Structural biology of transmembrane domains: Efficient production and characterization of transmembrane peptides by NMR

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Abstract

Structural characterization of transmembrane peptides (TMPs) is justified because transmembrane domains of membrane proteins appear to often function independently of the rest of the protein. However, the challenge in obtaining milligrams of isotopically labeled TMPs to study these highly hydrophobic peptides by nuclear magnetic resonance (NMR) is significant. In the present work, a protocol is developed to produce, isotopically label, and purify TMPs in high yield as well as to initially characterize the TMPs with CD and both solution and solid-state NMR. Six TMPs from three integral membrane proteins, CorA, M2, and KdpF, were studied. CorA and KdpF are from *Mycobacterium tuberculosis*, while M2 is from influenza A virus. Several milligrams of each of these TMPs ranging from 25 to 89 residues were obtained per liter of M9 culture. The initial structural characterization results showed that these peptides were well folded in both detergent micelles and lipid bilayer preparations. The high yield, the simplicity of purification, and the convenient protocol represents a suitable approach for NMR studies and a starting point for characterizing the transmembrane domains of membrane proteins.

Keywords: membrane protein; transmembrane peptide; maltose binding protein; solution NMR; solid-state NMR

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Abbreviations: CD, circular dichroism; DDM, n-Dodecyl β -D-maltoside; DMPC, dimyristoyl-phosphatidylcholine; DMPG, dimyristoyl-phosphatidylglycerol; DPC, dodecyl-phosphatidylcholine; DTT, dithiothreitol; GuHCl, guanidine chloride; HSQC, heteronuclear single-quantum coherence; IPTG, isopropyl-beta-D-thiogalactopyranoside; LIC, ligation independent cloning; LPPG, lysopalmitoyl-phosphatidylglycerol; MBP, maltose binding protein; NMR, nuclear magnetic resonance; PISA wheel, polar index slant angles wheel; PISEMA, polarization inversion spin exchange at magic angle; TCA, trichloroacetic acid; TEV, tobacco etch virus protease; TMPs, transmembrane peptides; SDS, sodium dodecyl sulfate.

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Small membrane proteins and membrane domains of large proteins represent particularly difficult structural challenges for current structural approaches. Such proteins and domains are significantly influenced by their environment, and consequently, the choice of a membrane mimetic environment is crucial. In addition, the expression, purification, and sample preparation for these constructs can be challenging. Here, we address this array of tasks from cloning to sample preparation for solution and solid-state NMR spectroscopy, as well as the initial structural characterization by NMR.

While membrane proteins represent 30% of most genomes, they represent the majority of pharmaceutical drug targets and yet <0.5% of the characterized protein

structures are membrane proteins. Because transmembrane domains have a highly hydrophobic amino acid composition and because of the near absence of water in the transmembrane environment, the whole balance of molecular interactions that stabilizes protein structure is altered. Hydrogen bonding within helical structures is strengthened in this low dielectric environment, but interhelical hydrogen bonding is relatively rare; instead, such helical interfaces are stabilized primarily by van der Waals interactions. Consequently, tertiary and quaternary structure is only modestly stabilized and often dynamics are observed between conformational substates. Multiple conformations and dynamics considerably increase the difficulty in characterizing membrane protein and membrane domain structure.

The challenge associated with transmembrane domains can, in some cases, be even more daunting when coupled with a large water-soluble domain requiring mixed sample requirements. Here, we advocate a “divide-and-conquer” approach for these transmembrane domains that function independently, and the approach is also applicable to small membrane proteins. For instance, it has been shown that the microenvironment of the transmembrane domain of Vpu, a membrane protein encoded by HIV and involved in the budding of new viral particles from the host cell, is quite similar to that of the full-length protein (Ma et al. 2002). In one of the recent crystal structures of a magnesium transporter, *Thermotoga maritima* CorA, it was shown that the structure of the isolated cytosol domain was so close to that of the full-length protein that it was used to help solve the full-length structure (Lunin et al. 2006). The tetrameric transmembrane domain of M2, a proton channel from influenza A virus, has been shown to be functional through single-channel conductance measurements (Duff and Ashley 1992) and through binding assays of the antiviral drug amantadine (Hu et al. 2006). Similarly, the second transmembrane helix of the human glycine receptor has been shown to form a functional homo-oligomer (Reddy et al. 1993). Indeed, there are many proteins where the transmembrane domain has been shown to be functional, although it is important to state that this will not always be the case, and therefore, when the approach is applied it will need to be justified. Furthermore, functional detail may be influenced not only by the cytoplasmic domain(s), but also by the protein’s membrane mimetic environment.

The TMPs studied here are from three membrane proteins, *Mycobacterium tuberculosis* CorA and KdpF as well as M2 from influenza A. The CorA family is a group of multiple-spanning integral membrane proteins which act as the main magnesium transporter constitutively expressed in nearly all the bacteria. The recent crystal structures of *T. maritima* CorA showed that there were two transmembrane helices in the transmembrane

domain which form a pore as a pentamer (Eshaghi et al. 2006; Lunin et al. 2006; Payandeh and Pai 2006). On the N-terminal side of the transmembrane domain, there is an amphipathic helix that forms a five helix bundle connecting the transmembrane and soluble domains. Between the two transmembrane helices is a highly conserved sequence that was not well resolved in crystal structures. The *M. tuberculosis* CorA studied here has considerable homology with the *T. maritima* CorA, especially in the transmembrane domain. M2 is an integral membrane protein with a single transmembrane helix from influenza A. The tetrameric M2 forms a proton channel in which the highly conserved His 37 in the middle of the transmembrane helix plays a critical role in the proton translocation (Hu et al. 2006). At the C terminus of the transmembrane helix, there is an 18-residue amphipathic helix that increases the stability of the transmembrane domain tetramer (Kochendoerfer et al. 1999; Tian et al. 2002). KdpF is a small membrane protein with a single transmembrane helix found in many bacteria. KdpF is a subunit of the high affinity K⁺-translocating Kdp complex and is thought to be a stabilizer for the multiple-subunit KdpFABC complex (Gassel et al. 1999). The transmembrane helical region of KdpF has much more hydrophobicity than the transmembrane helices from the channel-forming transmembrane domains of CorA and M2.

The synthesis of substantial quantities of TMPs, especially those that are larger than the peptides that can be conveniently synthesized by solid-phase peptide synthesis, is challenging. Recent progress in solid-phase synthesis has made the production of short peptides much easier than before; however, preparation of highly hydrophobic peptides with >50 residues is still difficult (Lindhout et al. 2003). For NMR studies, the requirement of isotopically labeled peptides also makes chemical synthesis costly and impractical for long peptides. Today, the most widely used method to produce these difficult peptides for NMR study is through expression in *Escherichia coli*. Due to instability and potential toxicity to the host cell, these peptides are usually expressed as a fusion with a carrier protein. In some systematic comparisons among different carrier proteins or tags, MBP has been shown to be one of the best for increasing the yield and solubility of the fusion product (Kapust and Waugh 1999; Korf et al. 2005; Nallamsetty and Waugh 2006). In addition, a few reports have confirmed some advantages for MBP, especially for the production of hydrophobic peptides and small proteins (Opella et al. 1999; Buck et al. 2003; Korepanova et al. 2007).

Recently, excellent progress has been made with the development of both solution and solid-state NMR spectroscopy for the structural characterization of membrane proteins (Ramamoorthy et al. 2004; Page et al. 2006). Solution spectroscopy requires the solubilization

of the proteins in a detergent micelle or small bicelle. Many detergents have been explored for this purpose, and it is clear that samples for different proteins are optimized with different detergents (Krueger-Koplin et al. 2004). Even SDS can be used to solubilize a functional tetrameric state of the K⁺ channel, KcsA (Yu et al. 2005; Chill et al. 2006), but more often DPC or LPPG appears to be effective. A set of HSQC spectra from 18 different membrane proteins ranging in molecular weight from 9 kDa to 29 kDa and from one to four transmembrane helices have recently been published (Page et al. 2006). In addition, there are already many solution NMR structures of small membrane proteins (Sanders and Sönnichsen 2006), such as phospholamban (Oxenoid and Chou 2005), MerFT (Howell et al. 2005), fd coat protein (Almeida and Opella 1997), and pardaxin (Porcelli et al. 2004) that have been deposited in the Protein Data Bank.

Solid-state NMR in using aligned samples of liquid crystalline lipid bilayers is the structural approach with the best membrane mimetic environment. Two approaches are typically used for alignment: either planar bilayers aligned between glass slides that will be used in this work, or magnetically aligned discoidal bicelles. Over the past decade this technology has evolved from the complete structure of gramicidin A (Ketchum et al. 1997) to the complete structure of the fd coat protein (Marassi and Opella 2003), the transmembrane domain of M2 protein from influenza A virus (Nishimura et al. 2002), and the M28 pentamer from the acetylcholine receptor (Opella et al. 1999) among the 10 membrane protein structures deposited in the Protein Data Bank

characterized by solid-state NMR. In addition, excellent aligned samples of full-length proteins, including a GPCR protein (Park et al. 2006), three proteins including two that have three transmembrane helices per monomer (Li et al. 2007), and even a protein with a relatively large soluble domain, cytochrome b₅ (Dürr et al. 2007), have recently been published.

By using MBP as the carrier protein and TEV as the cleavage enzyme, six TMPs with different lengths (25–89 residues) and hydrophobicity (0.3–1.7 for the mean hydrophobicity per residue) (Kyte and Doolittle 1982) are studied here. They have all been overexpressed, isotopically labeled, efficiently purified, and initially characterized by CD, solution NMR, and solid-state NMR in detergent micelles and lipid bilayers. Not only is this strategy efficient and reliable, but the samples generated are shown to have a high fraction of secondary structure, and appear, to the extent of the present assays, to be functional.

Results

Expression and purification of fusion proteins

The amino acid sequences of the peptides studied in this work are shown in Scheme 1. All of the fusion constructs from CorA, M2, and KdpF were overexpressed (Fig. 1) as protein fusions. It is remarkable that, for each construct, a considerable fraction of fusion protein is in the membrane fraction, especially for CorA–2TM and CorA–2TM+. For CorA–TM2, KdpF, and two M2 fragments, there is also

Scheme 1. (A) The nomenclature and sequences for the TMPs studied in the present work. The mean hydrophobicity per residue (Kyte and Doolittle 1982) for each transmembrane peptide is also listed at the end of the sequences in brackets. Based on TMHMM prediction (<http://www.cbs.dtu.dk/services/TMHMM/>, only for KdpF) and the experimental data (Tian et al. 2002; Lunin et al. 2006), the sequences corresponding to the transmembrane helices and the amphipathic helices are shaded and underlined, respectively. An additional three residues (SNA) at the N terminus of all peptides is from the TEV cleavage site, and is not shown in the sequences above. (B) Construction of the fusion protein expression plasmid. Both the MBP and TEV cleavage site are shaded. All the TMPs are cloned into the plasmid by LIC.

Figure 1. Expression and distribution of fusion proteins. All of the fusion proteins were expressed at 30°C in an overnight culture. Cells were collected by centrifugation and then sonicated (W). After centrifugation at 10,000g for 20 min, the inclusion body fraction (IB) was collected. The supernatant was ultracentrifuged at 100,000g for 30 min, and the new supernatant (cytoplasmic fraction, C) and pellet (membrane fraction, M, containing both outer and inner membranes) were collected, respectively. Note that the volumes of all the samples were the same, so that the distribution of each fusion protein in the fractions could be roughly compared.

some fusion protein found in the cytoplasmic fraction. The possibility that the fusion proteins were just associated with membranes, but not inserted into the membrane was excluded by washing the membranes with 5 M urea in a basic solution (used to remove proteins weakly associated with the membrane). The fusion proteins were still observed in the membrane fraction. Reduced inclusion body formation results from expression at 30°C overnight in both LB and M9 minimal media, especially for CorA-2TM and CorA-2TM+, and in this way ~50% more protein was produced. Yields of the MBP fusion constructs were typically 75–100 mg/L in LB media and 50–75 mg/L in M9 media (Table 1). By using Ni²⁺-NTA affinity chromatography, all the fusion proteins were purified to homogeneity (90%–95% in purity).

Table 1. Yields of fusion proteins, TMPs, and percent recoveries

	Yields of fusion proteins (mg) ^a	Yields of TMPs (mg) ^a	Percent recovery (%) ^b
KdpF	90	5.5	>80
M2 (22–46)	75	4.5	>80
M2 (22–62)	90	7	>80
CorA-TM2	110	6	>80
CorA-2TM	70	5	>50
CorA-2TM+	90	10	>50

^aThe yields were measured by UV absorption at 280 nm and the corresponding absorption coefficients were calculated based on the amino acid composition of the TMPs. The yield of purified KdpF was measured by a BCA assay. The values shown are typical yields.

^bThe percent recovery is obtained by dividing the theoretical yield (based on total fusion protein yield) by the observed yield of the peptide.

Cleavage and purification of the TMPs

As one of the most specific proteases, TEV recognizes seven sequential amino acids and has been widely used to digest fusion proteins (Parks et al. 1994). In our experiments, it was found that all of the fusion proteins could be digested by TEV, although the cleavage efficiencies varied. In preliminary experiments, it was found that the cleavage rate for CorA-2TM was too slow, and consequently, six glycine residues were inserted between the MBP domain and the TEV cleavage site. The reaction rate was then comparable with the other fusion proteins. All the fusion proteins here were successfully digested (≥80% cleavage) after an overnight reaction at room temperature.

Table 2. Characterization of TMPs by CD^a

	Protein concentration (μM)	Detergent	Literature value of helix content (%) ^b	CD helix content (%) ^c
KdpF	10	SDS	61 (Krogh et al. 2001)	60
M2 (22–46)	20	LPPG	89 (Tian et al. 2002)	80
M2 (22–62)	20	LPPG	82 (Wang et al. 2001)	80
CorA-TM2	44	SDS	47 (Lunin et al. 2006)	50
CorA-2TM	34	SDS	54 (Lunin et al. 2006)	60
CorA-2TM+	22	SDS	67 (Lunin et al. 2006)	60

^aAll the CD experiments were performed in 5% detergent indicated in the table, 5 mM acetic acid buffer at pH 4.5, and 25°C.

^bThe references are indicated behind the literature values.

^cCD estimates of helix content have an approximate error of ±10%.

Figure 2. Purification of TMPs. (A) Organic solvent extraction of short TMPs, including KdpF, M2 (22–46), M2 (22–62), and CorA–TM2. Lanes 1–3 show typical extraction results of CorA–TM2 from the organic solvent approach. Lane 1: before extraction; lane 2: the pellet after extraction; lane 3: purified peptide after extraction. (B) Accumulation of CorA–2TM and CorA–2TM+ by the denaturation/refolding approach. S, supernatant after refolding; P, precipitation after refolding. (C) Purified TMPs. Lanes 1–6 are M2 (22–46), M2 (22–62), KdpF, CorA–TM2, CorA–2TM, and CorA–2TM+, respectively.

For the short TMPs an organic solvent extraction method was used, resulting in >90% of the peptides being extracted in one step and the resultant purity was >95% (Fig. 2A,C). For longer peptides, a denaturation/refolding method was used resulting in >80% of the hydrophobic peptides forming a precipitate, while most of the MBP and TEV remain in solution. After solubilization of the precipitate in 6 M GuHCl and 0.5% DPC, the residual MBP, TEV, and uncleaved fusion protein (each with a His tag) were removed by Ni^{2+} -NTA agarose resin resulting in 90%–95% purity (Fig. 2B,C). Table 1 lists the yields of each peptide studied in this work. It should be noted that for M2 (22–62), the peptide on the SDS-PAGE showed a diffuse band, possibly suggesting exchange within an oligomeric mixture in SDS micelles. This would be consistent with the report that the amphipathic helix (45–62) following the transmembrane helix (26–44) can increase the stability of the oligomeric state (Kochendoerfer et al. 1999).

Structural characterization in detergent micelles

Preliminary conformational analysis of the TMPs was carried out by using CD spectra in 5% SDS or 5% LPPG micelles. As shown in Figure 3A–F, the CD spectra of the six peptides at pH 4.5 were characterized showing a helical content that varies from 50% to 80%. Fitting the CD data using the CDPro software package based on a standard reference set containing 13 membrane proteins, the helical contents of each peptide were estimated (Table 2) and compared well with values suggested by the literature.

As shown in Figure 3A–F, the HSQC spectra of the transmembrane peptides and domains in 5% SDS or 5% LPPG micelles at pH 4.5 and 50°C are all well resolved. Over 90% of the predicted resonances are observed in each spectrum (Table 3). The remaining signals may also be present but overlapping with those that were already counted. For the most part, spectral intensities are uniform

Figure 3. CD (inserted figures) and ^1H - ^{15}N HSQC spectra of TMPs. CD and NMR spectra were recorded under similar conditions (see Materials and Methods). The units in CD spectra are wavelength (nm) and molar circular dichroism, $\Delta\epsilon$ ($\text{M}^{-1}\cdot\text{cm}^{-1}$) for abscissa and ordinate, respectively. (A,B) M2 (22–46) and M2 (22–62) in 5% LPPG, respectively. (C) KdpF in 5% SDS. (D,E,F) CorA–TM2, CorA–2TM, and CorA–2TM+ in 5% SDS, respectively. The samples for NMR were in 10 mM DTT, 100 mM acetic acid buffer, pH 4.5, and the spectra were recorded at 50°C on a Varian Inova 500-MHz, 600-MHz, or 720-MHz spectrometer.

indicating little aggregation or conformational exchange in the samples. The dispersion of signals in the ^1H dimension (~ 1.25 ppm) is consistent with typical α -helical structures, substantiating the CD results. Overall, these peptides appear to have a high fraction of secondary structure with little or no β -sheet, which would generate a larger chemical shift dispersion and result in altered CD spectra. In addition, there is no evidence of conformational heterogeneity from ^1H - ^{15}N HSQC spectra of uniformly ^{15}N labeled samples as well as amino acid specific labeled samples (data not shown).

For CorA, the glycine regions in HSQC spectra from three different constructs are compared in Figure 4A and

B. The similarity of spectral resonances for the terminal CorA transmembrane helix (TM2) in each of the CorA constructs suggests that the structure and environment of this helix is very similar as in the isolated samples. For the M2 protein constructs, the spectra of the transmembrane domain in the isolated peptide (22–46) and in the transmembrane plus amphipathic helix (22–62) also show considerable similarity (Fig. 3A,B).

Solid-state NMR spectra in lipid bilayer

Figure 5A–C show the PISEMA spectra of uniformly aligned and uniformly ^{15}N labeled CorA–TM2, M2 (22–46),

Table 3. Characterization of TMPs by ^1H - ^{15}N HSQC^a

	Detergent	Percentage of expected cross-peaks in HSQC spectra (%) ^b
KdpF	SDS	97
M2 (22–46)	LPPG	93
M2 (22–62)	LPPG	93
CorA–TM2	SDS	91
CorA–2TM	SDS	93
CorA–2TM+	SDS	90

^a All the ^1H - ^{15}N HSQC spectra were recorded in 5% detergent indicated in the table, 10 mM DTT, 100 mM acetic acid at pH 4.5, and 50°C. The protein concentrations were 0.3–0.4 mM.

^b Percentage is calculated by dividing the observed number of cross-peaks in the HSQC spectrum by the expected number (total numbers of amino acid minus the numbers of the prolines and the amino terminates).

and KdpF in fully hydrated DMPC/DMPG bilayers in the liquid crystalline phase. The resonances from 100 to 200 ppm in the spectra indicate well aligned transmembrane helices in the lipid bilayer. These spectra show recognizable PISA wheels, in which the spectral resolution varies significantly between the three peptides. Fitting the data to theoretical PISA wheels shows that the tilt angles of the peptides relative to the normal angles of the lipid bilayer are 27°, 32°, and 34° for CorA–TM2, M2 (22–46), and KdpF, respectively. There are several factors that lead to an error bar for these characterizations, such as averaging of the spin interaction tensors and slight variations in the local backbone geometry (Wang et al. 2001), but these factors sum to no more than a $\pm 3^\circ$ error. The signals away from the PISA wheel are side-chain resonances and backbone resonances from the terminal domains of the peptide. The clear resonances in this region suggest that there are residues in the aqueous domain of each peptide that are well structured and well ordered in these fully hydrated lipid bilayer samples.

The varying spectral resolution among these peptides is caused by several factors. As the tilt of the helix becomes smaller, the PISA pattern becomes smaller, causing

increased congestion. Even the difference between 27° and 34° results in an increase in dipolar coupling variation of 5.8–6.6 kHz and an increase in chemical shift range from 45 to 53 ppm. This amounts to a 17% increase in the PISA wheel circumference with only a 7° increase in the helical tilt. In addition, resonance line widths can vary substantially, depending on sample alignment and more importantly on the dynamics of the polypeptide backbone.

Discussion

The structural characterization of small membrane proteins and small transmembrane domains is particularly challenging. Such systems have a relatively small molecular volume to lipid-exposed surface ratio. The structure of these proteins and domains is likely to be influenced by the quality of the membrane mimetic environment, because the small volume suggests weak stabilizing interactions within the protein or domain, and the large surface area suggests many protein–lipid interactions that will be dependent on the choice of the membrane mimetic environment. Weak stabilizing interactions may mean conformational heterogeneity or plasticity, and it may also mean that dynamics occur between low-lying conformational substates. All of which increase the complexity for elucidating structure–function relationships. The mean molecular weight of prokaryotic membrane protein monomers is small. For the *M. tuberculosis* genome it is 36 kDa, and >50% of the membrane proteins are predicted to have three or fewer transmembrane helices. Like M2 (one TM helix, tetrameric structure) and CorA (two TM helices, pentameric structure) many of these proteins will be oligomeric, and therefore, some of them may have large transmembrane domains even though the monomer is small. Here, the small domains of the functionally important M2 and CorA proteins as well as that of KdpF, which is part of a heteroprotein complex, are used to illustrate a strategy that could lead to structural characterization even in the presence of dynamics and/or heterogeneity that

Figure 4. The overlap of the glycine region in ^1H - ^{15}N HSQC spectra in SDS micelles at pH 4.5. (A) CorA–TM2 (black) and CorA–2TM (red); (B) CorA–2TM (red) and CorA–2TM+ (blue).

Figure 5. PISEMA solid-state NMR spectra of oriented CorA-TM2 (A), M2 (22–46) (B), and KdpF (C) in DMPC/DMPG (4:1) bilayer at 30°C. The calculated PISA wheels are overlaid on the spectra, and the tilt angles are 27°, 32°, and 34° for CorA-TM2, M2 (22–46), and KdpF, respectively. The PISA wheels were calculated using motionally averaged dipolar ($\nu_{\parallel} = 10.375$ kHz) and chemical shift tensors ($\sigma_{11} = 57$; $\sigma_{22} = 81$; $\sigma_{33} = 228$ ppm). Chemical shifts are referenced to liquid ammonia at 0 ppm via a saturated solution of $^{15}\text{NH}_4\text{NO}_3$ at 26 ppm.

are present in both the transmembrane domains of M2 and CorA.

Expression

For hydrophobic peptides containing 30–100 residues, expression coupled with a fusion protein has been shown to be very effective in *E. coli* (Korepanova et al. 2007). Here, this approach has been refined for enhanced yields, optimized cleavage, and for membrane insertion. Most of the published work for transmembrane protein expression has used carrier proteins that have a strong tendency to form inclusion bodies, such as KSI (Ma et al. 2005),

TrpΔLE (Estephan et al. 2005; Ma et al. 2005), and others (Thai et al. 2005). Although the formation of inclusion bodies can reduce the toxicity of the hydrophobic peptides by keeping the peptides away from the host cell membrane, and can decrease the degradation of the target protein, the insolubility of the fusion protein makes it difficult for protease cleavage under mild conditions. To release the peptides from the aggregated fusion protein in inclusion bodies, cyanogen bromide has often been applied to chemically cleave at the N terminus of an inserted methionine between the carrier protein and the passenger protein. However, this method is not appropriate for the transmembrane proteins containing a conserved methionine, and is also limited by potential side reactions and harsh reaction conditions (Düwel and Honek 1998).

MBP is a frequent choice as a fusion partner for cytosolic proteins, but has been less frequently used for membrane proteins (Opella et al. 1999; Buck et al. 2003; Korepanova et al. 2007). It has, however, been reported that MBP may act as a chaperone to enhance the solubility of the target protein and increase its yield (Pryor and Leiting 1997; Kapust and Waugh 1999; Riggs 2000). It is very important for the expression of hydrophobic peptides, since the fusion proteins with a hydrophobic tail usually have a tendency to form aggregates. Such chaperone-like effects are thought to include hydrophobic interactions between MBP and its passenger protein, but the interaction sites have yet to be determined (Kapust and Waugh 1999; Fox et al. 2001).

In our work, as shown in Figure 1, a considerable fraction of the MBP fusion proteins were expressed in the membrane fraction with high yields (Table 1), similar to a previous report where cytochrome b6, a small integral membrane protein, fused with MBP containing a signal peptide was overexpressed and inserted into membranes (Kroliczewski et al. 2005). In our constructs, the cloned MBP does not contain its signal peptide, and therefore, the mechanism for driving the fusion proteins into the *E. coli* membranes is not clear. Because a considerable quantity of the fusion protein is expressed in the membrane, it can be expected that the expressed peptides fused with MBP are well folded and potentially in a native-like conformation even before the TEV cleavage. Possibly due to the limited machinery for folding and insertion into the membrane environment, expression at low temperature, which decreases the expression rate, enhanced the yield in the membrane fraction and decreased the inclusion body formation.

Purification

While reverse-phase HPLC is a widely used method to separate the carrier and passenger proteins after protease digestion, the yield for hydrophobic peptides following

reverse-phase HPLC is typically low, possibly due to the irreversible adsorption on the hydrophobic solid phase (Jones et al. 2000; Fisher and Engelman 2001). In addition, the possible oligomerization or aggregation of the hydrophobic protein/peptide on the surface of the solid phase can also be a serious problem, resulting in poor resolution and low purity (Fisher and Engelman 2001). There is also considerable difficulty in scaling up such a purification protocol. Alternatively, the differences in solubility between MBP and the hydrophobic peptides may be convenient property for separation. However, following TEV cleavage the peptides expressed here do not readily precipitate despite peptide concentrations in the range of 0.1–1 mg/mL and low detergent concentrations (less than the critical micelle concentration). In addition, the peptides did not pass through an ultra-filtration tube despite the cutoff being much larger than the molecular weight of the peptide. The chaperone-like effects of MBP may be responsible for the increased solubility of the TMPs in the aqueous solution. Accordingly, the two purification methods, one involving solubilization of the transmembrane domains by organic solvents (Fig. 2A) and one utilizing high concentrations of GuHCl followed by rapid refolding (Fig. 2B), were employed in the present work to disrupt the interactions between the carrier and passenger proteins following cleavage for effective purification, and both worked equally well (Fig. 2C). Both of these protocols are dependent on having a purified fusion protein before TEV cleavage.

Biophysical characterization of the TMPs

CD and solution NMR (Fig. 3A–F; Tables 2,3) both show that these transmembrane peptides and domains have high helical content in detergent micelles as predicted for these proteins. Solid-state NMR confirms the helical content for several of these samples and further characterizes the helices as uniform, if not nearly ideal (i.e., $\phi = -60^\circ$ and $\psi = -45^\circ$) helical structures in a transmembrane configuration (Fig. 5).

For the three transmembrane constructs from CorA, the spectra overlap in the glycine region is so good that one can identify which glycines are from TM2, TM1, or the amphipathic helix with considerable certainty (Fig. 4A,B). Indeed, throughout the spectra the overlap clearly shows a uniformity of structure and environment for these three constructs. In the structural study of Vpu by solution NMR, the same phenomenon was observed when the spectra of the transmembrane peptide and that of the full-length protein were overlaid (Marassi et al. 1999; Ma et al. 2002). For CorA–TM2, the spectral overlap suggests that the interaction between TM1 and TM2 is relatively weak so that the helical backbone structures are essen-

tially independent of each other. While TM2 has two adjacent glycine residues in the transmembrane helix, which might suggest kinks or bends in the helix, the well-defined PISA wheel observed by solid-state NMR for TM2 (Fig. 5A) indicates the formation of a uniform helical structure with little variation in helical tilt from one side of the membrane to the other as has been analyzed in M2 (22–46) (Wang et al. 2001). The observed tilt of the isolated helix relative to the bilayer normal is likely to be the result of the hydrophobic mismatch with the DMPC/DMPG bilayer and not necessarily the tilt of the helix in the native *M. tuberculosis* membrane environment. The lack of the interaction with the first transmembrane helix can also influence the tilt angle. However, uniform helical structures, such as that observed for TM2, generate a relatively small available surface for interacting with another helix. Despite the presence of glycines there are no GxxxG or AxxxG type sequences that would suggest close packing of either TM2 with itself or with TM1, as in the glycophorin dimer (MacKenzie et al. 1997). In the crystal structures of *T. maritima* CorA, TM2 forms the outer ring in the pentameric structure and appears to interact only weakly with the first transmembrane helix and not at all with the other TM2 helices (Eshaghi et al. 2006; Lunin et al. 2006; Payandeh and Pai 2006).

The M2 transmembrane domain is functional without the N- and C-terminal domains (Duff and Ashley 1992), and has been structurally characterized by solid-state NMR with and without the antiviral drug amantadine bound (Wang et al. 2001; Hu et al. 2007a). The early structure of this domain without amantadine was obtained from samples of M2 (22–46) codissolved in organic solvents with lipid (Wang et al. 2001). More recently, it has been observed that when these samples are prepared from liposomes there are subtle and reproducible differences in both the structure and dynamics of this domain (C. Li and T.A. Cross, unpubl.). Here, the solid-state NMR data shows that the helical tilt is 32° (Fig. 5B), the same as the N-terminal helical tilt in the presence of amantadine, differing only slightly from the 38° tilt angle observed when organic solvents are used to cosolubilize the peptide and lipid (Wang et al. 2001). Here, the resonance line widths are broad in the PISEMA spectra, consistent with the efficient relaxation that has been observed for the samples prepared in this fashion, suggesting possible dynamic averaging of conformational substates (Hu 2005). The solution NMR spectroscopy of M2 goes beyond the transmembrane domain to include the amphipathic helix that binds to the surface of the lipid bilayer. Once again, the transmembrane domain and the extended construct show considerable similarity in the resonance frequencies for the transmembrane domain (Fig. 3A,B).

KdpF is one of many very small hydrophobic membrane proteins, such as the γ -subunit of the Na^+, K^+ -ATPase,

sarcophilin, and phospholamban, that regulate or stabilize transporter complexes. KdpF is a stabilizer of the K^+ transporter system, Kdp, composed of three additional proteins KdpA, KdpB, and KdpC, all three of which are transmembrane proteins. Here, KdpF, like CorA-TM2, is shown to have a nearly ideal helical structure in a lipid bilayer, as evidenced by the high-resolution PISEMA spectrum (Fig. 5C). Also like CorA-TM2, the helix tilt may be influenced by the hydrophobic mismatch and the lack of interaction with other subunits in the Kdp complex. For single transmembrane peptides experimental data (Duong-Ly et al. 2005; Park and Opella 2005; Ramamoorthy et al. 2007) indicate that the tilt angle can be significantly influenced by the hydrophobic mismatch between the hydrophobic length of the transmembrane peptide and the thickness of the lipid bilayers, except for situations where there are extensive helix-helix interactions. Future studies will focus on determining the binding partner or partners in the Kdp complex and to characterize the bound conformation of this small membrane protein. While sarcophilin has an equilibrium among different conformers that appears to be beneficial for its recognition of the transporter complex, there is no evidence in the solid-state NMR data that multiple conformations of KdpF exist (Buffy et al. 2006). However, the solution NMR spectra show relatively broad lines in SDS micelles (Fig. 3C), potentially reflecting exchange broadening that could result from a conformational equilibrium.

Starting point for characterizing the transmembrane domain

Although the six transmembrane peptides and domains studied in the present work vary in the length, sequence, and hydrophobicity, they have been efficiently expressed using a protocol outlined in Scheme 2, from genes to NMR samples and experimental data. This efficient protocol that generates high yields and high purity, as well as scalability for sufficient quantities of well-folded final products, represents a demonstrated approach for studying transmembrane peptides and domains.

Through screening a series of conditions for isolated transmembrane domains for functional activity, conformations will be selected for further investigation. The M2 transmembrane domain is a good example, since this domain has been shown to bind the antiviral drug, amantadine, and to conduct protons (Duff and Ashley 1992; Hu et al. 2006, 2007b). Consequently, its structure and dynamics in the model membranes are closely related with the function of the full-length M2 protein. Such a “divide and conquer” strategy for membrane proteins will speed up the progress toward characterizing these particularly challenging and often small membrane proteins, although this strategy may not be appropriate for all

Scheme 2. Pipeline for TMPs: from gene to NMR.

the membrane proteins, especially for those whose transmembrane domain is not distinguishable from the soluble domain or where there is an extensive interaction between the domains.

Materials and Methods

Cloning and plasmid construction

The DNA corresponding to each peptide were amplified by PCR and cloned into the modified pET30 vector containing a MBP fusion protein expression system by LIC (Dieckman et al. 2002).

In this system, the gene of MBP without its signal peptide was flanked with an N-terminal His tag and a C-terminal TEV cleavage site. An SspI cleavage site was directly inserted downstream of the TEV cleavage site and is used to insert the target gene by LIC (Scheme 1). For CorA-2TM, in order to increase the cleavage efficiency by TEV, six glycine residues were inserted just before the TEV cleavage site to increase the length of the linker between MBP and the target peptide. All constructions have been sequenced to verify their correct insertion.

Expression of fusion proteins in LB and M9 media

The plasmids encoding the fusion proteins were transformed into *E. coli* strain BL21 (DE3)-RP codon plus for expression. Usually, a single clone was picked and inoculated into 3-mL LB media with 100 µg/mL Ampicillin, and grown overnight at 37°C with shaking. The 3-mL culture was poured into 1-L LB media, and the culture was grown to an $OD_{600} = 0.6$ at 37°C with shaking. The culture was then cooled to 30°C, and expression was induced with 0.4 mM IPTG for 12–16 h at 30°C with shaking. In M9 media, the bacteria in a 3-mL overnight culture was collected by centrifugation and washed with M9 media once, then inoculated into 1-L M9 media. Other procedures were the same as for LB media.

Purification of fusion proteins

Before large-scale purification, the distribution of the fusion protein was studied. For a 10-mL overnight culture, cells were collected by centrifugation at 4000g for 10 min, then resuspended in 1-mL buffer containing 20 mM Tris-HCl, 50 mM NaCl, pH 8.0. After sonication for 30 sec on ice, the inclusion body fraction was collected as a pellet by centrifugation at 10,000g for 20 min at 4°C. To separate the cytoplasmic and membrane fractions, the supernatant was ultracentrifuged at 100,000g for 30 min at 4°C. The whole-cell sample, inclusion body fraction, cytoplasmic fraction, and membrane fraction were applied to SDS-PAGE to check the distribution of the fusion proteins. In addition, to test whether the fusion proteins were inserted in the membrane or simply associated with the membrane, the membrane fraction was extensively washed and sonicated in a basic buffer containing 5 M urea, 250 mM NaCl, 20 mM DTT, and 50 mM Na_2CO_3 , pH 10.0; the membrane fraction was again collected by ultracentrifugation as above.

For large-scale purification, cells were collected by centrifugation at 4000g for 10 min, and then washed once with a buffer containing 20 mM Tris-HCl, pH 8.0. Cooled cells were lysed by French press in the binding solution containing 50 mM NaCl, 20 mM Tris-HCl, pH 8.0, and the supernatant was collected after centrifugation at 10,000g for 20 min. DDM or DPC was then added to the supernatant to a final concentration of 0.87% for DDM or 0.5% for DPC to solubilize the fusion proteins in the membrane fraction. The supernatant was allowed to incubate with the Ni^{2+} -NTA agarose resin (QIAGEN) while gently shaking. After binding at 4°C overnight, the resin was washed with binding solution containing 20 mM imidazole followed by elution with an elution buffer containing 50 mM NaCl, 20 mM Tris-HCl, pH 8.0, 0.087% DDM, or 0.05% DPC and 300 mM imidazole. The fractions containing the fusion proteins were pooled and stored at 4°C temporarily.

TEV cleavage of fusion proteins

TEV was expressed as described previously, purified by Ni^{2+} -NTA agarose resin, and stored in 250 mM NaCl, 10 mM Tris-HCl, 50% glycerol, 5 mM DTT, 1 mM EDTA, and 0.05% Triton X-100, pH 8.0 at –20°C (Kapust and Waugh 2000). The TEV His tag was retained for later peptide purification schemes. To cleave the fusion protein, TEV was added to the freshly purified fusion proteins (2.5–5 mg/mL) at a ratio of 10:1 (fusion protein:TEV, by mass). Before addition of TEV, the fusion protein was usually diluted twofold by a solution containing 500 mM NaCl, 20 mM Tris-HCl, pH 8.0, to decrease the concentration of detergent that weakly inhibits the activity of TEV. The reaction was left at room temperature for 16–20 h.

Purification of the TMPs

Two complementary methods were used to purify the cleaved peptides. To purify KdpF, M2 (22–46), M2 (22–62), and CorA-TM2, the TEV cleavage reaction was stopped by addition of TCA at a final concentration of 6%. The precipitate was collected by centrifugation. After washing the pellet with water twice to remove residual TCA, the protein was lyophilized in a vacuum centrifuge. Then 20-mL methanol/1-L culture (for KdpF, M2 [22–46], and M2 [22–62]) or 20-mL mixed organic solvent containing 90% methanol, 9% chloroform, and 1% acetic acid per liter culture (for CorA-TM2) was added and mixed gently for several hours at room temperature. To remove the undissolved protein (MBP and TEV), the solution was centrifuged at 13,000g for 20 min, and the supernatant was carefully collected. The peptides were then lyophilized in a vacuum centrifuge and stored at –20°C.

To purify CorA-2TM and CorA-2TM+, the TEV cleavage reaction was stopped by the addition of GuHCl to a final concentration of 4 M, followed by rapid dilution using a buffer containing 50 mM NaCl, 20 mM Tris-HCl, pH 8.0 while mixing to decrease the concentration of guanidine to 0.5 M (the final concentration of proteins was around 0.3 mg/mL). After gently mixing overnight at 4°C, the precipitate was collected by centrifugation. The pellet was washed with water to remove GuHCl, and resolubilized with a small volume of 6 M GuHCl and 0.5% DPC. Then the solution was loaded onto a Ni^{2+} -NTA agarose resin pre-equilibrated with the same solution. After readorption at 4°C overnight, flow-through was collected and dialyzed against water to remove GuHCl and DPC. After exhaustive dialysis, the aggregated peptide was collected by centrifugation, lyophilized, and stored at –20°C.

CD experiments

The peptides were solubilized with a buffer containing 5% SDS for KdpF, CorA-TM2, CorA-2TM, and CorA-2TM+ or 5% LPPG for M2 (22–46) and M2 (22–62), respectively, and 5 mM acetic acid, pH 4.5. The concentration of peptides for CD studies was in a range from 10 µM to 50 µM, determined by the absorption at 280 nm, and double-checked by BCA assay (Pierce Inc.) by using bovine serum albumin in the corresponding detergents as the standard. The molar absorption coefficients were predicted based on the amino acid composition (Pace et al. 1995). CD experiments were performed using an AVIV 202 CD spectrometer with 0.1-cm quartz cuvettes. CD spectra were recorded from 260 nm to 200 nm in 0.5-nm steps and 1-sec integration time at 25°C. Each curve represents the average of

at least four scans and a blank containing the same buffer and detergent as the sample was subtracted from each of the spectra. The spectra were plotted as the molar circular dichroism ($\Delta\epsilon$ M⁻¹cm⁻¹). The helical content of the peptides was estimated by fitting the experimental data using the CDPPro software package.

Solution NMR

As previously, NMR samples were prepared by solubilization of peptides in 5% SDS (for KdpF, CorA-TM2, CorA-2TM, and CorA-2TM+) or 5% LPPG (for M2 [22–46] and M2 [22–62]), 10 mM DTT, 100 mM acetic acid, pH 4.5, and 10% D₂O (Page et al. 2006). The concentrations of peptides were 0.3–0.4 mM. All the ¹H-¹⁵N HSQC spectra were recorded at 50°C on a Varian Inova 500-MHz, 600-MHz, or 720-MHz spectrometer.

Solid-state NMR

All of the solid-state NMR samples were prepared with DMPC and DMPG in a 4:1 molar ratio, and the molar ratios of peptide to lipid in all the samples were 1:100. Proteoliposomes for KdpF, M2 (22–46) and CorA-TM2 were prepared somewhat differently. For KdpF, lyophilized peptide was solubilized in DPC micelles, and mixed with the preformed liposome. To prepare liposome, lipid was dissolved in chloroform in a round-bottom flask and the solvent evaporated in a rotary evaporator followed by vacuum drying overnight. The DMPC/DMPG film was then dissolved in deionized water at a final concentration of a 20 mg/mL bath, sonicated, followed by three freeze (liquid-nitrogen)–thaw (37°C) cycles for three times. Twenty percent octyl-glucoside (OG) was added to the mixture until the solution clarified. DPC and OG were removed by dialysis. For M2 (22–46) in methanol after the purification, dry lipid powder was directly added to the organic solution, followed by evaporation at room temperature. Then the M2 (22–46)/lipid powder was suspended in a phosphate buffer (50 mM, pH 8.0), sonicated, and dialyzed against water for 1 d. For CorA-TM2, the dry lipid powder was mixed with the peptide solubilized in 4% SDS solution. SDS was removed by exhaustive dialysis in the presence of Biobeads (Biorad). Proteoliposomes were collected by ultracentrifugation (196,000g for 90 min.) for all three preparations. The pelleted proteoliposomes were resuspended in a minimum of water or the phosphate buffer mentioned above for M2 (22–46), and deposited on the glass slides. The samples were dehydrated on the glass slides and rehydrated as previously described before sealing the samples in square glass tubing (Tian et al. 2002). The spectra were recorded at 30°C on a Bruker 600 MHz or an ultrawide bore 900-MHz solid-state NMR spectrometer using an NMR probe. Simulated PISA wheels for specific tilt angles of the transmembrane helices were overlaid on the resonances in the PISEMA spectra of uniformly ¹⁵N labeled peptides to determine the tilt angles with respect to the lipid bilayer normal.

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