Journal of Peptide Science
J. Pept. Sci. 2006; **12**: 808–822
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.816

JOURNAL OF PEPTIDE SCIENCE

Effects of *N*- and *C*-terminal addition of oligolysines or native loop residues on the biophysical properties of transmembrane domain peptides from a G-protein coupled receptor‡

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Received 20 April 2006; Revised 9 May 2006; Accepted 10 May 2006

Abstract: Transmembrane domains (TMDs) of G-protein coupled receptors (GPCRs) have very low water solubility and often aggregate during purification and biophysical investigations. To circumvent this problem many laboratories add oligolysines to the *N*- and *C*-termini of peptides that correspond to a TMD. To systematically evaluate the effect of the oligolysines on the biophysical properties of a TMD we synthesized 21 peptides corresponding to either the second (TPIFIINQVSLFLIILHSALYFKY) or sixth (SFHILLIMSSQSLLVPSIIFILAYSLK) TMD of Ste2p, a GPCR from *Saccharomyces cerevisiae*. Added to the termini of these peptides were either Lys_n ($n = 1,2,3$) or the corresponding native loop residues. The biophysical properties of the peptides were investigated by circular dichroism (CD) spectroscopy in trifluoroethanol–water mixtures, sodium dodecyl sulfate (SDS) micelles and dimyristoylphosphocholine (DMPC)-dimyristoylphosphoglycerol (DMPG) vesicles, and by attenuated total reflection Fourier transform infrared (ATR-FTIR) in DMPC/DMPG multilayers. The results show that the conformation assumed depends on the number of lysine residues and the sequence of the TMD. Identical peptides with native or an equal number of lysine residues exhibited different biophysical properties and structural tendencies. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: transmembrane peptides; circular dichroism; oligolysines; peptide structure

INTRODUCTION

The G-protein coupled receptor (GPCR) super family includes several thousand distinct but related proteins. They are found in a wide range of organisms and are involved in the transmission of signals across membranes [1]. Although the receptors are conserved in structure, the ligands span a large range of vastly diverse entities from peptides, small molecules to proteins [2]. They are composed of a single polypeptide chain containing seven regions of 20–28 hydrophobic amino acids that represent transmembrane domains (TMDs) [3]. The TM segments form α-helices, oriented roughly perpendicular to the membrane as shown in rhodopsin [4]. Structural analysis by techniques such as nuclear magnetic resonance (NMR) or X-ray crystallography of the TMDs of integral membrane proteins has traditionally been impeded by their hydrophobic nature and their tendency to form intractable aggregates in the presence of even relatively small amounts of water. This behavior is observed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and likely occurs on high performance liquid chromatography (HPLC) columns during peptide purification. Obtaining large amounts of TMD samples experimentally through biosynthesis or chemical synthesis is complicated by the fact that these polypeptides are inherently hydrophobic and often require nonstandard solubilization and purification strategies [5,6].

A number of studies have used charged/polar residues at the termini of hydrophobic peptides to prevent unwanted nonspecific aggregation of peptides [7–12]. Model TM peptides flanked with Lys residues have already been studied to investigate how TM peptides and bilayers influence each other's biophysical properties [13–16]. It has been reported that Lys residues added to the end of single-spanning TMDs

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‡ Dedication: Professor Murray Goodman initiated a multidisciplinary approach to study the biophysical properties of oligopeptides. His analyses contained careful design of model compounds, efficient synthetic routes and detailed biophysical analyses under a variety of conditions. His laboratory was the first to use fluoroalcohols in the conformational analysis of peptides paving the way for literally thousands of investigations using these versatile solvents, which are now considered to represent a membrane mimetic environment. The studies presented herein on peptides corresponding to transmembrane regions of a G-protein coupled receptor follow the 'Goodman method' and we will always remain indebted to our Professor and Teacher for his guidance and inspiration.

Table 1 Name and sequence of peptides corresponding to TM2 and TM6 of Ste2p

Abbreviation	Sequence	Molecular weight	
		Theoretical	Experimental
TM6-33	^{240}**QFD**SFHILLIMSSQSLLVPSIIFILAYSLK**PN**^{272}**Q**	3764.52	3764.00
TM6-32	^{241}**FD**SFHILLIMSSQSLLVPSIIFILAYSLK**PN**^{272}**Q**	3636.39	3636.42
TM6-31	^{242}**D**SFHILLIMSSQSLLVPSIIFILAYSLK**PN**^{272}**Q**	3489.21	3488.59
TM6-30	^{243}SFHILLIMSSQSLLVPSIIFILAYSLK**PN**^{272}**Q**	3374.12	3373.45
TM6-29	^{244}FHILLIMSSQSLLVPSIIFILAYSLK**PN**^{272}**Q**	3287.04	3286.63
TM6-28 (H)	^{245}HILLIMSSQSLLVPSIIFILAYSLK**PN**^{272}**Q**	3139.86	3139.57
TM6-28 (Q)	^{240}**QFD**SFHILLIMSSQSLLVPSIIFILAY^{267}S	3183.83	3182.72
KKKTM6KK	***KKK***^{243}SFHILLIMSSQSLLVPSIIFILAYSL^{269}***KKK***	3675.64	3675.13
KKTM6KK	***KK***^{243}SFHILLIMSSQSLLVPSIIFILAYSL^{269}***KKK***	3547.47	3546.99
KTM6KK	***K***^{243}SFHILLIMSSQSLLVPSIIFILAYSL^{269}***KKK***	3419.29	3418.84
TM6KK	^{243}SFHILLIMSSQSLLVPSIIFILAYSL^{269}***KKK***	3291.12	3290.82
KKKTM6KKK	***KKK***^{243}SFHILLIMSSQSLLVPSIIFILAYSL^{269}***KKKK***	3803.82	3802.98
KKTM6KKK	***KK***^{243}SFHILLIMSSQSLLVPSIIFILAYSL^{269}***KKKK***	3675.64	3673.21
KTM6KKK	***K***^{243}SFHILLIMSSQSLLVPSIIFILAYSL^{269}***KKKK***	3547.47	3545.12
TM6KKK	^{243}SFHILLIMSSQSLLVPSIIFILAYSL^{269}***KKKK***	3419.29	3418.46
TM2-30	^{75}**SRK**TPIFIINQVSLFLIILHSALYFKY**LL**^{104}**S**	3539.34	3538.80
TM2-28	^{76}**RK**TPIFIINQVSLFLIILHSALYFKY**L**^{103}**L**	3365.18	3365.44
TM2-26	^{77}**K**TPIFIINQVSLFLIILHSALYFKY^{102}**L**	3095.83	3094.93
KKKTM2KKK	***KKK***^{78}TPIFIINQVSLFLIILHSALYFK^{101}Y***KKK***	3623.55	3623.11
KKTM2KK	***KK***^{78}TPIFIINQVSLFLIILHSALYFK^{101}Y***KK***	3367.20	3366.28
KTM2K	**K**^{78}TPIFIINQVSLFLIILHSALYFK^{101}Y***K***	3110.85	3110.62

TM, Transmembrane domain; 6 and 2, order of the TMD in the receptor, next number represent the total residues in the peptide. Bold letters represent the native loop amino acids, bold and italic letters represent nonnative lysines. In the TM6 peptides Cys252 has been replaced with Ser.

of different integral membrane proteins including epidermal growth factor receptor (EGFR), glycophorin A (GpA) and influenza A virus M2 ion channel (M2) did not affect the dimerization or oligomerization propensity of these TMDs in SDS [12]. However, Iwamoto reported that Lys residues affect the dimerization of TMDs of fibroblast growth factor receptor 3 (FGFR3) [17]. These studies were done using CD spectroscopy in SDS micelles and SDS-PAGE as a method for probing TM helical packing and the nature of the oligomeric state.

Given these differences we decided to do a systematic study comparing CD and attenuated total reflection Fourier-transform infrared (ATR-FTIR) patterns of TM peptides containing the same core region and either Lys or natural loop residues at the termini. In this paper we focus on Ste2p, specifically on the second (TM2) and the sixth (TM6) TMDs of this GPCR, which is involved in yeast mating. We present a detailed CD analysis of these peptides in trifluoroethanol–water mixtures, SDS micelles and dimyristoylphosphocholine–dimyristoylphosphoglycerol (DMPC–DMPG) vesicles and an ATR-FTIR analysis of the orientation parameters of those peptides that assumed an α-helical structure. The analysis reveals that in micelles and lipid vesicles TM peptides have different conformational tendencies when native loop residues are replaced with Lys residues.

MATERIALS AND METHODS

Fmoc-protected amino acids and resins were purchased from Advanced ChemTech (Louisville, KY) except Fmoc-His(Trt)-OH, which was purchased from Calbiochem-Novabiochem Corp. (San Diego, CA) and Bachem Inc. (Torrance, CA). Diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), 2,2,2-trifluoroethanol (TFE), thioanisole, 1,2-ethanedithiol (EDT), SDS, and all other reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI). Solvents used for the synthesis and the purification were purchased from VWR Scientific (Piscataway, NJ) and Fisher Scientific (Springfield, NJ). DMPC and DMPG were purchased from Avanti Polar Lipids (Alabaster, AL).

Peptide Synthesis and Purification

The sequences and abbreviations of peptides examined in this study are shown in Table 1. All the peptides were synthesized on a 0.1 mmol scale, using an automated solid phase peptide synthesizer (Applied Biosystems Model 433A) starting with *N*-α-Fmoc-Gln(Trt)-Wang resin for TM6-33/28(H), N-α-Fmoc-Ser(tBu)-Wang resin for TM6-28(Q) and TM2-30, *N*-α-Fmoc-Leu-Wang resin for TM2-28/TM2-26 and *N*-α-Fmoc-Lys(Boc)-Wang resin for the remaining 11 peptides. The coupling strategy utilized FastMoc chemistry and double coupling was carried out for each residue using HBTU/HOBt activation; capping was accomplished with acetic anhydride in the presence of DIEA. The *N*-α-Fmoc-amino acids, employed for

DOI: 10.1002/psc

the synthesis, had the following side chain protection groups: Trt for Gln, His and Asn; OtBu for Asp; tBu for Ser, Thr and Tyr; and Boc for Lys. The Fmoc group was removed using 20% piperidine in NMP for 90 min. After completion of chain assembly, the *N*-terminal Fmoc protecting group was removed, the resin was washed with DCM and dried *in vacuo* overnight. The cleavage from the resin and the removal of the side chain protecting groups were performed simultaneously using a mixture of 10 ml TFA, 0.75 g phenol, 0.25 ml EDT, 0.5 ml thioanisole and 0.5 ml water for 2 h at room temperature. The cleaved resin was filtered, the filtrate was concentrated to a small volume and the crude peptides were precipitated by addition of diethyl ether.

Analytical RP-HPLC and semipreparative RP-HPLC were performed on a Vydac 259VHP54 polymer column (4.6 mm × 250 mm) and a Vydac 259VHP510 polymer column (10 mm × 250 mm) respectively, using water–acetonitrile linear gradients (containing either 0.025% or 0.1% TFA). Analytical chromatography was run at ambient temperature while semipreparative separations were at 50 °C. Detection was at 220 nm. To facilitate purification, concentrated solutions of 2–5 mg peptide/ml solvent were made by dissolving the crude peptides in either AcCN (40–50%):TFA (20–30%):H_2O (20–40%) or neat DMSO. The volume injected was adjusted so that 2–10 mg of crude peptide was purified in each run depending on the peptide solubility. All peptides were purified to over 95% homogeneity as judged by RP-HPLC. The identity and purity of the peptides were analyzed by reversed phase HPLC and electrospray ionization mass spectrometry (ESI-MS).

Circular Dichroism Spectroscopy

Sample preparation for CD analysis. TFE/H_2O. The purified lyophilized peptides (1 mg) were dissolved in 200 µl of 95% TFE to obtain stock solutions. Aliquots (25 µl) of the stock solution of each peptide were diluted to 500 µl with appropriate amounts of TFE and H_2O and the final concentration of the peptides in 25–50–95% TFE/H_2O was determined by UV spectroscopy by measuring the UV absorbance at 280 nm using an extinction coefficient of 1340/M/ cm for tyrosine.

DMPC/DMPG (4 : 1) vesicles or 0.5% SDS micelles. The purified lyophilized peptides (1 mg) were dissolved in 1 ml of TFE/H_2O (1 : 1) to obtain stock solutions. The concentrations of the stock solutions were determined by UV spectroscopy. Sufficient volumes of the stock solution to obtain a 50 µM final concentration of the peptides were added to either 2.5 mg of DMPC/DMPG (4 : 1) or 0.5% SDS in 500 µl of TFE/H_2O (1 : 1). The resulting solutions were frozen and lyophilized overnight. The peptide/lipid or peptide/detergent mixtures were resuspended in 500 µl of 10 mM phosphate buffer, pH 6.4, to give a final peptide concentration of 50 µM. The suspensions were sonicated at 50 °C for 60 min.

Spectroscopic Measurements. CD spectra were recorded on an AVIV model 6S DS CD instrument (AVIV associates, Lakewood, NJ) in the range of 185–280 nm at intervals of 1 nm with 5 s integration time at each wavelength, using quartz cuvettes with 0.1 or 0.02 cm path length for TFE/H_2O solutions and vesicles/detergent suspensions, respectively. Four scans were averaged and corrected for the contributions of the medium. The peptide-SDS ratios were 1/300 (mol/mol) at a peptide concentration of 50 µM and the peptide-lipid ratio was 1 : 140 (mol/mol) at a peptide concentration of 50 µM.

ATR-FTIR Spectroscopy

Preparation of lipid-peptide vesicles for ATR-FTIR. Peptides were first dissolved in 100% TFE, and water was added afterwards to reach 50% TFE. The appropriate volume of peptide solution to give 100 µM was added to 4 mg of DMPC/DMPG (4 : 1) previously dissolved in 50% TFE/water. The resulting solution was frozen and lyophilized and the peptide/lipid mixture was then resuspended in 1 ml of 0.01 mM sodium phosphate buffer, pH 6.3. The suspension was sonicated at 50 °C for 50 min in a W-383 sonicator (Misonix, Inc., Farmingdale, NY) equipped with a cup horn (40% output power). The vesicle preparation was exhaustively dialyzed against 0.01 mM sodium phosphate buffer, pH 6.3, using Spectrapor 6 dialysis tubing with a 1000 molecular weight cutoff (VWR Scientific Products). The resulting vesicles were then passed through a 0.45 µm polycarbonate filter. The calculated molar ratio of peptide to lipid was about 1 : 50.

ATR-FTIR spectroscopy. FTIR spectra were recorded at ambient temperature on a Nicolet Magna 550 spectrometer (Nicolet Analytical Instruments, Madison, WI) purged with N_2 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 mm × 10 mm × 4 mm with 45° beveled edges) were obtained by slowly evaporating the vesicle solution (250 µl) in the presence or absence of peptides. The ATR crystals were previously cleaned with methanol and chloroform, followed by 30 min plasma cleaning in a PDC-32G cleaner (Harrick, Ossining, NY). Following deposition, the ATR crystals were transferred to a desiccator in which the films were rehydrated by vapor diffusion in an atmosphere maintained at 98% relative humidity using a saturated solution of K_2SO_4 in water [18]. Rehydration was allowed to proceed for a minimum of 18 h at room temperature. The spectra for the respective phospholipid multilayers without peptide were subtracted to yield the difference spectrum of each peptide in the multilayer. Order parameters for the helical peptides were determined as before [19]. The order parameters and corresponding tilt angles were the average of three to five independent determinations.

RESULTS

Synthesis and Design of Transmembrane Peptides

Ste2p is a heptahelical GPCR [3]. On the basis of models from the Dumont, Konopka and our groups, the TM2 and TM6 cores would encompass ^{79}PIFIINQVSLFLIILHSALYFKYL^{103}L and ^{246}ILLIMSSQSLLVPSIIFILA^{266}Y, respectively. To ensure that we are extending into the hydrophilic loop region of TM6, we added three additional residues to each side of TM6, which we have previously found to have significant tendencies to aggregate [11], and chose a TM6 core extending from S243 to K269 (Table 1). In contrast to TM6, previous studies on TM2 indicated this

DOI: 10.1002/psc

domain had high helical tendencies and little predisposition to aggregate. Accordingly, we chose a shorter core extending from T78 to Y101 (Table 1).

Using an analysis developed by Liu and Deber [7] and the above cores we calculated that two lysine residues must be added to each side of TM2 to obtain optimum solubility, whereas three lysines are required on each side of the TM6 core. The peptides synthesized (*vide infra)* were prepared with the above design in mind. Our goal was to test the effect of different numbers of Lys residues on the biophysical properties of the TM core peptides and compare these effects with those of similar extensions by native receptor residues believed to be in the loop domains. Our design was somewhat challenged by the presence of Lys residues at positions 100 and 269 of the receptor. We did not count these residues as part of our extensions.

Peptide synthesis and purification. The purification of the peptides indicated in Table 1, in particular those derived from TM6, proved to be very difficult because the low solubility of the peptides caused frequent precipitation during HPLC purification. Purification was appreciably easier with the peptides containing nonnative Lys residues at the termini as compared with the native loop residues. The peptides derived from TM2 gave better results both in terms of yield of the synthesis and the ease of purification. Again the TM2 peptides with native loop residues were more difficult to purify than those with nonnative Lys residues at the termini. The final peptides all had the calculated MW as determined by ESI-MS and were highly homogeneous (Table 1). Figure 1 illustrates the HPLC traces of crude peptides from the TM2 series, run on a Vydac reverse phase polymer column using a water/acetonitrile gradient. The difference in elution time demonstrates that the Lys residues at the termini substantially reduce the overall hydrophobicity of the peptide. Moreover, the apparent quality of the crude Lys-capped peptides was significantly better than the same length crude peptide with native loop residues. In all cases, the final peptides subject to spectral analysis were >95% pure.

CD of TM6 peptides in trifluorethanol–water mixtures. TM6 peptides containing only native residues were helical in both TFE/water (95 : 5) and TFE/water (50 : 50) mixtures as judged by the strong n-π^* transition at 222 nm and the split $\pi \rightarrow \pi^*$ at 208 nm

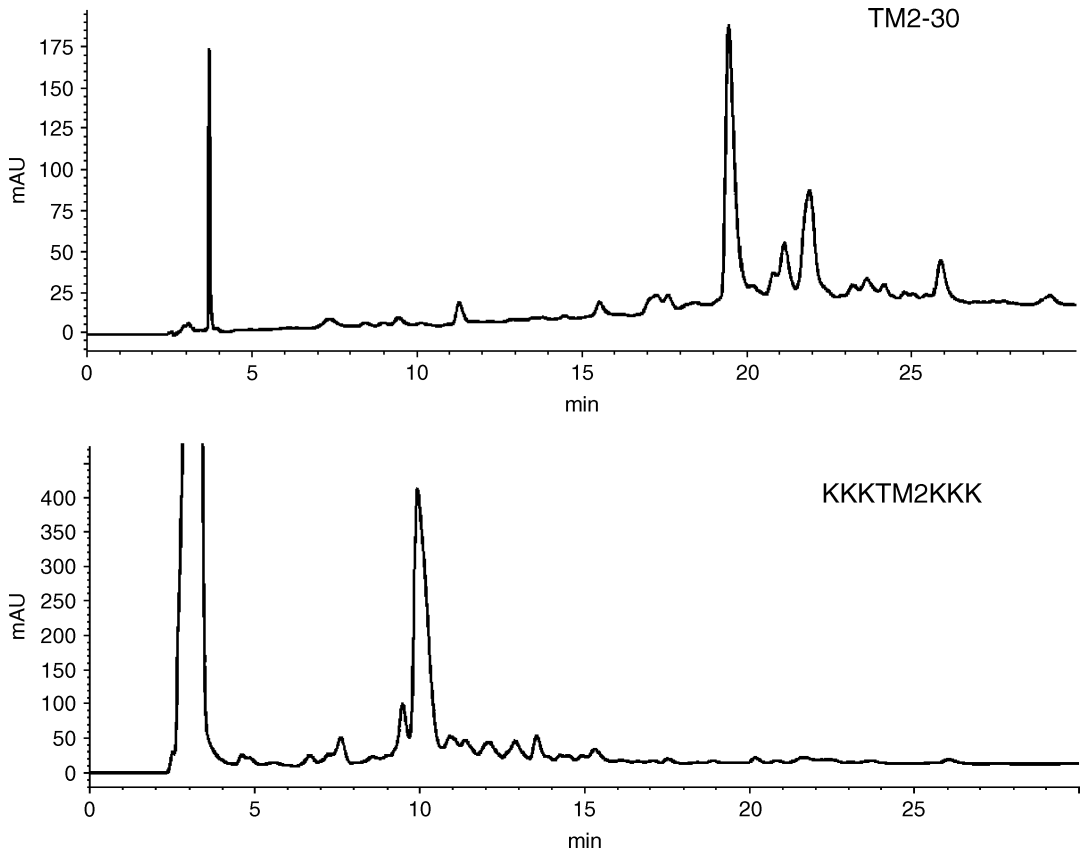

Figure 1 Analytical HPLC profiles of crude peptides corresponding to the TM2 of Ste2p from *Saccharomyces cerevisiae*. HPLC was run using a Vydac reverse phase polymer column eluted with a gradient from 30 to 60% acetonitrile–water containing 0.025% TFA at a flow rate of 1 ml/min over 30 min (detection: 220 nm). The main peaks correspond to the correct synthesis products, as identified by mass spectrometry. KKKM2KKK elutes earlier than TM2-30, indicating that the flanking nonnative Lysines reduce the overall hydrophobicity of the peptide.

DOI: 10.1002/psc

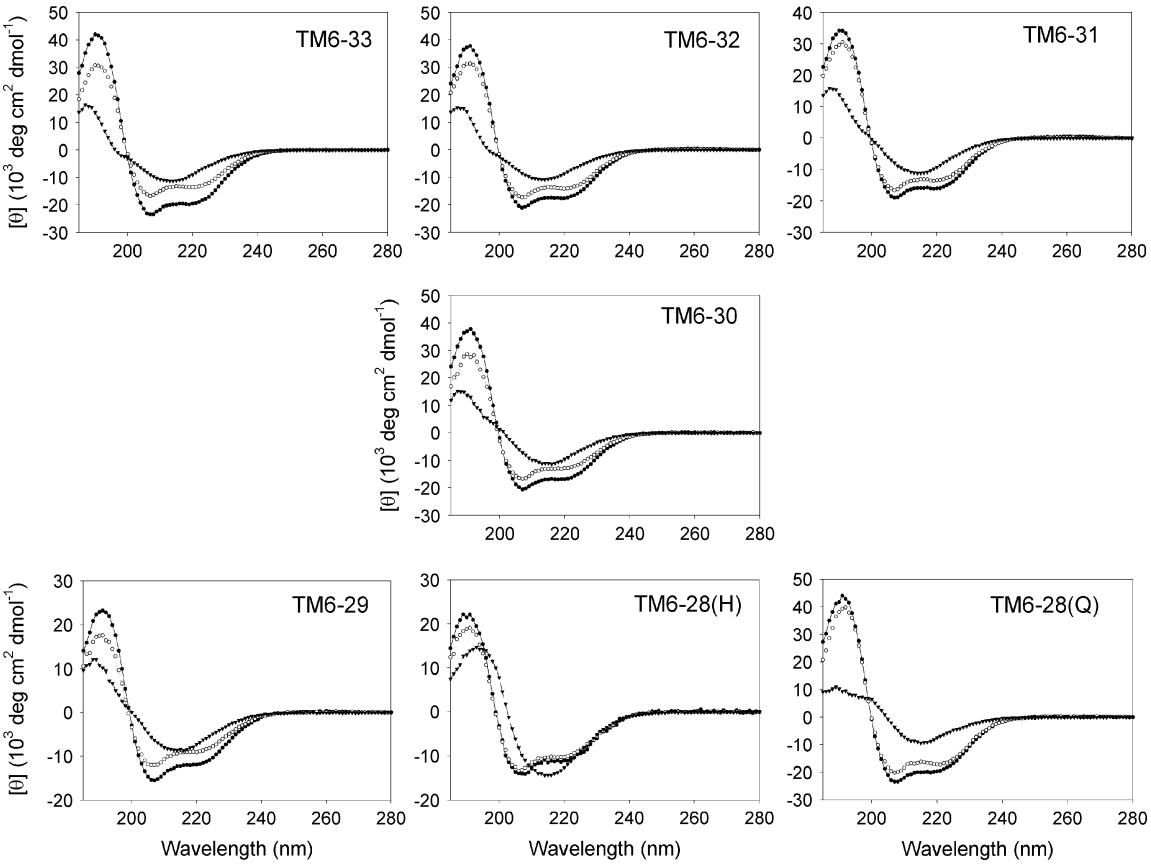

Figure 2 CD spectra of TM6 with natural loop amino acids at the termini in TFE-water mixtures. 95% TFE-5% water (v/v) (black circles), 50% TFE-50% water (v/v) (white circles) and 25% TFE-75% water (v/v) (black triangles).

and 192 nm (Figure 2). The ratio of the positive and negative $\pi \rightarrow \pi^*$ components (~2 : 1) is indicative of a well developed α-helix [20]. Interestingly, the two 28-residue peptides exhibited significantly different CD patterns with TM6-28(Q) being significantly more helical than TM6-28(H) as judged by the relative size of the 222-nm and 208 nm bands and the calculated mean residue ellipticities. In fact, the TM6-28(Q) peptide was as structured as the TM6-33 peptide based on peak shape and ellipticity values. All peptides in this series showed a markedly different CD pattern in TFE/water (25 : 75), exhibiting broad minima centered near 215 nm. This pattern is consistent with either a mixture of structures or the presence of peptide aggregates.

TM6 peptides with nonnative lysine residues added to a 27-residue core exhibited CD patterns typical of α-helices at all TFE/water ratios examined (Figure 3). When the amount of water was increased to 75% (v/v), we noticed a small decrease in the ellipticities, suggesting either a solvation effect, a perturbation of the helical structure by water or a precipitation of material in the more hydrophilic medium. Although some differences were observed for peptides containing different numbers of Lys residues at the termini, we judged these as insignificant from a structural perspective. In particular, in TFE/water mixtures TM6KK and KKKTM6KKK exhibited quite similar behavior.

TM6 peptides in micelles and bilayers. The CD patterns for the native TM6 sequences in the presence of SDS micelles or DMPC/DMPG (4 : 1) bilayers exhibited a marked reduction in intensity compared to TFE : H_2O solutions and the minima at 222 nm and 208 nm were not resolved (Figure 4). Sample preparation was difficult and it is not clear whether these peptides integrated fully into micelles or vesicles. Attempts to obtain the final peptide concentrations in the vesicles by quantitative amino acid analyses were inconclusive. The spectra in SDS obtained for TM6-33/29 showed very low helical content as judged by the broadness of the CD spectra between 208 and 220 nm, while for TM6-28(H) and TM6-28(Q), the conformational preferences were shifted toward the β-sheet structure (Figure 4). In DMPC/DMPG (4 : 1) all spectra suggest highly aggregated peptides. The presence of nonnative lysine residues at the end of the TMDs resulted in spectra that appeared to be more helical (compare Figure 5 KKKTM6KKK with Figure 4 TM6-33). This was particularly true in the presence of lipid vesicles. However, the ellipticities (−5000–10 000 deg-cm^2 decimol^{-1}) in the 208–222 nm range were significantly lower than found in aqueous

DOI: 10.1002/psc

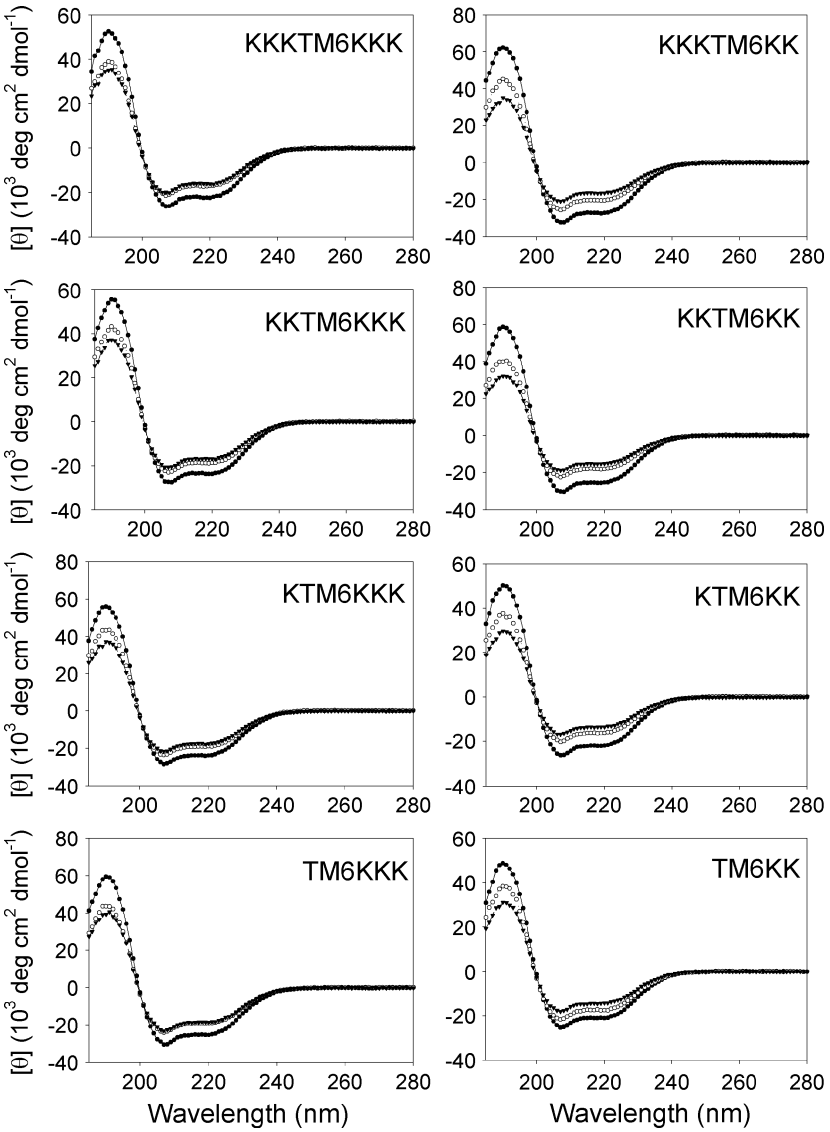

Figure 3 CD spectra of TM6 peptides with nonnatural Lys residues at the termini in TFE–water mixtures 95% TFE–5% water (black circles), 50% TFE–50% water (white circles) and 25% TFE–75% water (black triangles).

TFE. The CD patterns for most peptides in the K_nTM6K_n series in vesicles were indicative of a mixture of structures and suggest the aggregated state.

It is well documented that hydrophobic matching plays an important role in the way that a TM protein inserts into the surrounding lipid bilayer [21]. The hydrophobic thickness of the C-14 lipids DMPC and DMPG is 20 Å [14]. Assuming α helical structures and using the length of the hydrophobic core of TM6, we calculate that this peptide would span ~40 Å. To test for the influence of lipid bilayer thickness we also examined C16:0-C18:1 lipids, POPC and POPG, with a hydrophobic core of 27 Å [21] (Figure 6). The CD spectra of TM6 peptides in the presence of POPC/POPG vesicles exhibited mean residue ellipticities as high as $-20\,000$ deg-cm^2 dmol^{-1}. This is indicative of a much higher incorporation of peptide into the vesicle than in DMPC/DMPG vesicles. In no case, however, were distinct minima observed at both 208 nm and 222 nm. The shapes of all of the CD curves were indicative of a mixture of structures.

CD of TM2 peptides in trifluorethanol–water mixtures. CD spectra of the six TM2 peptides in aqueous TFE at the ratio of 95:5, 50:50 and 25:75 are shown in Figure 7. As judged by the double minima at 222 nm and 208 nm and the ratio of the magnitudes of the molar ellipticity at 195 nm and 222 nm, which is greater than or equal to 2:1, all the peptides show predominantly α-helical structures at 95% and 50% TFE–water (v/v). As the concentration of water increases to 75% (v/v) there were some minor changes in the CD spectra but the CD patterns, except for the case of TM2-26 and KTM2K, still exhibit two clear minima at 208 nm and 222 nm. These latter

DOI: 10.1002/psc

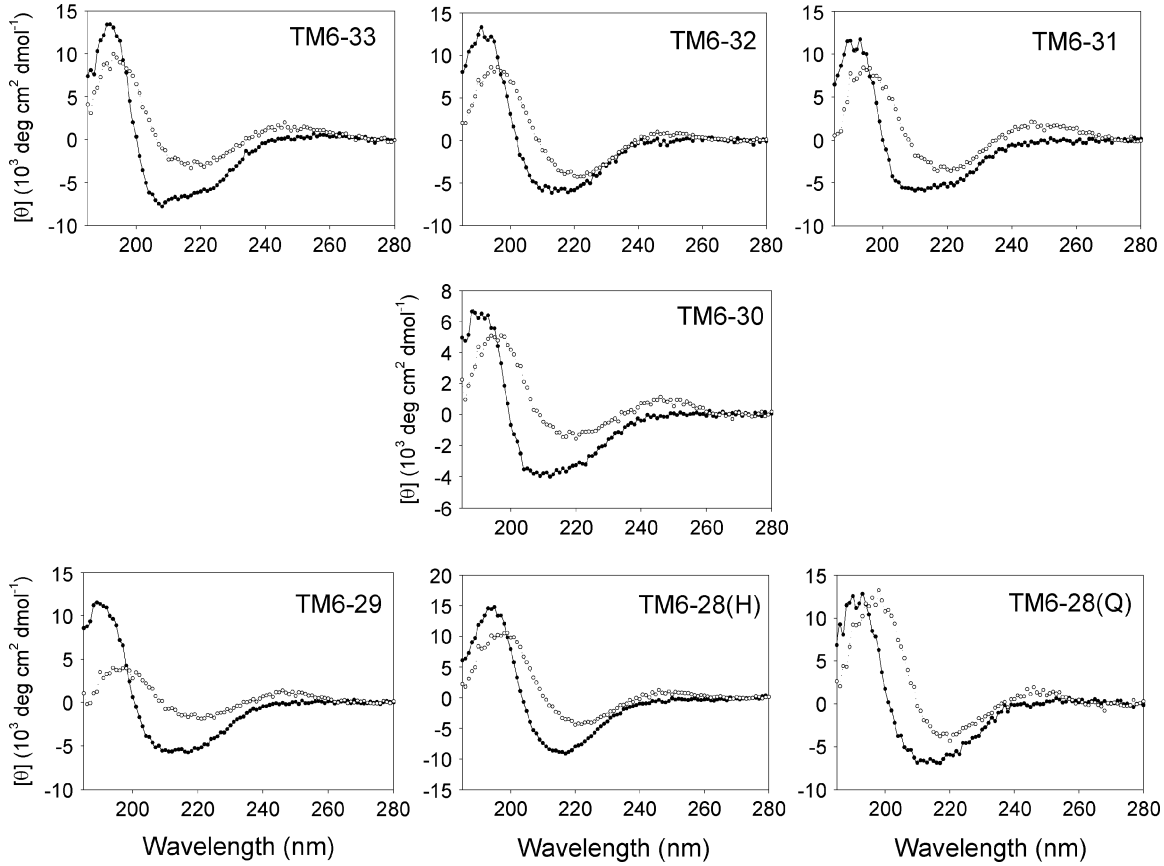

Figure 4 CD spectra of TM6 peptides with natural loop amino acids at the termini in 0.5% SDS micelles (black circles) and DMPC/DMPG (4 : 1) vesicles (white circles).

peptides show changes indicative of some loss of helical structure as judged by the disappearance of a clear 222 nm minimum.

TM2 peptides in SDS micelles and DMPC/DMPG vesicles. The secondary structures of the TM2 peptides were also examined in SDS micelles and lipid vesicles. In SDS micelles all TM2 peptides were helical as judged by the two minima at 208 and 220 nm. In comparison to the CD of these peptides in TFE/water, there was a noticeable reduction in the intensities of the two minima (Figure 8). Similar results were obtained for these peptides in DMPC/DMPG vesicles (Figure 8). Except for KTM2K and TM2-26 the differences between the peptides with nonnative lysine residues at the termini, when compared with peptides with natural loop residues at the termini, were relatively small.

ATR-FTIR spectroscopy for TM2 peptides. The correlation between the frequency of the amide I vibrational mode and the nature of the secondary structure is well established in the literature (reviewed in [22]). Frequencies in the regions of 1650–1660 cm^{-1} correspond to α-helical segments while absorbances in the 1630–1640 cm^{-1} and 1670–1685 cm^{-1} regions correspond to β-sheet elements. ATR-FTIR measurements complement and extend the CD data since polarization analyses for peptides in bilayers allow determination of the orientation of the peptide with respect to the multilayer. IR absorbance spectra of the TM2 peptides in DMPC/DMPG (4 : 1) multilayers using perpendicular or parallel-polarized light yielded average dichroic ratios for the 2918 cm^{-1} and 2850 cm^{-1} absorbances from 1.11 to 1.02 for different peptide preparations (Table 2). The resulting average order parameters, S_{lipid}, were between 0.72 and 0.77, and the average tilt angles, β, of the lipid CH_2 axis were between 23 °and 25° (Table 2). These results indicate that a well-ordered lipid multilayer is formed parallel to the ATR plate surface in the presence of the peptide [23].

ATR-FTIR spectra of the amide I region were measured for TM2 peptides in DMPC/DMPG (4 : 1) multilayers, and their Fourier self-deconvoluted (FSD) spectra were calculated (Figure 9). The dichroic ratios, R^{ATR}, calculated from the FSD α-helical amide I regions integrated from 1650 cm^{-1} to 1665 cm^{-1}, along with the respective order parameter S and tilt angles β, are summarized in Table 2. The dichroic ratio values, R^{ATR}, for TM2-30 and KKKTM2KKK were calculated to be 3.09 and 3.37, respectively, resulting in corresponding order parameters of 0.59 and 0.32. These results indicate that the helical region of TM2-30 and KKKTM2KKK orient at angles of 31° and 42°, respectively. In the

DOI: 10.1002/psc

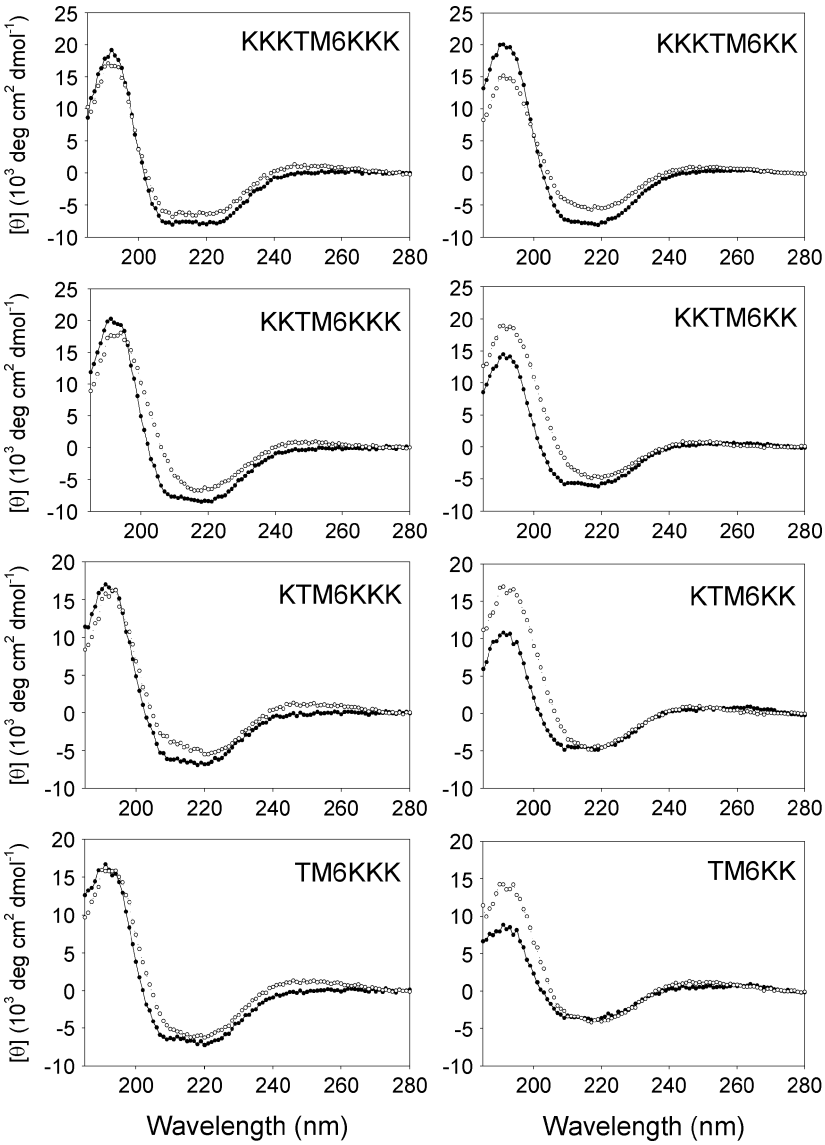

Figure 5 CD spectra of TM6 peptides with nonnatural Lys residues at the termini in the presence of detergents or vesicles. 0.5% SDS micelles (black circles) and DMPC/DMPG (4 : 1) vesicles (white circles).

same way, TM2-28 and KKTM2KK orient at angles of 30° and 26° and TM2-26 orients at an angle of 9°. There is no obvious correlation between the tilt angles and the number of Lys or natural residues at the termini of the TM2 peptides. Interestingly, KTM2K primarily showed an amide I intensity at 1631 cm^{-1}, indicating a predominantly β-sheet structure in the multilayers on crystals.

ATR-FTIR spectroscopy for TM6 peptides. Reconstitution of the TM6 peptides into vesicles was achieved by sonication of peptide/DMPC/DMPG in the presence of aqueous buffer followed by exhaustive dialysis of the vesicle suspension against aqueous buffer, which has been shown to be an efficient method to reconstitute these membrane peptides into vesicles [19]. In general, however, in the present study this method did not give good reconstitution of the TM6 peptides into DMPC/DMPG vesicles. After sonication, samples of peptides containing Lys residues were transparent. However, samples from peptides containing loop amino acids were turbid. Samples were first prepared at a molar ratio of peptide to lipid of 1 : 50 at 100 μM peptide; under these conditions the signal of the amide I was poor, which could be due to poor incorporation of the peptide into the multilayers probably due to peptide aggregation. Samples at a molar ratio of 1 : 100 at 50 μM peptide concentration gave better spectra, with a good signal in the amide I region (Figure 10). Peptides containing the natural loop residues at the termini did not integrate into any of the lipid vesicles and we did not observe any signal in the amide I region (data not shown). In contrast, for TM6 peptides with Lys residues at the termini, ATR-FTIR spectra in DMPC/DMPG (4 : 1) multilayers exhibited two maxima

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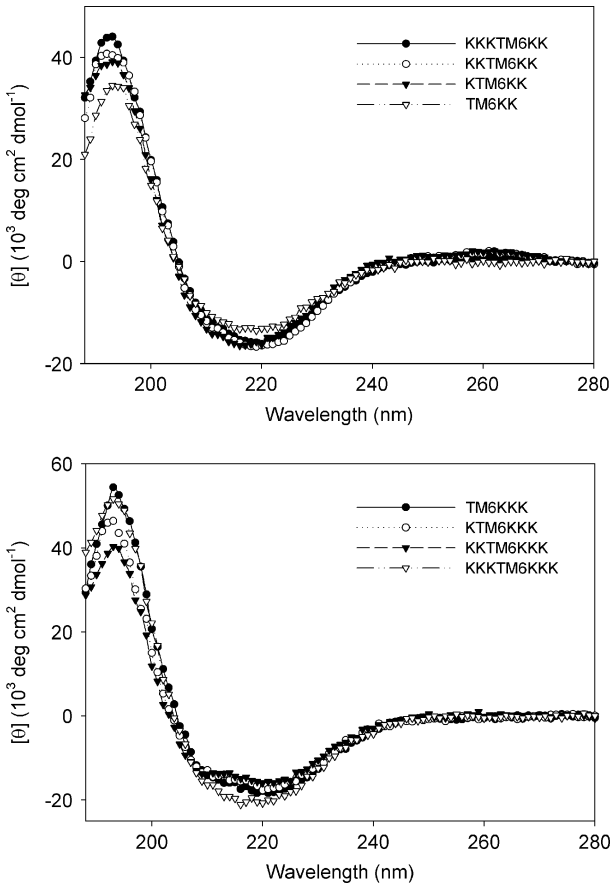

Figure 6 CD spectra of TM6 peptides with nonnatural Lys residues at the termini in POPC/POPG (4 : 1) vesicles.

in the amide I region, one at 1630 cm^{-1} indicating β-sheet and the other at 1659 cm^{-1} supporting the presence of an α-helical structure. In most cases, the β-sheet structure predominated, as seen in Figure 10. We also tried to reconstitute the TM6 peptides into POPC/POPG vesicles, which have a longer hydrophobic core. The results were not significantly different from those in DMPC/DMPG vesicles (data not shown).

DISCUSSION

GPCRs are found in virtually every eukaryotic cell type from single cell microorganisms such as yeast to humans. The ubiquitous nature of GPCRs and their large abundance and functional significance in many disease states make them an important target of structure–function investigation. Even though there is a debate about the use of peptide fragments for the study of structural details concerning GPCRs, many laboratories are using such peptides to learn about intact GPCRs [24–30] and other integral membrane proteins [31,32]. The synthesis of TM peptides is not routine as these molecules have very low water solubility and a pronounced tendency to aggregate. A number of groups have reported that solubility of the TMs can be increased by adding hydrophilic residues such as lysine to the termini [7–9,11–14,24,31]. Today this peptide design is aided using an algorithm that allows calculation of the number of lysine residues to be added to the end of the peptide to achieve solubility [7]. Although Lys residues clearly improve the solubility of TM peptides, these residues are not natural and an open issue is whether the addition of Lys residues at the termini of TMs will affect the biophysical tendencies of the TM core residues when compared with identical peptides that have natural loop residues at the termini.

In this study, a systematic evaluation of the influence of Lys or natural loop residues on the biophysical properties of peptide fragments corresponding to TM 2 and 6 of Ste2p from *S. cerevisiae* was carried out. It was immediately clear that, as reported for other systems (33 and references therein), adding Lys residues significantly decreased the lipophilicity of these TM peptides aiding in their synthesis and purification (Figure 1). The CD results comparing the influence of lysine residues and natural loop residues surrounding TM6 of Ste2p clearly indicate that the core peptide sequence has a greater tendency to aggregate in water and exhibits β-sheet-like CD patterns when it is extended by native loop residues than when it is surrounded by nonnative Lys residues (Figures 2–4).

When the CD analysis of these peptides was done in the presence of SDS micelles and DMPC/DMPG vesicles, which requires resuspension in aqueous buffer, we observed greater differences between the peptides with native loop residues and those with nonnative Lys residues (Figures 4 and 5). For example, KKKTM6KKK gave CD patterns in both vesicles and SDS micelles (Figure 5) that resembled that of an α-helix, whereas the patterns for TM6-33, a peptide with the same length but having native residue at the termini, indicated either mixed structures or a high tendency for aggregation (Figure 4). This conclusion was consistent with the results of ATR-FTIR studies in multilayers where the native loop residue containing peptides aggregated on the multilayers and gave virtually no signal while peptides containing nonnative Lys residues at the termini exhibited significant helical components (Figure 10).

Increasing the number of lysine residues is important to achieve a better solubilization of the peptide, which allows better incorporation of the peptide into the micelles or vesicles (Figure 5). Our studies on the TM6 peptides indicated that in vesicles and bilayers, the most helical patterns were obtained with three additional lysine residues at each terminus of the peptide. This was consistent with the calculations using Deber's algorithm [7]. Nevertheless, it is important to mention that none of the TM6 peptides containing Lys residues was 100% soluble in water.

In contrast to the TM6 series, all TM2 peptides were helical even at 25–75% TFE-water. Little difference

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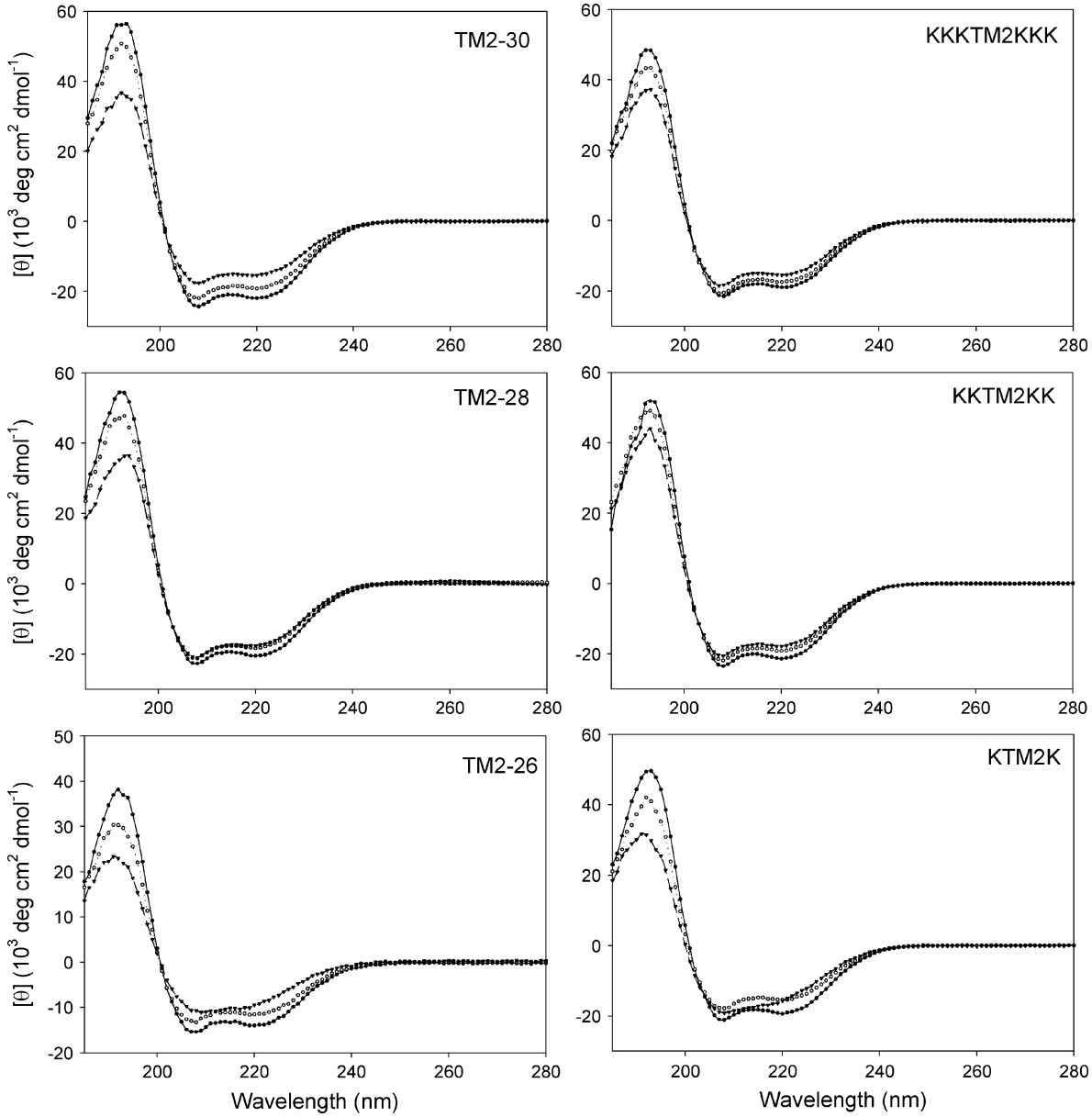

Figure 7 CD spectra of TM2 peptides in TFE/water mixtures. In each panel, the black circle represent 95% TFE-5% water, white circle represents 50% TFE-50% water and black triangle represents 25% TFE-75% water.

was observed between TM2 peptides containing natural loop amino acids and Lys residues. The CD analysis of these peptides in SDS micelles and DMPC/DMPG vesicles revealed that all the TM2 peptides remain predominantly α-helical in these membrane mimetic solvents (Figure 7). With the possible exception of KTM2K in SDS (see subsequent text), there were no significant differences between peptides containing loop amino acids and those with nonnatural Lys at the termini. However, we note that in the case of TM2 the natural loop residues at the *N*-terminus are SRK, which represent a hydrophilic sequence resembling KKK. In the case of the TM2 peptides the CD spectra for TM2-26 appeared to be as helical as those of KKKTM2KKK in both vesicles and micelles (Figure 8) and showed only a minor breakdown in helicity in 75% water/25% TFE. Thus, the peptide corresponding to the second TMD did not require the two additional Lys residues at each terminus as predicted by Deber's algorithm. The difference in behavior between TM6 and TM2 indicates that the sequence of the TM and its proximal loop residues will determine the necessity to add Lys residues at the termini of the hydrophobic core.

ATR-FTIR spectroscopy has been used to obtain information on the orientation of peptides interacting with membranes. Oriented multilayer systems were obtained by drying the samples on Ge ATR crystals and then rehydrating them in a high-humidity chamber. Order parameters of TM2 peptides (Table 2) were derived from the dichroic ratio of the amide I

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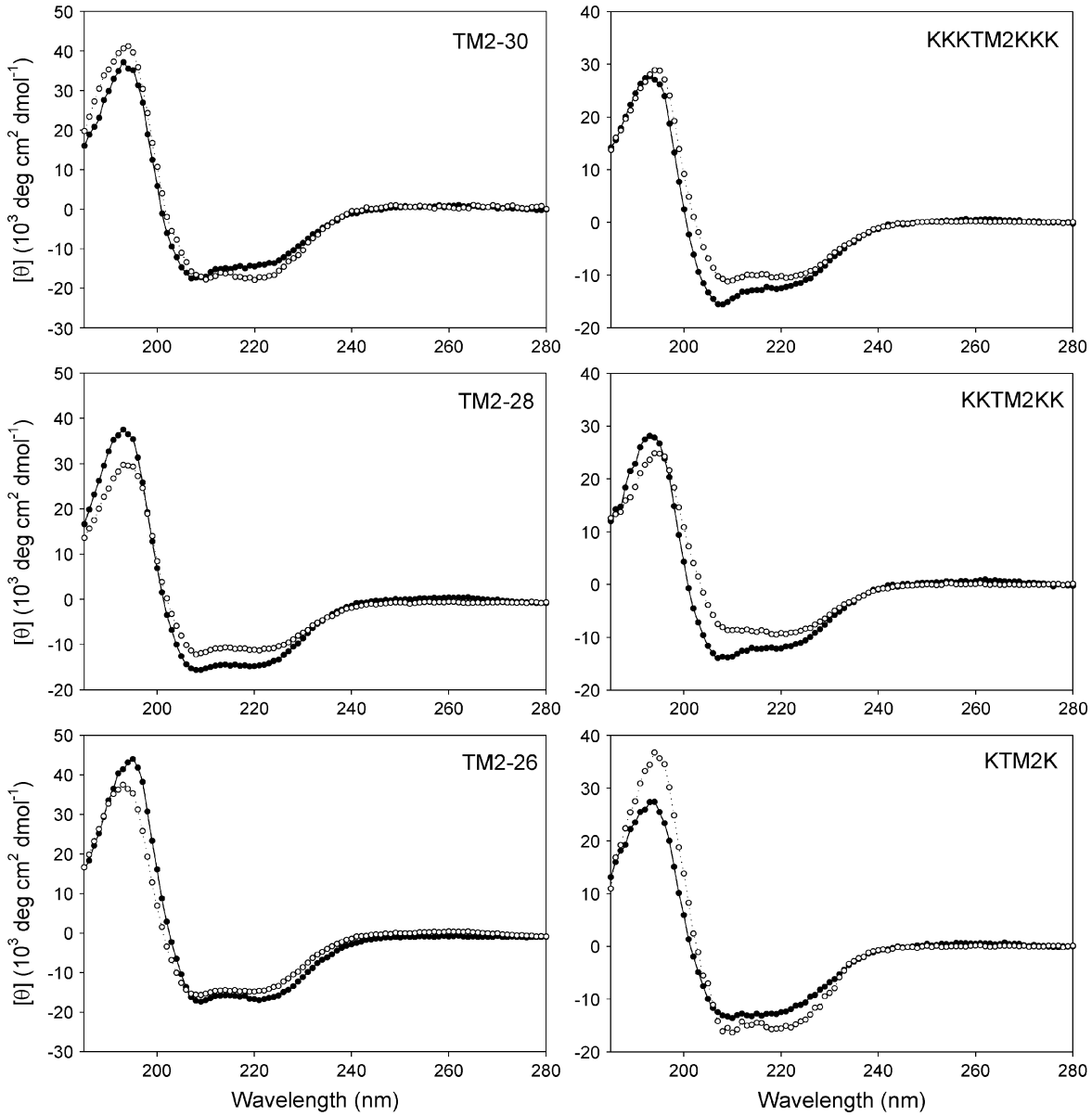

Figure 8 CD spectra of TM2 peptides in micelles and vesicles. 0.5% SDS micelles (black circles) and DMPC/DMPG (4 : 1) vesicles (white circles).

Table 2 Summary of orientation parameters of TM2 peptides in DMPC : DMPG (4 : 1) multilayers

	TM2-30	TM2-28	TM2-26	KKKTM2KKK	KKTM2KK	KTM2K
R^{ATR} of CH_2 2918 (cm^{-1})	1.11 ± 0.03	1.06 ± 0.02	1.06 ± 0.02	1.06 ± 0.04	1.08 ± 0.04	1.02 ± 0.01
R^{ATR} of CH_2 2850 (cm^{-1})	1.06 ± 0.01	1.06 ± 0.01	1.04 ± 0.01	1.06 ± 0.03	1.07 ± 0.02	1.06 ± 0.03
S of lipid	0.72 ± 0.04	0.75 ± 0.01	0.76 ± 0.02	0.75 ± 0.04	0.73 ± 0.03	0.77 ± 0.03
Tilt angle of lipid, β (deg)	25.4 ± 1.8	24.09 ± 0.8	23.72 ± 0.9	24.4 ± 2.0	25.0 ± 1.6	23.1 ± 1.6
Amide I band (cm^{-1})	1656	1656	1658	1656, 1632	1658, 1630	1631
R^{ATR} of amide I band	3.1 ± 0.3	3.15 ± 0.2	4.2 ± 0.1	2.51 ± 0.3	3.37 ± 0.7	NA
S of amide I band	0.59 ± 0.1	0.61 ± 0.1	0.97 ± 0.1	0.30 ± 0.2	0.70 ± 0.2	NA
Tilt angle of α-helix, β (deg)	31 ± 5	30 ± 4	9 ± 3	42 ± 7	26 ± 14	NA

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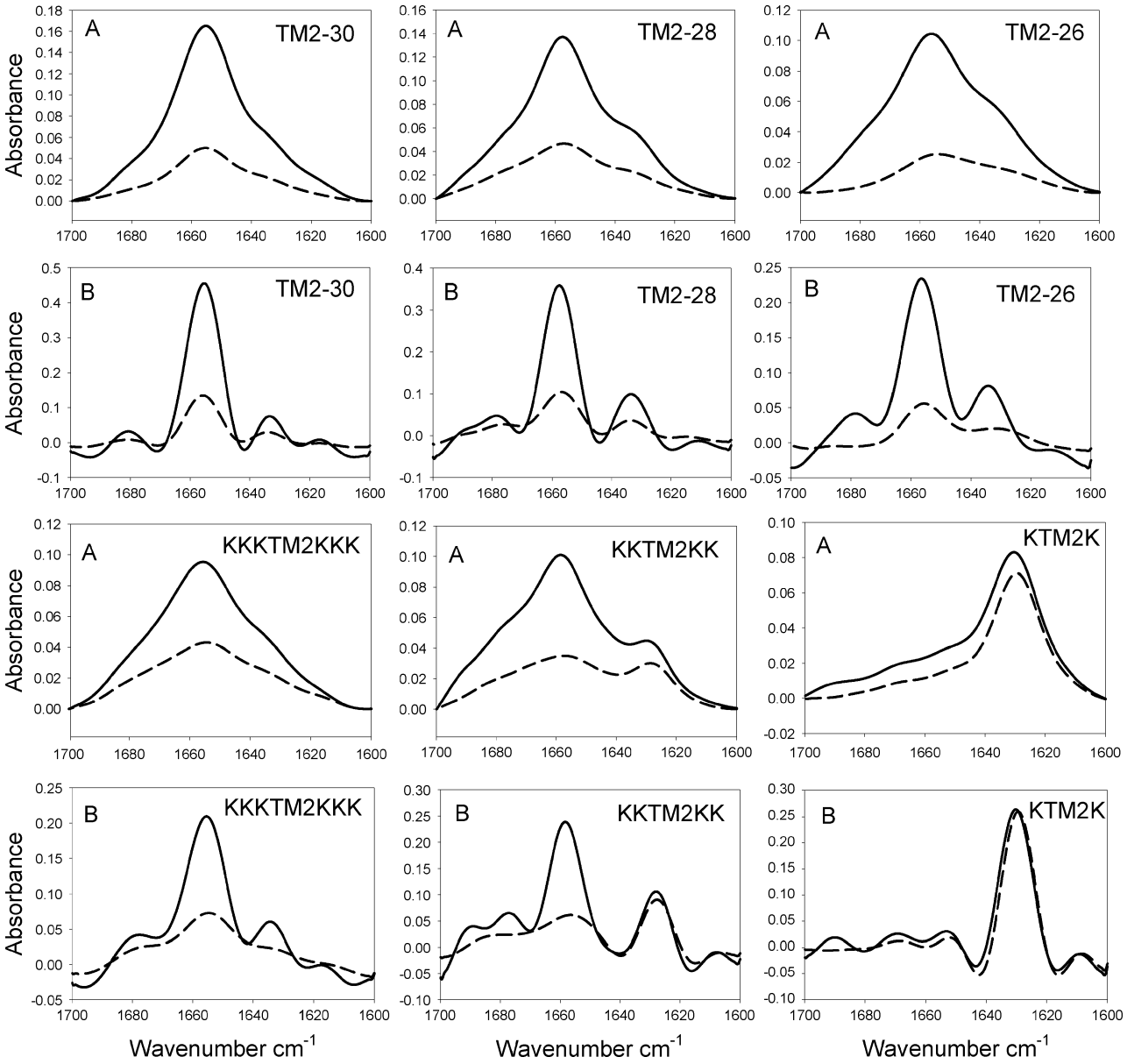

Figure 9 Amide I region of ATR-FTIR spectra of TM2 peptides in DMPC/DMPG (4 : 1) multilayers on Ge crystals. (A) parallel (solid line) and perpendicular (dashed line) polarized light. (B) Fourier self-deconvoluted spectra of (A) using a bandwidth at half-height of 13 cm^{-1} and an enhancement of 2.0.

bands using the equations described in previous publications [19]. The conformation of TM2 peptides is predominantly α-helical in membrane environments as determined by CD spectroscopy (Figure 8), with KTM2K exhibiting some tendency for β-sheet structure in SDS vesicles. When the same peptides were analyzed by ATR-FTIR spectroscopy, we noticed significant differences. Peptides with natural loop amino acids at the termini have one predominant band around 1656 cm^{-1} and a small band at 1630 cm^{-1}. The intensity of the 1630 cm^{-1} band decreased slightly as the chain length increased. For equivalent chain lengths, the same peptides with nonnative Lys residues at the termini showed a more pronounced band at 1631 cm^{-1} and the KTM2K peptide exhibited a high tendency to form a β-sheet structure in the multilayers on crystals (Figure 9). This observation indicates that when vesicles are slowly dried on Ge windows to form multilayers, the interaction of the peptide segments with each other or with the phospholipid may differ from those in suspended vesicles. Finally, as pointed out in the Results section, the tilt angles for these peptides did not reveal any correlation with chain length or with the peptide sequence. This calls into question the use of single TM peptides corresponding to regions of GPCRs to determine the way these regions orient in the membrane bilayer.

TM6 is perhaps the most important helix for coupling ligand binding on the extracellular side of GPCRs to a conformational change in the cytoplasmic loops.

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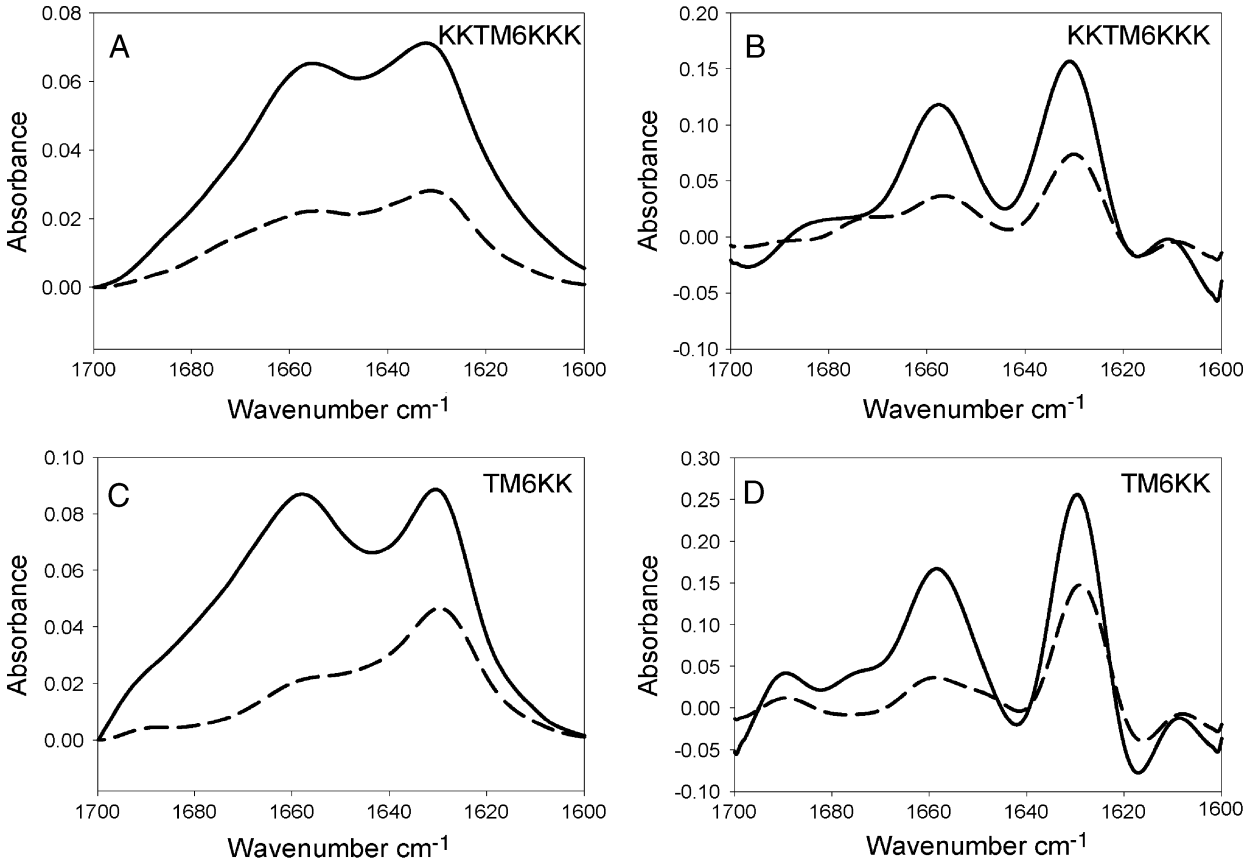

Figure 10 Amide I region of ATR-FTIR spectra of TM6 peptides in DMPC/DMPG (4 : 1) multilayers on Ge crystals. Parallel (solid line) and perpendicular (dashed line) polarized light (A) and (C). Fourier self-deconvoluted spectra of (A) and (C) using a bandwidth at half-height of 13 cm^{-1} and an enhancement of 2.0 (B) and (D).

Since TM6 connects to the third intracellular loop, which plays a big role in G-protein activation [34–36], movements in this TM helix are expected to propagate a signal. In Ste2p, TM7 and TM6 are proposed to interact at the Gln 253, Ser 285 and Ser 292 interface [37] and mutations that disrupt these interactions result in constitutive receptor activity [37,38]. Similarly, modeling studies suggest interactions between TM3 and TM6 that may be involved in coordinated movements during signal transduction [3,39]. On the basis of biophysical analyses of peptides corresponding to the seven TMs of the adenosine A_{2A} receptor, it was concluded that interactions between TMs are required for proper insertion and folding in the membrane [40]. Moreover, this study posited that TM peptides with weak helical tendencies might require interdomain interactions with partners to stabilize a helical structure in the membrane. It is clear that understanding the biophysical properties of TM6, its interactions with other TM peptides and the way it integrates into the membrane bilayer will be an important step in deciphering the signal transduction mechanism of Ste2p and other GPCRs. Although methods to improve the solubility of TM peptides are a necessary component of such studies, our investigation suggests that caution is necessary in interpreting the results of biophysical analyses of these membrane peptides. For example, it is not apparent why TM2-26 and KTM2K exhibit such striking differences in DMPC/DMPG multilayers in which the first peptide was predominantly helical and the latter peptide was completely a β-sheet (Figure 10). These peptides have nearly identical sequences differing only at the carboxyl terminus where there is a Leu to Lys change. It is surprising that the peptide with the Lys at its terminus aggregates into a sheet, although one would expect that Lys should be more stable than Leu interacting with the hydrophilic portion of the bilayer. We conclude that subtle changes in the structure of membrane peptides can markedly affect their conformational tendencies and this must be taken into account in the design and application of membrane peptides as surrogates for portions of integral membrane proteins.

In a previous work using IR techniques to investigate the secondary structure and orientation of the seven TMDs of Ste2p in phospolipid multilayers, it was demonstrated that TM6 forms mostly β-sheet structures [19]. Also, it was shown that TM6 assumes appreciable α-helical structure (~40%) in multilayers mimicking the composition of the *S. cerevisiae* cell membrane [19]. NMR and ATR-FTIR analysis of an 18-residue fragment of TM6 (TM6 252–269 C252A) showed

DOI: 10.1002/psc

that this fragment is mostly helical in a DMPC multilayer with a tilt angle of 8–12.5° between the helix and the bilayer normal [41]. The length of the TM is critical when the secondary structure is studied in a lipid environment [42] because hydrophobic matching plays an important role in the way a TM protein inserts in the surrounding lipid bilayer [14]. The design of a TM is done using hydropathy plots to help locate the beginning and end points of a particular TM segment that occurs in the context of a native protein. The core of the TM6 domain that we chose for analysis contained 27 residues. Perhaps the length of this particular domain makes the peptides we synthesized unsuitable for ATR-FTIR analysis in multilayers composed of C14 or C16 lipid chains. Preliminary CD results in a series of TM6 peptides recently prepared, in which the hydrophobic core is shorter (23 residues), show a more helical structure than the TM6 peptides analyzed in this work. We are currently embarking on a detailed analysis of these peptides.

To conclude, it is interesting to note that in the 1960s and 1970s Professor Goodman encountered similar solubility problems in attempting to analyze the conformational tendencies of homo-oligopeptides composed of L-alanine. Although studies on poly-L-alanine indicated it formed helices that were not water soluble, to increase the solubility of alanine oligopeptides Goodman and his coworkers added solubilizing groups such as oligomers of ethylene oxide and morpholino moieties to the peptide termini [43]. This approach allowed Goodman to show that alanine did have a tendency to assume helical structures at chain lengths exceeding seven residues and that they also could assume β-structures in weak solvents that allowed intermolecular hydrogen bonding [43]. These results proved to be consistent with later studies that showed that alanine peptides solubilized by lysine and glutamic acid residues assumed helices in water at low temperatures [44]. The careful use of model systems by Professor Goodman has always been a paradigm for his students and has inspired us to study biopolymers of increasing complexity, which by themselves were intractable to direct analysis. We remain indebted to this wonderful scientist for the rich inheritance he bequeathed to the field of peptide science.

Acknowledgements

This work was supported by research grants GM22086 and GM22087 from the National Institutes of Health.

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DOI: 10.1002/psc