ACCELERATED COMMUNICATION

The conformation of the pore region of the M2 proton channel depends on lipid bilayer environment

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Abstract

The M2 protein from influenza A virus is a 97-amino-acid protein with a single transmembrane helix that forms proton-selective channels essential to virus function. The hydrophobic transmembrane domain of the M2 protein (M2TM) contains a sequence motif that mediates the formation of functional tetramers in membrane environments. A variety of structural models have previously been proposed which differ in the degree of helix tilt, with proposed tilts ranging from ~15° to 38°. An important issue for understanding the structure of M2TM is the role of peptide–lipid interactions in the stabilization of the lipid bilayer bound tetramer. Here, we labeled the N terminus of M2TM with a nitroxide and studied the tetramer reconstituted into lipid bilayers of different thicknesses using EPR spectroscopy. Analyses of spectral changes provide evidence that the lipid bilayer does influence the conformation. The structural plasticity displayed by M2TM in response to membrane composition may be indicative of functional requirements for conformational change. The various structural models for M2TM proposed to date—each defined by a different set of criteria and in a different environment—might provide snapshots of the distinct conformational states sampled by the protein.

Keywords: M2 proton channel; EPR spectroscopy; site-directed spin labeling; membrane protein structure; peptide–lipid interactions; hydrophobic mismatch; helix tilt; lateral pressure

The M2 protein from influenza A virus is a 97-amino-acid protein with a single transmembrane helix that forms proton-selective channels essential to virus function. The hydrophobic transmembrane domain of the M2 protein (M2TM) contains a sequence motif that mediates the formation of functional tetramers in membrane environments. Energetics of formation of M2TM have been studied by using analytical ultracentrifugation (Salom et al. 2000; Howard et al. 2002) and thiol-disulfide equilibria (Cristian et al. 2003a,b). A high-resolution crystal structure has not been solved, although a variety of structural models have been proposed based on site-directed mutagenesis in conjunction with computer modeling (Pinto et al. 1997), molecular dynamics calculations (Zhong et al. 2000), infrared spectroscopy (Torres et al. 2000), and solid-state nuclear magnetic resonance spectroscopy (SSNMR) (Kovacs et al. 2000; Wang et al. 2001; Nishimura et al. 2002). The proposed structures are in good agreement with respect to the identities of the side chains lining the pore, the presence of a water-filled pore near the center of the channel, and the packing of monomers with a left-handed tilt. One detail in which models do differ is in the degree of helix tilt, with proposed tilts ranging from approximately 15° to 38°. The 38° angle structure is based on an abundance of high-resolution SSNMR orientational restraints and a single distance restraint (Nishimura et al. 2002), while the 15° angle structure is based on site-directed mutagenesis, and explains a large body of electrophysiological data for this channel (Pinto et al. 1997).

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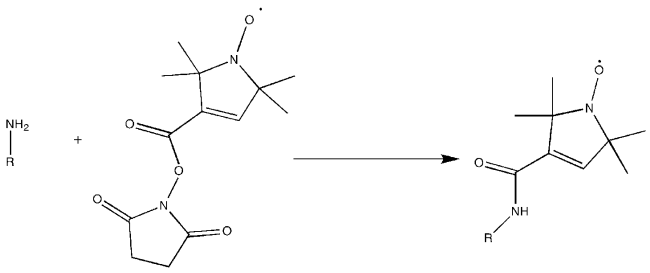

Figure 1. The spin-labeling reaction. The reaction of 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carboxylic acid N-hydroxysuccinimide ester to produce an N-terminal nitroxide labeled peptide.

Previous thiol-disulfide equilibria results indicate that the association of M2TM strongly depends on the thickness of the bilayer (Cristian et al. 2003b). Thus an important issue for understanding the structure of M2TM is the relative role of peptide–peptide versus peptide–lipid interactions in the stabilization of the lipid bilayer bound tetramer. A recent review highlights that lipid environment is important for structural integrity and optimal activity for a wide range of membrane bound proteins (Opekarova and Tanner 2003). Changing lipid bilayer morphology is known to affect conformational transitions related to the opening and closing of several channels (Perozo et al. 2002a; Yuan et al. 2004). For example, the stabilization of distinct conformations of the large mechanosensitive channel for *Escherichia coli* (MscL) was recently elegantly achieved by manipulating the nature and extent of lipid–protein interactions (Perozo et al. 2002a,b; Powl et al. 2003).

Here we ask whether lipid effects are able to shift the equilibrium structure of the M2 protein, which forms single channels in bilayers, which gate between open and closed states (Vijayvergiya et al. 2004). In this study we labeled the N terminus of the M2TM peptide with a nitroxide and studied the peptides reconstituted into different lipid bilayers by using electron paramagnetic resonance (EPR) spectroscopy. Analyses of spectral changes provide evidence that the lipid bilayer does influence the conformation of the channel.

Results and Discussion

The N terminus of the M2TM peptide was reacted with a nitroxide spin label as shown in Figure 1. Site-directed spin labeling has emerged as a powerful technique for studying membrane bound proteins within lipid bilayers, a feat that holds formidable challenges for traditional methods of high-resolution structural biology (Hubbell et al. 1998, 2000; Mchaourab and Perozo 2000). The EPR line shape of a spin label depends both on mobility and interactions with other nearby spins. We wish to focus on spin–spin interactions that can provide direct structural information through the distance dependence of dipolar coupling. Thus, underlabeled samples (with one or less spin label per tetramer) are compared with fully labeled samples (four spin labels per tetramer). Broadening in the fully labeled samples with respect to the underlabeled samples is due to spin–spin interactions.

Figure 2A presents an overlay of the under (black) and fully labeled (red) spectra for spin-labeled M2TM in four different bilayer environments. The experiments were all done under conditions where the protein was essentially fully tetrameric (Cristian et al. 2003b). Whereas there is little change between under and fully labeled spectra for 1,2,-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC), there is broadening of the fully labeled spectra with respect to the corresponding underlabeled spectra for 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). An estimate of the magnitude of spin–spin interaction is obtained from the ratio of central line amplitudes of normalized underlabeled and fully labeled spectra, Ω (Mchaourab and Perozo 2000). At large spin–spin distances, Ω is approximately one (no spin–spin coupling) but increases as spin labels approach each other. The observed pattern of spin interaction (Ω) shown in Figure 2B indicates that the spin labels are furthest in DLPC, approximately the same distance apart in DOPC and DMPC and closest in POPC.

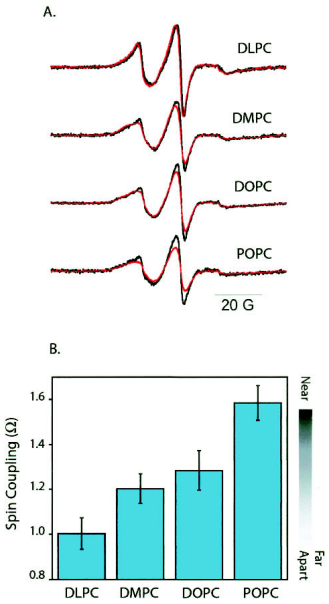

Figure 2. (*A*) X-band EPR spectra of spin-labeled M2TM reconstituted into DLPC, DMPC, DOPC, and POPC lipid bilayers. Black spectra are underlabeled (1:10, mol labeled/mol unlabeled), and red spectra are fully labeled. All spectra shown have been normalized to the same number of spins. (*B*) Analysis of the extent of spin–spin coupling in different lipid bilayers. Ω is the ratio of central line amplitudes (M = 0) of the normalized underlabeled and fully labeled spectra.

How different is the M2TM structure across the lipid systems studied? Due to the tetrameric geometry of the channel, two sets of possible distances between spin labels are present in the fully labeled samples (lateral and diagonal). Although quantitative distances cannot be accurately calculated due to the ambiguity introduced by both lateral and diagonal spin pairs, estimates are possible and informative in light of comparison with previously predicted structures. If spin labels are within 5–6 Å of each other, the resulting signal broadens extensively due to exchange mechanisms (Mchaourab and Perozo 2000). All current models for M2TM suggest longer interterminal distances than relevant for exchange mechanisms, consistent with the fact that none of our spectra exhibit extensive broadening. Previously published models for M2TM suggest a range of interlabel distances. When we model nitroxide labels onto the SSNMR structure determined in DMPC (Fig. 3A; Nishimura et al. 2002) we find an interlabel separation of ~18 Å, although this value is only one of a distribution that results from conformational flexibility of the Ser-Ser linker at the N terminus. Figure 3B shows that by systematically decreasing the tilt of helices from 35° to 15°, the labels approach each other, with an interlabel distance of ~11 Å for the 15° tilt structure, which is similar to the previously published model based on data collected in *Xenopus laevis* oocytes (Pinto et al. 1997). Ongoing studies with a rigid nitroxide label (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid [TOAC]) (McNulty and Millhauser 2000) at sites closer to the center of the transmembrane region will provide more highly constrained structural information.

We suggest that the observed differences in tilt angle in the literature are a consequence of the lipid bilayer used in data collection. Both acyl chain length and degree of unsaturation affect the hydrophobic thickness and membrane lateral pressure presented by a lipid bilayer to a membrane spanning peptide. Estimates of hydrophobic thickness for the lipids used here are DLPC, 19.5 Å; DMPC, 23.0 Å; DOPC, 27 Å; and POPC, 26.5 Å (de Planque and Killian 2003). With a hydrophobic stretch of 19 residues (~28.5 Å) (Cristian et al. 2003b), the length of the M2TM helix exceeds the bilayer thickness for all four lipids used in this study. Several possible adaptations for hydrophobic mismatch have been proposed and systematically studied for a range of systems (de Planque and Killian 2003). Tilting is one way a peptide can change its effective hydrophobic length to prevent exposure of hydrophobic amino acids to a polar environment. A simple geometrical calculation (helix length × cos [tilt angle]) for M2TM indicates an effective hydrophobic length of a 27.5 Å for a tilt of 15° and an effective hydrophobic length of 23.3 Å for a tilt of 35°.

Other mechanisms are possible for hydrophobic mismatch between peptide length and lipid bilayer thickness, such as peptide backbone adaptation, peptide aggregation, or lipid distortion (Killian 2003). However, we think a simple helix tilt model can satisfactorily account for the DLPC, DMPC, and POPC EPR data collected. The deformability of the lipid bilayer and the flexibility of the protein both determine the equilibrium state reached due to a mismatch between the hydrophobic portion of the protein and the hydrocarbon core of the bilayer. Previous studies have pointed out notable flexibility of the helix–helix interface in M2TM (Howard et al. 2002) and suggest that it is reasonable that mismatch energy goes into deforming the peptide conformation to match the dimensions of the lipid.

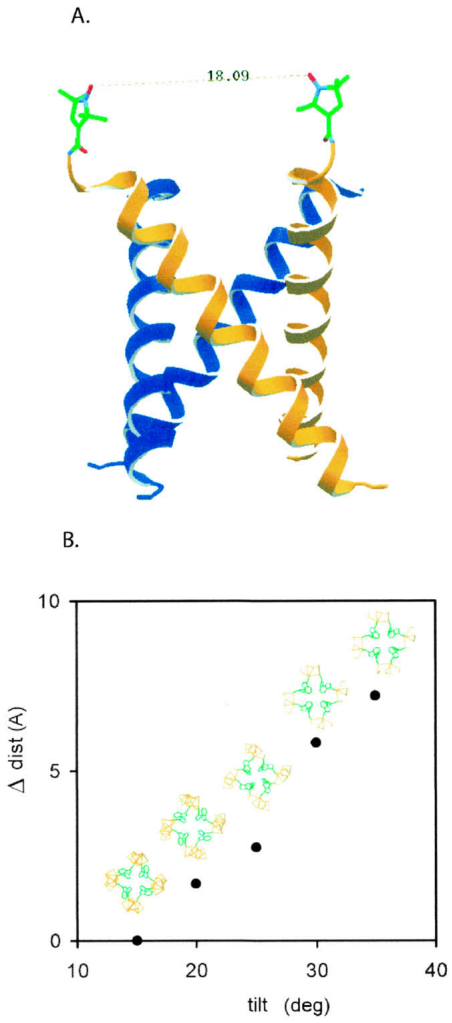

Figure 3. (*A*) Model of the nitroxide labeled M2TM using the SSNMR structure collected in DMPC (PDB code 1NYJ). The distance shown is for the lateral pair of helices, highlighted in orange. Diagonal helices would have a separation of ~25 Å (data not shown). (*B*) Computer models of M2TM calculated at various tilt angles demonstrate a monotonic increase in helix-axis separation with increasing tilt. The *Y*-axis of the figure represents changes in helix-axis separation relative to the separation at 15° tilt. Helix separation distances are calculated from helix-axis to helix-axis by averaging the coordinates of Cα for residues 22–27 at the N terminus. A 10 Å slab of each model is shown, centered on His37/Trp41, indicating the general structure of the gating interaction is intact for all tilt angles shown.

A cartoon model that shows one way M2TM could adapt to different hydrophobic thickness is shown in Figure 4. In this model, helices decrease their tilt angle as the bilayer thickens, resulting in shorter spin–spin distances and more broadening. This model accounts well for the EPR results collected for DLPC, DMPC, and POPC. Note that the structure pictured in Figure 3A was collected in DMPC and has a lateral interlabel distance of ~18 Å. Line broadening due to spin–spin interactions can typically be observed if spin labels are within ~20 Å (Mchaourab and Perozo 2000). Consistent with the cartoon model, data collected in DLPC would require an even bigger tilt angle than does DMPC and thus a greater interlabel distance—consistent with the negligible broadening observed for DLPC in this study.

We do note that there is a slight difference in the underlabeled lineshapes (black spectra in Fig. 2A), with DLPC being slightly sharper and POPC slightly broader than DMPC and DOPC. The spin labels in the underlabeled samples are isolated from nearby labels and spin coupling effects are not observed, although mobility effects are evident. The pattern of underlabeled lineshapes suggests that the spin label in the thinnest bilayer (DLPC) extends further from the surface of the membrane and is slightly more mobile, whereas the spin label in the thickest bilayer (POPC) is closer to the surface and is slightly less mobile. Thus although the broadening effects observed in the fully labeled samples are clearly consistent with the proposed helix tilt model, peptide–peptide interactions might still constrain the peptide tilt accessible to the channel, resulting in small differences across the lipids studied in the position of the N termini of the peptides with respect to the bilayer surface.

Previous work has indicated that flanking residues play an important role in determining the response of mismatch between peptide and lipid lengths (Killian 2003). For example, Trp residues in transmembrane proteins have been shown to have a preference for well-defined positions near the lipid carbonyls, contributing to interfacial anchoring. In M2TM there is a Trp residue near the C-terminal end of the transmembrane stretch. The N-terminal end of the M2TM transmembrane stretch does not have an anchoring residue and perhaps has flexibility in the exact interfacial position, thus enabling the peptide to tilt in response to different lipid environments. Another possibility is that the His residue at position 37, previously shown to be important for the stability of M2TM (Howard et al. 2002), serves as a pivot point about which helices move.

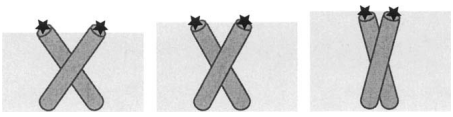

Figure 4. Cartoon model for helix tilt due to changing lipid bilayer thickness. Only two of the four helices of the tetramer are shown. As bilayer thickens (*left* to *right*), helices decrease their tilt angle to keep hydrophobic portion within the bilayer. As helices decrease their tilt angle, spin labels (*) get closer, increasing spin coupling and observed broadening.

Although DLPC, DMPC, and POPC data can be explained by tilting due to hydrophobic mismatch, DOPC has less broadening than would be predicted by such a model. In fact, the EPR data for DOPC and DMPC are quite similar despite their different reported hydrophobic thicknesses. The two acyl chains of DMPC (C14:0) are saturated, whereas DOPC (C18:$1_{\Delta 9}$) has a double bond in each of its acyl chains. A lipid bilayer is characterized by a distribution of lateral pressure densities that varies strongly with depth in the bilayer and depends on acyl chain length, degree and positions of unsaturation, and strength of head group repulsions (Cantor 1999). Recently, increasing attention has been paid to lateral pressure profiles in membranes and their effect on the conformations of membrane bound proteins (Cantor 2002; van den Brink-van der Laan et al. 2004). Although DOPC has a greater reported hydrophobic thickness than does DMPC, DMPC has a different shape than does acyl chain unsaturated DOPC. The shape of DOPC leads to increased lateral pressure in the acyl chain region of a bilayer with decreased lateral pressure in the head group region (Cantor 1999). Thus, the lateral pressure profile of a DOPC bilayer could energetically favor a M2TM conformation with a tilt angle similar to that found in DMPC bilayers. Peptide–lipid systems are complex, and conceivably several mechanisms, including both hydrophobic matching as well as lateral pressure, are operating simultaneously in determining equilibrium conformations. In fact, a combination of both effects was used to help explain lipid effects on the conformation of the large mechanosensitive channels for *E. coli* (MscL) (Perozo et al. 2002b).

EPR results reported here are consistent with previously published SSNMR studies on M2TM, which calculate the helix tilt to be 37° (±3) in DMPC and 33° (±3) in DOPC (Kovacs et al. 2000; Wang et al. 2001; Nishimura et al. 2002). Due to the small difference in observed tilt angle between DMPC and DOPC samples, the investigators concluded helix tilt is not dependent on the lipid environment and is predominantly dictated by peptide–peptide contacts (Kovacs et al 2000). Our work here considers additional bilayer environments and does indicate lipid differences. Like the SSNMR data, our EPR data show a slightly smaller tilt for DOPC than DMPC. This agreement is encouraging and suggests that, similar to the SSNMR data, the spin-labeled EPR experiments described here are quite sensitive to small changes in structure.

The structural plasticity displayed by M2TM in response to membrane composition, as well as mutations (Howard et al. 2002), may be indicative of functional requirements for conformational changes during packaging, gating, and proton transduction. The full-length M2 protein has a more favorable free energy of association than does M2TM (Kochendoerfer et al. 1999) and may not be as malleable

due to additional elements of conformational specificity beyond those in the transmembrane region. However, the various structural models for M2TM proposed to date—each defined by a different set of criteria and in a different environment—might provide snapshots of the distinct conformational states sampled by the protein.

Materials and methods

Synthesis, spin labeling, and purification of peptides

A synthetic peptide corresponding to the transmembrane segment (residues 22–46) of the M2 protein from influenza A was synthesized (SSDPLAVAASIIGILHLILWILDRL). The sequence corresponds to the chicken H5N1 strain of influenza A (Li 2004). This sequence, which contains a Val27Ala mutation, was chosen because previous studies had indicated this sequence was slightly stabilizing with respect to the Udorn strain (Howard et al. 2002).

Peptides were synthesized and cleaved from resin as previously described (Howard et al. 2002). The N terminus of peptide was spin-labeled according to the method in Figure 1 (Luneberg et al. 1995). The reaction was carried out at room temperature in a 1:1 mixture of DMSO and DMF. The synthetic crude peptide was first dissolved in DMSO. Next, the 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carboxylic acid N-hydroxysuccinimide ester was dissolved in DMF and added to the peptide/DMSO solution with stirring. N-methylmorphiline (NMM) was added with stirring.

The spin-labeled peptide was purified by reverse-phase HPLC on a Vydac preparative C-4 column by using a linear gradient at buffer B (6:3:1 2-propanol/acetonitrile/water) containing 0.1% TFA and buffer A (0.1% TFA in water). The identities of peptides were confirmed by using matrix-assisted laser desorption ionization mass spectrometry.

Sample preparation

Samples were prepared by using the following four lipids: DLPC, DMPC, DOPC, and POPC. All lipids were purchased from Avanti Polar Lipids. The same procedure was followed for each lipid, and conditions were such that the protein was essentially fully tetrameric (Cristian et al. 2003b). Peptide and lipid (molar ratio 1:100) were codissolved in TFE in a glass vial and then incubated for 1 h at room temperature. Solvent was then removed by using a gentle stream of nitrogen. The resulting peptide/lipid film was placed under high vacuum overnight to remove all traces of solvent. The films were then hydrated with buffer (100 mM Tris, 200 mM KC1, 1 mM EDTA at pH 8.6) and incubated at 37°C for 1 d.

EPR spectroscopy

X-band continuous wave EPR spectra were collected on a Bruker EMX spectrometer at 300 K, which is above the gel to liquid crystalline phase transition for all four lipids used. Each spectrum was collected with 2-mW incident power, 100-kHz modulation frequency, 1-G modulation amplitude, and a 150 G sweep width. For comparison of line shapes, each spectrum was double integrated and normalized to the same number of spins.

Model generation

M2TM is modeled as a C4 symmetric tetramer of straight helices using four parameters to define bundle geometry (Dieckmann and DeGrado 1997). The structure is optimized for each tilt angle using Monte Carlo Simulated Annealing (Metropolis et al. 1951; Kirkpatrick et al. 1983). Helix separation distances are calculated from helix-axis to helix-axis by averaging the coordinates of Cα for residues 22–27 at the N terminus.

Acknowledgments

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