Rotational orientation of monomers within a designed homo-oligomer transmembrane helical bundle

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Abstract

A peptide designed to form a homo-oligomeric transmembrane helical bundle was reconstituted into lipid bilayers and studied by using ²H NMR (nuclear magnetic resonance) with magic angle spinning to confirm that the helical interface corresponds to the interface intended in the design. The peptide belongs to a family of model peptides derived from a membrane-solubilized version of the water-soluble coiled-coil GCN4-P1. The variant studied here contains two asparagines thought to engage in interhelical hydrogen bonding critical to the formation of a stable trimer. For the NMR studies, three different peptides were synthesized, each with one of three consecutive leucines in the transmembrane region deuterium labeled. Prior to NMR data collection, polarized infrared spectroscopy was used to establish that the peptides were reconstituted in lipid bilayers in a transmembrane helical conformation. The ²H NMR line shapes of the three different peptides are consistent with a trimer structure formed by the designed peptide that is stabilized by interhelical hydrogen bonding of asparagines at positions 7 and 14.

Keywords: transmembrane peptides; helix association; ²H MAS NMR; ATR-FTIR

De novo design has proved a useful approach for testing features required for the folding of membrane proteins (DeGrado et al. 2003). A series of recent studies on homo-oligomerizing designed peptides has been used to decipher interactions that govern the association of transmembrane (TM) helical peptides (Zhou et al. 2001; Lear et al. 2003). To confirm and test design hypotheses, various biophysical methods, including gel electrophoresis, analytical ultracentrifugation, and fluorescence resonant energy transfer, have been used to characterize the oligomeric state of designed TM helical bundles. Essential for further analysis of de-

signed peptides and constructive feedback into the design process is the determination of molecular structure. Obtaining full three-dimensional high-resolution structures of membrane protein and peptide complexes is a challenge. However, local structural information on helix-helix interactions is very valuable and can often be readily obtained by using mutational or biophysical methods. Knowledge of the amino acids that mediate helix contacts in combination with data on the oligomeric state provides tight constraints on the structure of TM helical bundles in membrane bilayers.

Scanning mutagenesis along the length of the hydrophobic sequence, followed by gel electrophoresis, has been used to locate interfacial regions in TM helical bundles (Simmernan et al. 1996; Ruan et al. 2004). An alternative method that avoids the need for extensive peptide production is the use of ²H NMR spectroscopy (Ying et al. 2000). In this case, the motion of deuterated amino acid side-chains is measured around at least one turn of a TM helix. The ²H NMR line shape provides direct information on side-chain

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dynamics in hydrated lipid bilayers above the lipid phase transition temperature. The side-chains packed within a helix–helix interface exhibit restricted motion relative to side-chains oriented toward the surrounding lipid bilayer (Ying et al. 2000).

In this study, ^2H NMR is used to establish the rotational orientation of a TM peptide designed to form a homo-oligomeric helical bundle. The peptide belongs to a family of model peptides derived from a membrane-solubilized version of the water-soluble coiled-coil GCN4-P1 (Zhou et al. 2001; Lear et al. 2003). The variant studied here (GCN4MS1-N7N14) forms a stable trimer, as demonstrated by analytical ultracentrifugation and SDS–polyacrylamide gel electrophoresis (Lear et al. 2003). The specificity and strength of association of this particular peptide is attributed to two asparagine residues located at positions 7 and 14 that are located in “a” positions of the helical repeat and are thought to engage in interhelical hydrogen bonding (Fig. 1). For the ^2H NMR studies, three different peptides were synthesized, each with a single deuterium-labeled leucine at either position 8, 9, or 10 in the peptide sequence. These leucines correspond to positions “b,” “c,” and “d” of the helical repeat and are located between the two critical asparagines. L8 and L9 are in “b” and “c” positions and are expected to face the lipid bilayer, while L10 is in the “d” position and is predicted to be in the helical interface.

Results and Discussion

Polarized IR spectroscopy

Polarized infrared (IR) spectroscopy is an effective way to assess reconstitution of peptides embedded in lipid bilayers

Figure 1. Sequences of the three 29-residue GCN4MS1-N7N14 synthetic peptides studied. The deuterium-labeled leucine sites are shaded. The asparagines thought to be involved in interhelical hydrogen bonding are underlined. The helical wheel diagram shows one heptad of a three helical bundle viewed down the bundle axis. Sequences are labeled with their postulated corresponding positions in the heptad repeat. B represents the unnatural amino acid β -alanine.

Figure 2. Polarized IR spectra of GCN4MS1-N7N14. Spectra of GCN4MS1-N7N14 reconstituted into DMPC bilayers were obtained using parallel (solid line) and perpendicularly (dashed line) polarized light. The amide I vibration at 1655 cm^{-1} and the amide II vibration at 1546 cm^{-1} indicate that the peptide is helical. The dichroic ratio of the amide I vibration is 3.4 and indicates a transmembrane orientation.

(Fringeli 1993; Tamm and Tatulian 1997; Smith et al. 2002). Figure 2 presents attenuated total reflection (ATR) FTIR spectra of the homo-oligomeric TM helical bundle of GCN4MS1-N7N14 reconstituted into dimyristoylphosphatidylcholine (DMPC) bilayers. The spectra were obtained using light having perpendicular (dashed line) or parallel (solid line) polarization relative to the membrane bilayer surface. The region of the IR spectrum containing the amide I and amide II normal modes is shown. The amide I vibration at 1655 cm^{-1} and the amide II vibration at 1546 cm^{-1} are diagnostic of α -helical secondary structure (Tamm and Tatulian 1997). Although both the amide I and II normal modes are sensitive to protein secondary structure, the amide I vibration is the most widely used to determine protein conformation. The dichroic ratio of the amide I band is sensitive to the orientation of the GCN4MS1-N7N14 helix relative to the plane of membrane. The observed dichroic ratio of 3.4 corresponds to a helix tilt of $\sim 13^\circ$ relative to the membrane normal (see Materials and Methods). These data indicate that the GCN4MS1-N7N14 peptides have a helical conformation with a TM orientation in DMPC bilayers.

Deuterium magic angle spinning NMR spectroscopy

Deuterium NMR spectroscopy has been used extensively to study lipid dynamics (Bloom and Smith 1985). In contrast, ^2H NMR studies of membrane proteins reconstituted into lipid bilayers at high lipid–protein ratios have been limited by low sensitivity. To overcome this problem, we take advantage of the increased sensitivity afforded by magic angle spinning (MAS). MAS averages the quadrupolar interaction and reduces the usually broad deuterium line shape into a series of spinning side bands. The intensities of the side bands in the MAS deuterium spectrum trace out the broad deuterium line shape.

The strategy in these experiments is to deuterate consecutive leucines in the sequence of the GCN4MS1-N7N14 peptide. A single deuterated methyl group at the end of the highly flexible leucine side-chain provides a sensitive probe of molecular motion. We have previously shown that the motion of amino acids located in TM helix interfaces is restricted relative to amino acids oriented toward the lipid membrane (Ying et al. 2000). By characterizing side-chain motion as a function of the TM sequence, one can map out the interface of interacting TM helices.

^2H MAS spectra of the three leucine-labeled peptides incorporated into DMPC bilayers are shown in Figure 3. The MAS side bands are spaced at 3 kHz (the spinning frequency), and their intensities trace out the deuterium line shape. The intense resonance in the center of each spectrum is due to residual HDO, while the rest of the signal arises from a δ -methyl labeled (CD_3) leucine. Because of rapid methyl group rotation, the three deuterium sites in CD_3 are equivalent. Rapid methyl group rotation reduces the quadrupole coupling constant to a maximum value of ~ 40 kHz compared with the maximum theoretical value of ~ 127 kHz in the rigid limit (Keniry 1989). Additional rotations and librations about the $\text{C}\alpha\text{--C}\beta$ and $\text{C}\beta\text{--C}\gamma$ bonds, as well as rotational diffusion about the axis of the trimer, further reduce the quadrupolar interaction and produce the observed

Figure 3. Deuterium MAS NMR spectra of GCN4MS1-N7N14. MAS NMR spectra were obtained of GCN4MS1-N7N14 peptides specifically deuterated at a single methyl group of L8 (A), L9 (B), or L10 (C). The peptides were reconstituted into DMPC multilayers at a 1:100 peptide-lipid molar ratio and hydrated with ~ 50 weight% deuterium-depleted water. The NMR rotors typically contain ~ 3 mg peptide reconstituted with 60 mg lipid. The MAS spectra were obtained using single pulse excitation at a deuterium frequency of 55.2 MHz with the sample spinning at $3000 \text{ Hz} \pm 3 \text{ Hz}$.

Figure 4. Simulations of deuterium MAS NMR of GCN4MS1-N7N14 deuterium labeled at L8 (A), L9 (B), and L10 (C). The simulations were carried out by using the program SIMPSON version 1.1.0. The MAS rate was set to 3 kHz, and the asymmetry parameter was set to 1.0. The best fits to the observed sideband intensities were obtained by using quadrupole coupling constants of 14 kHz (A), 14 kHz (B), and 18 kHz (C).

line shapes (Batchelder et al. 1982). These motions result in narrowing of the spinning sideband manifold and a line shape with an asymmetry parameter (η) close to 1.0.

The high flexibility of the leucine side-chain limits analysis of the deuterium line shape by detailed spectral simulation. To quantify the observed differences in the breadth of the deuterium line shapes, MAS deuterium spectra were simulated by using the quadrupole coupling constant as the only free parameter. Figure 4 presents the simulated spectra that reproduce the spectral intensities observed in the experimental spectra presented in Figure 3. The L10 spectrum (Fig. 4C) has the broadest line shape, with a calculated quadrupole coupling of 18 kHz. The broader L10 line shape compared with that of L8 (Fig. 4A; 14 Hz) and L9 (Fig. 4B; 14 Hz) indicates a restriction in side-chain motion consistent with L10 interacting with protein side-chains on neighboring helices, whereas L8 and L9 are facing lipid.

Computational modeling

The GCN4MS1-N7N14 sequence was modeled on a parallel C3-symmetric left-handed trimeric coiled-coil. The choice of a parallel trimer was based on recent studies showing that the two N-terminal asparagines of GCN4MS1-N7N14 interact cooperatively relative to single and double mutants (Lear et al. 2003). Mutation of the asparagines reduces trimer stability, indicating they form interhelical hy-

drogen bonds. A parallel arrangement positions these asparagines such that the interhelical interactions can be formed. An antiparallel arrangement would require a large staggering of the bundle. Additionally, an antiparallel trimer lacks symmetry, which is incompatible with the observation of a single backbone asparagine NMR resonance for the Asn ^{15}N -labeled GCN4-MS1 trimer in micelles (Gratkowski et al. 2002). Preliminary model building suggested that given the heptad separation of the two asparagines, a left-handed coiled-coil would place the asparagines in the same position relative to the helix–helix interface. We therefore modeled the structure using a library of backbone left-handed coiled-coils, although we acknowledge that the practical difference between a coiled-coil and a gently curved α -helix is minor for the length of peptides studied here.

Figure 5 shows a cross section of a molecular model of GCN4MS1-N7N14 at the level of L8 to L10 that is consistent with the ^2H NMR data and the designed structure as pictured in Figure 1. L8 and L9 at the “b” and “c” positions of the helical wheel face outward from the bundle. L10 at the “d” position is packed in the interior of the helical bundle. N7 and N14 at the “a” positions above and below the targeted leucines form interhelical hydrogen bonded rings in the low energy structure.

Based on geometric considerations for optimal helix packing, Chothia et al. (1981) predicted that the crossing angles for right- and left-handed helices should be -52° and 23° , respectively. The helix tilt angle determined from the polarized IR measurements of 13° relative to the bilayer normal for GCN4MS1-N7N14 corresponds to a crossing angle of 26° , consistent with a left-handed crossing geometry. The left-handed computational model provides a good fit to a supercoiled bundle radius of $6.4 \text{ \AA}$. This is compatible with radii of other GCN4 derived water-soluble trimers

Figure 5. Cross sections of the computational model of the GCN4MS1-N7N14 trimer. L10 is in the bundle interior, while L8 and 9 are on the bundle surface (*left*). The Asn residues are located in the core of the bundle and form two interhelical hydrogen bonded rings. The N7 ring is highlighted in the cross section (*right*). Carbons are shown in green, oxygens in red, and nitrogens in blue. The N14 ring is below the N7 ring and shown in gray.

with polar core residue substitutions, which are $6.0\text{--}6.3 \text{ \AA}$ (Akey et al. 2001). The supercoil pitch of the water-soluble series of peptides varies between 90 and $100 \text{ \AA}$, which is smaller than the $180 \text{ \AA}$ used in the computational model presented here.

Conclusions

In summary, we have used ^2H MAS NMR spectroscopy to determine the rotational orientation of the TM helices within the trimer structure formed by the designed peptide GCN4MS1-N7N14. The ^2H NMR line shapes of the three consecutive leucines are consistent with a trimer structure that is stabilized by interhelical hydrogen bonding of asparagines at positions 7 and 14. More generally, the study describes a simple approach for mapping the interface of interacting TM helices. The approach complements gel electrophoresis, analytical ultracentrifugation, and fluorescence resonant energy transfer, which provide information on the oligomerization states of membrane proteins and TM peptides. Helix interactions are important in the folding of polytopic membrane proteins and in the oligomerization of membrane proteins and peptides having a single TM helix. De novo design in combination with ^2H MAS NMR provides a way to understand and test the interactions that govern the association of the TM helices in these proteins.

Materials and methods

Peptide synthesis

Peptides were synthesized by using Fmoc chemistry on an Applied Biosystems 433A peptide synthesizer as previously described (Lear et al. 2003) but with the following modification. L-Leu d3(5,5,5) was purchased from Cambridge Isotope Laboratories and the Fmoc group attached using Fmoc-OSu (9-fluorenylmethylsuccinimidyl carbonate). At positions to be isotopically labeled, the resin was removed from the synthesizer and the Fmoc-L-Leu d3(5,5,5) manually coupled before returning to the synthesizer for the completion of the synthesis. Peptides were purified using reversed-phase HPLC on a C4 preparative column using a linear gradient at 10 mL/min of buffer A (99.9% water, 0.1% TFA) and buffer B (60% 2-propanol, 30% acetonitrile, 10% water, and 0.1% TFA). The purity of peptides was assessed by analytical HPLC and the molecular weights confirmed with matrix-assisted laser desorption ionization mass spectrometry.

Reconstitution into lipid bilayers

HPLC purified peptides were cosolubilized with DMPC (Avanti Polar Lipids) in ethanol at a peptide–lipid molar ratio of 1:100. Ethanol was removed under a stream of nitrogen and the peptide/lipid films placed under high vacuum overnight to remove traces of solvent. The samples were then hydrated with deuterium-depleted water, vortexed, and incubated above the gel–liquid crystalline phase temperature of DMPC.

Polarized infrared spectroscopy

Polarized ATR FTIR spectra were obtained on a Bruker IFS 66V/S spectrometer. Multilamellar vesicles at a concentration of 10 mg lipid/mL were layered on a germanium internal reflection element using a slow flow of air directed at an oblique angle to the ATR plate to form an oriented multilamellar lipid-peptide film. ATR FTIR spectra were acquired at a resolution of 4 cm⁻¹ using parallel and perpendicularly polarized light. Each spectrum represents the average of 1000 transients, and a background spectrum of the germanium plate without sample was subtracted in each case.

The dichroic ratio (R^{ATR}) is defined as the ratio between absorption of parallel ($A_{||}$) and perpendicular ($A_{\perp}$) polarized light (Smith et al. 2002) and was used to calculate the order parameter S_{meas} using the equation,

$$S_{meas} = \frac{E_x^2 - R^{ATR} E_y^2 + E_z^2}{E_x^2 - R^{ATR} E_y^2 - 2E_z^2} = \left[\frac{3\cos^2 \theta - 1}{2} \right] \left[\frac{3\cos^2 \alpha - 1}{2} \right] S_{mem} \quad (1)$$

where θ is the angle between the helix director and the normal of the internal-reflection element, and α is the angle between the helix director and the transition-dipole moment of the amide I vibrational mode. The order parameter depends on the electric field amplitudes, $E_x = 1.399$, $E_y = 1.514$, and $E_z = 1.621$, in thick film limit, which is applicable for the concentration of lipid used here.

For our calculations, the value of α (42.4°) was derived from parallel experiments on bacteriorhodopsin, where the average helix tilt (14°) is known independently from the crystal structure (Goor-maghtigh et al. 1999). S_{mem} is an order parameter that accounts for disorder in the orientation of the reconstituted peptide. The value of α used for our calculations implies a value of S_{mem} between 0.8 and 0.9, corresponding the order parameter calculated for bacteriorhodopsin from measurements of mosaic spread (see Smith et al. 2002). We assume that the amount of order cannot be more than that for the highly ordered two-dimensional purple membranes containing bacteriorhodopsin. As a result, the value we estimate for the helix tilt of GCN4MS1-N7N14 (~13°) is an upper limit for the angle between the helix axis and the bilayer normal. If the peptide orientation is more disordered than bacteriorhodopsin, then the true helix tilt for GCN4MS1-N7N14 will be <13°. The dichroic ratios of the asymmetric and symmetric lipid CH₂ stretching modes at 2924 cm⁻¹ and 2852 cm⁻¹ were 1.19 and 1.09, respectively, which correspond to order parameters of 0.64 and 0.68, and are consistent with well-oriented lipids (Smith et al. 1994).

Deuterium NMR spectroscopy

Deuterium NMR spectra were obtained at a ²H frequency of 55.2 MHz on a Bruker Avance NMR spectrometer using a 4-mm MAS probe. A MAS frequency of 3 kHz was used to increase the number of spinning side bands. Single pulse excitation was employed using a 4.2-μsec 90° pulse, followed by a 30-μsec delay before data acquisition. The repetition delay was 0.25-sec. A total of 600,000–800,000 transients were averaged for each spectrum and processed using a 200-Hz exponential line broadening function. Spectra were obtained at 30°C.

MAS deuterium spectra were simulated using the program SIMPSON version 1.1.0 (Bak et al. 2000) with a MAS frequency of 3 kHz. The asymmetry parameter was set to 1.0 to reflect extensive motional averaging. Only the quadrupole coupling constant was varied to quantify the breadth of the line shape.

Molecular modeling

Computational searches were performed to obtain the lowest energy structure of the GCN4MS1-N7N14 trimer consistent with the NMR data. A library of possible trimer structures was generated from bundles of three ideal α -helices (3.5 residues/turn) that differed in the radius of the bundle and the rotational orientation of the individual helices. A supercoil pitch of 180 Å was subsequently applied to each bundle (Betz and DeGrado 1996; Dieckmann and DeGrado 1997; Pinto et al. 1997). Two sets of searches were performed: The first sampled over radii between 5.0 and 8.0 Å in 0.25 Å increments and over 360° of helix rotation in 10° increments, and the second search expanded on the global minimum of the first, sampling between 6.3 and 6.7 Å in 0.1 Å increments and over 20° of rotational space in 2° increments. For each backbone sampled, side-chain conformations were selected using the Goldstein Dead End Elimination criterion (Desmet et al. 1992; Goldstein 1994) followed by exhaustive enumeration of the remaining conformations. A backbone-dependent rotamer library was used to limit the conformational space to α -helix-compatible conformations (Bower et al. 1997). Only on-rotamer conformations were sampled, and the same library was used in both coarse- and fine-grained conformational searches. Energies were calculated using a van der Waals (vdW) 12-6 potential and a hydrogen bonding 12-10 potential function and a cosine angular dependence term based on donor and acceptor hybridization state (Gordon et al. 1999). Atom radii and well-depth parameters for vdW energies were taken from the united-atom set of AMBER parameters (Weiner et al. 1984). Hydrogen bonds were uniformly modeled with an ideal distance of 2.8 Å and a well depth of 2.0 kcal/mol. No electrostatics terms were included. All backbone generation and side-chain optimization was performed using the protCAD library of protein manipulation tools (Summa 2002). The lowest energy structure from the second search was subject to further minimization using DISCOVER97.0 (MSI/Biosym) and the AMBER force field (Weiner et al. 1984). The final structure had a radius of 6.4 Å and the helix rotation shown in Figure 5.

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