Retention of Native-like Oligomerization States in Transmembrane Segment Peptides: Application to the *Escherichia coli* Aspartate Receptor†

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ABSTRACT: Biophysical study of the transmembrane (TM) domains of integral membrane proteins has traditionally been impeded by their hydrophobic nature. As a result, an understanding of the details of protein−protein interactions within membranes is often lacking. We have demonstrated previously that model TM segments with flanking cationic residues spontaneously fold into α-helices upon insertion into membrane-mimetic environments. Here, we extend these studies to investigate whether such constructs consisting of TM helices from biological systems retain their native secondary structures and oligomeric states. Single-spanning TM domains from the epidermal growth factor receptor (EGFR), glycophorin A (GPA), and the influenza A virus M2 ion channel (M2) were designed and synthesized with three to four lysine residues at both N- and C-termini. Each construct was shown to adopt an α-helical conformation upon insertion into sodium dodecyl sulfate micelles. Furthermore, micelle-inserted TM segments associated on SDS−PAGE gels according to their respective native-like oligomeric states: EGFR was monomeric, GPA was dimeric, and M2 was tetrameric. This approach was then used to investigate whether one or both of the TM segments (Tar-1 and Tar-2) from the *Escherichia coli* aspartate receptor were responsible for its homodimeric nature. Our results showed that Tar-1 formed SDS-resistant homodimers, while Tar-2 was monomeric. Furthermore, no heterooligomerization between Tar-1 and Tar-2 was detected, implicating the Tar-1 helix as the oligomeric determinant for the Tar protein. The overall results indicate that this approach can be used to elucidate the details of TM domain folding for both single-spanning and multispanning membrane proteins.

Integral membrane proteins carry out a plethora of critical cellular functions including nutrient transport, ion pumping, and signal transduction (*1*). The majority of the transmembrane domains of integral membrane proteins consist of one or more ca. 20−30-residue hydrophobic segments (*2*) that span the lipid bilayer as α-helices. In addition to acting passively as membrane anchors, these segments contain specific arrays of side chains that serve as recognition elements for helix−helix packing within multispanning proteins. Specific interactions between TM[1] helices are also functionally important for TM domain oligomerization and underlie the formation of multimeric three-dimensional structures such as ion channels. In dimeric and multispanning membrane proteins, packing has been shown experimentally to occur via noncovalent, tertiary contacts of TM α-helices driven primarily by van der Waals interactions (*3*−*8*). However, since only a handful of membrane proteins have been solved to high resolution (reviewed in ref *9*), alternative approaches to the elucidation of the detailed nature of helix−helix interactions within membrane domains—and ultimately the rules which dictate the relevant interfaces—would be of considerable value.

Obtaining large amounts of TM domain samples experimentally through heterologous expression or synthesis is complicated by the fact that these polypeptides are inherently hydrophobic and often require nonstandard solubilization and purification strategies. The high tendency of hydrophobic peptides to adopt secondary structures and aggregate nonspecifically during synthesis has dictated the use of multiple couplings of amino acids and/or the use of elevated temperatures during synthesis (*10*−*14*). Synthetic procedures may also be complicated by binding of the peptide irreversibly to the stationary phase of HPLC columns during purification (*11*). Subsequent solubilization, and avoidance of aggregation, of these peptides may also require the use of harsh conditions or amphiphiles such as detergents, which must later be separated from the sample (*11*).

Previous workers have used charged/polar residues at the termini of hydrophobic peptides in order to prevent unwanted nonspecific aggregation of peptides (*15*−*17*). However, sequence-dependent specific oligomerization between TM

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[1] Abbreviations: TM, transmembrane; EGFR, epidermal growth factor receptor; GPA, glycophorin A; M2, influenza virus M2 protein; Tar, *E. coli* aspartate receptor; TAMRA, 5(6)-carboxytetramethylrhodamine; CD, circular dichroism; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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segments within these peptides has not been examined. Here, we examine the secondary structure and tertiary contacts of TM segment peptides with flanking lysine residues. Our approach involves predicting the TM residues from a given protein sequence using TM Finder, a program developed in our laboratory that uses experimentally derived dual requirements for both helicity and hydrophobicity to identify putative TM segments (*16*). Synthesis of the TM peptides thus identified is carried out with lysine tags ("Lys tags"), which overcome the inherent insolubility of TM segments and generally allow facile purification protocols. Subsequent structural characterization is then performed to determine the secondary and tertiary structures of the resulting TM peptides.

In the present work, peptides from three well-characterized single TM proteins were designed in this manner and found to retain native-like secondary structure and tertiary contacts when placed in membrane-mimetic environments. This finding reinforces the notion that individual TM segments can act as autonomous folding domains (*18*), thus emphasizing their role in guiding the folding of membrane proteins. The approach is then applied to the double-spanning membrane protein *Escherichia coli* aspartate receptor (Tar) (*19*) in order to characterize its individual TM segments in terms of secondary structure and oligomeric state(s).

MATERIALS AND METHODS

Materials. Fmoc amino acids were obtained from Nova Biochem (San Diego, CA), and PAL-PEG-PS resin was purchased from Applied Biosystems (Foster City, CA). Reagents used for peptide synthesis included *N*,*N*-dimethylformamide (Caledon, ON), piperidine (Acros), *N*,*N*-diisopropylethylamine (DIEA) (Aldrich), methanol (Caledon, ON), *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridino-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) (PerSeptive Biosystems). The peptide labeling reagent 5- (and 6-) carboxytetramethylrhodamine succinimidyl ester [TAMRA SE] was purchased from Molecular Probes, Eugene, OR. Reagents used in peptide cleavage from solid support were trifluoroacetic acid (TFA) (PerSeptive Biosystems), triisopropylsilane (TIPS) (Aldrich), phenol (Gibco), and ultrapure water. Diethyl ether (Caledon, ON) was used for peptide precipitation. Acetonitrile (MeCN) for HPLC purification was purchased from Caledon (Georgetown, ON). SDS detergent (lauryl sulfate) was purchased from Sigma. The Novex brand of precast gels and buffers (San Diego, CA) was used for SDS−PAGE. Reagents for the micro BCA assay were purchased from Pierce (Rockford, IL).

Peptide Synthesis. Peptides were synthesized using standard Fmoc chemistry on a PerSeptive Biosystems Pioneer peptide synthesizer. The synthesis employed the use of the Pioneer's standard (45 min) cycle. The HATU/DIEA activator pair was used with a 4-fold excess amino acid. A low-load (0.18−0.22 mmol/g) PAL-PEG-PS resin was used to produce an amidated C-terminus. Peptides were cleaved with a cocktail of 88% TFA/5% phenol/5% ultrapure water/2% TIPS. Cleaved peptides were precipitated with ice-cold diethyl ether. Centrifuged pellets were dried in a desiccator, redissolved in ultrapure water, and lyophilized.

Peptide Purification. Crude peptide powder was dissolved in water and 10 mg of peptide was loaded onto a C4 preparative RP-HPLC column. The major peak from a water/MeCN gradient was collected and lyophilized. Mass spectrometry was used to confirm the molecular weight of the purified peptide and the micro BCA assay was used to determine peptide concentration. Analytical HPLC chromatography was used to confirm the purity of peptides.

Peptide Labeling. Labeling with TAMRA SE was accomplished by reacting the free N-terminal amino group of the resin-bound peptide with 2-fold excess label under basic conditions (100 mM DIEA in DMF) overnight. Cleavage and purification of labeled peptides were done as described above.

Circular Dichroism Spectroscopy. CD spectra were recorded using a Jasco J-720 circular dichroism spectrometer. Samples were measured at peptide concentrations between 20 and 50 μM and were dissolved in a buffer containing 10 mM Tris/10 mM NaCl, pH 7.2, with or without 20 mM SDS. Measurements were taken using a quartz cuvette with a 0.1 mm path length. Spectral scans were performed from 250 to 190 nm with a step resolution of 0.2 nm, a speed of 20 nm/min, and a bandwidth of 1.0 nm. Temperature melting curves were obtained by monitoring the signal at 208 nm while the sample was incrementally heated using a water bath and a waterjacketed cell.

SDS−PAGE. Peptide samples were subjected to SDS−polyacrylamide gel electrophoresis using 10−20% tricine precast gels (Novex, San Diego, CA). Samples were boiled for 5 min prior to loading.

TM Finder. Transmembrane sequences were identified using the web-based program TM Finder (http://www.bioinformatics-canada.org/TM/) with the following parameters: for GPA, EGFR, and Tar, the N- and C-terminal windows were set at 3, the core length was set at 10, the gap was set at 3, and the segment length was set at 10. For M2, the N- and C-terminal windows were set at 2, the core length was set at 10, the gap was set at 3, and the segment length was set at 10 (*20*).

RESULTS

To demonstrate initially that TM peptides are capable of nativelike folding and oligomerization, we selected TM segments from three single TM membrane proteins, each with a distinct oligomeric state.

(*1*) *TM Domain of the Epidermal Growth Factor Receptor (EGFR).* Although intact EGFR is thought to be functionally dimeric in vivo, the association of EGFR monomers has been shown to be induced by binding of the EGF ligand (*21*) rather than via specific TM helix−helix interactions. Furthermore, the TM domain of EGFR fused to the carboxy terminus of the soluble nuclease from *Staphylococcus aureus* (*8*) was monomeric in SDS−PAGE experiments (*22*).

(*2*) *TM Domain of Glycophorin A.* The single-spanning sialoglycoprotein from human erythrocytes, glycophorin A (GPA), has been shown to form SDS-resistant dimers (*8*, *23*).

(*3*) *TM Domain of the Single-Spanning Influenza A Virus M2 Protein.* The M2 protein homooligomerizes via its TM domains to comprise a proton channel demonstrated to exist as a homotetramer (*24*).

Identification of TM Segments and Peptide Design. Output from the TM Finder program (*20*) was combined with

Table 1: Names, Sequences, and Oligomeric States of Peptides Derived from Transmembrane (TM) Segments of Various Membrane Proteins

protein	sequence[a]	peptide name	observed peptide oligomeric state[b]	reported oligomeric state (ref)
EGFR	KKKK-IATGMVGALLLLLVVALGIGLFM-KKKK	8K-EGFR	monomer	monomer (*21*, *22*)
GPA	KKKK-ITLIIFGVMAGVIGTILLISYGI-KKKK	8K-GPA	dimer	dimer (*8*)
M2	KKK-VAASIIGILHLILWILDRLFFKSIYRF-KKK	6K-M2	tetramer	tetramer (*24*)

[a] Underlined sequences represent residues predicted by TM Finder (*20*) as occurring in the protein TM segment. [b] Oligomeric state deduced from SDS−PAGE gels (see text and Figure 3).

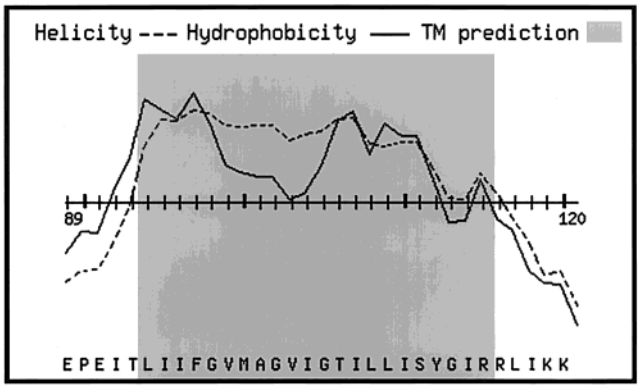

FIGURE 1: TM Finder output for glycophorin A. The full-length sequence of glycophorin A (accession code: NP_002090) was entered into the TM Finder input window (*20*). The predicted TM segment residues are LIIFGVMAGVGTILLISYGI. The TM Finder parameters used are given in the Materials and Methods section.

published experimental data on the individual systems involved to localize the final sequences of specific TM peptides (Table 1). As an example, Figure 1 shows the output from TM Finder for GPA; the program predicts the 22 amino acid TM segment LIIFGVMAGVIGTILLISYGIR. Published data, including the three-dimensional NMR structure of the TM region of the GPA dimer (*25*), indicate that residues I73-T74 likely also occur within the TM domain; the latter two residues were accordingly included in our peptide design. The output from TM Finder for EGFR was used directly. For the M2 peptide, the TM Finder output gave the following 20 amino acid sequence: VAASIIGILHLILWILDTLF. Although TM Finder did not include the relatively hydrophilic native C-terminal flanking residues FKSIYRF in the TM segment prediction, these residues were added into the synthesis to ensure that all TM-spanning residues were present in the peptide design. For the design of the M2 peptide, all included residues are native to the strain Udorn/72 except that the native Cys residue was changed to Ser. This was done to ensure that any oligomerization observed with this peptide was due to noncovalent helix−helix interactions. The C → S mutation was further justified by the influenza strain A/Hong Kong/470/97 that has such a mutation in its M2 TM domain (*26*).

Lys Tags. Lysine residues were added to the N- and C-termini of TM segment peptides of structure K_n-(TM)-K_n-amide to confer water solubility to the hydrophobic segments. To determine the number of lysines (n) needed solubilize a given TM segment, we first calculated the cumulative hydrophobicity of the TM domain using the Liu/Deber scale that is utilized by TM Finder (*16*); empirically, we find that a TM segment is rendered water soluble if at least one lysine is added to each terminus per 16 units of hydrophobicity. For example, the M2 segment exhibits a cumulative hydrophobicity of 48.9, and thus six Lys residues were added to its TM domain. Similarly, we tagged GPA (8K-GPA) and EGFR (8K-EGFR) each with eight lysines. The final sequences of the water-soluble Lys-tagged peptides are given in Table 1.

Peptide Synthesis and Purification. Multiple coupling steps, high temperatures, and/or alternating solvent systems have previously been employed to improve the synthesis of hydrophobic peptides (*14*, *27*). Our synthesis uses standard synthesis procedures with optimized selection of peptide design, solid support, coupling times, and appropriate activators (see Materials and Methods section). The N- and C-terminal lysine content was found not only to render the final peptide products soluble but also to eliminate aggregation of the growing peptides on the solid support. The "low load" PAL-PEG-PS resin was chosen for similar reasons, since this support had already been shown to be useful for the synthesis of hydrophobic peptides (*28*). Purification of the synthesized peptides was routinely carried out with a one-step RP-HPLC method using a C4 preparative column. Pure products were water soluble at concentrations up to 50 mg/mL.

Secondary Structure of Peptides. The secondary structures of TM peptides were examined using circular dichroism (CD) spectroscopy following solubilization in various solvent systems. Because the peptides were water soluble, it became possible to ascertain the secondary structures of native TM sequences dissolved in aqueous environments. Figure 2 illustrates the diversity of structures which arise: the 8K-EGFR peptide in aqueous buffer displays a spectrum that has been associated with a β-turn (*29*), while 8K-GPA was minimally structured and 6K-M2 was largely helical. The high helical content of the 6K-M2 peptide is in agreement with the Chou−Fasman prediction for this peptide in aqueous solution (*30*, *31*). CD spectra in Figure 2 further demonstrate the increase in helicity associated with insertion of the hydrophobic core of each peptide into the membrane-mimetic environment of SDS micelles, where all peptides became highly helical; flanking N- and C-terminal lysine residues presumably remain in the aqueous environment. As well, for the 6K-M2 peptide, the only peptide synthesized possessing a native Trp, the λ_{max} position of the Trp fluorescence was blue shifted upon addition of SDS from 347 to 333 nm, consistent with insertion into a hydrophobic micelle environment (data not shown).

Assessing the Peptide Oligomeric State Using SDS−PAGE. To test whether the TM segment peptides were able to self-associate in micelles, and what specific oligomerization states they displayed, peptides were assessed by SDS−PAGE. This method has been widely used to examine the association of transmembrane domains since SDS detergent micelles mimic the membrane environment (*8*, *22*, *23*, *32*−

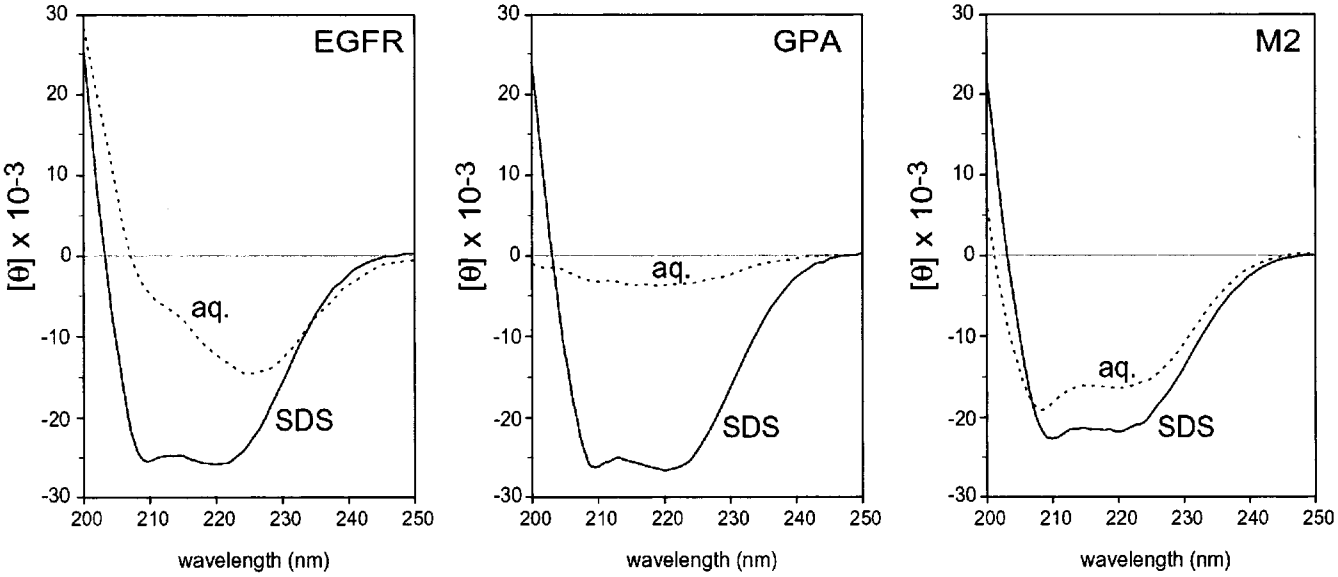

FIGURE 2: Secondary structure of TM segment peptides determined by circular dichroism spectroscopy. Spectra are shown for peptides dissolved at room temperature in aqueous buffer containing 10 mM Tris-HCl and 10 mM NaCl, pH 7.2 (dotted lines), and in detergent buffer containing 20 mM SDS, 10 mM Tris-HCl, and 10 mM NaCl, pH 7.2 (solid lines). Peptide names are as indicated in each panel. The peptide concentration was 50 μM in each case.

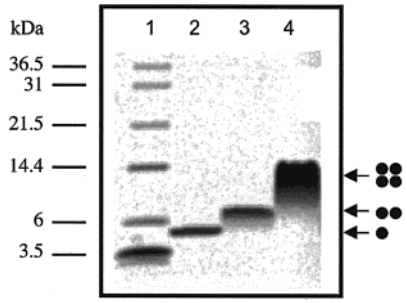

FIGURE 3: Oligomeric state of TM segment peptides probed by SDS−PAGE analysis. Peptides dissolved in SDS-containing sample buffer were boiled for 5 min prior to electrophoresis using 10−20% tricine gels. Molecular weight markers (lane 1) were used to determine the oligomeric states of peptides using regression analysis. R_f values were consistent with a monomeric state for 8K-EFGR (lane 2), a dimeric state for 8K-GPA (lane 3), and a tetrameric state for 6K-M2 (lane 4). See text for a further discussion.

34). In the present work, the gel system chosen is ideal for separation of species in the required molecular weight range. The gel patterns of the 8K-EGFR, 8K-GPA, and 6K-M2 peptides are shown in Figure 3. Note that the peptides vary slightly in molecular weights, so that comparisons of absolute migration positions among peptides are not made here. We found that all peptides designed in the present work migrated with their "wild-type" oligomeric states (Table 1). This result was established as follows: the logarithms of the MWs of protein standards were plotted versus the R_f value on SDS−PAGE, and an equation was derived using linear regression analysis (not shown). Through this procedure, an apparent molecular weight was calculated for each of the three peptides from their SDS−PAGE gel positions (Figure 3). Regression analysis showed that 8K-EGFR migrated as an apparent monomer of MW 4368 Da (1.3 × monomer MW), 8K-GPA migrated as an apparent dimer of MW 6430 Da (1.9 × monomer MW), and 6K-M2 migrated as an apparent tetramer of MW 15 438 Da (3.9 × monomer MW). The presence of a smear between 16 and 8 kDa for 6K-M2 may reflect an equilibrium between dimeric and tetrameric states on the time scale of the gel experiment.

In addition to showing that the TM peptides migrate to their relevant oligomerization states, the SDS−PAGE assay also showed that these peptides are thermostable. No differences in migration patterns were seen whether or not electrophoresis samples were boiled prior to loading. Furthermore, CD temperature melting experiments showed that only minimal helical content (∼15%) was lost upon heating of peptides to 90 °C for extended periods of time. The small amount of denaturation observed at elevated temperatures was shown to be fully reversible as pre- and postheating CD spectra were superimposable (data not shown).

TM Helix−Helix Interactions within the E. coli Aspartate Receptor. Having demonstrated the occurrence of wild-type oligomeric states in the above three peptide systems from single-spanning proteins, we extended these studies to investigate TM segment interactions within the double-spanning *E. coli* aspartate receptor (Tar). Tar contains an N-terminal TM helix (Tar-1) that is connected to a second TM segment (Tar-2) via a periplasmic domain (*19*). To model this system, peptides corresponding to Tar-1 and Tar-2 were synthesized, each with three N- and C-terminal lysines (Table 2). As with the previous peptide examples, these sequences were readily prepared, purified, and highly water soluble. The CD spectra of Tar-1 and Tar-2 Lys-tagged peptides in aqueous buffer and SDS are shown in Figure 4. The increase in helicity seen upon the addition of SDS detergent to samples is consistent with their insertion into the SDS micelles. Also, for Tar-2, which contains two Trp residues, the λ_{max} of the Trp fluorescence peak blue shifts from 353 to 333 nm upon the addition of SDS, further supporting peptide insertion into the micelle.

To assess the helix−helix interactions of 6K-Tar-1 and 6K-Tar-2, peptides were analyzed using SDS−PAGE (Figure 5A). 6K-Tar-1 migrates as a homodimer (lane 2), while 6K-Tar-2 migrates as a monomer (lane 3). A 1:1 admixture of the two peptides migrated with each at its original position,

Table 2: Names, Sequences, and Oligomeric States of Peptides Derived from Transmembrane (TM) Segments of the *E. coli* Aspartate Receptor (*19*)

TM segment	sequence[a]	peptide name	oligomeric state[b]
Tar (TM-1)	KKK-VVTLLVMVLGVFALLQLISGSLFF-KKK	6K-Tar-1	dimer
Tar (TM-2)	KKK-FAQWQLAVIALVVVLILLVAWYGI-KKK	6K-Tar-2	monomer

[a] Underlined sequences represent residues predicted by TM Finder (*20*) as occurring in the protein TM segments. [b] Oligomeric state deduced from SDS−PAGE gels (see text and Figure 5).

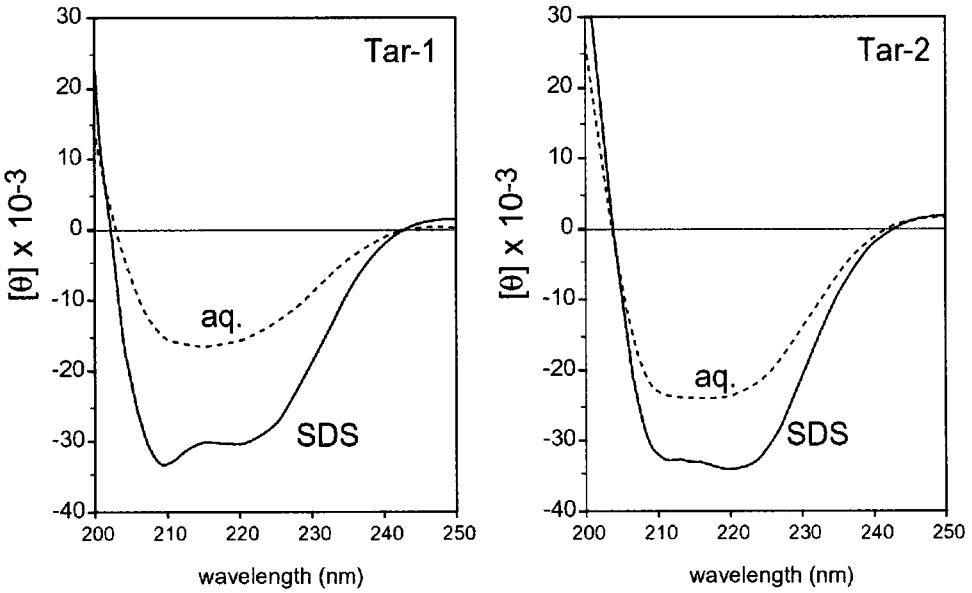

FIGURE 4: Secondary structure of 6K-Tar-1 and 6K-Tar-2 peptides derived from the *E. coli* aspartate receptor (*41*) determined by circular dichroism spectroscopy. Spectra are shown for peptides dissolved at room temperature in aqueous buffer containing 10 mM Tris-HCl and 10 mM NaCl, pH 7.2 (dotted lines), and in detergent buffer containing 20 mM SDS, 10 mM Tris-HCl, and 10 mM NaCl, pH 7.2 (solid lines). Left panel: 6K-Tar-1. Right panel: 6K-Tar-2. The peptide concentration was 20 μM in both cases.

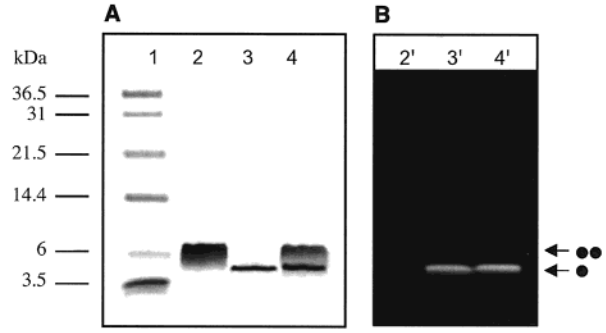

FIGURE 5: Oligomeric states of Tar TM segment peptides. Peptides dissolved in SDS-containing sample buffer were boiled for 5 min prior to electrophoresis using 10−20% tricine gels. (A) Coomassie-stained gel (followed by regression analysis; see text) showing that 6K-Tar-1 is dimeric (lane 2), while 6K-Tar-2 is monomeric (lane 3). 6K-Tar-1 and 6K-Tar-2 were mixed in water and then equilibrated in SDS buffer prior to electrophoresis (lane 4). (B) Ultraviolet light was used to visualize a gel identical to that in panel A in which fluorescent 6K-Tar-2, labeled with TAMRA, was run in the absence (lane 3′) and presence (lane 4′) of 6K-Tar-2. Unlabeled 6K-Tar-1 is not detected by UV light (lane 2′). No change in migration is evident between lanes 3′ and 4′, demonstrating the absence of heterodimerization between 6K-Tar-1 and 6K-Tar-2.

with no indication of mutual affinity or novel higher oligomers (lane 4). To further test whether any heterodimerization arose between 6K-Tar-1 and 6K-Tar-2, we labeled the N-terminus of 6K-Tar-2 with a fluorescent probe (TAMRA), which allowed us to visualize corresponding peptide bands on SDS−PAGE using ultraviolet light (Figure 5B). Any interaction between 6K-Tar-1 and 6K-Tar-2 would be expected to result in observation of a shift in the migration of the labeled peptide to a dimer position. As shown in Figure 5B, we found that labeled 6K-Tar-2 alone—and in the presence of 6K-Tar-1—migrated solely as a monomer. The migration position of the Tar-2-labeled peptide was confirmed to be monomeric by the usual Coomassie staining of the gel.

DISCUSSION

Our understanding of the rules that govern helix−helix packing within TM domains has lagged due to the sparse number of structures solved for membrane proteins (*9*). Accordingly, a simplified approach to this problem is required, and several groups have addressed this issue by studying individual TM segments of membrane proteins (*7*, *18*, *22*−*25*, *33*, *35*−*38*). However, studies of these highly hydrophobic TM domains have been limited due to the requirements for obtaining sufficient amounts of TM proteins by heterologous expression and/or the subsequent manipulation of samples for structural investigation. The engineering of Lys tags into TM peptides imparts water solubility, thereby facilitating their synthesis and purification in milligram quantities while still allowing studies of their structures and oligomeric states in membrane environments. In particular, the successful synthesis of the glycophorin A sequence with its many β-branched residues (50% of the TM residues) demonstrates the ability of this method to produce difficult sequences. Handling of peptides is simplified since they can be stored as stock solutions in water. Moreover, since TM

segments demonstrably behave as autonomous folding units that retain helix−helix recognition elements (*18*), the helical TM segments embedded in these constructs may profitably be studied in isolation and in recombination.

Helix−helix interactions via noncovalent packing of side chains were observed for the 8K-GPA and 6K-M2 peptides on SDS−PAGE, each of which migrated to molecular weights (homodimer and homotetramer, respectively) (Figure 3) consistent with those documented for the proteins from which they are derived (*8*, *24*). As well, 8K-EGFR migrated with an apparent MW consistent with that of a monomer, as observed for the wild-type EGFR protein (*21*, *22*). Importantly, the 6K-M2 peptide remained tetrameric even though the native Cys residue was mutated to Ser, negating the possibility of disulfide bonds as the source of the SDS−PAGE oligomerization we observed. The present observations of a 6K-M2 tetramer are in agreement with those of DeGrado (*24*). The formation of a functional tetrameric M2 channel in the absence of disulfide bonds is also supported by the naturally occurring influenza strains A/Hong Kong/470/97 and A/swine/Texas/4199−2/98 that have the mutations C50S and C50Y, respectively (*26*, *39*).

Our results also establish that the present peptide constructs obey the "two-stage" model of TM domain folding (*40*). The first stage of folding proposes that TM domains insert into membrane environments and fold into α-helices as a result of the hydrophobic effect. In the present work, secondary structural studies using CD spectroscopy demonstrated that the TM segment peptides were able to insert and fold independently into micelles as α-helices (Figure 2). In this process, the peptides also conform to the "threshold hydrophobicity" displayed by peptides which undergo spontaneous insertion into micellar particles (*16*). The second stage of the Popot−Engelman model consists of the lateral association of these helices, wherein specific interactions between the TM domains are proposed to be responsible primarily for the resulting biologically functional tertiary (and quaternary) structures. In our study, despite nearly identical helicity for the EGFR, GPA, and M2 peptides (Figure 2), each had a different oligomeric state that was consistent with establishment of tertiary contacts expected from previous reports on the corresponding proteins (*8*, *22*, *24*). These results reinforce the notion that CD spectra constitute an explicit measure of *secondary* structure for membrane-based proteins.

Following the confirmation of the retention of native-like oligomeric states for the TM peptides, we applied this approach to the bacterial aspartate receptor. The aspartate receptor of *E. coli* (Tar) contains two transmembrane domains (Tar-1 and Tar-2) and forms a homodimer in both the absence and presence of aspartate (*19*). Using the peptide construct approach, we synthesized both Tar-1 and Tar-2 in an effort to ascertain whether one of the TM helices serves as the recognition element in the Tar homodimer. Earlier disulfide cross-linking studies have suggested that a Tar-1 was adjacent to Tar-1′ from the equivalent monomer and that there was no such direct interaction between Tar-2 and Tar-2′ (*19*). We have shown here that the isolated Tar-1 TM domain forms an SDS-resistant homodimer while Tar-2 remains as a monomer (Figure 5). Mixing experiments on SDS−PAGE did not reveal any novel bands nor did they change the relative band intensities contributed by each peptide. This result suggested that peptides did not heterodimerize. However, given the similar molecular weights of both peptides, heterodimers might go undetected on the SDS−PAGE assay if a Tar-1/Tar-2 heterodimer interacted, ostensibly via the interactive face on the Tar-1 helix (otherwise, trimers would appear). To address this issue, we implemented a novel 5(6)-carboxytetramethylrhodamine assay for detecting heterodimerization of similarly sized peptides. This system demonstrated that fluorophore-labeled Tar-2 migrated as a monomer in the absence and presence of Tar-1, confirming that no heterodimerization occurred. These observations are in agreement with previous models based on disulfide cross-linking studies on the full-length protein (*19*, *41*−*44*) but further suggest that Tar-1 mediates the ligand-independent dimerization of Tar. The overall results are summarized schematically in Figure 6.

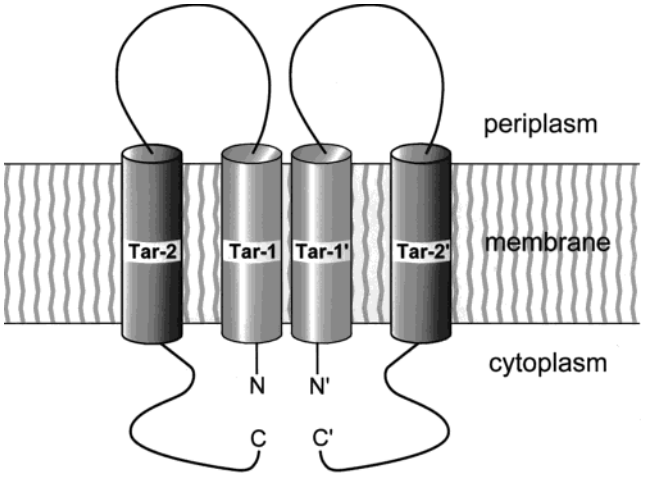

FIGURE 6: Schematic model of the aspartate receptor (adapted from ref *43*) as supported in the present work by CD spectroscopy and SDS−PAGE analysis of Tar-1 and Tar-2 TM segment constructs. Both TM domains were shown to insert into detergent micelles and fold into α-helices. Tar-1 and Tar-1′ likely associate in the receptor to form the dimeric interface of the ligand-independent Tar homodimer. Tar-2 does not interact with either Tar-2′ or Tar-1.

Previous studies have demonstrated that both GPA and M2 have sequence-specific interhelical interactions. For example, mutagenesis and subsequent NMR studies identified a "two-in−two-out" packing motif of LIxxGVxxGVxxT for GPA (*23*, *25*). Also, cross-linking studies done on M2 suggest that the packing of the four-helix bundle is based on a heptad repeat motif (*45*). While the present work does not identify the particular residues which participate in the helix−helix interactions that produce oligomers, our results do emphasize the fact that, in proteins for which oligomerization may be linked to function, TM sequences must be optimized for van der Waals packing, with the packing of sufficient net enthalpy to be detected as "SDS-resistant" dimers/oligomers on PAGE gels. The dimeric state established for Tar-1 in this study provides a new system in which such sequence-specific helix−helix packing may potentially be investigated.

In this context, a comparison of the TM sequences of EGFR and Tar-2 reveals certain common patterns of residue composition. Both of these peptides, which were found here to be monomeric on SDS−PAGE, contain a significant stretch of large, aliphatic hydrophobic residues centered near the TM core, i.e., EGFR contains LLLLLVV, while Tar-2

contains LVVVLILLV. Extensive stretches of such similarly sized residues may impede tight, knobs-into-holes packing and thereby mitigate against high-affinity interhelical associations. In both instances, there may indeed be functional reasons for promoting low-affinity helical association. In the case of EGFR, ligand-induced dimerization of the intact protein leads to cell signaling (*21*), and therefore TM helix affinities must be minimized in the absence of ligand to avoid constitutive receptor activity. The Tar-2 helix, on the other hand, is involved in transmitting the aspartate binding event across the membrane by changing its position in the bilayer relative to the unbound state (*44*). In this case, tight interhelical association could impede the conformational dynamics of the helix.

The peptide-based approach delineated here adds to the limited arsenal of techniques available for the study of TM segments, including the ToxR-based (*46*, *47*) and the staphylococcal nuclease fusion approaches (*8*), the latter two of which are valuable methods for assessing interhelical association. The Lys-tagged peptide approach has the specific advantage that it provides a straightforward construct in which the secondary structure and sequence-dependent oligomeric state(s) of a given TM segment (whether parallel or antiparallel) directly assessed in isolation without any background effects/signal from fusion protein domains. In addition, the peptides described here are highly thermostable, suggesting that they are robust systems capable of withstanding biophysical analysis under harsh conditions, a quality not typically seen in TM domains. The approach is also amenable, in principle, to biophysical assays that target the thermodynamics of helix−helix interactions in micelles, including through techniques such as ultracentrifugation and fluorescence resonance energy transfer (FRET). The observation that native-like folds and oligomerization states of the peptides are retained indicates their general utility as a tool for the structural analysis of the transmembrane domains of proteins.

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