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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. In order 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 to investigate the folding of a multi-domain 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) (Fig. 1A). The first four transmembrane segments form the voltage sensor domain (VSD) while the last two transmembrane segments form the pore domain (Fig. 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 have been carried out on K_v channels resulting in a wealth of information on 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, leads to diseases (4, 5). Studies on the folding of K_v channels reported until now 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 carried out using *in vitro* translation or by 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

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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 to monitor the folding process. Due to 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 the K_v channel K_vAP, from the archaeobacteria, *Aeropyrum pernix*. The K_vAP channel though archaeobacterial in origin, is functionally very similar to an 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 the K_v1.2–2.1 chimera channels (3, 23). In the crystal structures of the K_vAP channel, the VSD is distorted, which was demonstrated to be due to the absence of a lipid bilayer (24). A structural model for the native state of the K_vAP channel has been proposed based on the structure of the isolated VSD, structural homology to the K_v1.2 channel along with disulfide crosslinking and EPR spectroscopic data (24, 25). K_vAP has been used as a model K_v channel for biophysical and spectroscopic investigations due to 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).

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 identify 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 does 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 His₆ tagged K_vAP channel gene sub-cloned into the pQE60 plasmid (Qiagen) was kindly provided by Dr. Roderick MacKinnon (The Rockefeller University) (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 MgCl₂, DNase (5 µg/mL) and lysed by sonication. Unlysed cells and cell debris present was removed by centrifugation at 7500g, following which the cell membranes were pelleted by centrifugation at 100000g. The membranes were solubilized by decyl-β-D-maltoside (DM, 2% w/v) and the His₆ tagged K_vAP channel was purified by metal affinity chromatography (Talon, Clontech) and size exclusion chromatography (22). Size exclusion chromatography was carried out on a Superdex S200 column (GE Healthcare) using a running buffer composed of 50mM HEPES-KOH pH 7.5, 150mM 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, His₆ 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 N-terminal 20 amino acids were substituted by 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 crosslinking.

The GST-K_vAP fusion was expressed in *Escherichia 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 MgCl₂, 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 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 12000g for 10 min. The insoluble fraction, which contains the inclusion bodies, was washed 3 times with 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Tx-100. The inclusion bodies were resuspended in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% N-Lauryl Sarcosine (NLS) and digested with thrombin (Roche, 1U/L of culture) overnight, to cleave the K_vAP polypeptide from GST.

For purification of the K_vAP polypeptide, 1 vol of 50 mM Tris-HCl pH 7.5, 150 mM NaCl was added to the thrombin cleavage mixture. Tx-100 was added to 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 use in the refolding studies.

Unfolding of the K_vAP channel

A multi-step 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 2% (v/v) and the protein was precipitated by 15% trichloroacetic acid (TCA, w/v) for 15 min at 4 °C. The protein precipitate was collected by centrifugation at 3000g, washed twice with acetone + 0.1% trifluoroacetic acid (TFA), solubilized in 50% trifluoroethanol (TFE) + 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 dodecylsulfate (SDS), 10 mM β-mercaptoethanol. The concentration of the unfolded protein was determined by comparing the band intensities on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the unfolded protein to known concentrations of the native K_vAP channel. The lipids used: Asolectin (Soy Total Lipid Extract, Aso), 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-glycero-3-phosphoglycerol (POPG), 1-palmitoyl-2-oleoyl-glycero-3-phosphoethanolamine (POPE) and 1, 2-diphytanoyl-glycero-3-phosphocholine (DPhPC) were obtained from Avanti Polar Lipids. Lipids solutions in chloroform were dried under argon, re-dissolved in cyclohexane, and lyophilized. Lyophilized lipids were hydrated for 1 hour at a concentration of 5 mg/ml using 50 mM HEPES-KOH pH 7.5, 150 mM KCl, 10 mM β-mercaptoethanol and then sonicated for 5 × 30 sec pulses in a water bath sonicator to obtain 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 sec 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 crosslinking for 15 min at room temperature and the reaction was quenched by 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 crosslinking. We observed that in dodecylphosphocholine [Fos-12, 2% (w/v)], the native K_vAP channel is stable while non-specific aggregates dissociate into monomers. The glutaraldehyde crosslinking reaction was therefore carried out after the addition of 2% Fos-12, to distinguish the folded tetrameric species from the non specific aggregates. This step was also beneficial as the addition of Fos-12 served to quench the refolding reaction. The refolding samples (15–20 μgs of protein) were separated on a 10% SDS-PAGE gel and stained with coomassie blue. Following destaining, the gels were dried, 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 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, the folding reaction was quenched by Fos-12, followed by GA crosslinking and SDS-PAGE to determine the refolding yield at the various time points. The time course of the refolding showed mono-exponential 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 on the refolded K_vAP channel

Prior to electrophysiological measurements, the native and the refolded K_vAP channels were reconstituted into lipid vesicles composed of DPhPC, 10 mg/ml as previously described (22, 29). Functional measurements on the K_vAP channel were carried out using planar lipid bilayers composed of DPhPC. The recording solutions used for channel measurements consisted of 10 mM HEPES-KOH pH 7.5, 150 mM KCl on both the *cis* and *trans* sides. 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 of 200 ms duration. Normalized tail current amplitude (I/I_{\max}) was plotted against the test potential and fitted with a 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 sec 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 a single exponential equation, $i = A \exp(-t/\tau_{\text{inact}}) + C$, where i is the current at time t .

Cys protection assay using PEG maleimide

Single Cys substitutions at 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 carried out at room temperature as described previously, except that 1 mM DTT was used instead of 10 mM beta-mercaptoethanol. The unfolded Cys mutant proteins were diluted into lipids or into 2% Fos-12 (as a control). Immediately following dilution (< 30 sec), an aliquot of the refolding reaction 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 was also similarly treated with PEG-2K-mal. PEGylation was carried out for 30 min at room temperature and quenched by addition of 200mM 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 the unlabelled 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 on treatment with SDS (1% w/v), precipitation with TCA or with organic solvents (data not shown). To ensure extensive unfolding, the K_vAP channel was TCA precipitated, washed with acetone, dissolved in 50% TFE + 0.1% TFA and lyophilized. The lyophilized precipitate was dissolved in SDS (1% w/v) to provide the unfolded K_vAP used for the *in vitro* folding experiments. Unfolding of the K_vAP channel was confirmed by chemical crosslinking using glutaraldehyde. The native K_vAP channel is a tetramer and glutaraldehyde crosslinking of the native channel gives a protein band that migrates corresponding to a tetramer on SDS-PAGE (Fig. 1C). Unfolding results in loss of the native tetrameric state and glutaraldehyde crosslinking of the unfolded protein only gives a protein band that corresponds to the monomer on SDS-PAGE (Fig. 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 L compared to 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 based on their prior use in structural studies of the K_vAP channel (2, 24, 30, 31). Glutaraldehyde crosslinking 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 (Fig. 2). A dimeric species was 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). On dilution of the unfolded K_vAP polypeptide into asolectin vesicles, refolding was observed as indicated by the presence of a tetrameric species on glutaraldehyde crosslinking (Fig. 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 similar retention time on size exclusion chromatography compared to the native protein and glutaraldehyde crosslinking confirmed the tetrameric nature of the refolded K_vAP channel (Fig. 3A).

The CD spectrum of the refolded K_vAP channel is similar to the native channel (Fig. 3B). Calculation of the helical content using the k2d3 algorithm (<http://www.ogic.ca/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 + 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 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 maxima at 335 nm (Fig. 3C). Unfolding of the K_vAP channel results in a red-shift of the emission maxima 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 3 Trp residues, 2 in the pore domain and 1 in the voltage sensor domain. The similar fluorescence spectra, indicates a similar environment for the Trp residues in the native and the 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 Fig. 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 is similar to the native channel (Fig. 4B). Voltage dependence of channel opening for the refolded K_vAP channel was measured using macroscopic (multi-channel) currents (Fig. 4C). K_vAP is a depolarization activated K⁺ channel and channel openings increase sharply with depolarization (22). The data for channel opening versus voltage was fit to a two state Boltzmann distribution, to obtain the values for 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 ± 4.3 , z = 1.86 ± 0.36) were similar to the corresponding values (V_{0.5} = -40.7 ± 5.2 , z = 1.81 ± 0.25) for the native channel indicating similar gating behavior in these channels (Fig. 4D). Slow inactivation of the refolded K_vAP channel (287 ± 7 msec⁻¹, Fig. 4E) was also similar to the native channel (285 ± 20 msec⁻¹). The electrophysiological behavior of the refolded K_vAP channel is therefore essentially similar to the native channel.

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

Effect of lipid bilayer composition on the refolding of the K_vAP channel

In experiments described until now, 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 of defined composition. Unfolded K_vAP was diluted into lipid vesicles and the refolding yields were evaluated after a 2 hour incubation at room temperature by glutaraldehyde crosslinking (Fig. 5A, B). In POPC lipid vesicles, the extent of refolding was very low compared to asolectin vesicles ($7 \pm 3\%$ versus $30 \pm 4\%$). Negatively charged lipids have been shown to be important for the structure and function of certain membrane proteins, for examples see (33, 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 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 ($15 \pm 2\%$) compared to POPC vesicles. POPC and DPhPC have the same head group, therefore the differences in refolding of the K_vAP channel in these lipids must arise either due to a specific requirement for a lipid with an isoprenyl tail (similar to archaeal lipids) or due to 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 carried out the refolding using lipid vesicles with varying ratios of POPC and POPE (Fig. 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 greater than 40% due to the interference of POPE with the glutaraldehyde crosslinking reaction. We established that POPE, at percentages greater than 40%, interferes with the glutaraldehyde reaction by evaluating the crosslinking 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 crosslinking. The time course of folding of K_vAP in POPC, DPhPC and POPC: POPE (6: 4) lipids are shown in Fig. 5E. The rate of folding of the K_vAP channel in POPC is very slow while a higher rate and extent of folding is observed in DPhPC and in 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 ($t_{1/2} = 65.8$ 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 (*Aeropyrum pernix*) (39), we investigated the effect of elevated temperatures on the *in vitro* folding process. Refolding of K_vAP was carried out 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 glutaraldehyde crosslinking (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 (Fig. 6A, B). At the highest temperature tested, the refolding yield was 73 ± 4% after 10 minutes compared to a yield of 15 ± 2% after a 2 hour incubation at room temperature. The time course of folding of K_vAP in DPhPC vesicles at room temperature and 80 °C is shown in Fig 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 K_vAP 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 hours 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 (Fig. 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 arise because Cys residues within the lipid bilayer show a lower reactivity compared to 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. 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 at residues 101 in the S3 helix and 127 in the S4 helix to monitor the incorporation of the VSD into the lipid bilayer while Cys substitutions at residues 161 in the S5 helix and 214 in the S6 helix were generated to monitor the incorporation of the pore domain (Fig. 7A). Cys substitutions at residues 139 in the S4-S5 loop and at 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 sec) and treated with PEG-2K-mal to test incorporation of the K_vAP polypeptide into the lipid bilayer.

On addition to lipid vesicles, Cys residues in the transmembrane segments (Cys 101 in S3, Cys 127 in S4, Cys 161 in S5 and Cys 214 in S6) were immediately protected from reaction with PEG-2K-mal (Fig. 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) or DPhPC lipid vesicles. In contrast, the Cys139 and Cys 175 in the aqueous exposed regions were not protected from modification by PEG-2K-mal on the 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 external to the membrane (Fig. 7C). We observe complete labeling of Cys139 and Cys 175, 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 due to the residual detergent (SDS, 3.5 mM) during refolding. The similar labeling of Cys 139 and Cys 175 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 rapidly incorporates into lipid vesicles with a “native-like” 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) or 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 L of the culture medium. *In vitro* folding of the K_vAP channel provides an alternate 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 mgs of the purified refolded K_vAP channel per L of the culture medium, ~8 fold higher than the protein yield 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 indicate 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 and so it is expected that the channel can fold at an elevated temperature. However, to 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 to obtain 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 lipid effects on structural and functional properties of the channel as unlike the protein purified from *Escherichia coli* membranes, the refolded K_vAP channel is devoid of any lipid contaminations of unknown nature (29, 41, 42). Further, *in vitro* folding also sets the stage for semisynthesis of the K_vAP channel, an important technique that enables 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 of 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 the insertion takes place with a “native-like” 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 channel assembly within the lipid bilayer. Channel assembly within the lipid bilayer consists of the folding of the VSD, the folding and tetramerization of the pore domain, and the inter subunit 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 or lipid bilayer composition makes the K_vAP channel a good model system for investigating the assembly pathway of a multi-domain membrane protein.

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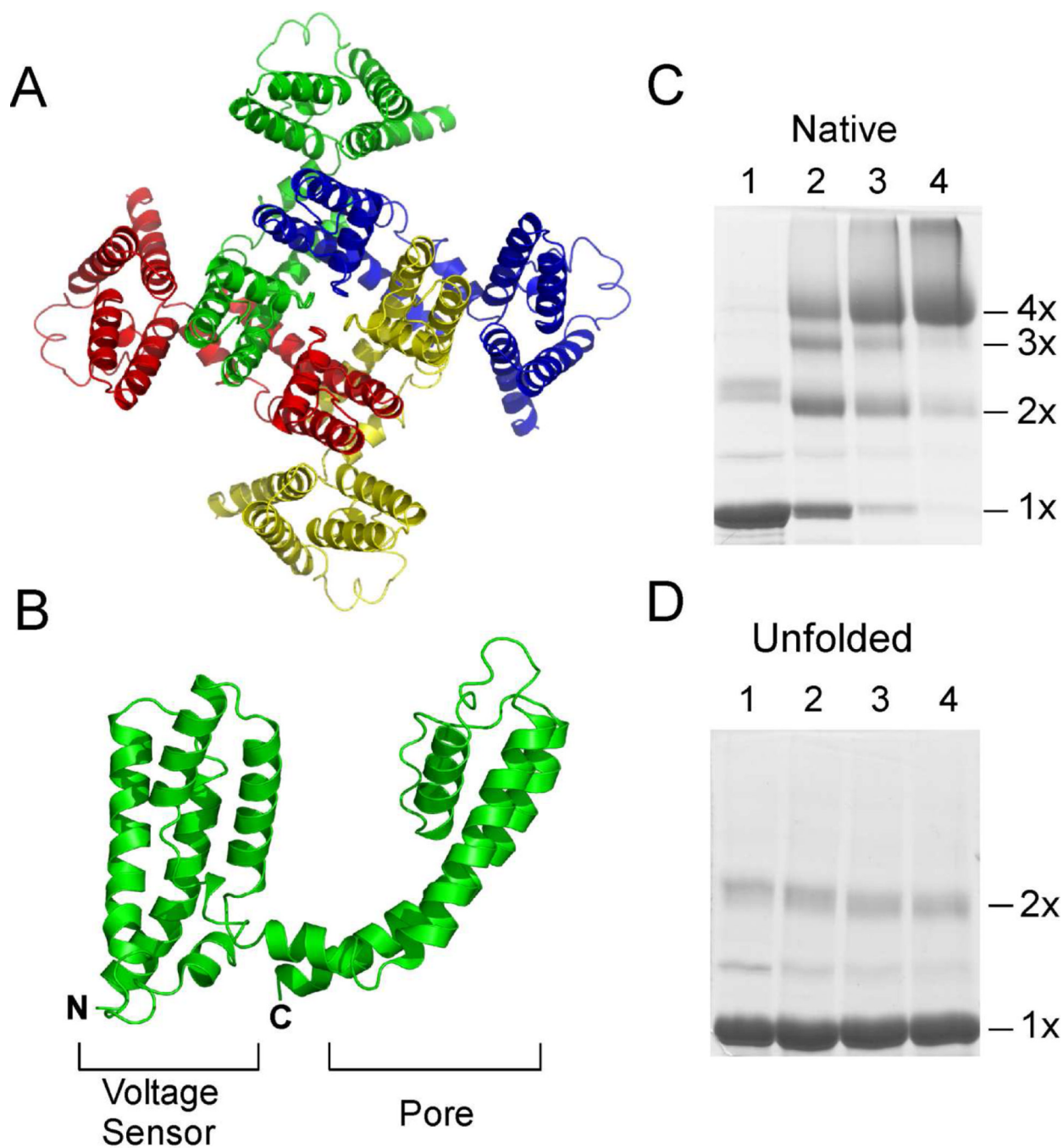


Fig. 1. Structure of the K_v AP channel

(A) Top view of the tetrameric K_v AP channel. The structural model of the K_v AP channel as described in (24) is shown. (B) Structure of a single subunit of the K_v AP channel. Each subunit consists of two domains, the sensor domain and the pore domain. SDS-PAGE gel showing glutaraldehyde crosslinking of the native K_v AP channel (C) and the unfolded protein (D). Lanes 1) without crosslinker; 2) 0.025%; 3) 0.05% and 4) 0.1% glutaraldehyde. The oligomeric nature (1 \times , 2 \times , 3 \times & 4 \times) of the crosslinked bands is indicated.

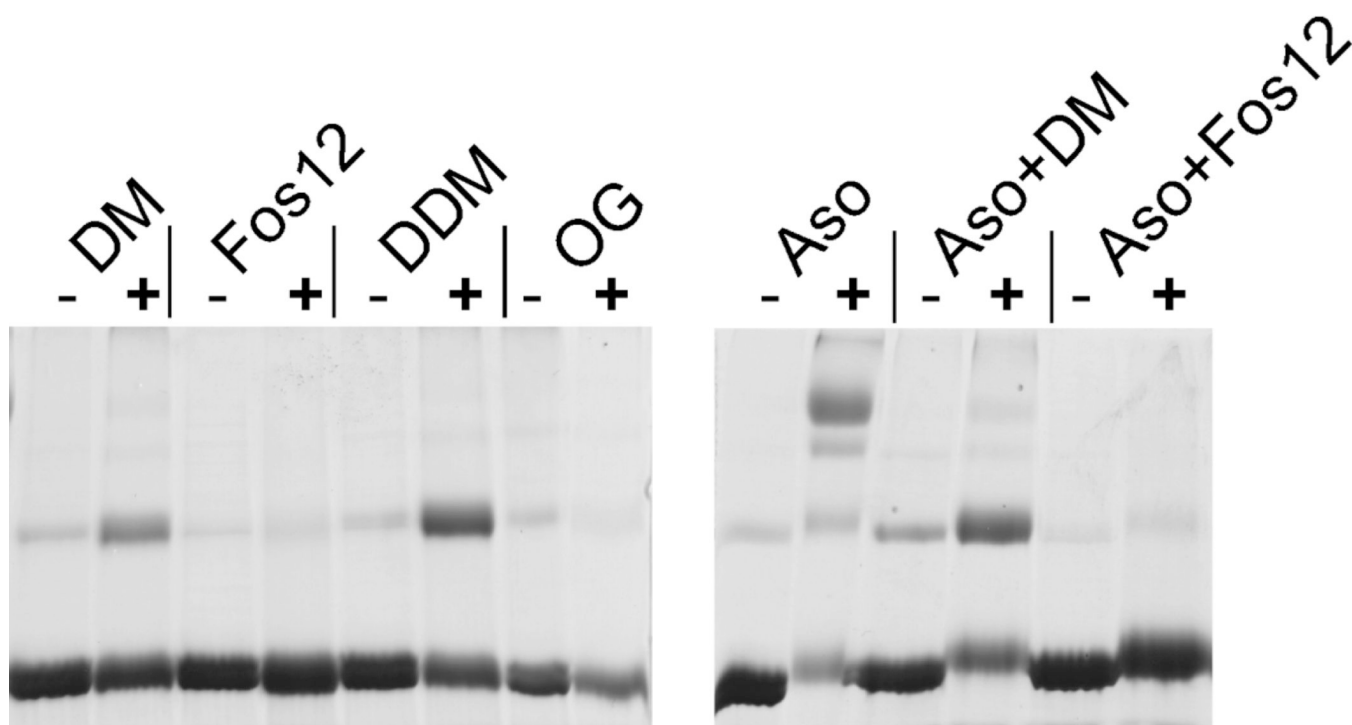


Fig. 2. *In vitro* folding of the K_vAP channel

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 crosslinking followed by SDS-PAGE was used to determine formation of tetrameric species. (-) without crosslinker and (+) 0.1% glutaraldehyde.

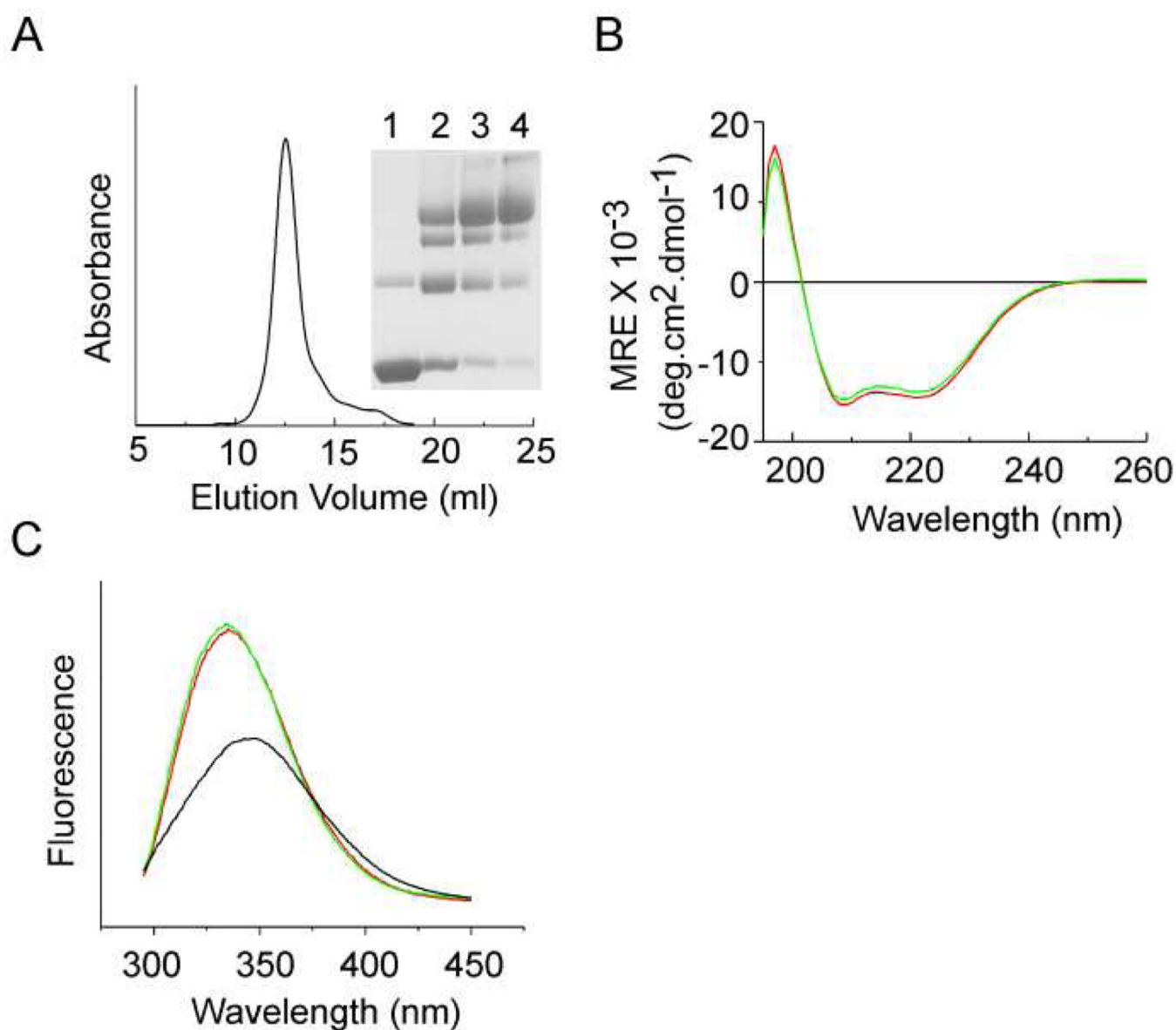


Fig. 3. Characterization of the refolded KvAP channel

(A) Size exclusion chromatography of the refolded KvAP channel. Elution volume observed = 12.5 ml. Inset: Glutaraldehyde crosslinking of the peak fraction. Lanes 1) without crosslinker; 2) 0.025%; 3) 0.05% and 4) 0.1% glutaraldehyde. (B) CD spectra of the refolded KvAP channel. CD spectra reported as mean residue ellipticity (MRE) for the native (red) and the refolded KvAP channel (green) in 10mM sodium phosphate buffer pH 7.5, 150mM KCl, 0.25% DM. A protein concentration of 450 $\mu\text{g}/\text{ml}$ was used. (C) Fluorescence spectra of the refolded KvAP channel. Intrinsic fluorescence spectra (excitation = 280 nm) for the native (red), refolded (green) recorded in 50 mM HEPES pH 7.5, 150 mM KCl and 0.25% DM and unfolded KvAP in 100 mM sodium phosphate pH 7.5, 1% SDS (black). A protein concentration of 25 $\mu\text{g}/\text{ml}$ was used.

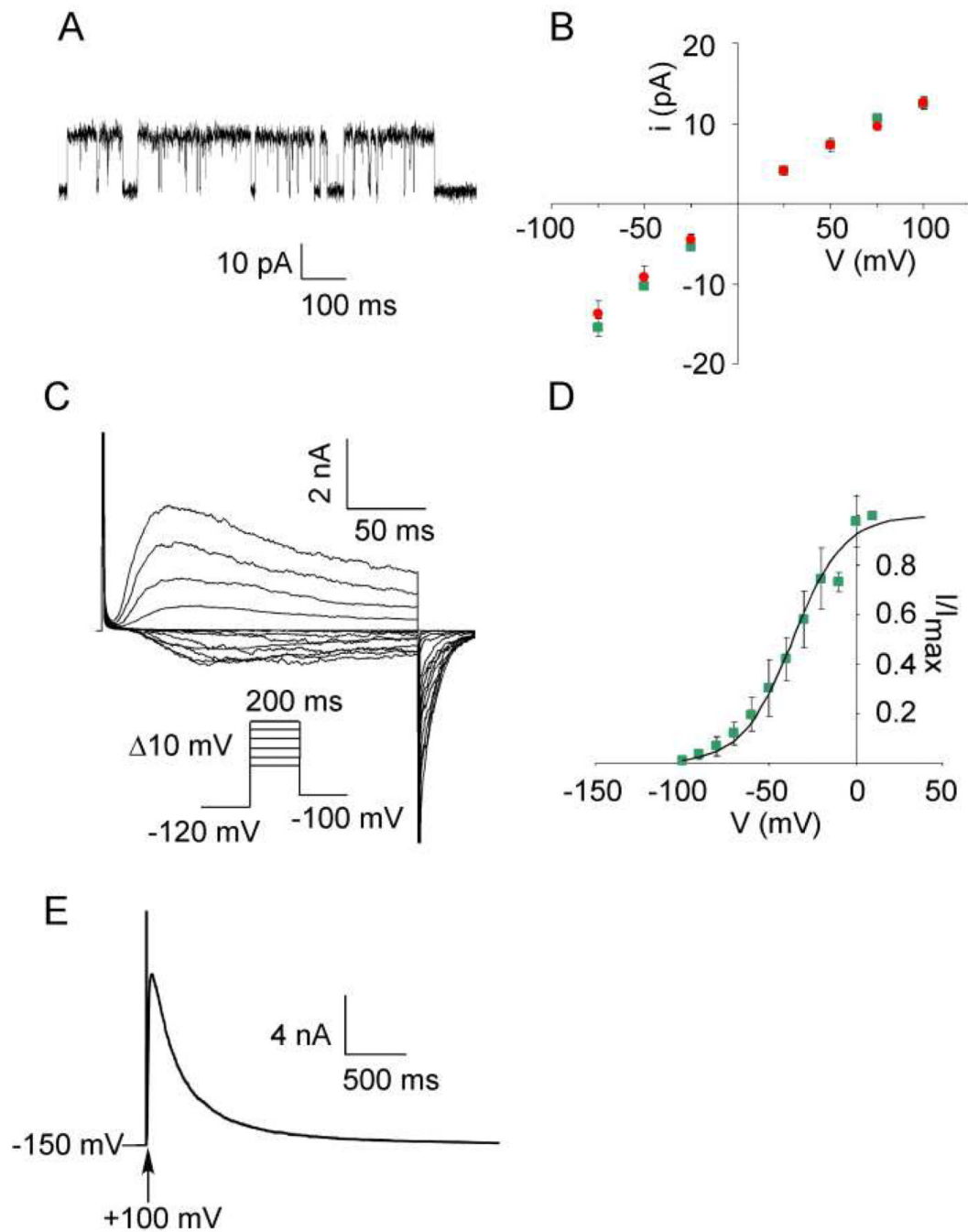


Fig. 4. Functional characterization of the refolded KvAP channel

(A) Single channel trace for the refolded KvAP channel recorded at +100 mV. (B) Single channel current as a function of voltage for the native (red) and refolded (green) KvAP channels. (C) Voltage activated macroscopic currents from the refolded KvAP channel recorded using the voltage protocol shown (inset) (D) Voltage gating of the refolded KvAP 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 $V_{0.5} = -36.1 \pm 4.3$ and $z = 1.86 \pm 0.36$. (E) Slow inactivation of the refolded KvAP channel. KvAP 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 carried out in 150 mM KCl, 10 mM HEPES-KOH pH 7.5. For Panels B and D, the error bars indicate the standard deviation for 3 or more experiments.

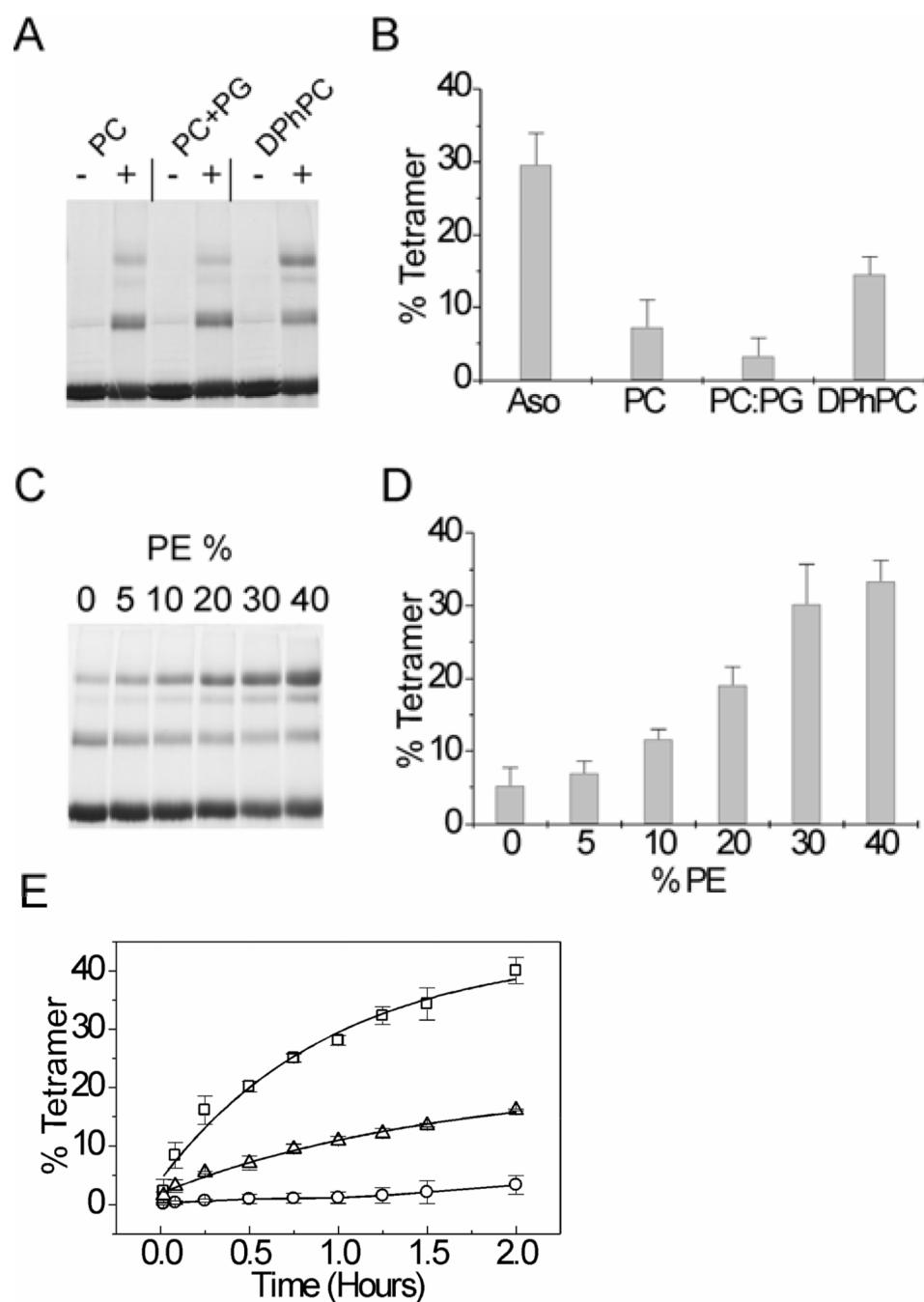


Fig. 5. Refolding of the K_v AP channel in various lipids

(A) SDS-PAGE gel showing glutaraldehyde crosslinking of the K_v AP channel refolded in POPC, POPC:POPG (75:25) and DPhPC lipid vesicles for 2 hours at room temperature (–, without crosslinker and +, 0.1% glutaraldehyde) and (B) bar graph comparing the refolding yields in the various lipids. Refolding yields of K_v AP in asolectin vesicles (Aso) under similar refolding conditions is 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_v AP channel. The extent of refolding after 2 hours at room temperature is shown. (E) Time course of refolding of the K_v AP channel in POPC (circles), DPhPC (triangles) and POPC:POPE (60:40, squares) lipid vesicles. The smooth lines for DPhPC and POPC:POPE

corresponds 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 3 or more experiments.

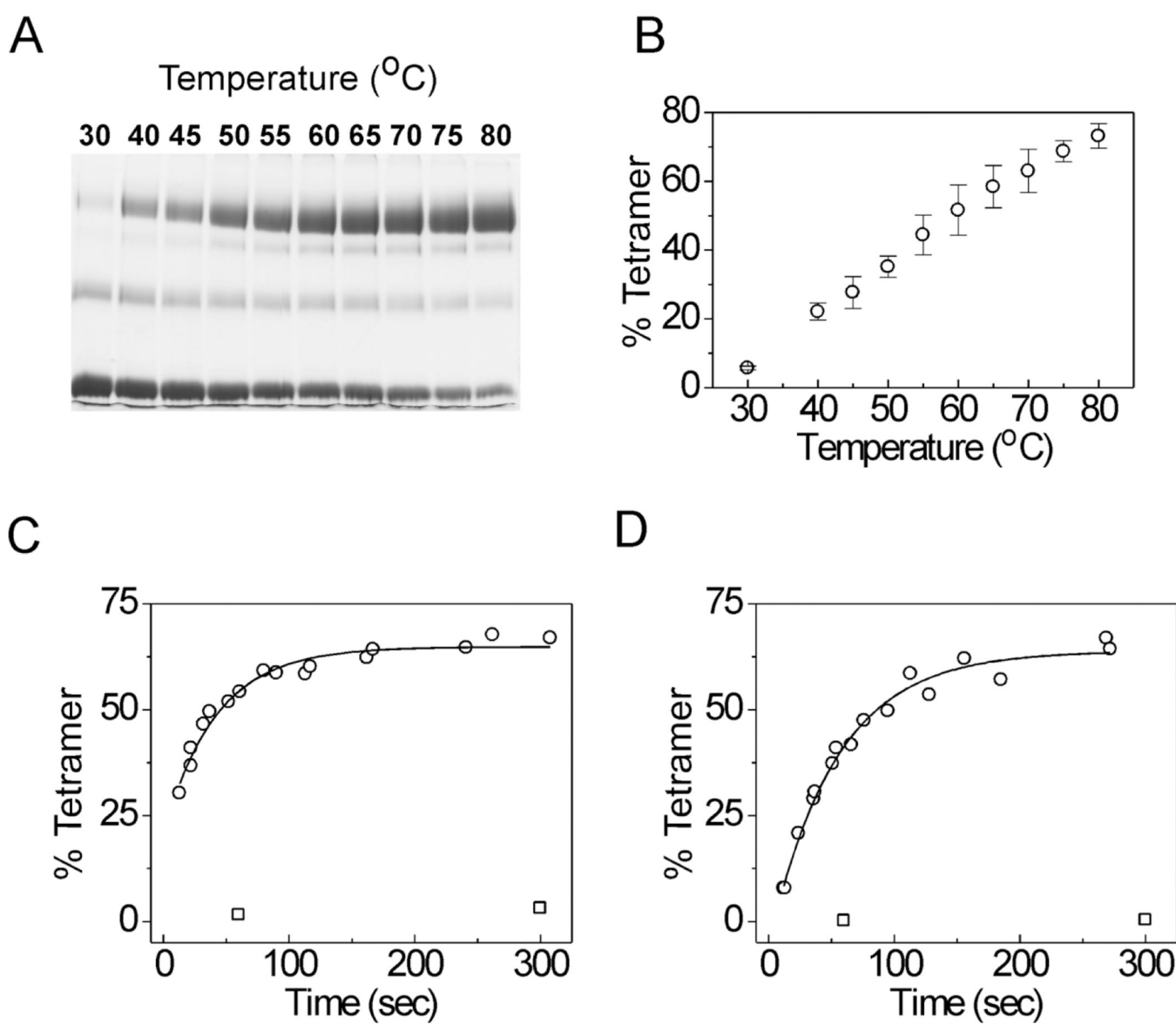


Fig. 6. Effect of temperature on refolding of the K_v AP channel

(A) SDS-PAGE gel showing refolding of K_v AP in DPhPC lipid vesicles after 10 min incubation at the indicated temperatures. (B) The refolding yields after 10 min are plotted as a function of temperature. Error bars indicate standard deviation ($n=3$). Time course of refolding of the K_v AP channel in DPhPC (C) and POPC (D) at 25°C (squares) and 80°C (circles). The solid lines represent single exponential fits to the data.

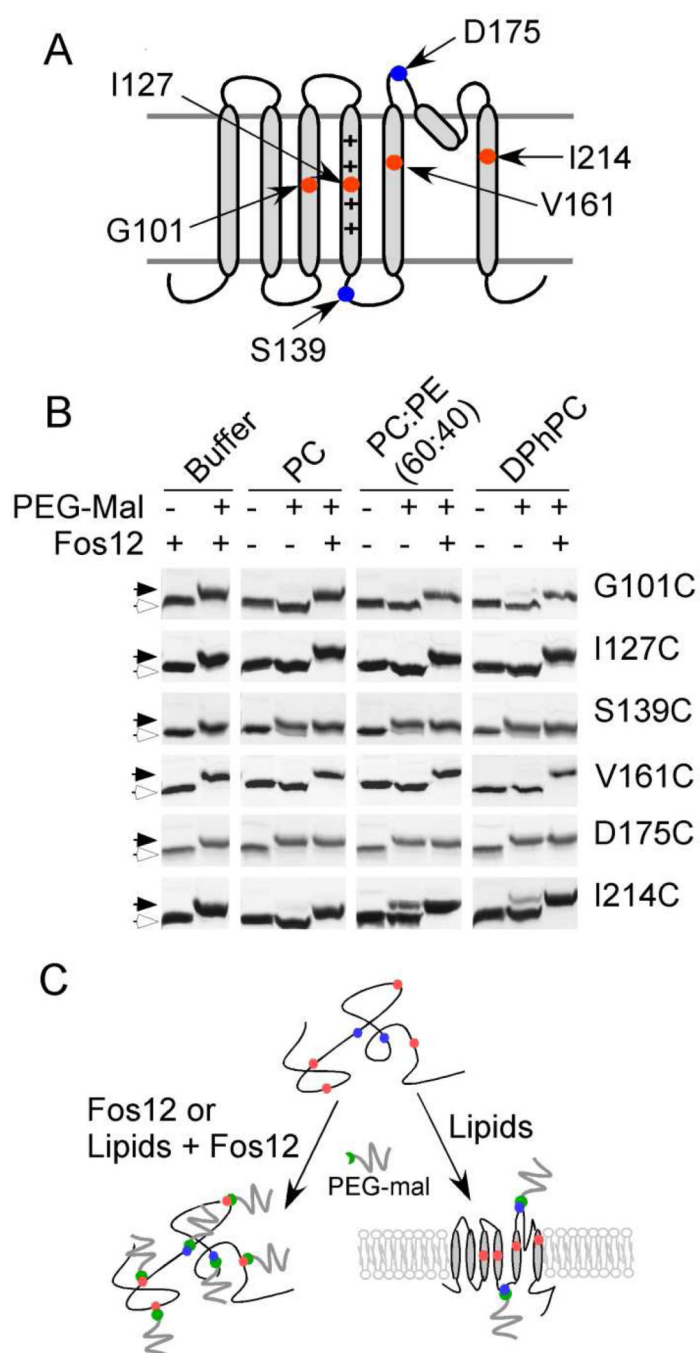


Fig. 7. Insertion of the K_v AP polypeptide into the lipid bilayer during refolding
(A) Cartoon depicting the location of Cys residues introduced into the K_v AP channel for the PEGylation experiment. **(B)** Accessibility of Cys residues to PEGylation immediately after dilution of unfolded protein into lipid vesicles or into buffer containing 2% (w/v) Fos12 at room temperature. SDS-PAGE gels showing modification of the single Cys mutants of K_v AP by PEG-2K-mal. The slow migrating PEGylated protein (solid arrows) and fast migrating protein without modification (open arrows) are indicated. **(C)** Summary of the PEGylation experiment. Upon dilution of the unfolded K_v AP 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 Fos12 (2%, w/v).