The Chain Length Dependence of Helix Formation of the Second Transmembrane Domain of a G Protein-coupled Receptor of Saccharomyces cerevisiae* 

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The chain length dependence of helix formation of transmembrane peptides in lipids was investigated using fragments corresponding to the second transmembrane domain of the α-factor receptor from Saccharomyces cerevisiae. Seven peptides with chain lengths of 10 (M2-10; FKLLSNYSS), 14 (M2-14), 18 (M2-18), 22 (M2-22), 26 (M2-26), 30 (M2-30) and 35 (M2-35; RSRKTPIFIINQVSLFLIILH-SALYFKYLLSNYSS) residues, respectively, were synthesized and all of the other peptides assumed partially helical structures in this environment. Another homologous 30-residue peptide (M2-30B), missing residues SNYSS from the N terminus and extending to RSRRKT on the C terminus, was helical in lipid bilayers, suggesting that residues at the termini of transmembrane domains influence their biophysical properties. Attenuated total reflection Fourier transform infrared spectroscopy revealed that M2-10, M2-14, M2-18, and M2-30 assumed β-structures in this environment. Another homologous 30-residue peptide (M2-30B), missing residues SNYSS from the N terminus and extending to RSRRKT on the C terminus, was helical in lipid bilayers, suggesting that residues at the termini of transmembrane domains influence their biophysical properties. Attenuated total reflection Fourier transform infrared spectroscopy revealed that M2-10, M2-14, M2-18, and M2-30 assumed β-structures. These studies show that chain length must be taken into consideration when using peptides representing single transmembrane domains as surrogates for regions of an intact receptor. Furthermore, this work indicates that the tilt angle and conformation of transmembrane portions of G protein-coupled receptors may be estimated by detailed spectroscopic measurements of single transmembrane peptides.

The folding and structure of integral membrane proteins is driven by entropic factors that cause the apolar side chains of many amino acid residues to seek the interior of lipid bilayers and enthalpic factors that require that the hydrogen bond potential of the peptide group be satisfied in the low dielectric membrane interior (1). Thermodynamic analyses indicate that the α-helix is the most favored conformation of membrane-spanning regions of proteins. Indeed, it is believed that preassembled α-helices form in the aqueous cytoplasm of the cell and then insert into the bilayer (2). The thickness of the hydrocarbon milieu of the bilayer depends on the fatty acid composition and is often assumed to be about 30 Å. On this basis, the minimum number of residues that can form an α-helix and span the bilayer is predicted to be 20. However, this prediction assumes that the peptide inserts into the membrane parallel to the bilayer normal.

Recent x-ray studies on integral membrane proteins and in particular on rhodopsin show that not all of the transmembrane helices of this protein orient normal to the bilayer (3, 4). Thus, in some cases, more than 20 residues will be required to span the bilayer. Similar conclusions are reached upon inspection of other crystal structures (5–7). Unfortunately, the number of crystal structures for membrane proteins remains very small in comparison with the proportion of these molecules in cells (8, 9). Accordingly, many investigators are using peptide fragments as model compounds to examine the biophysical and structural tendencies of transmembrane regions of these proteins (10, 11).

An unresolved question in these studies is the appropriate length of the model peptide and the influence of length on the ability of the peptide to assume a stable helix in bilayers. This question is important because amino acid residues in transmembrane regions of a protein are often those, such as Val, Ile, and Phe, that assume β-sheet structures in globular proteins (12). Although Deber and co-workers (13, 14) have demonstrated that the secondary structural preferences of amino acid residues are context-dependent and that these residues have high helix-forming tendencies in membrane mimetic solvents, this correlation was determined using peptides of a constant length. Studies with short peptides indicate that in solution β-sheet formation is maximum for peptides containing 8–12 residues (15).

We have been conducting a detailed biophysical analysis on synthetic peptides corresponding to putative domains of the α-factor receptor of the yeast Saccharomyces cerevisiae. This G protein-coupled receptor recognizes the tridecapeptide phero-
mone (WHWLQKPGPMM-y-factor) and triggers a cascade of intracellular events ultimately resulting in sexual conjugation and diploid formation (16, 17). Studies on fragments of the receptor using CD (18, 19), IR (20), and NMR spectroscopies (21, 22) have revealed the conformational tendencies of the seven transmembrane domains and, in the case of IR analysis, gave information on the orientation of the peptides in lipid bilayers.

In the present communication, we focus on the second transmembrane domain of Ste2p. Peptides of various chain lengths corresponding to the sequence of this domain were synthesized (see Table I for the nomenclature used in this paper and the sequences of the peptide fragments) and examined using both CD spectroscopy and IR spectroscopy to determine their secondary structural preferences and orientation in lipid bilayers. The results provide insights into the influence of peptide chain length on both the propensity of these peptides to form helices in lipid bilayers and their helical orientation in multilayers.

**EXPERIMENTAL PROCEDURES**

**Materials**—Wang resin, Fmoc-protected amino acids, O-benzotriazolyl-N,N,N′,N′-tetramethyluronium hexafluorophosphate, and hydroxybenzotriazole were purchased from Advanced ChemTech (Louisville, KY). Diisopropylmethanamine, diisopropylethylamine, dicyclohexylcarbodiimide, trifluoroacetic acid, thiourea, 1,2-ethanedithiol, and 4-N,N′-dimethylaminopyridine were purchased from Aldrich. Solvents used for syntheses and purifications were purchased from VWR Scientific Products (Piscataway, NJ) and Fisher. Dimethylsulfoxide containing dimethylsulfoxide sodium salt were purchased from Avanti Polar Lipids (Alabaster, AL).

**Peptide Synthesis**—Eight peptide fragments with different chain lengths corresponding to the sequence of the second Ste2p transmembrane domain were synthesized on a Wang resin using Fmoc protection strategies. For the synthesis of all peptides except for M2-30B (Table I), the first Fmoc-Ser(tBu)-OH (20-fold excess) was loaded manually on the Wang resin (0.4 mmol of O-H/g) using the dicyclohexylcarbodiimide/4-N,N′-dimethylaminopyridine strategy in dichloromethane. Small amounts of unreacted OH groups on the Wang resin were capping using a 100-fold excess of acetic anhydride in the presence of 4-N,N′-dimethylaminopyridine. After the peptide chain was manually elongated to three amino acid residues (i.e., Fmoc-Tyr(tBu)-Ser(tBu)-Ser(tBu)-Wang resin), the resin was loaded on a solid phase peptide synthesizer (model 433A; Applied Biosystems). The coupling strategy utilized FastMoc chemistry with the use of extended coupling times. Double coupling was carried out for each residue using O-benzotriazolyl-N,N,N′,N′-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazole activation, and capping was accomplished with acetic anhydride in the presence of diisopropylethylamine. Approximately 15% of each peptide was separated from the reaction vessel when the peptide chain length reached 10, 14, 18, 22, 26, and 30 residues, respectively. Ultimately, the peptide was elongated to 35 residues. In this strategy, seven portions of resin with peptide chain lengths of 10, 14, 18, 22, 26, 30, and 35 were obtained during one batch synthesis. The synthesis of M2-30B was carried out using a Wang resin (0.4 mmol of OH/g) where the first Fmoc-Leu-OH was loaded manually as described above. The resulting Fmoc-Leu-Wang resin was then transferred to the synthesizer, and chain assembly was completed.

Each portion of resin was treated with a cleavage solution containing 0.38 g of phenol, 0.25 ml of thioanisole, 0.12 ml of 1,2-ethanedithiol, 0.25 ml of water, and 5 ml of trifluoroacetic acid. The cleavage reaction was carried out at room temperature for 1.5 h. The reaction mixture was filtered, and the filtrate was concentrated to a small volume on a rotary evaporator at room temperature. The crude peptides were precipitated by the addition of anhydrous ether and collected by filtration. The crude peptides were lyophilized from TFE/H2O (1:4) before purification.

**Peptide Purification**—Peptides were purified on a semipreparative μBondapak C18 HPLC column (19 × 300 mm) at room temperature with elution solvents of water (0.1% trifluoroacetic acid) plus acetonitrile (0.1% trifluoroacetic acid) at gradients from 10 to 80% acetonitrile over 90–120 min. All peptides were purified to over 98% homogeneity as judged by analytical reverse phase HPLC on a μBondapak C18 column (3.9 × 300 mm) at room temperature with linear gradients of water (0.05% trifluoroacetic acid) plus acetonitrile (0.025% trifluoroacetic acid) or water (0.025% trifluoroacetic acid) plus methanol (0.025% trifluoroacetic acid). Detection was at 220 nm.

**Mass Spectrometry Characterization**—The final products were assessed by electrospray mass spectrometry to verify correct intact molecular weight and sequence. Peptides were dissolved in acetonitrile/H2O/acetic acid (50:50:1) at ~5 μmol/μl. The solution was directly applied to a flow rate of 5 μl/min into an electrospray ion trap (LCQ-DECA Thermo Finnigan) operating in the positive ionization mode. All peptides were found to be within 0.5 daltons of the expected mass. The correct sequence order was confirmed by collision-induced dissociation (MS/MS) on the M2-35 peptide. Since all peptides except for M2-30B were synthesized on the same template, portions of the sequence of the M2-35 peptide are replicated in each of the shorter peptides. The +3 and the +4 charge state of the M2-35 peptide were individually isolated and fragmented. The resultant product ions were compared with the theoretical ions from the M2-35 peptide. Fragment ions corresponding to 26 of the 35 amino acids were identified. Furthermore, all ions above the background could be attributed to the M2-35 sequence. No diagnostic product ions were observed from the first 8 amino acids from the N terminus or the last two from the C terminus. Further, the amino acids of C terminus are bulkier, so the order is equivalent, and the presence of two series was confirmed by an agreement between calculated and measured values for the predicted fragment ion from the third residue from the C terminus. The sequence of the 8 amino acids of the N terminus was confirmed by 1H NMR spectroscopy. The longest peptide with 35 residues had previously been confirmed by amino acid analysis (19). The peptide synthesized in this study was identical with the authentic sample as judged by co-injection on analytical HPLC. Thus, the sequencing of all peptides examined in this investigation have been proven.

**Preparation of Lipid-Peptide Vesicles for CD, FTIR, and ATR-FTIR**—Peptides corresponding to the second Ste2p TMD (0.17–0.57 mg in 0.10 ml of TFE/H2O (10:1)) were added to 4 mg of DMPC/DMPG (4:1) in 1 ml of CHCl3/CH3OH (4:1). The resulting solution was dried under N2 flow with a blast of organic solvent. The residue was reconstituted in film under vacuum overnight, and the lipid was then resuspended in 1 ml of 0.1 M phosphate buffer, pH 6.3. The suspension was sonicated at ~50 °C for 60 min in a W-385 sonicator (Misomix, Inc., Farmingdale, NY) equipped with a cup horn (40% output power). The vesicle preparation was exhaustively dialyzed three times into 600 ml of 0.1 M phosphate buffer, pH 6.3, using Spectrapor 6 dialysis tubing with a molecular weight cut-off of 12000. VR Scientific Products Water Defense System was used for the final dialysis. The lipid vesicles were then passed through a 0.45-μm polycarbonate centrifugal filter. The calculated molar ratio of peptides to lipid was about 1:50. The final peptide concentrations ranged from 0.08 to 0.13 mM.

**Circular Dichroism Measurements**—The CD spectra of the peptides were recorded on an AVIV model 62 DS CD instrument (AVIV Associates, Lakewood, NJ), which was interfaced with a computer used for all mathematical calculations. Two cuvettes with light path lengths of 0.02 and 0.1 cm were used for vesicle suspensions and TFE/H2O solutions, respectively. All spectra were the average of five scans between 300 and 190 nm at an interval of 1 nm with a 1-s integration time at each wavelength. The bandwidth for each measurement was 1 nm. Peptide concentrations in solution and vesicle suspension were determined from UV absorbance using an extinction coefficient of 1540 cm−1 M−1 for a single tyrosine residue at 280 nm (23). Prior to calculation of the final ellipticity, all spectra were corrected by subtracting the reference spectra of TFE/H2O at the ratio of 99:1 or 50:50 or of a DMPC/DMPG (4:1) vesicle suspension in 0.1 mM phosphate buffer (pH 6.3) without peptides. CD intensities are expressed as mean residue ellipticities (deg cm2 dmol−1).

**Estimation of α-Helical Percentage**—Estimation of α-helical percentages was made using a method initially suggested by Fasman and Fasman (24) and later modified by Wu et al. (25) and Chen et al. (26). These methods use ellipticities at either 208 or 222 nm and calculate fractional helicities as follows:

\[
\theta_\alpha = \theta - \theta_i \left[ \theta_i^{100} - \theta \right] 
\]

where \(\theta\) represents the experimentally observed mean residue ellipticity; values for \(\theta_i^{100}\) and \(\theta_i^{100}\) corresponding to 0 and 100% helical
content at 222 nm were estimated to be 2000 and 30,000 degrees-cm²/dmol, respectively (25, 26).

**FTIR and ATR-FTIR Spectroscopy**—FTIR spectra were recorded at ambient temperature (−20 °C) on a Nicolet Magna 550 Fourier transform infrared spectrometer (Nicolet Analytical Instruments, Madison, WI) purged with N₂ and equipped with a DTGS detector and an ATR accessory (Pike Technologies, Inc., Madison, WI). The infrared beam was polarized using a 1-inch diameter wire grid ZnSe polarizer. For each sample, 1000 interferograms were averaged at a spectral resolution of 4 cm⁻¹ and processed using one-point zero filling and Happ-Genzel apodization. For orientation studies, lipid films on the top surface of germanium ATR crystals (55 × 10 × 4 mm with 45° beveled edges) were obtained by slowly evaporating the vesicle suspension (250 μl) in the presence or absence of peptides. The ATR crystals were previously cleaned with chloroform, TFE, and methanol, followed by 30-min plasma cleaning in a PDC-32G cleaner (Harrick, Ossining, NY). Following deposition, the ATR crystals or CaF₂ windows were transferred to a desiccator, where the films were rehydrated by vapor diffusion in an atmosphere maintained at 98% relative humidity using a saturated solution of K₂SO₄ in water (27). Rehydration was allowed to proceed for a minimum of 18 h at room temperature. The spectrum for the respective phospholipid multilayers without peptide was subtracted to yield the difference spectrum of each peptide in the multilayer.

**Peptide Amide H/D Exchange Experiment**—100 μl of the preformed lipid vesicle with or without peptide was dried on the surface of the CaF₂ window (25-mm diameter). The windows containing lipid films were rehydrated using the method described above. After recording FTIR spectra from the hydrated film, it was transferred to a dry box (i.e. an environment maintained at less than 10% relative humidity using P₂O₅), where it was dehydrated by storage at room temperature overnight. After recording FTIR spectra from the dry film in order to confirm dehydration, 8 μl of D₂O was added directly to its surface. The sample was tilted until the water had wet the entire surface of the film and then incubated in the dry environment for 30 min in order to allow most of the excess D₂O to evaporate. Whereas some of bulk D₂O was still visible on the surface of the film, it was transferred to a sealed chamber maintained at a relative humidity of 98% (in D₂O) by a saturated solution of K₂SO₄ in D₂O. The amide exchange was allowed to proceed for 18 h. After all of the bulk D₂O had evaporated from the surface of the film, FTIR spectra of the samples were recorded in a N₂ environment.

**Analysis of Orientation from ATR-FTIR Dichroism**—Order parameters for the helical peptides were determined using established methods (28–30). The measured dichroic ratio, R^{ATR}, defined as the ratio between the absorption of light polarized parallel and perpendicular to the surface of the internal reflection element, was used to calculate an order parameter (S) using Equation 2,

\[ S = \frac{(E_y - R^{ATR} E_x + E_z)}{(E_y - R^{ATR} E_x - E_z)} \times \frac{3(\cos^2 \alpha - 1)}{2} \]  

(Eq. 2)

in which Eₓ, Eᵧ, and E𝑧 are the integrated absorption coefficients (31). If the film refractive index is independent of the wavelength, Equation 2 can be written in a simplified form (Equation 3) by setting Eₓ = 1.388, Eᵧ = 1.516, and E𝑧 = 1.625 (32),

\[ S = \frac{(R^{ATR} - 2)}{(R^{ATR} + 1.45)} \times \frac{3(\cos^2 \alpha - 1)}{2} \]  

(Eq. 3)

in which α represents the angle between the principal transition dipole moment and the molecular director; α = 99° for the amide I mode (28). The order parameter S is related to the tilt angle β from the normal of the internal reflection element by Equation 4.

\[ S = \frac{(3\cos^2 \beta - 1)}{2} \]  

(Eq. 4)

Order parameters for the lipids are obtained from the symmetric and asymmetric stretching modes of the lipid methylene groups by setting α = 90°.

**RESULTS**

**Peptide Design and Synthesis**—A peptide representing the second transmembrane domain of Ste2p was designed to have the sequence RSRKTPIFIQVSLFLIIHSLASYFFKYLLNS-YSS that extends from Arg⁷⁴ to Ser¹⁰⁸ of the receptor. This peptide includes the entire transmembrane hydrophobic region plus 6 extracellular and 5 cytoplasmic residues (19). The complete peptide with the wild-type sequence was synthesized previously and found to form a highly α-helical secondary structure in aqueous TFE, SDS micelles, DMPC vesicles, and multilayers (19, 20). Therefore, the above region of Ste2p was chosen to study the effect of chain length on the secondary structures assumed by membrane peptides. We synthesized seven peptide fragments with 10, 14, 18, 22, 26, 30, and 35 residues, respectively, starting from the carboxyl-terminal serine (Ser¹⁰⁸). These peptide fragments were named M2-10, M2-14, M2-18, M2-22, M2-26, M2-30, and M2-35, respectively, in which M2 represents the second transmembrane domain and the number following M2 represents the chain length of these peptide fragments (19). The peptides were prepared using solid-phase peptide chemistry. To examine the influence of loop residues on the biophysical properties of the second transmembrane domain, one peptide lacking SNYSS (M2-30B) was also synthesized.

Membrane peptides usually contain regions that are predominantly hydrophobic, and peptide chains that are assembled on a resin matrix can aggregate either with other peptide chains or with the polymer support. Therefore, low loading substitution of the resin (less than 0.4 mmol/g) was selected for this synthesis. Fmoc-Sert(Bu)-Sert(Bu)-Wang resin was not stable during the standard deprotection in the presence of 20% piperidine/N,N-dimethylformamide, possibly due to diketopiperazine formation upon Fmoc removal. It was determined that using 10% piperidine/N,N-dimethylformamide (5 ml for 0.1-mm scale of resin) for 10 min to deprotect Fmoc-Sert(Bu)-Sert(Bu)-Wang resin followed by immediate reaction with the next amino acid residue minimized the loss of peptide chains. Completion of the deprotection and subsequent coupling reaction were carefully monitored by UV absorbance of the dibenzofulvene-piperidine adduct.

**CD Analysis**—The shape and intensity of a CD spectrum between 180 and 240 nm are sensitive to protein secondary structure. As the Ste2p receptor protein is localized to the plasma membrane in Saccharomyces cerevisiae, all of the fragments were analyzed using CD spectroscopy in membrane mimicetic media including aqueous TFE and DMPC/DMPG (4:1) vesicles. Qualitative analysis of the α-helicity of a peptide by CD spectroscopy is based on the discernment of characteristic peak shapes. In particular, double minima at 222 and 208 nm and a ratio of the magnitudes of the molar ellipticities at 222 nm is negligible (37, 38).

Quantitative analysis of these CD spectra was carried out using the ellipticity at 222 nm to approximate percent α-helicities as described under “Experimental Procedures.” Estimated α-helicities are listed in Table I. It should be noted that calculations based on the mean residue ellipticity at 222 nm are not significantly affected by the presence of His residue, since the contribution of the imidazole chromophore to the CD spectrum at 222 nm is negligible (37, 38).

**CD Spectroscopy of Peptides in Aqueous TFE**—CD spectra of all the peptide fragments in aqueous TFE at the ratio of 99:1 and 50:50 are presented in Fig. 1. As judged by the double minima at 222 and 208 nm and the ratio of the magnitudes of the molar ellipticities at 195 and 222 nm, all of the peptides except for M2-10 show predominantly α-helical secondary structure in 99% TFE/H₂O. The M2-10 peptide is predominantly disordered in both solvent mixtures. None of the α-helical peptides was less than 44% helical in this medium (Table I). With the exception of M2-30, increasing the concentration of water from 1 to 50% did not significantly affect the CD spectra.
of the M2 peptides (Fig. 1). The CD spectrum of M2-30 undergoes a shift from that of an α-helix to that of a β-sheet-like structure based on a minimum near 215 nm and the absence of the double minima at 208 and 222 nm. The results showed that in aqueous TFE the formation of α-helix begins at M2-14 and increases with chain length with the obvious exception of M2-30. The conformation of the latter peptide is dependent on the water concentration.

**CD Spectroscopy of Peptides in DMPC/DMPG (4:1) Vesicles**—CD analysis of the eight peptides in DMPC/DMPG (4:1) vesicles indicates that M2-10, M2-14, M2-18, and M2-30 exhibit a broad minimum near 215 nm and a maximum near 193 nm (Fig. 2). In contrast, M2-22, M2-26, M2-30B, and M2-35 have CD patterns characterized by double minima at 208 and 222 nm, a maximum near 195 nm, and ratios of the molar ellipticity at 195 nm to that at 222 nm of 3.3, 3.3, 2.9, and 2.5, respectively. The estimated α-helical contents for M2-22, M2-26, M2-30B, and M2-35 are 58, 61, 58, and 92%, respectively (Table I). Control studies showed that except for M2-10, all of the other peptides were nearly insoluble in phosphate buffer. The 10-residue peptide had very low solubility, and the small amount of sample that dissolved gave a random coil CD pattern (data not shown). The results indicate that there is a critical chain length between 18 and 22 residues for α-helix formation for peptides corresponding to the second transmembrane domain of Ste2p in the lipid-like environment. Notably, M2-30 did not form a stable α-helical secondary structure in DMPC/DMPG (4:1) vesicles and exhibited a CD pattern indicative of a β-sheet structure (Fig. 2).

**Secondary Structures of Peptides in DMPC/DMPG Multilayers**—The correlation between the frequency of the amide I (primarily a carbonyl stretching mode) vibrational mode and the secondary structure of a polypeptide has been well established in the literature (39). Frequencies in the region of 1650–1660 cm−1 correspond to α-helical segments, while modes vibrating in the region of 1630–1640 cm−1 and 1670–1685 cm−1 correspond to β-sheet elements. FTIR spectra of all the peptides in DMPC/DMPG (4:1) multilayers are presented in Fig. 3 and Table II. In the case of M2-14 and M2-18, this absorbance dominates the amide I region, whereas for M2-10 and M2-30 additional absorbances were noted at 1664 and 1657 cm−1, respectively. A shoulder at 1657 cm−1 for the amide I of M2-30 probably reflects a small amount of α-helical structure. The shoulder at 1646 cm−1 for the amide I of M2-10 may be due to ordered chains. The FTIR spectra suggest that all of these peptides assume significant amounts of β-sheet structures in DMPC/DMPG multilayers. In contrast, M2-22, M2-26, M2-30B, and M2-35 all exhibit major absorbances at 1657 or 1658 cm−1, indicating predominantly α-helical secondary structures for these peptides in DMPC/DMPG (4:1) multilayers. These results are fully in concordance with the CD analyses in the presence of vesicles (Fig. 2).

**Amide Proton H/D Exchange in Peptide/DMPC/DMPG Multilayers**—Amide proton H/D exchange occurs slowly in the hydrophobic environment of the multilayer, since this would require disruption of the backbone hydrogen bonding pattern and exposure of the highly polar peptide bonds in an extremely hydrophobic environment (40–42). Thus, measurement of the extent of such exchange by observing the reduction in the amide II mode (a coupling of N-H rocking and C-N stretching modes) provides a reliable indication of the penetration of the peptide into the membrane (43). Accordingly, we used amide H/D exchange to estimate the percentage of the residues in peptide fragments of the second domain of the Ste2p receptor, which were protected by DMPC/DMPG (4:1) multilayers. The results from equivalent experiments on M2-10, M2-14, M2-18, M2-22, M2-26, M2-30, M2-30B, and M2-35 in DMPC/DMPG (4:1) multilamellar films are presented in Fig. 3 and Table II. For all of the peptides, between 58 and 95% of the amide protons are protected from H/D exchange during an 18-h exposure of the peptides in phospholipid multilayers to 98% D2O relative humidity. The protected residues must be participating in some kind of structural interaction that prevents their backbone protons from exchanging with bulk solvent. One explanation is that significant proportions of the peptides were buried in the hydrophobic multilayers.

**ATR-FTIR Spectroscopy of M2-22, M2-26, and M2-30B in DMPC/DMPG (4:1) Multilayers**—M2-35 is predicted by hydrophathy analysis to have 24 transmembrane, 5 cytoplasmic, and 6 extracellular residues. Previously, its α-helix was found to orient at an angle of 34° relative to the multilayer normal in DMPC/DMPG multilayers (20). To investigate the orientations of the predominantly α-helical M2-22, M2-26, and M2-30B with respect to the multilayer normal (28, 32, 39, 44, 45), we recorded their ATR-FTIR spectra in DMPC/DMPG multilayers. The orientations of the lipid chains of dry films in the presence of peptides were determined from the dichroism of the CH2 stretching vibrations at 2850 cm−1 (symmetric) and 2918 cm−1 (asymmetric) in the ATR-FTIR absorbance spectra. The dichroic ratios averaged from four independent experiments were between 1.01 and 1.11 (Table III). Assuming that the transition dipole moment of the symmetric CH2 stretching vibration is perpendicular to the molecular axis, the resulting calculated order parameters, S, of the acyl chains were between 0.73 and 0.78, and the average tilt angles, β, of the lipid’s CH2 axis were between 25 and 23°, in agreement with the results for well ordered fatty acid chains in the gel phase (46). These results indicate that the peptides examined do not affect either the structure or the orientation of the multilamellar phase.

**ATR-FTIR spectra of the amide I regions were measured for M2-22, M2-26, and M2-30B in DMPC/DMPG (4:1) multilayers,**

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**Table I**

| Peptides | Sequences | TFE/H2O ratio | DMPC/DMPG vesicles (4:1) |
|----------|-----------|--------------|------------------------|
| M2-10    | FKYLLSNYS | 100          | 100                    |
| M2-14    | SALYFKLLS | 44           | 44                     |
| M2-18    | ILLSALFYK | 52           | 52                     |
| M2-22    | LFILLLYKL | 67           | 72                     |
| M2-26    | INQVLILLYK| 82           | 85                     |
| M2-30    | PIFIINQV  | 83           | 61                     |
| M2-30B   | RSRTPIFIM | 88           | 58                     |
| M2-35    | RSRTPIFIM | 81           | 72                     |

* a, not calculated due to absence of double minima at 208 and 222 nm.
and their Fourier self-deconvoluted spectra were calculated (see Fig. 4) (47). The dichroic ratios, $R_{\text{ATR}}$, calculated from the Fourier self-deconvoluted $\alpha$-helical amide I vibrational regions integrated from 1650 to 1665 cm$^{-1}$, along with the respective order parameter $S$ and tilt angles $\beta$, are summarized in Table III. The dichroic ratio values, $R_{\text{ATR}}$, for M2-22, M2-26, and M2-30B were calculated to be 4.13, 4.07, and 2.85, respectively, resulting in corresponding order parameters of 0.94, 0.92, and 0.49. These results indicate that the axis of the $\alpha$-helices of M2-22, M2-26, and M2-30B orient at angles of 12, 13, and 36°, respectively, with respect to the multilayer normal. For reference purposes, the tilt angle of M2-35, reported to be 34° (20), was confirmed in this work (data not shown).

**DISCUSSION**

The major objective of this investigation was to determine the chain length dependence of helix formation and tilt angles for transmembrane domains in membrane multilayers. As a paradigm, we chose the second transmembrane domain of the $\alpha$-factor receptor of *Saccharomyces cerevisiae*, which had previously been found to be highly helical (19, 20). CD results showed that in 99% TFE/H$_2$O and in 50% TFE/H$_2$O solutions, the critical chain length for helix formation by these peptides was between 10 and 14 residues. In contrast, in DMPC/DMPG (4:1) vesicles or multilayers, ~8 more residues were necessary to form a helical structure. ATR-FTIR results indicated that helices of M2-22 and M2-26 oriented nearly parallel to the
multilayer normal, whereas M2-35 tilted at an angle of 34°. Most notable is the observation that M2-30 appeared to assume a β-like structure both in 50% TFE/H₂O and in the presence of DMPC/DMPG (4:1) vesicles or multilayers.

The critical chain length for helix formation of oligopeptides in solution has been extensively investigated by Goodman and co-workers (48). Homo-oligopeptides often begin forming helical structures at 7 residues in organic solvents. In aqueous medium, the chain length required for helix formation is appreciably longer. Baldwin and co-workers (49) demonstrated that at low temperature, a 17-residue peptide composed predominantly of alanine residues could assume an α-helical structure. In other studies, slightly shorter peptides could form helices in aqueous environments by forming a four-helix bundle (50). The formation of the α-helix for polypeptides has also been reported to have a critical chain length in the solid state (51–54). The exact chain length required for helix formation depended on sequence and sample preparation. Oligomers below the critical chain length or in a nonequilibrium state assume β-aggregates. Longer peptides generally form more stable α-helices.

Detailed information on the three-dimensional structure of the Ste2p is not yet available due to its refraction to crystallization. Indirect information on the structure of this GPCR has been obtained by investigation of seven putative TMDs obtained by chemical synthesis. CD analyses on these peptides have been conducted in both phospholipid bilayers and aqueous TFE solutions (19). TFE is a well known inducer of α-helices for peptides (55). Previously, we found that titration of TFE-peptide solutions with water reduced the helicities of the synthetic proteins.

FIG. 2. CD spectra of synthetic peptides of the second Ste2p TMD in DMPC/DMPG (4:1) vesicles suspended in 0.1 mM phosphate buffer at pH 6.3. The peptide lipid molar ratio was about 1:50, and the final peptide concentrations ranged from 0.08 to 0.13 mM.
TMDs of the α-factor receptor and that the secondary structures of TMDs in DMPC bilayers were often similar to those observed in certain TFE/H$_2$O mixtures. For example, a 31-residue peptide corresponding to the sixth transmembrane domain of Ste2p was 57% helical in TFE but had CD patterns indicative of the presence of β-structures in both 25% TFE/H$_2$O and in DMPC bilayers (19). These results suggest that useful information about the conformational preferences of transmembrane regions in membrane lipids can be discerned from studies in aqueous trifluoroethanol. Indeed, a detailed NMR study of the seven TMDs of Ste2p in TFE/H$_2$O (4:1) has provided new insights into the secondary structures assumed by specific amino acids in these membrane peptides.2

In the present study, the critical chain lengths for structure formation of peptides representing the second transmembrane domain of Ste2p were between 10 and 14 residues in aqueous TFE and between 18 and 22 residues in DMPC/DMPG (4:1) as

2 B. Arshava and F. Naider, unpublished data.

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**TABLE II**

Amide I and II frequencies and H/D exchange data for fragments of the second Ste2p TMD in DMPC/DMPG (4:1) multilayers

| Fragment | Amide I band (cm$^{-1}$) | Amide II band (cm$^{-1}$) | Amide II exchange (%) |
|----------|--------------------------|---------------------------|-----------------------|
| M2-10    | 1621, 1664               | 1548                      | 21%                   |
| M2-14    | 1623                     | 1548                      | 5%                    |
| M2-18    | 1623                     | 1535                      | 17%                   |
| M2-22    | 1658                     | 1547                      | 9%                    |
| M2-26    | 1657                     | 1546                      | 17%                   |
| M2-30    | 1657                     | 1546                      | 36%                   |
| M2-30B   | 1657                     | 1545                      | 42%                   |
| M2-35    | 1657                     | 1546                      | 30%                   |

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**FIG. 3.** Amide I and amide II regions of the FTIR spectra of seven fragments of the second transmembrane domain of Ste2p in phospholipid multilayers on CaF$_2$ windows. Spectra were measured in DMPC/DMPG (4:1) multilayers hydrated in 98% H$_2$O relative humidity (solid line) or in 98% D$_2$O relative humidity (dashed line).
judged by both CD and IR analyses. Three groups have reported hydropathy analyses on Ste2p (18, 56, 57). These analyses differ very slightly in the residues placed at the membrane interface and the number of residues actually in the bilayer. Two of the analyses predicted 24 transmembrane residues in the second TMD (18, 56), whereas the third predicted that only 21 residues of this domain would be in the bilayer. Given these predictions and assuming that the SNYSS sequence could enter the hydrophobic region of the bilayer, only M2-22, M2-26, M2-30, and M2-35 have sufficient residues to fully span the lipid. The shorter peptides M2-10, M2-14, and M2-18 would not be able to form helical structures that completely cross the lipid bilayer. Thus, on first analysis the ability of the M2 peptides to form helical structures is correlated partially to the need to form a helical structure that crosses the bilayer.

A previous study on synthetic peptides composed of Leu and Ala and flanked by Trp residues (WALP peptides) concluded that hydrophobic mismatch was a determinative factor in the integration of peptides into bilayers (58). Peptides forming /H9251/H9251-helices that were significantly shorter or significantly longer

|                | M2-22 | M2-26 | M2-30B |
|----------------|-------|-------|--------|
| Lipid CH₂      | 2918 cm⁻¹ | 2850 cm⁻¹ | 2918 cm⁻¹ | 2850 cm⁻¹ | 2918 cm⁻¹ | 2850 cm⁻¹ | 2918 cm⁻¹ | 2850 cm⁻¹ |
| Lipid CH₂      | 2918 cm⁻¹ | 2850 cm⁻¹ | 2918 cm⁻¹ | 2850 cm⁻¹ | 2918 cm⁻¹ | 2850 cm⁻¹ | 2918 cm⁻¹ | 2850 cm⁻¹ |
| Amide I        | 1.08 ± 0.02 | 1.05 ± 0.03 | 1.04 ± 0.04 | 1.01 ± 0.02 | 1.11 ± 0.04 | 1.05 ± 0.02 | 2.85 ± 0.08 | 0.74 ± 0.03 | 0.94 ± 0.04 | 0.78 ± 0.04 | 0.92 ± 0.05 | 0.73 ± 0.05 | 0.49 ± 0.03 |
| R_ATR          | 1.08 ± 0.02 | 1.05 ± 0.03 | 1.04 ± 0.04 | 1.01 ± 0.02 | 1.11 ± 0.04 | 1.05 ± 0.02 | 2.85 ± 0.08 | 0.74 ± 0.03 | 0.94 ± 0.04 | 0.78 ± 0.04 | 0.92 ± 0.05 | 0.73 ± 0.05 | 0.49 ± 0.03 |
| S              | 25 ± 2 | 12 ± 0 | 23 ± 2 | 13 ± 5 | 25 ± 3 | 36 ± 2 |
| Tilt angle β (°) | 25 ± 2 | 12 ± 0 | 23 ± 2 | 13 ± 5 | 25 ± 3 | 36 ± 2 |

*R_ATR* of lipid is the dichroic ratio determined from the peak height of the CH₂ stretching absorption at 2918 cm⁻¹ or 2850 cm⁻¹. *S* is the order parameter calculated from the average dichroic ratio *R_ATR*. *S* of the lipid is the averaged value from the *R_ATR* values at 2918 and 2850 cm⁻¹.

A previous study on synthetic peptides composed of Leu and Ala and flanked by Trp residues (WALP peptides) concluded that hydrophobic mismatch was a determinative factor in the integration of peptides into bilayers (58). Peptides forming /H9251-helices that were significantly shorter or significantly longer...
than the hydrocarbon span of the lipid were excluded from the membrane (58). In the present study, it is important to note that only peptide associated with the lipid was examined spectroscopically. The dialysis step used in the sample preparation removed free peptide, and it was determined that, with the exception of M2-10, the other peptides did not have any significant solubility in phosphate buffer. Our finding that M2-10, M2-14, and M2-18 all form mostly β-sheet structures in both the presence of DMPG/DMPG lipid vesicles (CD studies) and in lipid multilayers (IR studies) probably results from a hydrophobic mismatch that causes these peptides first to aggregate into sheet structures followed by interaction of the sheet structures with the lipids. We did not ascertain whether the aggregates penetrate into the bilayers or interact with the head groups. The low deuterium/proton exchange rates observed for these peptides (Table II) is consistent both with membrane penetration or extensive intermolecular hydrogen bonding.

The difference in the tilt angles of the four peptides that form helical structures in the vesicles and multilayers (M2-22, M2-26, M2-30B, and M2-35) may also be the result of hydrophobic mismatch. If one assumes that the hydropathy predictions are correct (18, 56, 57), then only about 17, 21, 25, and 25 residues, respectively, of these peptides should be in the hydrocarbon domain of the membrane. The SNYSS sequence should be outside the membrane core. If this is the case, then it is likely that M2-22 forms a helix that does not completely span the bilayer, that M2-26 just spans the bilayer, and that both M2-30B and M2-35 would be too long for the bilayer. Thus, the orientation studies that show almost no tilt for M2-22 and M2-26 and tilt angles of 36 and 34° for M2-30B and M2-35 suggest that the shorter helices integrate perpendicular to the bilayer, whereas the longer helix can only be accommodated if they tilt to relieve the hydrophobic mismatch that would result. This conclusion is consistent with the findings on the WALP peptides that reported small tilt angles (−10–15°) for peptides shorter than the bilayer and large tilt angles (−30°) for peptides expected to be significantly longer than the bilayer (58). It is also consistent with results on synthetic peptide segments of bacteriorhodopsin that contained 35–45 residues and also exhibited tilt angles between 30 and 40° (59) and with findings on an 18-residue peptide fragment of the sixth TMD of Ste2p that had a very small tilt angle (22).

One can compare the results obtained herein with the M2 peptides with those found from the crystal structure on rhodopsin (3, 4). Of the seven transmembrane helices of this GPCR, four have tilt angles of 25–33°, and three have tilt angles of from 1 to 9°. It is intriguing that with the exception of helix VI, the domains with the largest number of residues (30–34 amino acids) have large tilt angles. In contrast, the domain with the smallest tilt angle (domain IV) has the fewest residues (19 amino acids) in the lipid environment. It is difficult to analyze the rhodopsin transmembrane domains from the perspective of hydrophobic mismatch, because the light reception receptor was crystallized from mixed micelles. Furthermore, many of the domains of this GPCR are highly kinked. Nevertheless, the fact that the synthetic peptides corresponding to transmembrane domain 2 of Ste2p exhibit tilt angles that are in the range of those found for the transmembrane regions from the crystal structure of rhodopsin is very interesting, leading to speculation that studies with single transmembrane domains may indeed mirror tilt angles of transmembrane domains in the native proteins.

The most significant anomaly in the present study was the observation that M2-30 formed a β-sheet structure in TFE/H2O (50:50) in the presence of DMPG/DMPG vesicles and in DMPC/DMPG multilayers. The finding again points to the relevance of results from studies in TFE water mixtures to the lipid milieu. In the case of M2-30, the peptide is predicted to have 4–7 residues that are in the extracellular loop of Ste2p and 23–26 residues in the bilayer (18, 56, 57). It is likely that this span is too long for the bilayer, thereby causing a hydrophobic mismatch. Unlike M2-35, M2-30 does not contain the hydrophilic sequence (RSRKT) at the amine (cytosolic) end. This positively charged sequence is expected to interact favorably with the negatively charged lipid head groups and to stabilize the tilted M2-35. Lack of this end group in M2-30 leads to unfavorable interactions at the N terminus of the peptide and exclusion from the membrane. This exclusion results in an aggregated peptide that then somehow associates with the DMPC/DMPG vesicles and multilayers. The fact that no CD or IR spectra could be measured for M2-30 in phosphate buffer excludes the possibility that free peptide is contributing to the spectra examined. In the study of the WALP peptides cited above, it was found that replacement of the WW sequence at the termini of the peptides with KK sequences resulted in a marked increase in the peptide insertion in the bilayer (58). This supports the contention that the hydrophilic residues at the peptide termini can provide thermodynamically favorable interactions with the lipid head groups that can overcome hydrophobic mismatch.

The importance of the nature and placement of the residues at the termini is illustrated by M2-30B. This 30-residue peptide contains the same hydrophobic core residues as M2-30, but five cytoplasmic loop residues (RSRKT) were included and five extracellular loop residues (SNSYSS) were omitted. As judged by both CD and IR analysis, M2-30B forms a helical structure in bilayers (Figs. 2–4). This suggests that loop residues on both termini of a transmembrane peptide are not required for membrane insertion and helix formation. However, the fact that M2-30B but not M2-30 was helical in lipid bilayers suggests that residues at the N and C termini of a transmembrane domain influence its biophysical properties, in particular, its interaction with lipid membranes. In the case examined herein, the highly cationic RSRKT sequence of the cytoplasmic loop appears to interact favorably with the negatively charged phospholipid head groups stabilizing the insertion of the tilted M2-30B core. In contrast, the polar SNSYSS sequence in M2-30 apparently does not interact strongly enough to compensate for the hydrophobic mismatch of the same core residues. If this finding proves to be general for other transmembrane domains, it may have implications for the attachment of lysine residues at both termini of a transmembrane core. Such lysine attachment is now commonly used to increase membrane peptide solubility and thereby facilitate biophysical studies on these peptides (60, 61).

In conclusion, the results of this study show that the chain length chosen for peptide surrogates used to examine regions of GPCRs is a critical variable. In the case of the second transmembrane domain of Ste2p, helices could not begin forming with peptides containing fewer than 22 residues. This critical chain length was characteristic of the lipid environment, because the same peptides formed helical structures in TFE and in TFE containing up to 50% water. For longer peptides, the residues at the termini played an important role in determining whether helices could integrate into the membrane. Thus, the choice of the termini of peptide fragments should be done judiciously and following information learned using hydropathy analysis. Since both the overall conformational tendency of the peptide and the tilt angle in the membrane bilayer is influenced by length and termini, these factors should be considered in applying results on model peptides to the intact integral membrane protein.
