Helical Structure and Orientation of Melittin in Dispersed Phospholipid Membranes from Amide Exchange Analysis in Situ†

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ABSTRACT: A trapping method combined with high-resolution nuclear magnetic resonance spectroscopy is described for the measurement of hydrogen–deuterium exchange rates for individual amides of polypeptides bound to fully hydrated, dispersed phospholipid bilayers. Exchange rates were measured for 22 of the 24 amide hydrogens of bee venom melittin bound to bilayers composed of egg phosphatidylcholine/phosphatidylserine (88:12, mol/mol) dispersed in 20 mM sodium acetate, pH 4.0. Amides of residues 5–11 and 16–22 had exchange rates suppressed by between 30- and 1000-fold, and the rate suppression exhibited a helical periodicity with amides on the hydrophobic helix face up to 20-fold more stable than those on the hydrophilic face of the helix. These results demonstrate that under the conditions studied melittin adopts a helical conformation with stable helical hydrogen bonds extending to residue 22 and that the helix is oriented with the hydrophobic face directed toward the membrane interior.

The analysis of the kinetics of hydrogen exchange from peptide amides of proteins using NMR[1] spectroscopy has made significant contributions to the study of protein structure, dynamics, and folding. The method relies on the findings that the dominant contribution to the suppression of hydrogen exchange from backbone peptide amides is their participation within the hydrogen bonds that define protein secondary structure and that exchange of hydrogen-bonded amides occurs during transient backbone fluctuations that free the amide for exchange with solvent, either by local or global unfolding of secondary structure into a solvent-accessible state or by more local structural fluctuations involving permeation of solvent to the site of exchange (Woodward et al., 1982; Wagner, 1983; Englander & Kallenbach, 1984). Measurement of hydrogen exchange kinetics for all the amides resolvable in the high-resolution NMR spectrum of a small soluble protein can yield detailed information on hydrogen-bond stabilities and backbone fluctuations at potentially every amide site throughout the protein structure (Wagner, 1983; Wüthrich et al., 1984; Dempsey, 1986; Torchia et al., 1989; Gooley et al., 1992).

The application of amide exchange analysis to membrane proteins has not followed the advances brought about by the use of NMR spectroscopy because high-resolution NMR methods that allow the resolution of individual amide resonances cannot be applied to membrane systems. Studies of amide exchange from proteins in membranes using bulk tritium or deuterium exchange have shown that the number of slowly exchanging amides in bacteriorhodopsin corresponds to the number expected to be hydrogen bonded within transmembrane helices (Downer et al., 1986), that some side-chain amide groups, probably hydrogen-bonded within the membrane domain, exchange slowly from rhodopsin (Englander et al., 1982), and that several amides within the transmembrane domain of erythrocyte glycophorin may be virtually unexchangeable (Sami & Dempsey, 1988). Definition of the exchange-stable amides in these proteins should contribute to the definition ofthe topology of the proteins in the membrane. To date, however, detailed amide-resolved exchange kinetics from membrane peptides and proteins have only been measured after isolation into "isotropic" media as described for melittin and an analogue in methanol (Dempsey, 1988, 1992) and the M13 viral coat protein in detergent micelles (Henry & Sykes, 1990, 1992).

In this report we describe an approach to measuring amide exchange kinetics for individual backbone amides from bee venom melittin reconstituted in fully hydrated, dispersed phospholipid bilayer membranes. Melittin (Habermann, 1972) is a 26 amino acid membrane-active peptide that induces voltage-dependent ion conductance at nanomolar concentrations, hemolysis at micromolar concentrations, and reversible disc micellization of membranes composed of saturated phosphatidylcholine at high peptide to lipid ratios [reviewed in Dempsey (1990)]. Despite conclusions made from ion channel recordings together with consideration of the crystal structure of the aqueous tetramer (Terwilliger & Eisenberg, 1982) that the ion-conducting state consists of associated helical monomers in a transbilayer orientation (Tosteson & Tosteson, 1981; Hanke et al., 1983), biophysical studies in a number of systems have yielded conflicting interpretations on the resting state of the peptide in the absence of a membrane potential that are only now being resolved in terms of variations in the structural features of melittin–lipid complexes that depend on the physical state (largely the degree of hydration) of the bilayers and (apparently) errors in interpretation of fluorescence energy transfer in membranes using chemically modified melittin (Frey & Tamm, 1991; John & Jahnig, 1991). Here we describe the measurement of amide exchange kinetics for 22 of the 24 backbone peptide amides of melittin reconstituted in fully hydrated, dispersed phospholipid bilayer membranes using a trapping technique combined with high-resolution ^{1}H NMR spectroscopy. A marked helical periodicity in the exchange rates of the membrane-bound peptide allows the extent of helical hydrogen bonding and the orientation of the helix in the membrane to be defined.

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[1] Abbreviations: 2D, two dimensional; ECOSY, exclusive correlated spectroscopy; HOHAHA, homonuclear Hartmann–Hahn experiment; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PS, phosphatidylserine.

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MATERIALS AND METHODS

Melittin was purified from bee venom by ion-exchange chromatography on SP-Sephadex C25 (Gauldie et al., 1976). All membrane preparations were free of phospholipase A_2-derived lipid hydrolysis products as determined by thin layer chromatography following exchange experiments. Bee venom phospholipase A_2 is inactive at the pH (pH 4.0) of the experiments described here (Dempsey & Watts, 1987). Egg phosphatidylcholine (PC, grade 1) and phosphatidylserine (PS) were purchased from Lipid Products, South Nutfield, Redhill, Surrey, U.K.

Amide Exchange Experiments. Membranes were prepared by mixing lipids [PC/PS (88:12, mol/mol)] in $CHCl_3/CH_3OH$ (2:1 v/v) and drying under high vacuum before resuspending at 125 mg mL^{-1} in H_2O (1 mM EDTA) containing sufficient melittin to give the required peptide to lipid ratio. The bilayers were incubated for 1 h at 30–35 °C and freeze-thawed six times before lyophilization. Deuterium NMR experiments have shown that melittin partitions completely into negatively charged membranes under these conditions (Dempsey et al., 1989). Hydrogen–deuterium exchange was initiated by resuspending the dried membranes into exchange buffer (20 mM sodium acetate, pH* 4.0, in D_2O). Samples were removed throughout a time course of exchange at 21 °C, rapidly frozen in a bath of dry ice–isopropanol, and lyophilized. During final lyophilization, the frozen exchange samples were maintained at a temperature of −10 °C by immersing the containers in partially frozen 2 M NaCl in order to limit any artifactual exchange during the drying process that occurred if the drying samples were left at room temperature. NMR spectra were measured immediately after dissolving the dried lipid/melittin residue in deuteromethanol containing a precalibrated concentration of DCl sufficient to bring the pH of the methanolic solution to a pH where amide exchange from melittin is slow (Dempsey, 1988). In the exchange experiments described here, the final membrane composition was PC/PS/melittin (88:12:2, mol/mol/mol), and the lipid concentration was 125 mg mL^{-1} in exchange buffer. Results obtained for membrane-bound melittin at 1 mol % relative to total lipid (94% egg PC and 6% PS), which is so far the lowest melittin to lipid ratio accessible using the method described, were similar to those described in the following sections for melittin at 2 mol %.

NMR Spectroscopy. One-dimensional (1D) ^1H NMR spectra were obtained at 25 °C with a JEOL alpha spectrometer operating at 500 MHz for protons, using Guassian selective excitation pulses of 2 ms, centered around 7.9 ppm. The pulse attenuation was calibrated to give 270° pulses which results in refocusing of large frequency-dependent phase errors arising from long pulses (Emsley & Bodenhausen, 1989). Bessel frequency filters set at 1000 Hz either side of the excitation frequency were used for additional suppression of signals outside the spectral region of interest. 1D spectra were obtained in about 3 min (64 scans with a 0.7-s relaxation delay into 16K complex data points). Two-dimensional (2D) phase-sensitive ECOSY spectra (Griesinger et al., 1985) and 2D HOHOHA spectra (Davis & Bax, 1983) were measured at 15 °C using nonselective excitation pulses, and the spectra were carefully phased to allow observation of the weak cross peaks appearing at very low contour levels. The spectra were obtained in 6 h with 256 time increments of 2048 complex points. The data were zero filled twice in t_1 and Fourier transformed after applying shifted sine bell window functions in t_1 and t_2. All spectra were processed using JEOL EDL software. Amide exchange rates were determined from semilogarithmic plots of amide signal intensity in the ^1H NMR spectra of melittin solubilized after increasing periods of exchange in the membrane. The exchange rates were corrected for sequence-dependent inductive contributions to exchange by using the data of Molday et al. (1972).

RESULTS AND DISCUSSION

To determine the structural properties of melittin in fully hydrated, dispersed bilayers using the amide exchange method, we have chosen to study the peptide in membranes composed of phosphatidylcholine with sufficient phosphatidylserine to neutralize the excess positive charge density arising from the presence of melittin (six positive charges at pH's below pH 6.5) in the bilayer. This allows the direct comparison of experiments at different melittin concentrations without altering the net electrostatic charge at the membrane surface which can affect base-catalyzed amide exchange rates due to concentration of exchange catalyst (hydroxide anion) at the membrane surface in solutions of low ionic strength and stabilization of negatively charged exchange intermediates (Englander & Kallenbach, 1984). Melittin partitions completely into these membranes when dried lipid is hydrated in melittin-containing buffer, and deuterium NMR experiments on membranes of similar composition have shown that the charges on PS and melittin are effectively neutralized by matching the molar composition of the charged species but that this charge neutralization does not involve discrete PS–melittin complexes with lifetimes greater than microseconds (Dempsey et al., 1989); i.e., all components of the membrane are in diffusional exchange in the plane of the membrane.

Dissolving the lyophilized membranes in methanol results in cosolubilization of the phospholipids, giving rise to large (up to 10^5-fold) excess intensity in the lipid signals resulting in degradation of the melittin amide signals by dynamic range effects. Although widely dispersed over a chemical shift range between about 0.8 and 5.5 ppm, the lipid signals occur well away from the peptide amide signals (downfield of 7.5 ppm) allowing selective excitation of the amide region of the ^1H NMR spectrum using phase-refocusing 270° Gaussian excitation pulses. Figure 1 shows a series of ^1H NMR spectra of the amide region of melittin from deuteromethanol-solubilized melittin–lipid complexes obtained after increasing periods of hydrogen–deuterium exchange in the membrane-associated state. The amide signals in the ^1H NMR spectrum of melittin in methanol have been assigned to individual amino acids in the peptide sequence (Bazzo et al., 1988), and the hydrogen exchange properties of individual amides in that solvent are well characterized (Dempsey, 1988, 1992). Despite the presence of up to 100-fold molar excess of lipids, the resolution of the spectra and the characteristic chemical shifts of the amide signals are comparable to those for melittin alone, indicating that the peptide does not form complexes with the lipids in methanol that perturb the spectral properties of the amide signals.[2] The differences between the zero time spectrum and the spectra at increasing times result only from the different periods of exchange in the membrane-bound state, and individual exchange rates for the 16 melittin amides that are resolvable in the one-dimensional spectra were obtained directly from these spectra. Multiexponential decays of signal intensity arising from unresolved amide signals can

[2] Although the NH signals of melittin are unperturbed by the presence of 100-fold molar excess of codissolved membrane lipids, melittin is more soluble in CD_3OD in the presence of lipids, indicating that some degree of association may occur.

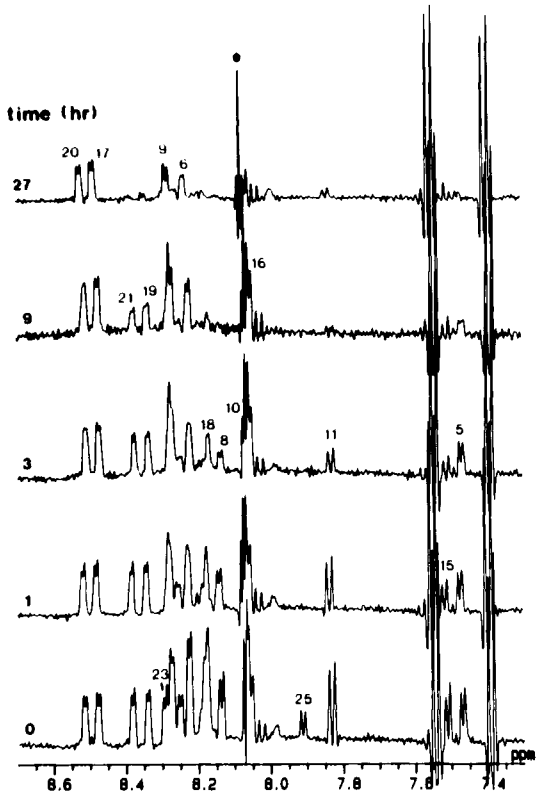

FIGURE 1: Amide region of 500-MHz ^{1}H NMR spectra of melittin solubilized into deuteromethanol after increasing periods of hydrogen–deuterium exchange in the membrane-bound state. The amide signals are numbered according to the amino acid sequence in the spectrum in which the signal is last observed. The signal marked with an asterisk is an impurity.

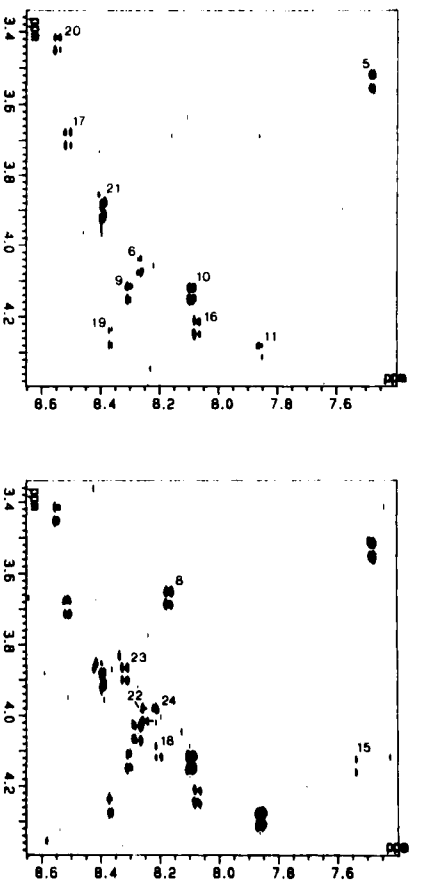

FIGURE 2: Fingerprint region of 2D ECOSY spectra of melittin solubilized into deuteromethanol after 0 (bottom) and 3 h (above) of amide exchange in the membrane-bound state.

be resolved if the decay rates differ by at least 5-fold, and the rates can be assigned to specific amides by measuring two-dimensional spectra throughout the exchange time course (Dempsey, 1992). Figure 2 shows, for example, that L9 is the slowest exchanging amide of the unresolved signals at 8.28 ppm and that S18 is the slowest of the unresolved signals at 8.19 ppm. The exchange rates of rapidly exchanging amides were more conveniently monitored by exchange-in experiments in which backbone-deuterated peptide (prepared by incubating melittin in D_2O at 50 °C, pH 7, for 1 h) was allowed to exchange on the bilayer with acetate buffer in H_2O. Figure 3 shows the time-dependent increase in amide signal intensity in deuteromethanol-solubilized membranes during an exchange-in experiment. 2D ECOSY spectra obtained from exchange-in samples were used to confirm the assignments shown in the one-dimensional spectra of Figure 3. Both the NH and CHα resonances of L6 and K7 are unresolved in methanol (Bazzo et al., 1988), precluding assignment of exchange rates for these resonances from the fingerprint region of ECOSY spectra. The NH signals of L6 and K7 were distinguished using 2D HOHAHA experiments which identified L6 as the slowly exchanging amide in exchange-out experiments from identification of the side-chain β proton signal and K7 as the rapidly exchanging amide in exchange-in experiments from identification of the β- and δ-methylene signals of the K7 side chain (not shown). Comparison of one- or two-dimensional spectra of CD_3OD-solublized bilayer complexes after long exchange-out (Figures 1 and 2) and short exchange-in periods (Figure 3) qualitatively distinguishes classes of amides in membrane-bound melittin having high and low exchange stabilities, respectively. Exchange rates for all the melittin amides were determined except for those of G12 and L13 which are unresolved in the ^{1}H NMR spectrum

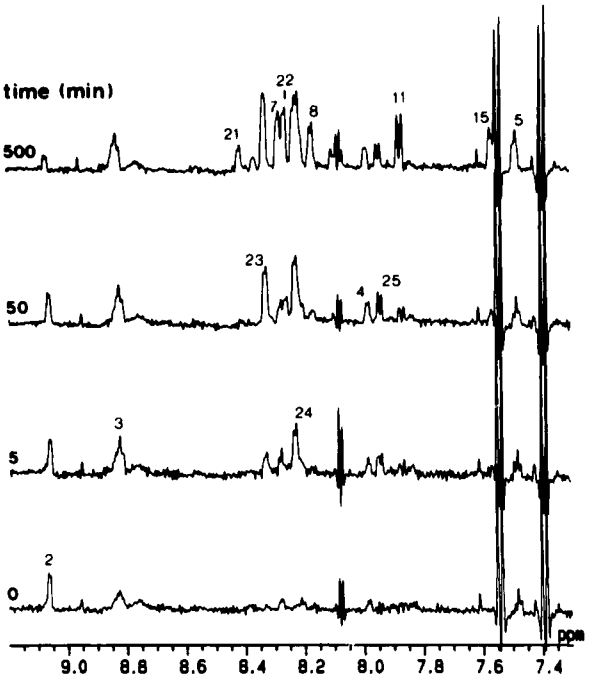

FIGURE 3: Amide region of 500-MHz ^{1}H NMR spectra of melittin solubilized into deuteromethanol after increasing periods of exchange-in (see text) in the membrane-bound state.

of melittin in deuteromethanol and also exchange too quickly for assignment by two-dimensional NMR in that solvent (Dempsey, 1988).

When the exchange rates of the amide hydrogens of membrane-bound melittin are plotted along the peptide sequence, two features are apparent (Figure 4a): amides of

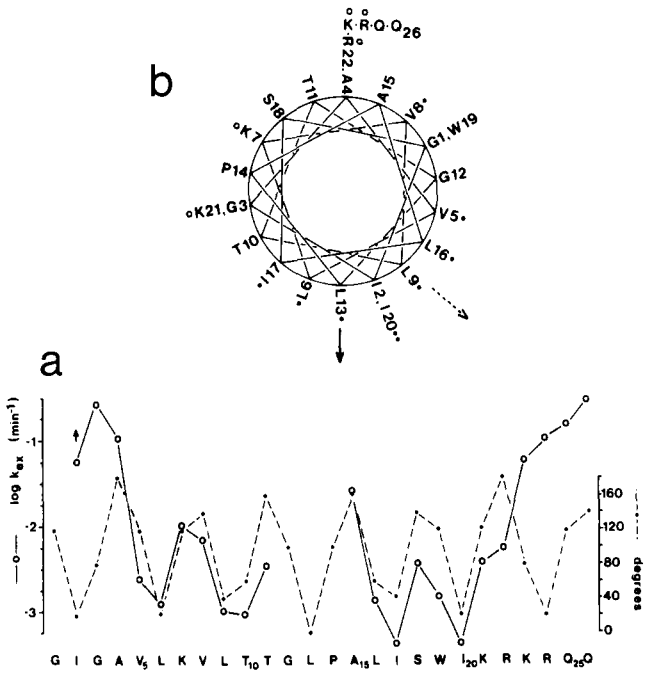

FIGURE 4: (a) Profile of the logarithms of the amide exchange rates of membrane-bound melittin at pH 4.0 as a function of amino acid sequence (○) fitted to the angular variation of amino acid residues around an ideal α-helix (●). The exchange rates have been corrected for sequence-dependent inductive effects using the data of Molday et al. (1972). The vertical arrow above I2 indicates that this amide exchanges with a rate faster than the maximum rate experimentally measurable. (b) Helical wheel distribution of amino acids around an ideal melittin α-helix illustrating features determined in the present study. Helical hydrogen bonding stops at residue R22, and the side of the helix containing the exchange-stable amides is shown with a heavy arrow. Amino acids with very nonpolar side chains are marked with a dot; small open circles mark amino acids with polar side chains. The direction of the amphipathic moment for an ideal α-helix encompassing residues 1–22 is indicated with a dotted arrow.

residues 5–11 and 16–22 have rates suppressed by 30–1000-fold relative to the terminal residues (2–4 and 23–26), and the stable group of amides exhibit a marked periodicity in their exchange rates. This periodicity maps closely to the periodicity of an α-helix (Figure 4a). The exchange stabilities and helical periodicity of the nonterminal residues is consistent with the formation of an α-helix starting at the N-terminus (the amide of Ala-5 is the first NH expected to be hydrogen bonded in an α-helix) and extending to the NH of R22. The effect of P14 on the hydrogen-bond properties of the helix was not determined because of the absence of exchange data for G12 and L13 NH's, but the low exchange stability of A15 NH indicates that, as in methanol (Dempsey, 1992), P14 causes the expected destabilization of helical hydrogen bonds around residues 12–15. Amides in the helical regions having enhanced exchange stabilities are expected to be on the side of the helix extending away from contact with water above the membrane. The best fit of the experimental to the helical periodicity was estimated by sliding the experimental profile of rates horizontally across the helical periodicity and choosing the register at which differences between the two periodicities were minimized. The best fit (Figure 4a) orients the helix so that L13 is pointing down into the membrane (solid arrow in Figure 4b). This orientation differs somewhat from that expected if the helix were oriented along the direction of the amphipathic moment (Eisenberg et al., 1982) for an α-helix encompassing residues 1–22 (dotted arrow of Figure 4b). The difference may be due to the uncertainty in fitting the experimental periodicity to the helical periodicity and to the fact that the amino acid side chains are oriented along the radius enclosing the backbone CHα for calculating the amphipathic moment. Reorientation of the long side chains of R and K residues which make a large contribution to the amphipathic moment may cause a significant shift in the direction of the real amphipathic moment of the peptide. The disruption of stable C-terminal helical hydrogen bonding after R22 keeps the charged side chains of K23 and R24 from projecting onto the hydrophobic face of the helix (Figure 4b). In a study of the accessibilities of spin-labeled membrane-bound melittins to aqueous spin relaxation probes (Altenbach & Hubbel, 1988), evidence was obtained that the side chain of K23 was oriented away from contact with water, consistent with the continuation of the helix of a surface-lying peptide at least through residue 23. In that study the K23 side chain was modified with a nitroxide spin label converting the very polar side chain into a nonpolar one. This modification may be sufficient to promote the continuation of the peptide helix through residue 23 in the membrane-bound peptide. In native melittin, the rapid exchange of K23 NH [and the remainder of the C-terminal residues (Figure 4a)] indicates that the last four residues do not adopt stable helical structure in the native peptide in charge-neutralized membranes.

The structural features described here refer to melittin in charge-neutralized PC/PS bilayers. In pure egg phosphatidylcholine bilayers, there is no particular distribution of exchange rates among the melittin amides which all exchange about 10^4 times more quickly than the most stable amides described here (not shown). This is not necessarily inconsistent with strong evidence that melittin adopts an α-helical conformation in egg PC bilayers (Drake & Hider, 1979; Vogel, 1981) but indicates that the hydrogen-bonded structure is less stable in PC membranes without the presence of negatively charged lipids and that the dominant conformational fluctuation(s) that limits amide exchange involves cooperative unfolding of structure perhaps accompanying transient dissociation from the bilayer into an unstructured state in solution. In contrast to the isolated melittin helix in methanol where cooperative helix fluctuations probably underlie amide exchange (Dempsey, 1988, 1992), the helical periodicity in exchange from the PC/PS membrane-bound peptide demonstrates that amide exchange is limited by multiple structural fluctuations and perhaps variations in water penetration to the site of exchange. The latter situation is similar to the amide exchange properties of a covalently stabilized coiled-coil leucine zipper peptide dimer in which a helical periodicity in exchange rates was also observed (Goodman & Kim, 1991).

Because the in situ amide exchange procedure described here is a trapping method in which the exchange properties of individual amides are assayed by extraction into an isotropic medium, the structural features measurable by amide exchange analysis may be determined under any chosen condition. The method may be useful for studying topological properties of small membrane proteins, many of which have ^{1}H NMR spectral assignments in organic solvents that solubilize membranes (Moody et al., 1987; Tappin et al., 1988; Bruch et al., 1989; Barsukov et al., 1990). The extreme exchange slowing factors for peptide amides hydrogen bonded within transmembrane helical regions (Sami & Dempsey, 1988) indicates that the transmembrane helices of these proteins may be identified as well as the conformational properties of the linking regions in the aqueous phase above the membrane.

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