-12 (as a control). Immediately following dilution (<30 s), an aliquot of the refolding reaction mixture was incubated with 8 mM PEG-2K-mal (Creative PEGWorks) directly or after solubilization of the lipid vesicles with 2% Fos-12. The control reaction mixture was also similarly treated with PEG-2K-mal. PEGylation was conducted for 30 min at room temperature and quenched by addition of 200 mM DTT followed by SDS–PAGE loading buffer. The extent of labeling was determined by electrophoresis on a 12% SDS–PAGE gel to separate the labeled and unlabeled proteins.

RESULTS

Unfolding of the K_vAP Channel. Two different approaches were used to obtain the unfolded K_vAP polypeptide that was required for the in vitro folding experiments. In the first approach, the K_vAP polypeptide was obtained by unfolding the native channel. The native K_vAP channel was expressed and purified as previously described.[22] Unfolding of the native K_vAP channel takes place upon treatment with SDS (1%, w/v) or precipitation with TCA or organic solvents (data not shown). To ensure extensive unfolding, the K_vAP channel was precipitated with TCA, washed with acetone, dissolved in 50% TFE and 0.1% TFA, and lyophilized. The lyophilized precipitate was dissolved in 1% (w/v) SDS to provide the unfolded K_vAP used for the in vitro folding experiments. Unfolding of the K_vAP channel was confirmed by chemical cross-linking using glutaraldehyde. The native K_vAP channel is a tetramer, and glutaraldehyde cross-linking of the native channel gives a protein band that migrates like a tetramer on SDS–PAGE (Figure 1C). Unfolding results in the loss of the native tetrameric state, and glutaraldehyde cross-linking of the unfolded protein gives only a protein band that corresponds to the monomer on SDS–PAGE (Figure 1D).

The second approach used to obtain the unfolded K_vAP polypeptide was to express the K_vAP channel as a fusion with GST. Appending GST at the N-terminus of K_vAP results in directing protein expression to inclusion bodies. Following expression, the inclusion bodies were isolated and the K_vAP polypeptide was released from GST by proteolysis. The K_vAP

polypeptide was then purified by metal affinity chromatography and subjected to the extensive unfolding protocol described for native K_vAP prior to in vitro folding experiments. The yield of the unfolded K_vAP polypeptide using this approach was 10−12-fold higher per liter compared to the yield of native expression followed by unfolding.

Refolding of the K_vAP Channel. For assembly of the native tetrameric K_vAP channel from the unfolded polypeptide in SDS, we initially tested refolding by dilution of the denaturant (SDS) with mild detergents. The detergents [DM, *n*-dodecyl β-D-maltopyranoside (DDM), *n*-octyl β-D-glucopyranoside (OG), and Fos-12 (2%, w/v)] were selected on the basis of their prior use in structural studies of the K_vAP channel.[2,24,30,31] Glutaraldehyde cross-linking was used to check for refolding, which was indicated by the presence of a tetrameric species on SDS−PAGE. Refolding by dilution of the unfolded polypeptide into a detergent solution was not successful as the tetrameric species was not observed with any of the detergents tested (Figure 2). A dimeric species was

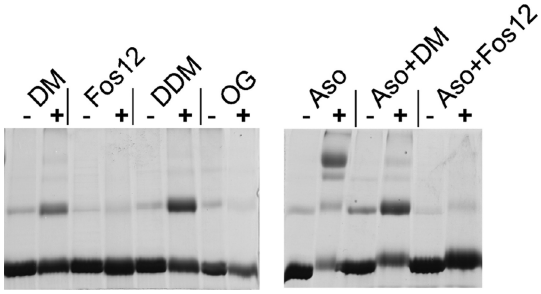

Figure 2. In vitro folding of the K_vAP channel. Refolding was tested after dilution of the unfolded protein into detergents [DM, Fos-12, DDM, and OG (2%, w/v)], asolectin lipid vesicles (Aso), or asolectin lipid vesicles solubilized with DM or Fos-12 (2%, w/v). Glutaraldehyde cross-linking followed by SDS−PAGE was used to assess the formation of tetrameric species: (−) without cross-linker and (+) with 0.1% glutaraldehyde.

observed in the case of DM and DDM. Dimerization of the isolated VSD in DM has been previously reported and was suggested to take place through the S4 segment.[32] We anticipate that the dimerization of the K_vAP polypeptide in DM and DDM is similarly mediated through the S4 segment. Next, we investigated lipid vesicles for refolding of the K_vAP channel. Lipid vesicles have successfully been used for refolding of the KcsA and NaK channels from the extensively unfolded state.[14,21] Upon dilution of the unfolded K_vAP polypeptide into asolectin vesicles, refolding was observed as indicated by the presence of a tetrameric species on glutaraldehdye cross-linking (Figure 2). Refolding of the K_vAP channel was, however, not observed when the asolectin vesicles were solubilized by detergents, indicating that the refolding process requires an intact lipid bilayer.

Characterization of the Refolded K_vAP Channel. The K_vAP channel obtained after in vitro folding was purified by metal affinity chromatography followed by size exclusion chromatography. The refolded K_vAP channel had a retention time on size exclusion chromatography similar to that of the native protein, and glutaraldehyde cross-linking confirmed the tetrameric nature of the refolded K_vAP channel (Figure 3A).

The CD spectrum of the refolded K_vAP channel is similar to that of the native channel (Figure 3B). Calculation of the helical content using the k2d3 algorithm (http://www.ogic.ca/

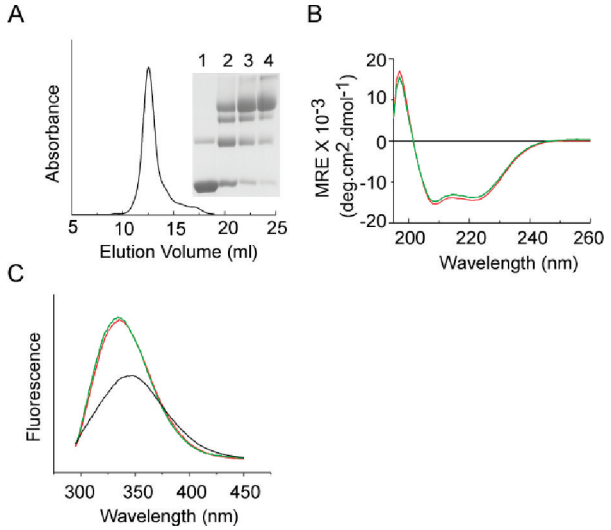

Figure 3. Characterization of the refolded K_vAP channel. (A) Size exclusion chromatography of the refolded K_vAP channel. The observed elution volume was 12.5 mL. The inset shows glutaraldehyde cross-linking of the peak fraction: lane 1, without cross-linker; lanes 2−4, 0.025, 0.05, and 0.1% glutaraldehyde, respectively. (B) CD spectra of the refolded K_vAP channel. CD spectra reported as mean residue ellipiticity (MRE) for the native (red) and refolded K_vAP (green) channel in 10 mM sodium phosphate buffer (pH 7.5), 150 mM KCl, and 0.25% DM. A protein concentration of 450 μg/mL was used. (C) Fluorescence spectra of the refolded K_vAP channel. Intrinsic fluorescence spectra (excitation at 280 nm) for the native (red) and refolded (green) channel recorded in 50 mM HEPES (pH 7.5), 150 mM KCl, and 0.25% DM and unfolded K_vAP channel in 100 mM sodium phosphate (pH 7.5) and 1% SDS (black). A protein concentration of 25 μg/mL was used.

projects/k2d3/orainaldia.html) gave similar values, 40% for the native and 37% for the refolded K_vAP channel. In contrast, the K_vAP polypeptide has a helical content of 70% in 50% TFE and 0.1% TFA and 47% in 1% SDS (data not shown). These CD experiments therefore indicate a similar secondary structure for the native and refolded K_vAP channels and show that there are changes in the secondary structure during the refolding process.

The intrinsic fluorescence spectrum of the native K_vAP channel shows an emission maximum at 335 nm (Figure 3C). Unfolding of the K_vAP channel results in a red shift of the emission maximum to 345 nm and is accompanied by a 50% decrease in the fluorescence intensity (at 335 nm). The fluorescence spectrum of the refolded K_vAP channel is similar to that of the native protein. The K_vAP channel consists of three Trp residues, two in the pore domain and one in the voltage sensor domain. The similar fluorescence spectra indicate a similar environment for the Trp residues in the native and refolded K_vAP channels pointing to the structural similarity of these proteins.

The purified refolded K_vAP channel was reconstituted into DPhPC planar lipid bilayers for measurement of channel activity. Single-channel openings for the refolded K_vAP channel are shown in Figure 4A. The single-channel conductance (124 ± 6 pS for refolded vs 125 ± 8 pS for the native channel at 100 mV in 150 mM K^+) and the single-channel current−voltage curve for the refolded K_vAP channel are similar to those of the native channel (Figure 4B). The voltage dependence of channel opening for the refolded K_vAP channel was measured using macroscopic (multichannel) currents (Figure 4C). K_vAP is a depolarization-activated K^+ channel, and the number of channel

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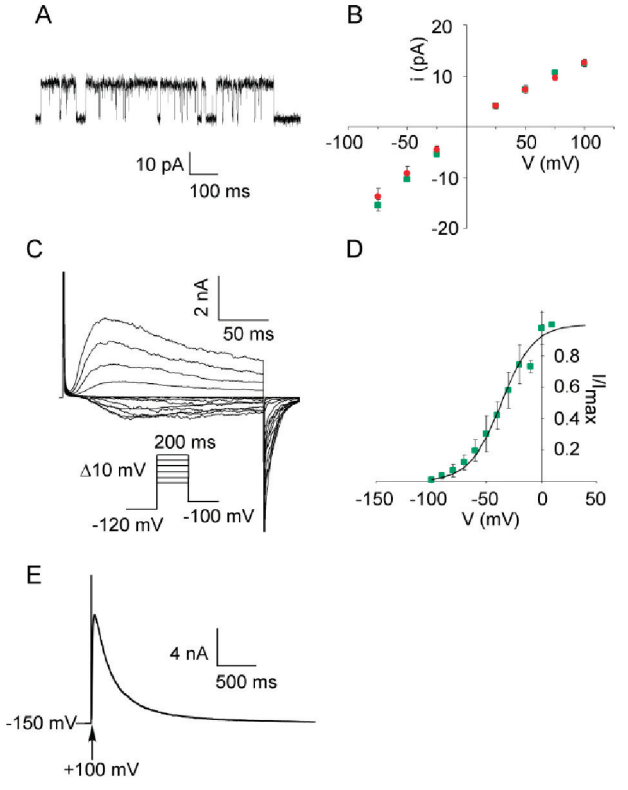

Figure 4. Functional characterization of the refolded K_vAP channel. (A) Single-channel trace for the refolded K_vAP channel recorded at 100 mV. (B) Single-channel current as a function of voltage for the native (red) and refolded (green) K_vAP channels. (C) Voltage-activated macroscopic currents from the refolded K_vAP channel recorded using the voltage protocol shown (inset). (D) Voltage gating of the refolded K_vAP channel. The fraction of the maximal current observed was plotted as a function of the test potential (see Methods). The smooth line corresponds to a Boltzmann function with a $V_{0.5}$ of -36.1 ± 4.3 and a z of 1.86 ± 0.36. (E) Slow inactivation of the refolded K_vAP channel. K_vAP currents were elicited from a holding potential of -150 mV by a depolarization to 100 mV. The inactivation time constant was determined by fitting the decay in current after peak activation to a single-exponential function. The recordings were conducted in 150 mM KCl and 10 mM HEPES-KOH (pH 7.5). For panels B and D, the error bars indicate the standard deviation for three or more experiments.

openings increases sharply with depolarization.[22] The data for channel opening versus voltage were fit to a two-state Boltzmann distribution, to obtain the values of $V_{0.5}$, the voltage for half-maximal opening, and z, the apparent gating charge. The values for the refolded K_vAP channel ($V_{0.5} = -36.1 \pm 4.3$, and $z = 1.86 \pm 0.36$) were similar to the corresponding values ($V_{0.5} = -40.7 \pm 5.2$, and $z = 1.81 \pm 0.25$) for the native channel, indicating similar gating behavior in these channels (Figure 4D). Slow inactivation of the refolded K_vAP channel [287 ± 7 ms^{-1} (Figure 4E)] was also similar to that of the native channel (285 ± 20 ms^{-1}). The electrophysiological behavior of the refolded K_vAP channel is therefore essentially similar to that of the native channel.

The similar biochemical behavior and spectroscopic and electrophysiological characteristics all suggest that the refolded K_vAP channel is similar to the native channel.

Effect of Lipid Bilayer Composition on the Refolding of the K_vAP Channel. In previously conducted experiments, asolectin vesicles were used for the refolding of the K_vAP channel. Asolectin is a heterogeneous mixture of soybean lipids. To investigate if specific lipids are required, we tested in vitro folding of the K_vAP channel using lipid vesicles with a defined composition. Unfolded K_vAP channel was diluted into lipid vesicles, and the refolding yields were evaluated after a 2 h incubation at room temperature by glutaraldehdye cross-linking (Figure 5A,B). In POPC lipid vesicles, the extent of refolding

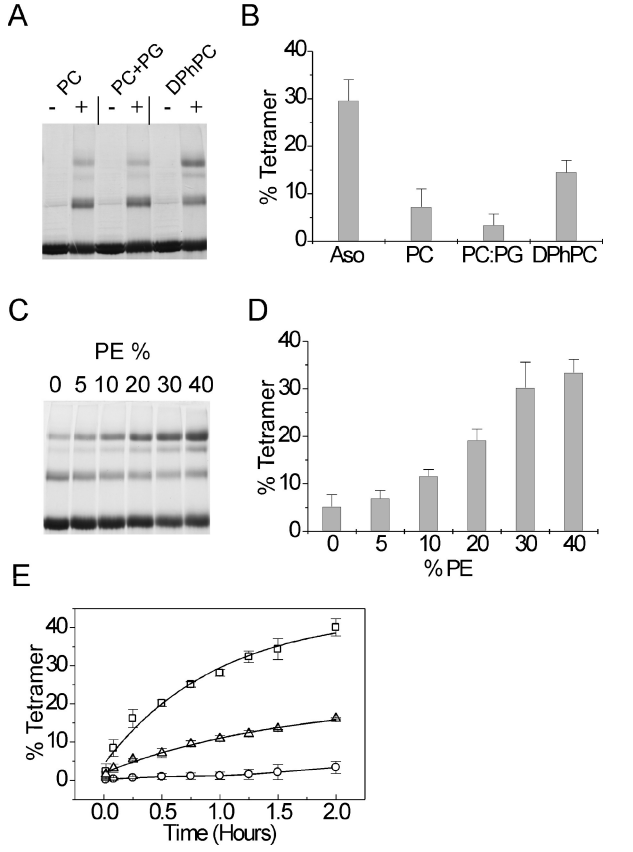

Figure 5. Refolding of the K_vAP channel in various lipids. (A) SDS–PAGE gel showing glutaraldehyde cross-linking of the K_vAP channel refolded in POPC, POPC/POPG (75:25), and DPhPC lipid vesicles for 2 h at room temperature: (−) without cross-linker and (+) with 0.1% glutaraldehdye. (B) Bar graph comparing the refolding yields in the various lipids. Refolding yields of K_vAP in asolectin vesicles (Aso) under similar refolding conditions are also shown. (C) SDS–PAGE gel and (D) bar graph showing the effect of increasing the ratio of POPE in POPC/POPE lipid vesicles on refolding of the K_vAP channel. The extent of refolding after 2 h at room temperature is shown. (E) Time course of refolding of the K_vAP channel in POPC (○), DPhPC (△), and 60:40 POPC/POPE (□) lipid vesicles. The smooth lines for DPhPC and POPC/POPE vesicles correspond to a single-exponential fit, while a line joining points is shown for POPC. For panels B, C, and E, the error bars indicate the standard deviation for three or more experiments.

($7 \pm 3\%$) was very low compared to that in asolectin vesicles ($30 \pm 4\%$). Negatively charged lipids have been shown to be important for the structure and function of certain membrane proteins (for examples, see refs 33 and 34). To determine if negatively charged lipids are required, we tested in vitro folding of the K_vAP channel in a mixture of POPC and POPG. We observed that the inclusion of POPG did not improve the extent of refolding ($3 \pm 2\%$), indicating that the low extent of refolding observed in the POPC lipid vesicles is not due to the absence of negatively charged lipids. As the K_vAP channel is

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archaeal in origin, we tested refolding in DPhPC lipid vesicles. DPhPC contains isoprenyl tails similar to archaeal lipids.[35,36] Our tests indicated a higher extent of folding in the DPhPC lipid vesicles (15 ± 2%) than in POPC vesicles. POPC and DPhPC have the same headgroup; therefore, the differences in refolding of the K_vAP channel in these lipids must arise either because of a specific requirement for a lipid with an isoprenyl tail (similar to archaeal lipids) or because of differences in the bilayer properties of POPC and DPhPC lipids.

Lipid bilayer properties such as the bilayer curvature stress can strongly influence the folding of membrane proteins.[37,38] The curvature stress of the lipid bilayer can influence the folding of a membrane protein either by an effect on the insertion into the lipid bilayer or through an effect on the packing of the transmembrane helices during folding. The curvature stress of the bilayer can be altered by varying the ratio of phosphoethanolamine lipids (PE) in a PC/PE lipid bilayer.[37] To determine how the curvature stress of the bilayer influences the refolding of the K_vAP channel, we conducted the refolding using lipid vesicles with varying ratios of POPC and POPE (Figure 5C,D). We observed a steady increase in the refolding yield with an increase in the POPE ratio. We could not determine the effect of POPE percentages of >40% due to the interference of POPE with the glutaraldehyde cross-linking reaction. We established that POPE, at >40%, interferes with the glutaraldehyde reaction by evaluating the cross-linking of the native K_vAP channel in lipid vesicles with varying ratios of POPC and POPE (data not shown). In the presence of 40% POPE, the extent of refolding was higher than the extent of refolding observed in DPhPC lipids and equals the extent of refolding observed in asolectin vesicles.

The kinetics of in vitro folding of the K_vAP channel was determined by quenching the folding reaction at various time points by the addition of Fos-12 (2%, w/v), followed by glutaraldehyde cross-linking. The time courses of folding of K_vAP in POPC, DPhPC, and POPC/POPE (6:4) lipids are shown in Figure 5E. The rate of folding of the K_vAP channel in POPC is very slow, while a higher rate and a higher extent of folding are observed in DPhPC and POPC/POPE (6:4) lipid vesicles. In these vesicles, the time course of folding was well fit by a single exponential and these fits were used to obtain the half-time ($t_{1/2}$) for the reaction. The higher rate of folding observed in POPC/POPE (6:4) lipid vesicles ($t_{1/2}$ = 40.8 min) compared to DPhPC vesicles ($t_{1/2}$ = 65.8 min) suggests that lipids with isoprenyl tails are not essential for the refolding of the K_vAP channel. The increase in the rate of refolding with the ratio of POPE in a POPC/POPE lipid bilayer suggests that increasing the membrane curvature stress of the lipid bilayer favors the refolding of the K_vAP channel.

Effect of Temperature on the Refolding of the K_vAP Channel. As the K_vAP channel was cloned from a hyperthermophilic archaeon (*A. pernix*),[39] we investigated the effect of elevated temperatures on the in vitro folding process. Refolding of K_vAP was conducted at temperatures ranging from 30 to 80 °C for 10 min, and the folding yields were determined by quenching the folding reaction with Fos-12 (2%, w/v), followed by glutaraldehdye cross-linking [0.1% (w/v) at room temperature for 15 min]. We used DPhPC lipid vesicles for our initial experiments as DPhPC lipid bilayers do not show a phase transition over this temperature range.[35] With an increase in temperature, we observed a dramatic improvement in the extent of folding (Figure 6A,B). At the highest temperature tested, the refolding yield was 73 ± 4% after 10 min compared

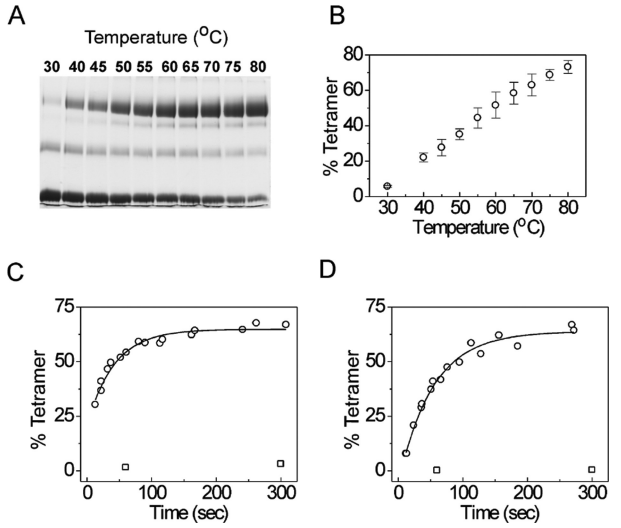

Figure 6. Effect of temperature on refolding of the K_vAP channel. (A) SDS–PAGE gel showing refolding of the K_vAP channel in DPhPC lipid vesicles after a 10 min incubation at the indicated temperatures. (B) Refolding yields after 10 min plotted as a function of temperature. Error bars indicate the standard deviation (n = 3). (C and D) Time courses of refolding of the K_vAP channel in DPhPC (C) and POPC (D) at 25 (□) and 80 °C (○). The solid lines represent single-exponential fits to the data.

to a yield of 15 ± 2% after a 2 h incubation at room temperature. The time course of folding of K_vAP in DPhPC vesicles at room temperature and 80 °C is shown in Figure 6C. Single-exponential fits to the data indicate that the half-time ($t_{1/2}$) for the reaction changes from 65.8 min at room temperature to 0.48 min at 80 °C.

A similar influence of temperature was also observed for the refolding of the K_vAP channel in POPC lipid vesicles. The extent of refolding of the K_vAP channel in POPC lipid vesicles was 63 ± 5% after 10 min at 80 °C compared to a yield of 7 ± 3% after 2 h at room temperature. The rate of refolding in POPC lipid vesicles at 80 °C ($t_{1/2}$ = 0.61 min) approaches the rate of refolding observed for DPhPC lipid vesicles (Figure 6D). These observations indicate that temperature has a major influence on the in vitro folding of the K_vAP channel.

Membrane Insertion of the K_vAP Channel during Refolding. One mechanism by which the composition of lipid vesicles and temperature can alter refolding rates of the K_vAP channel is through an effect on the insertion of the unfolded polypeptide into the lipid bilayer. To investigate this possibility, we developed an assay to monitor the incorporation of the K_vAP polypeptide into lipid bilayers. Our assay is based on the approach developed by Deutsch et al.[40] In our assay, we introduced Cys residues into various segments of the K_vAP channel and determined the accessibility of these Cys residues to modification by PEG-2K-mal in the presence and absence of lipid vesicles. If the Cys-containing segment of the K_vAP polypeptide is incorporated into the lipid bilayer, then it is protected from modification by PEG-2K-mal, while a Cys residue in a segment that is not incorporated into the lipid bilayer is accessible to modification. The difference in modification by PEG-2K-mal arises because Cys residues within the lipid bilayer are less reactive than aqueous exposed Cys residues. Further, the modifying reagent used, PEG-2K-mal, is highly water soluble, and therefore, concentrations within the lipid bilayer are expected to be extremely low.

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Modification by PEG-2K-mal is detected by a shift in the mobility on SDS−PAGE, and the extent of modification reports on the degree of incorporation of that segment of the K_vAP polypeptide into the lipid bilayer.

We generated Cys substitutions of residue 101 in the S3 helix and residue 127 in the S4 helix to monitor the incorporation of the VSD into the lipid bilayer, while Cys substitutions of residue 161 in the S5 helix and residue 214 in the S6 helix were generated to monitor the incorporation of the pore domain (Figure 7A). Cys substitutions of residue 139 in the S4−S5 loop and residue 175 in the S5−pore helix loop were generated to serve as markers for the aqueous exposed segments of the K_vAP channel. The Cys mutants of the K_vAP channel were purified and unfolded as previously described. The unfolded K_vAP polypeptides were diluted into lipid vesicles for refolding at room temperature, and an aliquot was immediately withdrawn (∼30 s) and treated with PEG-2K-mal to test incorporation of the K_vAP polypeptide into the lipid bilayer.

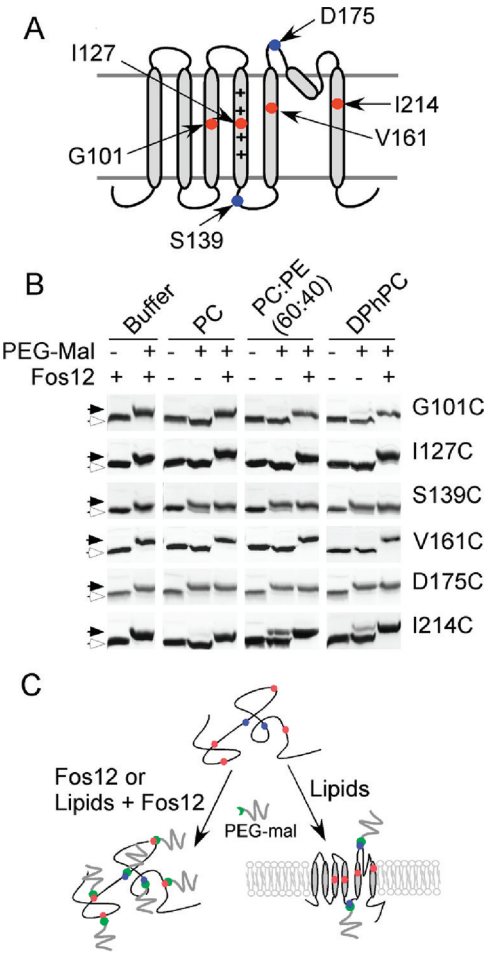

Figure 7. Insertion of the K_vAP polypeptide into the lipid bilayer during refolding. (A) Cartoon depicting the location of Cys residues introduced into the K_vAP channel for the PEGylation experiment. (B) Accessibility of Cys residues to PEGylation immediately after dilution of unfolded protein into lipid vesicles or into buffer containing 2% (w/v) Fos-12 at room temperature. SDS−PAGE gels showing modification of the single-Cys mutants of K_vAP by PEG-2K-mal. The slowly migrating PEGylated protein (solid arrows) and fast migrating protein without modification (open arrows) are denoted. (C) Summary of the PEGylation experiment. Upon dilution of the unfolded K_vAP polypeptide into lipid vesicles, the Cys residues in the transmembrane segments (red) became inaccessible to PEGylation while the Cys residues in the loops (blue) remain accessible. In contrast, all the Cys residues are accessible to PEGylation when the unfolded K_vAP polypeptide is diluted into detergent micelles or into lipid vesicles solubilized by Fos-12 (2%, w/v).

Upon addition to lipid vesicles, Cys residues in the transmembrane segments (Cys101 in S3, Cys127 in S4, Cys161 in S5, and Cys214 in S6) were immediately protected from reaction with PEG-2K-mal (Figure 7B). This protection was not observed when the K_vAP polypeptides were diluted into buffer (in the absence of lipid vesicles) or when the lipid vesicles were solubilized with detergent. The composition of the lipid bilayer does not influence the protection of the Cys residues, and almost complete protection of these transmembrane Cys residues from modification was seen for POPC, POPC/POPE (6:4), and DPhPC lipid vesicles. In contrast, Cys139 and Cys175 in the aqueous exposed regions were not protected from modification by PEG-2K-mal upon addition of the K_vAP polypeptide to lipid vesicles. A similar extent of modification was observed for these Cys residues in the presence of lipid vesicles compared to the absence of lipid vesicles or when the lipid vesicles were solubilized with detergent.

These experiments indicate that upon addition of the unfolded K_vAP polypeptide to lipid vesicles, the transmembrane segments are immediately incorporated into the lipid bilayer while the loop regions stay outside the membrane (Figure 7C). We observe complete labeling of Cys139 and Cys175, residues in loop regions that are expected to be on opposite sides of the membrane. This observation suggests that PEG-2K-mal is able to permeate into the lipid vesicles, probably because of the residual detergent (SDS, 3.5 mM) during refolding. The similar labeling of Cys139 and Cys175 indicates that we cannot differentiate between Cys residues exposed to the exterior and the lumen of the lipid vesicles by using the PEG-2K-mal reagent.

Our results suggest that during refolding, the unfolded K_vAP polypeptide is rapidly incorporated into lipid vesicles with a "nativelike" topology. The extent of incorporation is independent of the composition of the lipid bilayer as similar incorporation is seen in the case of POPC, POPC/POPE (6:4), and DPhPC lipid vesicles. This rapid incorporation of the K_vAP polypeptide into lipid vesicles is observed at room temperature, at which the rate of refolding is very low. These Cys protection experiments therefore suggest that the two factors, composition of the lipid bilayer and the temperature, that are important for the in vitro folding of the K_vAP channel do not influence the insertion of the K_vAP polypeptide into the lipid bilayer but instead influence a later folding step that takes place within the lipid bilayer.

High-Level Expression of the K_vAP Channel. A major challenge in investigations of membrane proteins is obtaining sufficient quantities of the native protein for structural and biochemical analysis. The general approach used is expression in the native state in a heterologous system. In the case of the K_vAP channel, expression in the native state in *E. coli* membranes provides ∼1 mg of the purified protein per liter of culture medium. In vitro folding of the K_vAP channel provides an alternative to native expression. The GST fusion approach provides high-level expression of the K_vAP polypeptide as inclusion bodies, which using the optimal refolding conditions identified can be efficiently folded to the native state. Using this approach, we were able to obtain 8 mg of the

purified refolded K_vAP channel per liter of culture medium, an amount ~8-fold greater than the amount of protein obtained by native expression.

DISCUSSION

In this study, we demonstrate that the K_vAP channel can be folded in vitro from the extensively unfolded state. Biochemical and functional characterization of the refolded K_vAP channel indicates that it is similar to the native channel. While we have established that the unfolded K_vAP polypeptide is monomeric, the extent of residual structure in this state is not known. We anticipate that the folding process described corresponds to rearrangement of secondary structure and the formation of the tertiary and quaternary structure. We show that in vitro folding of the K_vAP channel requires lipid bilayers. A lipid bilayer requirement has been demonstrated for the refolding of the KcsA and NaK ion channels.[14,21] The similar architecture of the pore domain in these channels and the K_vAP pore suggests that the lipid bilayer requirement for the folding probably originates from the pore domain.

The refolding of the K_vAP channel in lipid vesicles is influenced by the curvature stress of the lipid bilayer. An increase in the curvature stress of the lipid bilayer increases the refolding yield of the K_vAP channel. The major factor influencing the refolding of the K_vAP channel is temperature, and at elevated temperatures, close to quantitative refolding of the K_vAP channel is observed. The K_vAP channel was cloned from a hyperthermophile, so it is expected that the channel can fold at an elevated temperature. However, to the best of our knowledge, this is the first report of the necessity of elevated temperatures for the efficient folding of a membrane protein. The increase in efficiency of in vitro folding with temperature is probably a feature unique to hyperthermophilic membrane proteins like K_vAP. The efficient in vitro folding of the K_vAP channel combined with high-level expression of the K_vAP polypeptide as inclusion bodies provides an easy means of obtaining high yields of the K_vAP channel. The higher yields obtained using the in vitro folding approach will be useful in isotopic labeling of the K_vAP channel for NMR studies. This approach will also be useful in investigating the specific effects of lipids on the structural and functional properties of the channel as unlike the protein purified from *E. coli* membranes, the refolded K_vAP channel is devoid of any lipid contamination of an unknown nature.[29,41,42] Further, in vitro folding also sets the stage for semisynthesis of the K_vAP channel, an important technique that allows the incorporation of unnatural amino acids and peptide backbone modifications for structure–function investigations.[43]

The in vitro folding of the K_vAP channel can be considered to consist of two stages, with the first stage corresponding to the insertion of the unfolded polypeptide into the lipid bilayer and the second stage comprising channel folding and assembly within the bilayer. Insertion of the K_vAP polypeptide into the lipid bilayer was probed using the Cys protection assay. The assay demonstrates that the insertion of the unfolded K_vAP polypeptide into lipid bilayers is quite rapid and takes place with a nativelike topology. The rapid insertion of the K_vAP polypeptide into the lipid bilayer is probably related to the presence of residual detergent (SDS, 3.5 mM) during refolding, which may facilitate the insertion process.

As membrane insertion is relatively fast, the rate-limiting step in K_vAP refolding must be the assembly of the channel within the lipid bilayer. Assembly of the channel within the lipid bilayer consists of the folding of the VSD, the folding and tetramerization of the pore domain, and the intersubunit association of the VSD and the pore domain. Further experiments will be necessary to determine the order of these steps in the in vitro folding of the K_vAP channel and to pinpoint the specific step that is enhanced by elevated temperatures and the composition of the lipid bilayer. The efficient in vitro folding of the K_vAP channel and the ability to manipulate the folding rate by experimental conditions such as temperature and lipid bilayer composition make the K_vAP channel a good model system for investigating the assembly pathway of a multidomain membrane protein.

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