Deuterium/Hydrogen Exchange Factors Measured by Solution Nuclear Magnetic Resonance Spectroscopy as Indicators of the Structure and Topology of Membrane Proteins

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ABSTRACT Deuterium/hydrogen exchange factors (χ) were measured for the backbone amide sites of the membrane-bound forms of the 50-residue fd coat protein and the 23-residue magainin2 peptide in lipid micelles by solution nuclear magnetic resonance spectroscopy. By combining kinetic and thermodynamic effects, deuterium/hydrogen exchange factors overcome the principal limitations encountered in the measurements of kinetic protection factors and thermodynamic fractionation factors for membrane proteins. The magnitudes of the exchange factors can be correlated with the structure and topology of membrane-associated polypeptides. In fd coat protein, residues in the transmembrane helix have exchange factors that are substantially smaller than those in the amphipathic surface helix or the loop connecting the two helices. For the amphipathic helical peptide, magainin2, the exchange factors of residues exposed to the solvent are appreciably larger than those that face the hydrocarbon portion of membrane bilayers. These examples demonstrate that deuterium/hydrogen exchange factors can be measured by solution NMR spectroscopy and used to identify residues in transmembrane helices as well as to determine the polarity of amphipathic helices in membrane proteins.

INTRODUCTION

The structural analysis of helical membrane proteins begins with the identification of those residues in hydrophobic transmembrane helices. Many algorithms have been applied to the open reading frames of genome sequences for this purpose (Deber et al., 2001; Feng and Zhang, 2000; Jacoboni et al., 2001; Lecompte et al., 2001; Liakopoulos et al., 2001; Lolkema and Slotboom, 1998; Rayan et al., 2000; Tusnady and Simon, 2001). On the other hand, experimental determinations of the structure and topology of helical membrane proteins have been limited by the availability of samples suitable for structure determination by nuclear magnetic resonance (NMR) spectroscopy or x-ray crystallography. Even in those cases where it has been possible to obtain resolved and assigned two-dimensional solution NMR spectra of membrane proteins in lipid micelles, it has been difficult or impossible to perform exchange experiments that yield measurements of kinetic protection factors or fractionation factors throughout the polypeptide. Under most experimental conditions, residues in amphipathic helices exchange too rapidly for the measurement of kinetic protection factors and those in hydrophobic helices too slowly to reach equilibrium for the measurement of thermodynamic fractionation factors. In this article, we describe an adaptation of widely used solution NMR exchange experiments that provides measurements of deuterium/hydrogen exchange factors that are highly informative about the structure and topology of helical membrane proteins.

The 50-residue coat protein is the major structural component of fd bacteriophage, and large amounts accumulate in the cell membrane prior to virus assembly. It is a typical membrane protein. It has a short amphipathic helix that lies in the plane of the bilayer and a relatively long hydrophobic transmembrane helix that spans the membrane bilayers; the two helices are connected by residues that form a structured loop; residues at both the N and C termini are mobile on most timescales; and there is evidence of conformational flexibility in the loop connecting the two helices on relatively slow timescales (McDonnell et al., 1993; Almeida and Opella, 1997). Dynamics are essential to the biological roles of this protein because its structure undergoes a major change during assembly, as the amphipathic helix tilts approximately 90° from the perpendicular to become almost parallel to the transmembrane helix.

The kinetics of deuterium/hydrogen exchange at many individual backbone amide sites of fd coat protein has been characterized (Henry and Sykes, 1990; O'Neil and Sykes, 1988). The finding that residues 28 to 45 undergo very slow exchange, whereas residues 7 to 20 exchange more rapidly, but not as fast as unstructured residues, is consistent with the dynamics characterized from line-shape and relaxation measurements on the protein in membrane environments. Although the characterization of its motions over a wide range of time scales complements the experimentally determined structure of the protein in describing its biological functions, its three-dimensional structure is essential for the interpretation of its dynamics. Because the determination of the complete three-dimensional structures of membrane proteins remains a difficult and lengthy task, it would be highly desirable to be able to characterize key aspects of

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their structures at an early stage of the investigations through exchange measurements.

Hydrophobic transmembrane helices are the dominant structural element of many membrane proteins. Transmembrane helices are generally very stable, primarily because of the large amount of energy needed to break individual hydrogen bonds in the polypeptide backbone (White and Wimley, 1999a,b). Hydrogen bonds not only stabilize the secondary structure but also contribute to tertiary folding and assembly, conferring on membrane proteins exceptional thermal stability and resistance to denaturing agents. Therefore, the strength of the hydrogen bonds in the polypeptide backbone of these proteins provides a source of structural information, independent of other experimental data or calculations. Because most of the membrane protein structures determined so far have limited resolution, it is not possible to identify all of the hydrogen bonds with confidence. Only in the recently determined 1.55-Å resolution structure of bacteriorhodopsin can the role of the network of hydrogen bonds in stabilizing the protein structure be fully appreciated (Luecke et al., 1999).

NMR spectroscopy is a well-established method for characterizing hydrogen bonds in proteins. Hydrogen bonds can be identified from characteristic patterns of homonuclear ^{1}H nuclear Overhauser enhancements (NOEs) (Wagner and Wuthrich, 1982; Wuthrich, 1986), kinetic protection factors (Englander and Kallenbach, 1984; Li and Woodward, 1999 and references therein), thermodynamic fractionation factors (Kreevoy and Liang, 1980; Loh and Markley, 1994; Harris and Mildvan, 1999), magnetization transfer (Spera et al., 1991; for review, see Dempsey, 2001), and more recently from scalar couplings (Cornilescu et al., 1999). Unfortunately, all of these high-resolution NMR approaches have substantial limitations in the case of membrane proteins, which require the presence of lipids for stabilization of their native conformations and functions. Although lipids that form micelles can be used to solubilize membrane proteins for solution NMR studies, the aggregates are relatively large and have inconvenient relaxation properties. The difficulties arising from the broad line widths are compounded by the limited chemical shift dispersion caused by their predominantly α-helical secondary structure, and by the relatively high temperatures and pH values that are typically necessary to obtain high-resolution solution NMR spectra. Therefore, the high-resolution studies of H/D exchange are limited to very few examples (Henry and Sykes, 1994; Morein et al., 1996; Sessions et al., 1998; Williams et al., 1996; Zhang et al., 1995).

One promising approach for characterizing H/D exchange in membrane-bound proteins is the so-called "exchange trapping method" (Dempsey, 1998, 2001; Dempsey and Handcock, 1996; and references therein). This method relies on the relative high solubility of membrane proteins in organic solvents, "trapping" the lipid-peptide complexes in solvents where the amide exchange is intrinsically slow. Although, this method and its variants have been used to characterize the hydrogen exchange of membrane bound peptides and specific sites in small membrane proteins (Dempsey, 1998, 2001; Dempsey and Handcock, 1996; Czerski et al., 2000), it requires the optimization of both protein reconstitution in the appropriate lipid environment and an additional organic solvent scan to solubilize the protein-lipid complexes. In the trapping method, it is crucial to identify the correct mixture of organic solvents for a correct solubilization of the protein-lipid complexes to avoid the appearance of structural heterogeneity (e.g., multiple peaks in the heteronuclear single quantum correlation (HSQC) spectra) or the unwanted posttrap H/D exchange that would complicate the data analysis (Czerski et al., 2000).

A more robust method to determine the H/D exchange has been reported by Cross and co-workers using solid-state NMR spectroscopy in lipid reconstituted membrane proteins (Cotten et al., 1999a,b). By monitoring ^{15}N-D dipolar interactions in orientated samples, these researchers have characterized the details of the hydrogen-deuterium exchange for the insertion of the dimeric gramicidin into a bilayer environment (Cotten et al., 1999a,b; Huo et al., 1996).

Through the use of uniformly isotopically labeled proteins and careful optimization of sample conditions, including temperature and pH, we have been able to resolve and assign all backbone resonances in two-dimensional HSQC spectra of a variety of helical membrane proteins with between 23 and 122 residues in lipid micelles (Opella, 1997). Because the intensity of a correlation resonance directly reflects the amount of ^{1}H bonded to the ^{15}N, these spectra can be used to determine the relative amounts of deuterium or hydrogen bonded to each amide nitrogen in the protein backbone.

Deuterium/hydrogen fractionation has been widely used to characterize the hydrogen bond strength of alcohols, phenols, carboxylic acids, and other compounds (Kreevoy and Liang, 1980). It has also been used to determine the stability of transition states in enzymes (Loh and Markley, 1994; Harris and Mildvan, 1999). D/H fractionation methods are based on thermodynamic rather than the more commonly used kinetic criteria. The exchange reaction described by Eq. 1 is carried out in solutions prepared with known amounts of D_2O and H_2O. The equilibrium constant (Φ) of the reaction is called the fractionation factor.

$$NH + D\ (bulk) = ND + H\ (bulk) \quad (1)$$

$$\Phi = [ND][H]/[NH][D] \quad (2)$$

Φ values greater than 1.0 indicate that the amide nitrogen site prefers deuterium to hydrogen. The equilibrium position of the reaction depends on the relative zero-point vibrational energies of the N-H and N-D bonds. Amide nitrogens that participate in strong hydrogen bonds preferentially

bind ^1H over ^2H in mixed D_2O/H_2O solutions and have correspondingly larger resonance intensities in NMR spectra (Edison et al., 1995a,b).

The measurement of exchange factors, as described in this article, combines kinetic protection factors and thermodynamic fractionation factors to show more accurately the structural features of helical membrane proteins in lipid micelles. The method consists of measuring the protection factors after a specific amount of time has been allowed to elapse in samples containing different fractions of D_2O and H_2O. In the case of both fd coat protein and magainin2, a 30-min incubation period at each temperature was necessary to reach a quasistationary state. Peak volumes did not change significantly during the next 3-h period, enabling the HSQC spectra to be acquired under stable conditions. The optimal time interval is likely to be protein specific, however, we found that the same 30-min interval worked well for these two quite different helical membrane proteins. The results for fd coat protein in lipid micelles show a remarkable correlation between the measured D/H exchange factors and the structures determined by NMR spectroscopy (Almeida and Opella, 1997). Moreover, the effects of temperature can be measured and the activation energies of the exchange processes calculated. In addition to the examples in the figures, we have obtained preliminary results on membrane proteins with between one and four transmembrane helices that suggest that measurements of D/H exchange factors may be generally applicable to the identification and characterization of transmembrane helices in membrane proteins.

D/H exchange factor measurements can also be applied to amphipathic in-plane helices, the second major type of secondary structure in membrane proteins. Magainin2 is a 23-residue antibiotic peptide associated with membranes (Zasloff, 1987). It is soluble and unstructured in aqueous solution but folds into an amphipathic helix in the presence of lipids (Gesell et al., 1997). It is among the best-characterized membrane associated peptides, and its structure has been determined in micelles by solution NMR spectroscopy. There is a strong correlation between the hydrophobicity of its residue side chains as plotted on a helical wheel and their exchange factors. Further, these results are consistent with the structural findings from solid-state NMR spectroscopy showing the polarity of the magainin2 helix that lies in the plane of the membrane bilayer (Marassi et al., 2000).

MATERIALS AND METHODS

Sample preparation

Uniformly ^{15}N-labeled fd coat protein was expressed and purified as previously described (McDonnell et al., 1993). The samples for NMR spectroscopy were prepared in 5-mm tubes (Shigemi, Allison Park, PA). The protein concentration was 1.9 mM; the d_{25}-sodium dodecyl sulfate (Cambridge Isotope Laboratories, Woburn, MA) concentration was 480 mM in aqueous solution; the pH was 4.0; and the sample volume was 0.3 ml. Magainin2 samples were prepared by dissolving 5 mg of peptide and 80 mg of d_{25}-sodium dodecyl sulfate in 0.5 ml of water. The solution was 20 mm in NaCl, 5 mM in citrate, and the pH of the sample was adjusted to 4.0 (Gesell et al., 1997).

Samples were lyophilized and resuspended in solutions containing 5%, 10%, 30%, 50%, 70%, and 90% D_2O, respectively, and the HSQC spectra were obtained after a fixed 30-min incubation period. This period was sufficient for the intensities to reach a stationary state, because the peak volumes did not change significantly during the next 3-h period.

NMR spectroscopy

The NMR experiments were performed on a Bruker DMX 600 NMR spectrometer. The two-dimensional correlation spectra were acquired using the fast-HSQC pulse sequence (Mori et al., 1995) with a gradient water flip back. A relatively long recycle delay (4 s) was used to minimize the effects of the increasing amount of deuterium in the solution. A typical data set consists of 512 complex points in the acquisition dimension and 256 t_1 increments. The data were processed using the FELIX97 software package (MSI, San Diego, CA). A sine bell window function shifted by 90° was applied in both dimensions. Zero filling was performed in both dimensions to give a final data set with 1 K × 512 real points.

Measurements of D/H exchange factors

The volumes of the amide resonances in the two-dimensional spectra were measured using the routines in FELIX97. The values were normalized to those found in the sample with 5% D_2O and 95% H_2O. The peak volumes were plotted as a function of the mole fraction of H_2O in the solution, and the exchange factors, χ, were determined using Eq. 3.

$$(y\mathrm{C})^{-1} = \chi(1 - X)/X + 1 \tag{3}$$

in which y is the peak volumes and X the mole fraction of H_2O in the solution. The χ values plotted in the figures represent the mean of two separate experiments. The experiments were performed at 35°C, 40°C, 45°C, 55°C, and 65°C, respectively, for fd coat protein and at 30°C for magainin2.

RESULTS

The backbone amide $^1H/^{15}N$ correlation resonances of uniformly ^{15}N-labeled fd coat protein in micelles have been assigned (Almeida and Opella, 1997). The resolution of the two-dimensional HSQC spectrum is such that the volumes for all of the resonances could be measured reliably from spectra obtained between 35°C and 50°C. At temperatures higher than 55°C, most of the resonances from residues in the loop and the amphipathic helix disappear, whereas resonances from residues in the transmembrane helix are still present and undergo only minor changes in chemical shift frequencies. At temperatures lower than ~35°C, many of the resonances are broad. To obtain high-resolution NMR spectra, it is therefore necessary to limit the H/D exchange factor measurements to the 35 to 50°C temperature range.

The method consists of measuring the amide resonance volume as a function of the increasing amount of D_2O after waiting a fixed amount of time (30 min) to reach a quasistationary state. The experiment is then repeated at various

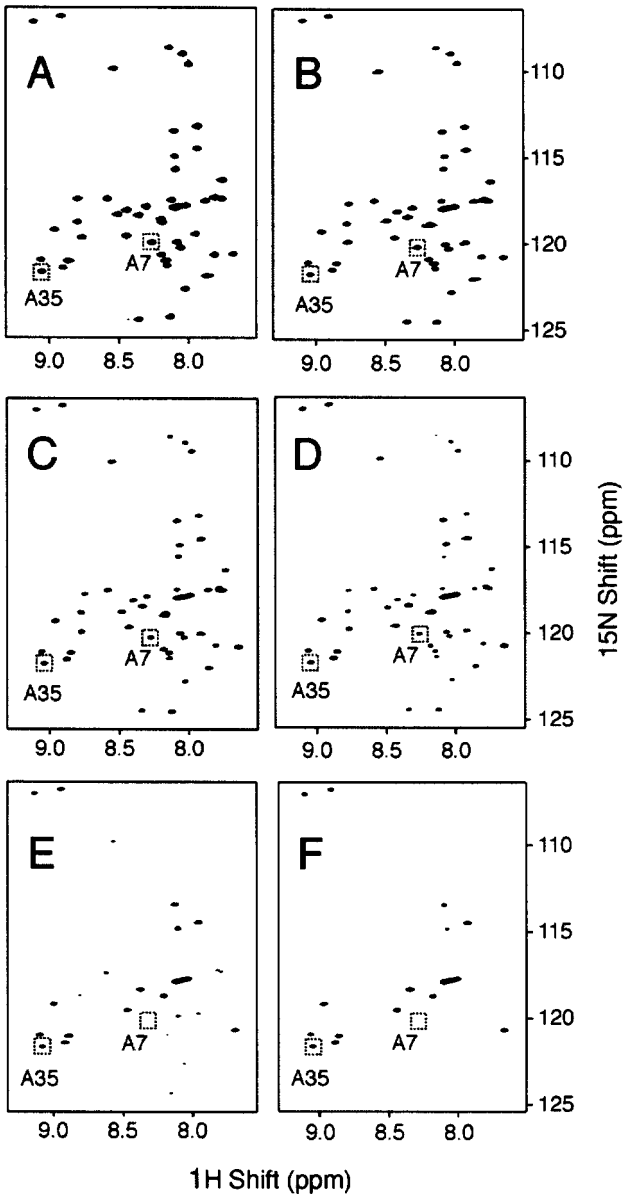

FIGURE 1 Two-dimensional HSQC spectra of fd coat protein in lipid micelles at 5%, 10%, 30%, 50%, 70%, and 90% D_2O, respectively. Residues A7 and A35, in the amphipathic and transmembrane domains, respectively, are indicated by the dotted boxes.

temperatures. Fig. 1 presents two-dimensional HSQC spectra of fd coat protein in lipid micelles at 35°C. In the HSQC spectra the resonances from two alanine residues of fd coat protein are highlighted, one in the transmembrane helix (A35) and the other near the end of the amphipathic helix (A7). The data in Fig. 1 illustrate that, upon increasing of D_2O concentration, there is a dramatic variation in peak volumes that reflects the strengths of hydrogen bonds at each site. The exchange factors (χ) calculated according to Eq. 3 are not related to the type of residue, because both are alanines, but rather reflect the protein structure.

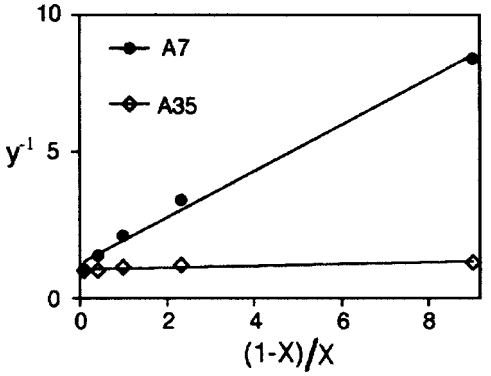

FIGURE 2 The relative NH peak volumes of A7 and A35 plotted in the form given by Eq. 3. The experimental errors for are 0.04 as estimated from reproducibility of duplicate measurements.

Fig. 2 contains a plot of the normalized volumes of the A7 and A35 resonances as a function of the H_2O mole fraction at 35°C. The slopes of the plots for these two residues are quite different, yielding exchange factor values of 0.43 and 0.20 for A7 and A35, respectively.

The relationship between fd coat protein secondary structure and the χ values is illustrated by the bar graph in Fig. 3 *A*. Because residues 1 to 5 are mobile and unstructured on most timescales (Almeida and Opella, 1997), and proline 6 does not have an observable resonance in HSQC spectra, the data for structural analysis are limited to residues 7 to 50. From the analysis of the bar graph at each temperature, it is possible to distinguish two different populations of χ values (higher values for the N and C terminus, the structured loop, and the amphipathic helix) and lower values for the transmembrane domain (residues 27–42). The mean value of χ in the transmembrane domain is 0.2 at 35°C and 0.007 at 45°C, which is dramatically lower than the χ values of other residues located in both the amphipatic helix or the loop between the helices. These structural features identified by the exchange factors show a remarkable correlation with the structure of fd coat protein in micelles (Almeida and Opella, 1997).

The calculated χ values display significant temperature dependence. The mean value of χ ranges from 0.75 at 35°C to 1.4 at 55°C, which is significantly larger than the fractionation factors measured for random coil polypeptides (Bowers and Klevit, 1996). As with the fractionation factors (Lin et al., 1998), upon increasing the temperature over a range from 35°C to 65°C, the values for the exchange factors first decrease and then increase. At low temperatures, the dipolar contribution to the NMR signal is predominant and results in a positive slope, whereas at higher temperatures the exchange contribution of the proton with the solvent prevails and gives a negative slope. Conse-

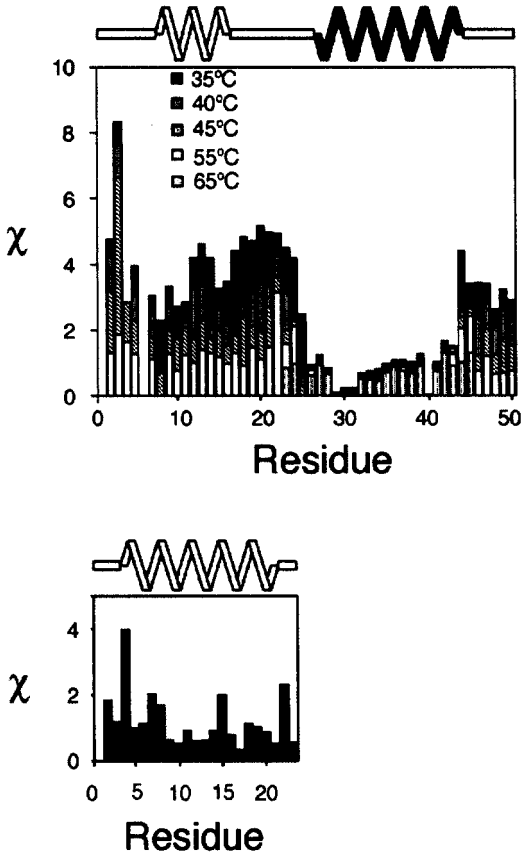

FIGURE 3 Exchange factors for backbone amide hydrogens. (*A*) fd coat protein in lipid micelles at different temperature. (*B*) Magainin2 in lipid micelles at 35°C.

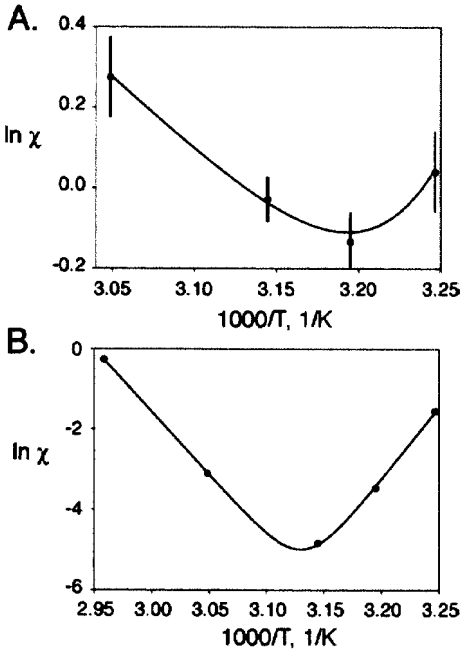

FIGURE 4 (*A*) Temperature dependence of exchange factors for the amphipatic and loop residues. The values for the constants were 11 and −37 kcal/mol for C_d and C_{ex}, respectively. (*B*) Temperature dependence of structural factors for the TM helix. The values for the constant were 92 and −118 kcal/mol for C_d and C_{ex}. The error bars in *A* represent the rmsd of the exchange factors from the average value calculated over the amphipathic and loop residues. In *B* the deviations from the average are contained within the dots representing the experimental points.

quently, the H/D total activation (ΔE_{tot}) of the process can be divided into two dominant contributions:

$$E_{tot} = E_d + E_{ex} \quad (4)$$

in which E_d is the activation energy for the dipolar term and E_{ex} is the activation energy for the exchange process. Therefore, it is possible to rewrite the Arrhenius equation:

$$\ln \chi = \ln[\exp(-E_d/RT + C_d) + \exp(-E_{ex}/RT + C_{ex})] \quad (5)$$

in which C_d and C_{ex} are constants and represent the intercepts of the two lines at infinite temperatures in the plot ln χ versus $1/T$. The values for the activation energies were calculated using a nonlinear least squares fitting routine in the Sigmaplot software package (SPSS Inc., Chicago, IL). The activation energy calculated for the fd coat protein transmembrane helix is 62 kcal/mol, corresponding to a contribution of ~3.9 kcal/mol for each residue in the helix. The activation energy for the amphipathic helix and the loop is 7 kcal/mol, corresponding to a contribution of 0.24 kcal/mol for each residue.

The temperature dependencies of the average exchange factors of the amphipathic and hydrophobic helices are shown in Fig. 4, *A* and *B*, respectively. The inverse temperature dependence is biphasic. To determine the calorimetric enthalpy, the same approximation used in the determination of the activation energy for kinetic exchange is used (Harris et al., 1997; Harris and Mildvan, 1999). The two contributions to the total calorimetric activation energy, dipolar and exchange, can be calculated from Eq. 5. The curve calculated from the transmembrane helix (Fig. 4 *A*) has a minimum at ~47.5°C. In the case of the amphipathic helix and the connecting loop (Fig. 4 *A*), the minimum is shifted to ~40.0°C. As for the kinetics analysis, the two terms, dipolar and exchange, have unequal and opposite temperature dependencies. The values for E_d and E_{ex} are listed in Table 1. The calorimetric activation energy for the exchange contribution is ~9 times higher for the transmembrane helix than

TABLE 1 Activation energies for fd coat protein in lipid micelles

	Average E_{ex} (kcal/mol)	Average E_d (kcal/mol)	Number of residues
Amphyphatic helix + loop	7 ± 1	−44 ± 2	29
Transmembrane helix	62 ± 2	−72 ± 2	16

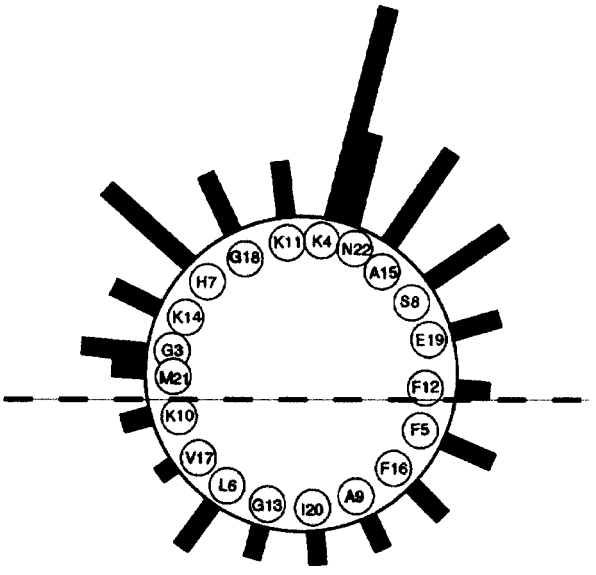

FIGURE 5 Helical wheel representation of magainin2 with exchange factors. The hydrophobic residues are colored in gray.

for the amphipathic helix, whereas their dipolar contributions differ by only approximately a factor of 1.6.

The exchange factors for fd coat protein were determined starting with fully deuterated amide sites (obtained by preexchanging the protein in D_2O) and increasing the amount of H_2O until reaching a final solution containing 95% H_2O 5% D_2O. The values of the exchange factors were similar for all sites, indicating that the measurements can be made starting with either hydrogens or deuterons on the amide groups in the backbone of the protein.

Magainin2 gives well-resolved two-dimensional homonuclear ^1H NMR spectra in micelles (Gesell et al., 1997), making it possible to measure peak volumes for essentially all of the amide backbone sites. Experiments were performed on samples of magainin2 containing a range of ratios of D_2O and H_2O at 30°C. The bar graph in Fig. 3 *B* shows the fractionation factors measured for each residue. Unlike χ values of the fd transmembrane helix, χ values for magainin2 have an apparent periodicity. The helical wheel representation of the exchange factors (Fig. 5) is revealing, showing a correlation between the exchange factors and the hydrophobicity of the residue.

Residues on the hydrophobic side of the amphipathic helix have a mean exchange factor of 0.7. In contrast, residues on the amphiphilic side have a value of 1.5. This is a significant difference, and gives an indication of the polarity of the helix on the membrane surface. Magainin2 is a highly symmetric helical molecule positioned with its hydrophobic side toward the bilayer, as shown in Fig. 5.

DISCUSSION

The kinetic exchange data of the amide hydrogens for the membrane-bound form of fd coat protein are in agreement with the data showing two helices (Almeida and Opella, 1997). However, the H/D exchange factors give more details about the nature and the strength of the hydrogen bonds. The most prominent feature of the exchange process is the direct correlation between the low exchange factors determined for the transmembrane helix and the length of the helix. These data suggest that there are 16 residues with very strong hydrogen bonds (residues 28–42). Our finding that there are 16 residues in the transmembrane helix is in agreement with those of Wimley and others (1998) who report 16 as a critical number for the formation of a transmembrane α-helix. It is also consistent with our solid-state NMR measurements on numerous membrane proteins, which generally have 16 resolved resonances in the transmembrane helical region of two-dimensional spectra.

The structural features of magainin2 have been extensively investigated by both solution and solid-state NMR (Gesell et al., 1997; Ramamoorthy et al., 1995; Marassi et al., 2000). In contrast to most membrane-associated peptides, magainin2 is soluble but unstructured in aqueous solution. In the presence of lipids, magainin2 assumes a helical conformation. Solution and solid-state NMR data are in agreement in identifying a helix between residues 2 and 20. In sodium dodecyl sulfate (SDS) and dodecyl phosphocholine (DPC) micelles, the helix can be extended up to residue 23 (Gesell et al., 1997). In the case of membrane-associated peptides, the definition of their orientation respect to the membrane is crucial to understanding their function. Recently, the orientation of magainin2 has been determined experimentally using solid-state NMR data obtained on an oriented sample in lipid bilayers (Marassi et al., 2000). Remarkably, the same information can be extracted independently from the D/H exchange factor measurements for a sample in lipid micelles. The residues in the hydrophobic face of the molecule show lower values for the exchange factors than do the hydrophilic residues with χ values of the hydrophilic residues twice as large as those of the hydrophobic residues.

We have measured the D/H exchange factors for fd coat protein in micelles at a variety of temperatures. The differences in χ values do not appear to be a function of amino acid type, and it is possible to observe a strong correlation between the secondary structure elements and the χ values. In particular, the transmembrane helix has χ values significantly smaller than 1.0. Higher χ values are observed for the amphipathic helix and the loop. In addition, the variations in exchange factors for the hydrophobic and amphiphilic residues of magainin2 are significantly larger than the variations of fractionation factors typically observed for the different secondary structures of globular proteins in solution (Khare et al., 1999), making these measurements a useful method for positioning amphipatic helices on the surface of the micelle (see Fig. 5).

Finally, another advantage of the proposed method is that, once the conditions for the high-resolution of the

HSQC spectra are found, it does not require further optimization of the sample conditions, and the analysis of the exchange data can be carried out in a straightforward manner.

In conclusion, we have presented a new and simple method based on D/H exchange factors measurements that allows amide exchange measurements for membrane bound proteins in conditions in which the time-resolved methods fail. This method can reveal the two principal structural features of helical membrane proteins, identifying the residues in transmembrane helices and the orientation of amphipathic helices on the surface of membranes.

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