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Structure and Topology of a Peptide Segment of the 6th Transmembrane Domain of the *Saccharomyces cerevisiae* α -Factor Receptor in Phospholipid Bilayers

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Abstract

A detailed analysis of the structure of an 18-residue peptide AQSLLVPSIIFILAYSLK [M6(252–269, C252A)] in 1,2-dimyristoyl-sn-glycero-phosphocholine bilayers was carried out using solid state NMR and attenuated total reflection Fourier transform infrared spectroscopy. The peptide corresponds to a portion of the 6th transmembrane domain of the α -factor receptor of *Saccharomyces cerevisiae*. Ten homologs of M6(252–269, C252A) were synthesized in which individual residues were labeled with ¹⁵N. One- and two-dimensional solid state NMR experiments were used to determine the chemical shifts and ¹H–¹⁵N dipolar coupling constants for the ¹⁵N-labeled peptides in oriented dimyristoylphosphatidylcholine bilayers on stacked glass plates. These parameters were used to calculate the structure and orientation of M6(252–269, C252A) in the bilayers. The results indicate that the carboxyl terminal residues (9–14) are α -helical and oriented with an angle of about 8° with respect to the bilayer normal. Independently, an attenuated total reflection Fourier transform infrared spectroscopy analysis on M6(252–269, C252A) in a 1,2-dimyristoyl-sn-glycero-phosphocholine bilayer concluded that the helix tilt angle was about 12.5°. The results on the structure of M6(252–269, C252A) in bilayers are in good agreement with the structure determined in trifluoroethanol/water solutions (B. Arshava et al. *Biopolymers*, 1998, Vol. 46, pp. 343–357). The present study shows that solid state NMR spectroscopy can provide high resolution information on the structure of transmembrane domains of a G protein-coupled receptor.

Keywords

6th transmembrane peptide; *Saccharomyces cerevisiae*; α -factor receptor; phospholipid bilayers; ATR-FTIR; solid state NMR

INTRODUCTION

G protein-coupled receptors (GPCR) constitute a large family of integral membrane proteins of profound biological importance that are involved in many physiological processes.^{1–4} As determined by hydropathy analysis, all members of this family contain seven transmembrane domains, generally presumed to be helical, connected by either extracellular or cytoplasmic loops. The low-resolution structure of these receptors is often assumed similar to those of the well-studied proteins bacteriorhodopsin and rhodopsin that serve as model systems for GPCR topology.

Despite the fact that a few high-resolution electron and x-ray diffraction structures have been reported, the need for biophysical data on the structure of integral membrane proteins remains great. Unfortunately, GPCRs are notoriously difficult to crystallize and solution-phase NMR experiments cannot at present yield detailed structures of proteins in membrane bilayers. In contrast, solid state NMR methods are being developed that overcome the inherent problems of membrane protein structure determination. The structure of the M2 transmembrane segment of the acetylcholine receptor was found to form an α -helix with a 12° tilt with respect to the bilayer.⁵ The orientation of the four helical bundle of the M2 protein channel of influenza A was found to have a 30° tilt relative to the bilayer.⁶ Structural details of several membrane proteins have been elucidated by solid state NMR experiments on oriented lipid bilayer samples. These include Vpu, a membrane protein from HIV-1,⁷ cecropin A,⁸ colicin E1,⁹ and magainin,¹⁰ which are antibiotic peptides; mellitin, a pore forming peptide,¹¹ gramicidin A, a β -sheet peptide that traverses the bilayer,¹² and the amino terminal fragment of alamethicin.¹³

The methodology for solid state NMR spectroscopy to solve protein structures requires that the protein be immobilized on a time scale of 10^{-3} s and uniaxially oriented with respect to the magnetic field axis. The correlation time condition is met in lipid bilayer preparations of membrane peptides; the orientation is achieved during hydration of the bilayer/ peptide preparation on stacked glass plates under controlled humidity. Indeed, the orientation of the peptide during hydration of the bilayer is analogous to the self-assembly of a membrane protein inside a biological membrane. Immobilization and orientation of a peptide or protein allows the measurement of orientation dependent spin interaction frequencies that describe the three-dimensional positioning of the peptide bond planes relative to a magnetic field vector. The three-dimensional structure can be elucidated when sufficient data are collected on the protein in the bilayer environment.⁵ The resulting protein structure is described by the φ and ψ . Ramachandran angles linking successive peptide planes displaying the topological orientation of the protein with respect to the membrane bilayer.

We report here the structure determination of a model peptide, AQSLVPSIIFILAYSLK [M6(252–269, C252A)], comprising a portion of the 6th transmembrane domain of the Ste2p α -mating factor receptor of *Saccharomyces cerevisiae*. M6(252–269, C252A) indicates residues 252–269 in the sequence of the receptor using 1 for the amine terminal residue. The naturally occurring Cys in position 252 has been changed to Ala to eliminate disulfide formation during biophysical studies. The intact mutant receptor with the Cys to Ala replacement is functional in signal transduction.¹⁴ Ste2p, a member of the GPCR family, has served as a useful paradigm for understanding the function of GPCRs in

numerous studies including extensive molecular biological investigation (for reviews, see Refs. 15–20). The yeast receptor and G protein subunits are in some cases functionally interchangeable with components of mammalian G protein signaling systems.^{21, 22} Most pertinent to the approach taken in this study, fragments of the receptor have been found to assemble into functional protein capable of binding ligand and triggering signaling transduction.¹⁴ We report on the solid state NMR and attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) studies of the synthetic peptide with the sequence AQSLVPSIIFILAYSLK [M6(252–269, C252A)] where 10 specific amide backbone sites were individually labeled with ¹⁵N. These experiments allowed us to determine the structure and orientation of this fragment of the 6th transmembrane domain of Ste2p in lipid bilayers.

EXPERIMENTAL PROCEDURES

Synthesis and Purification of ¹⁵N-Labeled M6(252–269, C252A)

All reagents and solvents for solid phase peptide synthesis were analytical grade. N- α -(Fmoc)-N- ϵ -(Boc) lysine *p*-ben-zyloxybenzyl alcohol polystyrene (Wang resin; Fmoc: 9-flourenylmethoxycarbonyl; Boc: *t*-butyloxycarbonyl) was prepared by the DCC-DMAP method and loading capacity 0.14 mMol/g was determined by uv spectroscopy.²³ [¹⁵N]-L-Leu, Fmoc-[¹⁵N]-L-Ile and Fmoc-[¹⁵N]-L-Ala were purchased from Cambridge Isotope Laboratory (Andover, MA). Fmoc-[¹⁵N]-L-Phe and Fmoc-[¹⁵N]-L-Val were purchased from ISOTEC, Inc. (Miami, OH). Fmoc-[¹⁵N]-L-Leu was prepared by the FmocOSu method.²⁴

The labeled M6(252–269, C252A) peptides had the sequence AQSLVPSIIFILAYSLK corresponding to a portion of the 6th transmembrane domain of the α -factor receptor of *Saccharomyces cerevisiae*. All peptides were synthesized on a Fmoc-Lys-(Boc)Wang resin in a stepwise manner on a 0.1 mmol scale using an Applied Biosystems, Inc., Model 433A synthesizer. The protocol utilized for the synthesis of the peptides was the FastMoc routine using the basic conductivity monitoring option of the synthesizer. Each amino acid residue was double coupled, the coupling and the deprotection time was increased compared to the standard ABI-433A protocol in order to improve the coupling efficiency. Free α amino groups that still failed to couple were capped with acetic anhydride after each amino acid was incorporated. These strategies minimized impurities found in the crude peptide recovered after cleavage of the hydrophobic transmembrane peptide from the resin. Peptides with ¹⁵N labeled amino acids at specific amino acid residues were prepared by directly incorporating Fmoc-protected ¹⁵N-labeled amino acids into the peptide chains. After the completion of assembly of the peptide chain, the resin was treated with 95% trifluoroacetic acid (TFA), 2.5% 1,2-ethanedithiol, and 2.5% water at room temperature for 1.5 h. The reaction mixture was filtered to remove the resin. The crude peptide was precipitated by adding cold anhydrous ether to the concentrated filtrate.

The peptides were purified using preparative reversed phase high performance liquid chromatography (HPLC) on a Hewlett Packard 1050 HPLC system using Waters μ -Bondapak C4 column (19 $\times$ 300 mm) with water (+0.025% TFA) and acetonitrile (+0.025% TFA) as eluent. Preparative HPLC used a linear gradient from 10 to 70% acetonitrile over 100 min. Analytical HPLC used a Waters μ -Bondapak C-18 column (3.9 $\times$ 300 mm) and linear gradient from 10 to 100% acetonitrile over 30 min. The uv detector monitored at 220 nm. Peptide with greater than 98% homogeneity was obtained after two cycles of preparative HPLC purification. The synthetic yield of the peptide was estimated to be ca. 40% by analytical HPLC of the crude peptide before purification. After two purification cycles on HPLC the yield of final product was about 17%. The purity of the final products was assessed by analytical HPLC, amino acid analysis, electron spray mass spectrometry,

and ^1H NMR spectroscopy of representative peptides in solution. All of the labeled peptides had identical retention time on HPLC, the correct amino acid ratios, the expected ^1H NMR spectrum and a molecular weight within 1 Da of the calculated value.

Solid-State NMR Spectroscopy

Solid-state ^{15}N NMR experiments were performed on samples of the M6(252–269, C252A) peptide fragment of the Ste2p receptor in lipid bilayers. The peptides contain a single specific ^{15}N -labeled amino acid at the position Leu4, Leu5, Val6, Ile9, Ile10, Phe11, Ile12, Leu13, Ala14, or Leu17. The lipid bilayer was characterized by ^{31}P solid state NMR spectra.

The solid state NMR experiments were performed on a home-built spectrometer with a 700/62 Magnex magnet (Magnex Scientific Ltd. Oxfordshire, UK). The samples were placed in a flat-coil double-resonance $^{15}\text{N}/^1\text{H}$ probe with the coil dimension of $11 \times 11 \times 3$ mm; a separate flat-coil probe double tuned for $^{31}\text{P}/^1\text{H}$ was used for the ^{31}P NMR measurements. The one-dimensional ^{31}P NMR spectra of the oriented lipid bilayers with embedded peptide M6(252–269, C252A) on glass plates were obtained using single pulse excitation with ^1H decoupling during acquisition. One-dimensional ^{15}N chemical shift spectra of the same peptide in lipid bilayer preparations were recorded using the mismatch optimized (CPMOIST) cross-polarization technique^{25,26} with a 52 kHz cross-polarization field strength and 62.5 kHz ^1H decoupling field strength during the 5 ms. acquisition time. A total of 2000 transients were collected with a recycle time of 7 s to reduce radio frequency heating of the hydrated samples. All spectra were recorded at 5°C .

The two-dimensional polarization inversion spin exchange at the magic angle (PISEMA) spectra²⁷ of the peptide were obtained using similar field strengths and signal averaging as in the one-dimensional experiments. The fast-switched Lee–Goldberg condition was set up with a 34.5 kHz frequency jump to effect the ^1H spin-lock at the magic angle. The ^1H – ^{15}N dipolar frequency was allowed to evolve for 32 t_1 increments for a total of 1.25 ms. The two-dimensional data sets were processed using Felix97 (Molecular Simulations, Inc., San Diego, CA). The data were zero filled twice in the t_2 dimension and four times in the t_1 dimension yielding a 512×512 real matrix. The apodization in t_2 was 400 Hz exponential multiplication with a sine bell apodization applied in t_1 . The ^{15}N chemical shift frequencies were measured relative to external saturated ammonia.

Oriented Sample Preparation for Solid State NMR Studies

The purified ^{15}N -labeled M6(252–269, C252A) peptide (2–4 mg) was dissolved in 2 mL of CHCl_3 containing 50 mg of 1,2-dimyristoyl-sn-glycero-phosphocholine (DMPC) and 0.3 mL of trifluoro ethanol (TFE). The solution was sonicated in a bath sonicator until clear and spread evenly onto 20 etched glass plates $11 \times 11 \times 0.07$ mm (Marienfeld Glassware, Bad Margenheim, Germany). The glass plates were etched prior to use in a 50% solution of hydrogen fluoride, rinsed twice in deionized water, and finally in 95% ethanol. The plated solution was dried to evaporate the solvent, stacked and hydrated in a flask maintained at 94% relative humidity (RH) using saturated ammonium phosphate. The hydration was allowed to proceed at 42°C overnight. To prevent sample dehydration during the NMR experiment, the stacked glass plates were wrapped in Parafilm and heat-sealed in a polyethylene bag before being placed in the flat coil double-resonance NMR probe. The molar ratio of peptide to lipid was between 1:100 and 2:100.

Unoriented Sample Preparation for Solid-State NMR Studies

The purified ^{15}N -labeled M6(252–269, C252A) peptide (2–4 mg) was dissolved in 2 mL of CHCl_3 containing 50 mg of DMPC and 0.3 mL of TFE. The solution was sonicated in a bath sonicator until clear. The solution was dried to evaporate the solvent and hydrated in a flask

maintained at 94% RH using saturated ammonium phosphate at 42°C overnight. To prevent sample dehydration during the NMR experiment, the sample was wrapped in Parafilm and heat-sealed in a polyethylene bag before being placed in a 5 mm round coil double-resonance NMR probe. The molar ratio of peptide to lipid was 2:100.

ATR-FTIR Dichroism Spectroscopy

Infrared spectra were recorded at ambient temperature (~20°C) on a Nicolet Magna 550 Fourier-transform infrared spectrometer (Nicolet Analytical Instruments, Madison, WI) equipped with DTGS detector and an ATR accessory (Pike Technologies, Inc., Madison, WI). The infrared beam was polarized using one 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 level of zero filling (that doubles the number of points), ATR correction, and Happ–Genzel apodization. Fourier self-deconvolution with a band width at half height of 13 cm⁻¹ and a resolution enhancement parameter of 2.0 was used to resolve overlapping peaks in the Amide I region of the spectrum.²⁸

Oriented Sample Preparation for ATR-FTIR Dichroism Studies

M6(252–269, C252A) peptide (0.13 mg) in 0.13 mL TFE was added to 4 mg of DMPC in 0.5 mL CHCl₃. The resulting solution was dried under N₂ flow. Residual traces of organic solvent were removed by placing the dried film under vacuum overnight and the sample was suspended in 1 mL of 0.1 mM phosphate buffer, pH 6.3. The suspension was sonicated at approximately 50°C for 60 min in W-385 (Misonix, Inc., Farmingdale, NY) sonicator equipped with a cup horn (at 40% output power). The resulting vesicles were then passed through a 0.45 μm polycarbonate centrifugal filter. The calculated molar ratio of peptide to lipid was 1:100.

Lipid films on the top surface of germanium ATR crystals (55 × 10 × 4 mm with 45° beveled edges) were obtained by slowly evaporating 250 μL of the preformed lipid vesicles with or without M6(252–269, C252A) peptide. The crystals were previously cleaned with chloroform, TFE, and methanol, followed by 30 min plasma cleaning in PDC-32G plasma cleaner (Harrick, Ossining, NY). Following deposition, the ATR crystals were transferred to a desiccator where the films were rehydrated by vapor diffusion in an atmosphere maintained at 98% relative humidity by a saturated solution of K₂SO₄ in water.²⁹ Rehydration was allowed to proceed for a minimum of 18 h at room temperature.

RESULTS

Solid State NMR spectroscopy

The solid state ³¹P NMR spectra of DMPC bilayers with embedded M6(252–269, C252A) peptide on glass plates were used to characterize the quality of orientation of the lipid bilayers. The appearance of a single resonance at 30 ppm relative to 85% phosphoric acid as an external standard, indicates that the orientation of the nonesterified O—P—O plane of the phosphate head group aligns perpendicular to the bilayer normal.³⁰ placing the long axis of the lipid parallel to the applied magnetic field (data not shown). These spectra revealed that the bilayers are well oriented (>90%) with the dominant peak at 30 ppm. Approximately 10% of the integrated intensity in the ³¹P spectrum is attributed to a phosphate axially symmetric powder pattern spanning from 30 to -14 ppm, indicating very small amounts of the lipid randomly distributed on the glass plates. The ¹⁵N NMR spectrum of an unoriented sample of the ¹⁵N-Leu-4 M6(252–269, C252A) peptide inserted into lipid bilayers and hydrated in a multilamellar phase had the full breadth of a static ¹⁵N amide chemical shift powder pattern with the principal elements of $\sigma_{11} = 60$ ppm, $\sigma_{22} = 78$ ppm, and $\sigma_{33} = 225$ ppm (Figure 1, top left). The presence of the full asymmetric powder pattern for a ¹⁵N

resonance obtained on an unoriented sample confirms the immobility of the peptide amide backbone sites in hydrated lipid bilayers on the time scale of the ^{15}N chemical shift interaction.

Figure 1 contains the one-dimensional solid state ^{15}N NMR spectrum of each of the specifically ^{15}N -labeled M6(252–269, C252A) peptides in oriented DMPC bilayers. Each oriented peptide gives a unique chemical shift frequency. However, they are all similar and within the region of the ^{15}N chemical shift powder pattern consistent with an α -helix oriented parallel to the magnetic field, that is a transmembrane helix in the lipid bilayer. The ^{15}N chemical shift frequencies for each of the labeled sites are listed in Table I.

The one-dimensional spectrum of the ^{15}N Ile-10 M6(252–269, C252A) peptide repeatedly gave a spectrum that was a combination of oriented ^{15}N chemical shift superimposed on an ^{15}N chemical shift powder pattern. This can be seen as the intensity near the σ_{11} and σ_{22} discontinuities of the ^{15}N powder pattern. The source of this powder pattern is attributed to aggregation of the peptide as it was dissolved with the DMPC. The oriented chemical shift frequency was reproducibly measured from this spectrum as the intense peak that was present near the σ_{33} discontinuity in repeated sample preparations from this labeled sample. The standard deviation for the measured oriented frequency was found to be ± 2.5 ppm from repeated measurements of freshly prepared oriented samples for this label as well as the other labeled samples. The ^1H — ^{15}N dipolar couplings and the ^{15}N chemical shift frequencies were measured using the two-dimensional PISEMA experiment.²⁷ The spectra for each of the labeled sites are shown in Figure 2 and the frequencies listed in Table I. These spectra were collected as a symmetric data set about the zero frequency in the dipolar dimension, and are displayed as one-half of the full dipolar frequency range. The correlation cross peaks measured in the PISEMA spectra gave a unique ^{15}N chemical shift and ^{15}N — ^1H dipolar coupling for each oriented site. These cross peaks fall in the region of the PISEMA spectrum that is also consistent with an α -helix traversing the bilayer. The multiple cross peaks close to the zero dipolar axis represent an artifact of the experiment due to a slight mismatch of the Lee–Goldberg off-resonance spin–locking fields that result in an exponential decay of the ^{15}N magnetization, hence a zero dipolar frequency.

The dipolar frequency for ^{15}N -Leu-4 M6(252–269, C252A) peptide was measured using a one-dimensional ^{15}N spectrum recorded with the Lee–Goldberg decoupling to remove the homonuclear ^1H — ^1H contribution to the dipolar splitting.³¹ The dipolar frequency for this residue was narrow at 3.5 kHz, which was not sufficiently resolved from the zero-frequency artifact in the two-dimensional PISEMA experiment.

Structure Calculations

The ^{15}N chemical shift and ^{15}N — ^1H heteronuclear dipolar coupling frequencies provide the orientational constraints for structure determination of the peptide in lipid bilayers. These two interactions are described as second-rank tensors in three-dimensional space. The principal elements of the chemical shift tensor are measured from the discontinuities in the powder pattern. The orientation of the tensor in the molecular frame is described by a set of Euler angles relating two orthogonal axis systems and has been characterized in crystalline model peptides.³² The ^{15}N chemical shift frequency measured in an oriented sample can be evaluated according to

$$\sigma_{\text{obs}} = \sigma_{11} \sin^2 \theta \cos^2 \varphi + \sigma_{22} \sin^2 \varphi \sin^2 \theta + \sigma_{33} \cos^2 \theta \quad (1)$$

where σ_{11} , σ_{22} , and σ_{33} are the principle elements of the chemical shift tensor, and θ and φ are the Euler angles relating the tensor to the laboratory frame.

The ^1H — ^{15}N dipolar interaction is characterized by an axially symmetric tensor with one of the elements of the principal axis system lying along the bond vector connecting the two nuclei. The dipolar interaction results in a doublet, with maximal splitting when the bond vector is aligned parallel to the applied field, and half maximal when the bond vector is aligned perpendicular to the applied field. The evaluation of the dipolar splitting from the orientational parameter is given by

$$\Delta\nu_D = \nu_H(3 \cos^2\theta - 1) \quad (2)$$

where $\nu_H = \gamma_H \gamma_N / r^3$ and γ_H and γ_N are the gyromagnetic ratios of the ^1H and ^{15}N nuclei, respectively and θ describes the angle the ^1H — ^{15}N bond vector makes with respect to the applied magnetic field.

The program Restrict³³ was used to define in three-dimensional space the allowed orientations of the individual peptide planes from the experimental frequencies. The two measured frequencies, ^{15}N chemical shift and the ^1H — ^{15}N dipolar coupling, from each peptide plane were used to generate α, β restriction plots, where α and β are the polar angles relating the molecular frame of the peptide to the laboratory frame. The planar orientations α and β from the two measured frequencies are described in a common axis system in the peptide plane; α defines the angle the N—H bond vector makes with respect to the projection of the magnetic field vector into the peptide plane; β is the angle the vector orthogonal to the peptide plane makes with respect to the magnetic field (Figure 3).

The results of the restriction analysis for the ten measured peptide planes of the M6(252–269, C252A) peptide are shown graphically in Figure 4. The combination of the two experimental frequencies greatly limits the orientation of the individual peptide planes in α, β space.

The allowed regions of the peptide plane orientations were used to determine all possible linkages of proper tetrahedral geometry at the $\text{C}\alpha$ atom joining successive peptide planes using the program FISI.³⁴ These linkages are the ψ, ϕ Ramachandran dihedral angles. Peptide structures were assembled from all possible combinations of sequential ψ, ϕ angles using the experimental data from the ten ^{15}N -labeled backbone sites. The program FISI was applied to determine the $\text{C}\alpha$ linkages of these 10 residues. The region of the peptide lacking experimental constraints containing residues 7 and 8 and 15 and 16 were modeled. This resulted in 32 structures assembled from the 7 pairs of ψ, ϕ dihedral angles consistent with the NMR constraints. These structures were then evaluated for a minimum energy conformation by applying the Xplor protocol for simulated annealing (SA), followed by an energy minimization.³⁵ The lowest energy structure was selected and its dihedral angles were used as the constraints for further energy minimization. A set of 50 structures was produced using the Xplor protocol for simulated annealing. An extended template for the 18-residue peptide was used as the starting structure. The simulated annealing was run at an initial temperature of 500°C using the dihedral constraint constant set at 400 with a tight range of $\pm 1^\circ$ around the set of 7 determined dihedral angles. The dihedral constraints for residues 15 and 16 were set to the α -helical values of $\phi = -65^\circ$ and $\psi = -40^\circ$ with a generous range set at $\pm 20^\circ$. The proline residue at position 7 in the helix was modeled in a minimum energy conformation corresponding to the most extended helix. This form of the helix used the left-handed helical region in the Ramachandran plot for the residue 6 preceding proline ($\phi = 55^\circ$ and $\psi = 90^\circ$) and a compact conformation ($\phi = -60^\circ$ and $\psi = -55^\circ$) for the proline residue.³⁶ The limits around these dihedral constraints were set loose as well to $\pm 20^\circ$. A gentle SA refinement protocol at 300°C was applied to further refine the 50 structures generated from the initial SA. The same dihedral constraints were applied. An

average structure was generated for the 47 refined structures consistent with the NMR dihedral constraints.

The lowest energy structure was then evaluated for the fit to the NMR data using the program BACK-TOR. This program used the ϕ and ψ angles from the peptide structure and a peptide plane that has a fully determined α,β angular orientation and then calculates sequentially the ^{15}N and $^{15}\text{N}\text{—}^1\text{H}$ frequencies. In this case, the peptide plane corresponding to Leu4 was used to calculate through Val6, then the calculation was restarted at the next set of consecutive measurements at Ile9 and calculated through to Leu17. The ^{15}N chemical shift frequencies and $^1\text{H}\text{—}^{15}\text{N}$ dipolar couplings for residues 5–6 and 10–17 in the peptide were calculated. The measured frequencies are compared to the calculated values in Table I. The experimental data fit to ± 2.4 ppm for the ^{15}N chemical shift and ± 0.4 kHz for the $^1\text{H}\text{—}^{15}\text{N}$ dipolar couplings. These values are within the experimental error of the measurements providing a confirmation for the average structure generated from the NMR constraints.

The orientation of the helix axis can be determined from the refined low energy structure with the backbone atoms displayed using the program Insight-II, as shown in Figure 5.

An alternate axis is assigned as the molecule axis at the peptide plane of Phe11. The molecule is then rotated by $\alpha = 187.2^\circ$ about z and $\beta = 84.6^\circ$ about y . A protein data base format file is output that matches this transformed structure. The orientation of the helix axis formed by the N—H vectors for residues 9–14 from this structure was evaluated relative to the bilayer normal (laboratory Y axis). The helix axis spanning residues 9–14 was found to make an angle of 8° with respect to the bilayer normal. Figure 5 shows the structure and topology of the M6(252–269, C252A) peptide determined from the experimental solid state NMR data.

An alternative way to determine of the topology of the helix axis is illustrated using the analysis of the “PISA wheel” presentation of the PISEMA spectra.^{37,38} Figure 6 shows calculated spectra for a series of tilt angles of a right-handed α -helical structure.

The panel labeled TM6 represents a superposition of the experimental data from the two-dimensional PISEMA spectra of the M6(252–269, C252A) peptide. By comparing the pattern labeled TM6, especially the dipolar frequency range, which is the more reliable parameter, to the calculated PISA wheel patterns, it could be determined that the helix tilt axis lies between 5° and 10° relative to the bilayer normal, in good agreement with the calculation of the helix tilt for residues 9–14 from the refined structure.

The solution NMR structure of the M6(252–269, C252A) peptide has been solved in a TFE/water (80%:20%) solvent system.³⁹ The comparison of the solid state NMR structure with this structure gives an overall RMSD of the backbone atoms of $1.15 \text{ \AA}$ and an RMSD for the backbone atoms of the helical span from residues 9–14 of $0.24 \text{ \AA}$. The comparison in Figure 7 demonstrates that the helical portions of the two structures are similar, although the N-terminal residues differ, largely because they are only loosely constrained by the solution NMR data.

ATR-FTIR Dichroism Studies

To gain independent insights into the structure of the M6(252–269, C252A) peptide in DMPC an infrared dichroism analysis was carried out. Our approach involved measuring order parameters for the peptide using ATR-FTIR methods.^{11, 40, 41} 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

$$S = [(E_x^2 - R^{\text{ATR}} E_y^2 + E_z^2) / (E_x^2 - R^{\text{ATR}} E_y^2 - 2E_z^2)] / [(3 \cos^2 \alpha - 1) / 2] \quad (3)$$

where E_x , E_y , and E_z are the integrated absorption coefficients, α is the angle between the principal transition dipole moment and the molecular director.⁴²

If we presume the film refractive index is independent of the wavelength, $E_x = 1.398$, $E_y = 1.516$, $E_z = 1.625$, Eq. (3) can be written in a simplified form⁴³:

$$S = [(R^{\text{ATR}} - 2) / (R^{\text{ATR}} + 1.45)] / [(3 \cos^2 \alpha - 1) / 2] \quad (4)$$

Angle $\alpha = 39^\circ$ for the amide I mode.⁴² The order parameter S is related to the tilt angle β from the normal of the internal reflection element by the following equation⁴²:

$$S = (3 \cos^2 \beta - 1) / 2 \quad (5)$$

Order parameters for the lipids are obtained from the symmetric and asymmetric stretching modes of the lipid methylene groups by setting $\alpha = 90^\circ$.

Figure 8A shows the measured ATR-FTIR absorbance spectra obtained between 3000 and 2800 cm^{-1} of the M6(252–269, C252A) peptide in DMPC multilayers using perpendicular or parallel polarized light. The peak at 2957 cm^{-1} represents lipid CH_3 asymmetric stretching vibrations. Absorbance maxima at 2919 and 2850 cm^{-1} represent the asymmetric and symmetric lipid CH_2 stretching vibrations, respectively.

Dichroic ratios and order parameters were calculated using each of these peaks. As seen in Table II, the averaged dichroic ratios for 2919 and 2850 cm^{-1} absorbencies are 1.10 and 1.05, respectively. The resulting averaged order parameters S_{lipid} for the 2919 and 2850 cm^{-1} peaks are 0.70 and 0.76, respectively. The averaged S for the lipid is 0.73. These results indicate that a well-ordered lipid multilayer is formed on the Ge crystal surface with a tilt angle of 25.1° from the normal of the multilayer.

The amide I absorbance maximum of M6(252–269, C252A) in the DMPC lipid multilayer (cf. Figure 8B) is at 1661 cm^{-1} supporting the presence of an α -helical secondary structure.⁴⁴ Shoulders at 1635 and 1685 cm^{-1} indicate a small amount of β -sheet in this sample. To eliminate contributions from the β -sheet structures the dichroic ratio of the amide I vibrational mode was measured using the deconvoluted spectra shown in Figure 8C. As summarized in Table II, the averaged dichroic ratios for amide I band is 4.09. The resulting order parameter for the amide I band averaged from 5 independent preparations of the peptide in DMPC multilayers is 0.93. Using this value the helix tilt angle relative to the membrane normal is calculated to be 12.5° . Due to the fact that an order parameter is influenced by both orientation and sample order, these results only indicate a maximum angle of 12.5° between the helix and the bilayer normal.

DISCUSSION

The study of peptides corresponding to sequences of integral membrane proteins and particularly of G protein-coupled receptors is gaining increased acceptance as a way to characterize the three dimensional structure of the native proteins.^{45, 46} Nonetheless, there

are only a few high-resolution studies of peptides from GPCRs in bilayers and even fewer comparisons between solution and bilayer results on the same peptide. Most detailed studies on segments of GPCRs involve peptide sequences of rhodopsin.^{47–49} These investigations concluded that the isolated loops of this receptor assume specific conformations in phosphate buffer pH 5.9, and that a 15 residue peptide representing about two-thirds of the 6th transmembrane domain assumes a helical structure in DMSO. NMR studies in solution have also been reported on peptide segments of a number of other membrane proteins^{50,51} and references therein.

It is widely assumed that ligand binding has an effect on the conformation of GPCRs resulting in the initiation of the signal transduction cascade. Transmembrane domains of GPCRs are increasingly seen as key elements in modulating signaling transduction. In fact, many constitutively active receptor mutants^{52–64} (receptor variants that do not require ligand to activate signal transduction due to a mutation that places them in the activated state) contain mutations in the sixth transmembrane domain. Ste2p is among those receptors that have been used to study constitutive mutations, and the 6th transmembrane domain has been found to harbor the strongest and most frequently identified constitutive mutations.^{65–69} Thus, elucidation of its structure by biophysical techniques is a step toward elucidating the structural basis for the key regulatory role played by this region of the receptor.

Previously, we reported biophysical studies on peptides corresponding to loops and the 6th and 7th transmembrane domains of Ste2p.^{39,70} CD studies indicated that the transmembrane peptides were helical in aqueous TFE and other organic solvents and had some tendency to form β -like structures in SDS and lipid vesicles. An NMR analysis of M6(252–269, C252A) in 20% aqueous TFE resulted in a high resolution structure that was helical at the carboxyl terminus and extended after the proline residue in the middle of the peptide.³⁹ Conditions suitable for studying this peptide in lipid micelles have not been identified yet.

Solid-state NMR was able to determine the three-dimensional structure of the M6(252–269, C252A) receptor domain in lipid bilayers. The structure was found to contain a right handed α -helix in the carboxy terminal residues 9–17 with a bend at proline resulting in an extension of the helix to the amino terminal end. This amino terminus forms an extended helix at residues 4–6. The helix axis at the carboxyl terminus was found to tilt at an angle of 8° with respect to the bilayer normal. The overall features of this high-resolution structure are quite similar to those found for this same peptide in 20% aqueous TFE. CD studies concluded that this peptide is not highly helical in 20 mM SDS.³⁹ Furthermore, this region of the receptor is thought to be conformationally sensitive.^{39, 67}

The solid state NMR results can be compared to those from ATR-FTIR dichroism for determining helix axis orientation. The ATR-FTIR measurements determined overall helix axis orientation using the amide I vibrational modes. This method resulted in a helix tilt of about 12.5°. The solid state NMR measurements are assigned to individual peptide planes in the structure. Therefore the absolute helix axis can be evaluated for the specific regions of the peptide that span a right-handed α -helix. It was determined that the helix axis spans residues 9–17. As discussed above the helix tilt for this region of the peptide is 8° relative to the bilayer normal. The good agreement between these independent methods provides confidence that M6(252–269, C252A) assumes the structure calculated from the solid state NMR study. However, it is important to note that whereas solid state NMR is looking at individual amide sites, ATR-FTIR is evaluating an ensemble average of all amide bond orientations. Nevertheless, recent advances in site-directed infrared dichroism theory and selective labeling with ¹³C have enabled the helical tilt angle and pitch for specific residues in the M2 protein from influenza A virus to be determined.^{28, 71} Given the difficulty in

studying intact receptors in membranes it would seem logical to apply multipronged spectroscopic approaches to study their structures.

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FIGURE 1.

One-dimensional solid state ^{15}N NMR spectra of specifically labeled M6(252–269, C252A) peptides oriented in DMPC bilayers at 5°C. ^{15}N -labeled sites are indicated on the spectra. A 1 ms phase alternated mixing time at a 52 kHz field strength was used for cross-polarization, with a 63 kHz field strength used for ^1H decoupling during the 5 ms acquisition time. Approximately 20,000 transients were collected for each spectrum on a home-built spectrometer with a ^1H resonance frequency of 700 MHz.

FIGURE 2.

Two-dimensional PISEMA spectra collected of specifically labeled M6(252–269, C252A) peptide oriented in DMPC bilayers at 5°C. For each of 32 t_1 increments, 1024 transients were collected. A 7 s recycle time was employed to avoid sample heating. A 1 ms mixing time at a 52 kHz field strength was used for cross-polarization, with a 63 kHz field strength used for ^1H decoupling during the 5 ms acquisition time. Data were processed using Felix-ND (Biosym) with an exponential apodization of 400 Hz applied to the t_2 dimension. The matrix was zero filled to 512×512 real data points. ^{15}N -labeled sites are indicated on the spectra.

FIGURE 3.

The representation of the α, β angles relating the molecular axis system to the laboratory axis frame (X, Y, Z) for a peptide bond.

FIGURE 4.

The α , β restriction plots of peptide planar orientations corresponding to the measured ^{15}N chemical shift and ^1H - ^{15}N dipolar coupling frequencies for the residues of the M6(252–269,C25A) peptide indicated above each plot.

FIGURE 5.

Lowest energy refined structure of M6(252–269,C25A) in oriented lipid bilayers. Red NH amide bonds indicate the measured sites from solid state NMR constraints. The helix axis relative to the bilayer normal is indicated in green.

FIGURE 6.

The “Pisa wheel” representation of the PISEMA spectra for a right-handed α -helix with the specified tilt axis relative to the bilayer normal. The top left spectrum is the experimental values measured for the M6(252–269, C252A) peptide.

FIGURE 7.

The comparison of (A) the solution NMR structure of M6(252–269, C252A) in 20% TFE to (B) the solid state NMR structure of M6(252–269, C252A) in DMPC bilayers.

FIGURE 8.

(A) Parallel (solid line) and perpendicular (dotted line) polarized light ATR-FTIR spectra of the methylene stretching modes region of the DMPC bilayers on germanium surface in the presence of M6(252–269, C252A) peptide. (B) Parallel (solid line) and perpendicular (dotted line) polarized light ATR-FTIR spectra of the amide I region of dried M6(252–269, C252A) peptide in DMPC bilayers on germanium surface. (C) Fourier self-deconvoluted spectrum of B using a band width at half height of 13 cm^{-1} and a resolution enhancement²⁸ of 2.0.

Table I

^{15}N Chemical Shift Frequencies and ^{15}N — ^1H Dipolar Coupling Frequencies of the Selectively ^{15}N -Labeled Amide Sites in the M6(252–269, C252A) Peptide in Oriented DMPC Bilayers

Residue	^{15}N Chemical Shift (ppm)		^{15}N — ^1H Dipolar Frequency (kHz)	
	Experimental	Calculated ^a	Experimental	Calculated ^a
Leu-4	155.0 ± 2.5	—	3.2 ± 0.5	—
Leu-5	219.6 ± 2.5	217.3	16.6 ± 0.5	14.4
Val-6	202.1 ± 2.5	208.3	16.9 ± 0.5	17.2
Ile-9	214.6 ± 2.5	—	16.6 ± 0.5	—
Ile-10	219.4 ± 2.5	219.7	18.0 ± 0.5	18.2
Phe-11	219.5 ± 2.5	220.8	19.5 ± 0.5	18.8
Ile-12	215.3 ± 2.5	218.4	18.1 ± 0.5	17.6
Leu-13	209.8 ± 2.5	214.4	17.6 ± 0.5	18.2
Ala-14	210.2 ± 2.5	208.8	15.2 ± 0.5	15.4
Leu-17	207.0 ± 2.5	206.6	15.9 ± 0.5	14.2

^aCalculated values for ^{15}N frequencies and ^{15}N — ^1H dipolar frequencies are derived from the minimized refined NMR structure for the M6(252–269, C252A) peptide using the program BACKTOR.

Table II

ATR-FTIR Dichroic Ratios R^{ATR} , Order Parameters S , and Calculated Tilt Angles β of DMPC Lipid Multilayer and M6(252–269, C252A) Peptide

Parameters	Vibration Mode		
	Peptide Amide I α -Helix 1661 cm^{-1}	Lipid CH_2 2919 cm^{-1}	Lipid CH_2 2850 cm^{-1}
$R^{\text{ATR},a}$	4.09 ± 0.17	1.10 ± 0.01	1.05 ± 0.02
S^b	0.93 ± 0.05	0.70 ± 0.02	0.76 ± 0.02
Tilt angle β (deg)	12.5 ± 4.9	26.6 ± 0.9	24.0 ± 1.0

^a R^{ATR} is the average observed dichroic ratio based on peak area from 1650 to 1675 cm^{-1} for peptide and peak height at 2919 and 2850 cm^{-1} for lipid from five independent experiments.

^b S is the order parameter calculated from the average dichroic ratio R^{ATR} .