Hydrogen Bond Stabilities in Membrane-Reconstituted Alamethicin from Amide-Resolved Hydrogen-Exchange Measurements

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ABSTRACT Amide-resolved hydrogen–deuterium exchange-rate constants were measured for backbone amides of alamethicin reconstituted in dioleoylphosphatidylcholine vesicles by an exchange-trapping method combined with high-resolution nuclear magnetic resonance spectroscopy. In vesicles containing alamethicin at molar ratios between 1:20 and 1:100 relative to lipid, the exchange-rate constants increased with increasing volume of the D_2O buffer in which the vesicles were suspended, indicating that exchange under these conditions is dominated by partitioning of the peptide into the aqueous phase. This was supported by observation of a linear relationship between the exchange-rate constants for amides in membrane-reconstituted alamethicin and those for amides in alamethicin dissolved directly into D_2O buffer. Significant protection of amides from exchange with D_2O buffer in membrane-reconstituted alamethicin is interpreted in terms of stabilization by helical hydrogen bonding. Under conditions in which amide exchange occurred by partitioning of the peptide into solution, only lower limits for hydrogen-bond stabilities in the membrane were determined; all the potentially hydrogen-bonded amides of alamethicin are at least 1000-fold exchange protected in the membrane-bound state. When partitioning of alamethicin into the aqueous phase was suppressed by hydration of reconstituted vesicles in a limiting volume of water [D_2O:dioleoylphosphatidylcholine:alamethicin; 220:1:0.05; (M:M:M)], the exchange-protection factors exhibited helical periodicity with highly exchange-protected, and less well-protected, amides on the nonpolar and polar helix faces, respectively. The exchange data indicate that, under the conditions studied, alamethicin adopts a stable helical structure in DOPC bilayers in which all the potentially hydrogen-bonded amides are stabilized by helical hydrogen bonds. The protection factors define the orientation of the peptide helix with respect to an aqueous phase, which is either the bulk solution or water within parallel or antiparallel transmembrane arrays of reconstituted alamethicin.

INTRODUCTION

Alamethicin (Fig. 1) is one of the most widely studied examples of a group of peptides from microorganisms and animal venoms that diffuse into cell membranes and induce voltage-dependent ion channel activity (Boheim, 1974; Woolley and Wallace, 1992; Sansom, 1993). There is considerable interest in determining the molecular mechanisms underlying voltage-gated ion channel activity in excitable membranes, and alamethicin and related molecules have been used as models for exploring these events. Interest in the mechanism of ion channel formation by alamethicin has focused on the membrane-bound structure of the peptide and the voltage-induced transition from a pre-pore to a conducting channel state. There is little doubt that the conducting state consists of helical alamethicin molecules in a transmembrane array forming the lining of an aqueous pore through which ions permeate. Multiple conductance levels arise from the recruitment or loss of monomers from the conducting array, and measurements of the conductance at fixed voltage as a function of the peptide concentration indicate that 4–14 molecules can organize into conducting channels (reviewed in Sansom, 1993).

More controversial is the nature of the voltage-dependent transition from nonconducting to conducting states. Much of the discussion of this question centers on the interaction of the peptide helix dipole with the membrane potential; in the absence of fixed charges on the peptide (the Q18 variant of alamethicin is active) the helix dipole is the only source of electrostatic interaction with the membrane potential. Three main models are accommodated within the helix dipole interaction: the peptide either undergoes reorientation from a surface to a transmembrane orientation (Baumann and Mueller, 1974; Boheim, 1974; Huang and Wu, 1991) or a voltage-dependent reorientation of antiparallel helices yielding a net conducting parallel association (Boheim et al., 1983) or the peptide associates with its N-terminal helix inserted into the membrane as a barrel array and the voltage-dependent step recruits nonhelical structure at the C terminus into an extended helix, augmenting the helix dipole and forcing the array further into the membrane (Fox and Richards, 1982; Hall et al., 1984; Cascio and Wallace, 1988). Augmentation of the helix dipole may have a smaller contribution from bending around the Pro-14 residue of the peptide. Definition of one of these models requires a better determination of the conducting and pre-

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Abbreviations used: CD, circular dichroism; DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; HPLC, high-performance liquid chromatography; FTIR, Fourier transform infrared; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PDLA, poly-D, L-alanine; PF, amide exchange-protection factor; U, one-letter code for α-aminoisobutyric acid (Aib); Phol, phenylalaninol.

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1 5 10 15 20
Ac U P U A U A Q U V U G L U P V U U Q Q Phol

FIGURE 1 Amino acid sequence of alamethicin. Peptide having the sequence shown and a variant having an A-to-U replacement at residue 6 (alamethicin A6U) were used in the present study. Some variants have E replacing Q at residue 18. Phol is phenylalaninol; U is α-aminoisobutyric acid (Sansom, 1993).

conducting states of the peptide in membranes. However, spectroscopic analysis of membrane-reconstituted alamethicin has not yielded an accurate determination of the extent of stable helix in pre-pore states (Vogel, 1987; Cascio and Wallace, 1988; Haris and Chapman, 1988; Huang and Wu, 1991; Woolley and Wallace, 1993).

We recently described an exchange trapping procedure for measuring amide-resolved hydrogen–deuterium-exchange kinetics from membrane-reconstituted polypeptides (Dempsey and Butler, 1992; Dempsey, 1994). Amides that are hydrogen bonded within secondary structure have suppressed exchange kinetics, whereas non-hydrogen-bonded amides exchange with kinetics similar to those of model unstructured peptides (Bai et al., 1993). Comparison of measured exchange kinetics with kinetics calculated from model amides allows an exchange-protection factor to be determined, which, if it is sufficiently large, indicates that the amide is protected within a hydrogen bond. We present a study of amide-resolved hydrogen-exchange kinetics from alamethicin reconstituted in phospholipid bilayer membranes under a variety of conditions. The exchange data are interpreted in terms of the general helical structure of alamethicin in membranes and lead to a model for stable hydrogen bonding that provides constraints on possible structures of membrane-bound states.

MATERIALS AND METHODS

An extract of alamethicin from *Trichoderma viride* was purchased from Sigma Chemical Co. (Poole, UK). Dioleoylphosphatidylcholine (DOPC) and dimyristoylphosphatidylcholine (DMPC) were obtained from Lipid Products (South Nutfield, UK). All other reagents were of research grade or higher.

Purification of alamethicin

We separated the major components of the Sigma alamethicin preparation separated on a semipreparative Vydac C4 column, using a linear gradient of water (0.1% trifluoroacetic acid) and acetonitrile (0.1% TFA) (Fig. 2). The two major components were recovered by evaporation of the eluting solvents, and the peptides were stored at 4°C as solutions in methanol. We determined the concentrations of stock solutions of alamethicin in methanol by circular dichroism after dilution with methanol, using a value for the mean residue ellipticity ($[\theta]_{220}$) of -12,750° cm^{-2} $dmol^{-1}$ at 20°C (Rizzo et al., 1987). The final peptide:lipid ratio in the exchange samples was determined after completion of exchange measurements by NMR integration of the nonexchangeable Phol20 ring proton signals of alamethicin and the terminal methyl signal of the lipid acyl chains (Fig. 3).

Amide-exchange experiments

Amide exchange was initiated after the peptide was reconstituted at various peptide:lipid molar ratios. Lipid (DOPC) was initially dried from $CHCl_3$:

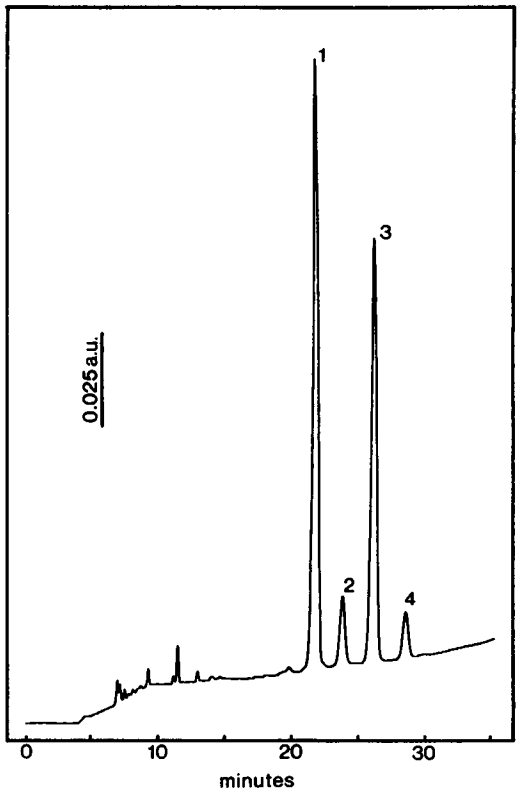

FIGURE 2 HPLC UV elution profile at 225 nm obtained during purification of the Sigma preparation of alamethicin. Peptide (2 mg) was chromatographed on a Vydac C4 semipreparative column eluting with a gradient of acetonitrile (45% to 65%) in water (0.1% trifluoroacetic acid) between 5 and 35 min.

MeOH solutions and excess solvent removed by pumping under high vacuum for several hours. The dried lipid was rehydrated in buffer (30-mM sodium acetate, pH 4.0–5.0, 30-mM sodium maleate, pH 6.0, or 50-mM sodium phosphate, pH 7.0) at a concentration of 80 mg ml^{-1} and tip sonicated on ice to produce small unilamellar vesicles. Alamethicin was carefully dried from methanolic solutions in 0.5-mg aliquots as a thin film around the lower half of 1.5-ml Eppendorf tubes by blowing with nitrogen.

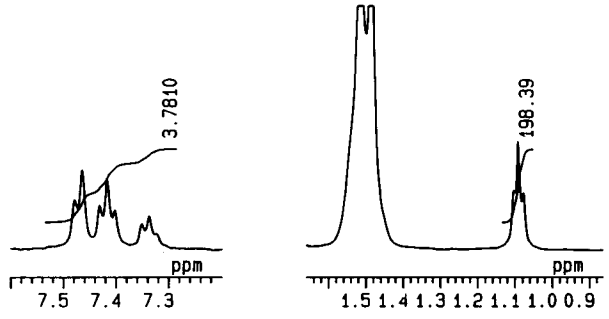

FIGURE 3 High field (right) and low field (left, at 100-fold vertical expansion) sections of the NMR spectrum of an exchange-trapped alamethicin:DOPC reconstitution with starting peptide:lipid ratio of 1:40 (M:M), after dissolving in deuteromethanol. The integrals of the signals from the lipid acyl chain terminal methyl groups (1.1 ppm; six protons) and the nonexchangeable ring protons of Phol20 (7.3–7.5 ppm; five protons) are shown and indicate a final peptide:lipid ratio of 1:44 (M:M).

It was important to ensure very thin, even distribution of the peptide as a surface film for efficient incorporation into vesicles; peptide deposited by centrifugal evaporation could not easily be reconstituted into vesicles by diffusion. Small aliquots of the sonicated DOPC vesicles were added to the tubes to give the required peptide:lipid molar ratio, and the tubes were vigorously vortexed and allowed to stand at room temperature for 1 h. The peptide was taken up into the vesicles by this procedure, as shown by circular dichroism experiments (see below) and by determination of the alamethicin:lipid molar ratio by NMR following exchange experiments (Fig. 3); preparation of reconstituted vesicles in this way avoided the introduction of methanol into the vesicle suspension. The peptide:lipid ratio determined after exchange experiments was always within 15% of the starting peptide:lipid ratio.

In most experiments, amide exchange from reconstituted alamethicin was initiated by dilution of the vesicles into a 10-fold excess of buffer made in D_2O (Method 1). The exchange buffer was 20-mM sodium acetate (pH* 4.0–5.0, where pH* is the directly measured pH using a hydrogen electrode calibrated with aqueous pH buffers), 20-mM sodium maleate (pH* 6.0), or 50-mM sodium phosphate (pH* 7.0–7.5). Individual samples were incubated at 26°C in a shaking water bath for various time periods to sample an exchange time course. Exchange was quenched by rapid freezing of the samples in dry ice–isopropanol, and all solvent was removed by lyophilization. The frozen samples were maintained at -10°C (by immersion of the lyophilizer containers in a frozen slurry of 2-M KCl) to limit artifactual exchange during lyophilization, and the lyophilized samples were kept at -20°C until NMR spectra were measured. In one series of experiments in which the ratio of water to membrane lipids was reduced to suppress partitioning of alamethicin into the aqueous phase, alamethicin was reconstituted into DOPC vesicles in water (rather than in buffer), the reconstituted vesicles were freeze dried, and amide exchange was initiated by resuspension of the dried membranes directly in buffer prepared in D_2O (Method 2). In these experiments the molar ratio of water to lipid was 550:1 or 220:1. Exchange was quenched in these samples in the same way as described for the samples in which exchange was initiated by dilution into D_2O buffer. FTIR spectra of alamethicin in reconstituted DOPC vesicles prepared by Method 1 and Method 2 were indistinguishable (unpublished results), indicating that the conformation of the reconstituted peptide is unaffected by the method of reconstitution and initiation of amide exchange. The FTIR spectra were very similar to those previously described for alamethicin in DMPC (Haris and Chapman, 1988). Previous studies have also shown that the CD spectrum of alamethicin in DMPC bilayers (Cascio and Wallace, 1988) and its voltage-dependent channel activity in black lipid membranes (Mueller and Rudin, 1968) are the same in samples in which peptide is added to preformed membranes and in those in which reconstitution is achieved through cosolubilization of peptide and lipid in solvent followed by drying and hydration.

Amide-resolved hydrogen exchange kinetics for alamethicin in D_2O buffer were measured by exchange trapping because of the limited aqueous solubility of the peptide, which precluded direct time-resolved measurement of the loss of amide NMR signal intensity within a single exchanging sample. Previous studies have shown that alamethicin is soluble in water at concentrations of at least 86 μM (Rizzo et al., 1987), although the peptide may be partially self-associated under these conditions (e.g., McMullen and Stirrup, 1971). A sample of the peptide in methanol was diluted into sodium acetate buffer, pH* 4.0, in D_2O at 26°C to a final concentration of 50 μM. The residual methanol concentration was 2% by volume. Aliquots were removed throughout a time course of exchange, rapidly frozen and lyophilized as described above, and kept at -20°C until NMR spectra were run.

NMR and circular dichroism spectroscopy

NMR spectra were measured using the JEOL α 500 MHz spectrometer of the Bristol Centre for Molecular Recognition. Before the exchange samples were measured, an identical dried lipid:peptide sample was dissolved in 0.56-ml deuteromethanol and the concentration of deuterium chloride required to bring the measured pH (pH*) to a value for which amide hydrogen exchange from alamethicin is intrinsically slow (pH* 3–5; Dempsey, 1995) was determined. Each of the dried exchange samples was dissolved in 0.56 ml of deuteromethanol containing the precalibrated concentration of DCl and put into a 5-mm NMR tube for immediate spectral acquisition. NMR spectra were obtained at 20°C by use of truncated Gaussian 270° selective pulses (Emsley and Bodenhausen, 1989) of 2 ms centered on the amide region of the NMR spectrum to limit excitation of the cosolubilized lipid signals (Dempsey and Butler, 1992; Dempsey, 1994) after adjustment of the shimming, tuning, excitation frequency, and selective pulse attenuation with the dummy sample. Generally, each spectrum was obtained within 4–7 min following dissolution of the dried lipid:peptide complex in the acidic deuteromethanol. Exchange-trapped samples of alamethicin in D_2O buffer without lipids were measured with nonselective excitation pulses.

CD spectra were obtained on a Jobin-Yvon CD6 spectra polarimeter. Samples for CD were prepared as described above, and spectra were obtained at 25–30°C in 2- or 0.1-mm CD cuvettes, depending on the peptide and vesicle concentrations. Raw data from the average of three scans were imported into Sigmaplot (Jandel Scientific, Erkrath, Germany), and we baseline corrected the unsmoothed data by zeroing the ellipticity at 260 nm before plotting.

Measurement of amide-exchange-rate constants and determination of exchange-protection factors

Pseudo-first-order exchange-rate constants were determined by fitting the amide signal intensity in the ^1H NMR spectra obtained after increasing exchange times to curves defining first-order decay by use of Sigmaplot. Standard deviations in the fits are not shown in the figures for clarity but were always less than ±25% of the stated or displayed value.

An amide-exchange-protection factor (PF) is taken to be the ratio of the amide-exchange-rate constant for poly-D, L-alanine (k_{PDLA}) at the pH* and temperature of the exchange measurement divided by the corrected amide exchange-rate constant for the backbone amide under consideration (k_{corr}); i.e., $PF = k_{PDLA}/k_{corr}$. We determined the rate constant for PDLA (k_{PDLA}) from the equation

$$k_{PDLA} = k_A \times 10^{-pH^*} + k_B \times 10^{(pH^*-pK_D)} + k_W \qquad (1)$$

(where k_A, k_B, and k_W are the rate constants for catalysis by acid, base, and water, respectively), using values given in Table 3 of Bai et al. (1993) for the rate constants, activation energies, and dissociation constant of D_2O (pK_D). In using this equation we corrected the pH measured with a hydrogen electrode calibrated with aqueous pH buffers (pH*), using pH*corr = pH* + 0.4 (Bai et al., 1993). In the pH* range of the data presented here (pH*corr = 4.4–7.4) the calculated exchange-rate constant for PDLA is dominated by the contribution from base-catalyzed exchange. Experimental exchange-rate constants for the alamethicin amides were corrected for sequence-dependent inductive and steric contributions by use of the data in Table 2 of Bai et al. (1993). The sequence-dependent contribution for Aib has not been determined in water and for the purpose of this study was taken to be (for base-catalyzed exchange) [log k_{ex}(Aib) - log k_{ex}(Ala) = -0.7 (L); = -0.35 (R); in the notation of Bai et al. (1993)]. These values were estimated from the effects of Aib (relative to those of Ala and Gly) on the acid- and base-catalyzed exchange-rate constants of protected dipeptides in methanol (Dempsey, 1995) in which Aib caused an approximate fivefold suppression of base-catalyzed exchange of its own amide proton relative to that of Ala.

Exchange data were obtained for all the amides except Aib1 NH, which has unfavorable exchange properties in methanol because of its lack of exchange protection by hydrogen bonding and the large shift of pH_{min} to low pH relative to the other amides in that solvent (Dempsey, 1995). At pH* values in which the *C*-terminal amides exchange sufficiently slowly to allow unperturbed intensity measurements in exchange-trapped samples (pH* 4 - 5.5), the amide NH of Aib1 exchanges too quickly for reliable intensity measurements.

RESULTS

Purification of alamethicin

The Sigma preparation of alamethicin consists of two major and two minor components readily separated by HPLC under the conditions described in Materials and Methods. Because of the limited amount of peptide available and the requirement for relatively large amounts for the amide-exchange measurements (0.5 mg per exchange time point), each of the two main fractions of alamethicin was isolated and used in a separate exchange experiment. The two major components are alamethicin having the sequence of Fig. 1 (peak 1, Fig. 2) and alamethicin with Aib replacing Ala-6 (peak 3, Fig. 2). Characterization of the HPLC fractions was confirmed by assignment of the respective high-resolution two-dimensional NMR spectra in methanol and is consistent with the characterization of related HPLC fractions described by Kelsh et al. (1992). No attempt was made to determine the composition of the minor components of the mixture (peaks 2 and 4, Fig. 2).

Characterization of vesicles containing alamethicin

Many studies characterizing the interaction of alamethicin with lipid membranes have used DOPC as the membrane lipid. We have attempted to study amide exchange from membrane-reconstituted alamethicin under conditions that match those previously studied, within the constraints of the methods employed, in particular in the work described in Rizzo et al. (1987), Huang and Wu (1991), and Woolley and Wallace (1993), in which strong partitioning of alamethicin into DOPC is demonstrated. In addition, each of these studies indicates significant effects (on peptide orientation and on aggregation) depending on the peptide:lipid ratio, and we have studied amide exchange from DOPC-reconstituted alamethicin between two extremes accessible to the practicalities of the amide-exchange protocol. These are 1:20 and 1:100 (alamethicin:DOPC; M:M).

The CD spectra of samples of alamethicin reconstituted in DOPC vesicles at 1:20 and 1:100 in acetate buffer, pH* 4.0, are shown in Fig. 4. These samples were prepared by diffusion of alamethicin, dried as a thin film, into sonicated DOPC vesicles and are very similar to the samples prepared in the same way for amide-exchange experiments. Previous studies have shown that membrane lipids maintain bilayer configuration over this range of peptide:lipid ratio (Banerjee et al., 1985). The CD spectra are similar to those interpreted by Woolley and Wallace (1993) as arising from peptide self-associated in membranes. The major feature of these spectra is the large excess ellipticity of the band near 224 nm ($n\pi^*$ transition) over the band around 208 nm ($\pi\pi^*\|$) characteristic of parallel helical coiled coils (Lau et al., 1984; Zhou et al., 1992). Woolley and Wallace (1993) have argued that the increase of this ratio ($\epsilon_{224}/\epsilon_{208}$) on increasing the peptide:lipid ratio, or on decreasing the temperature at constant peptide:lipid ratio, results from alamethicin self-association in the membrane. The spectra in the present study show only a small increase in the ratio of $\epsilon_{224}/\epsilon_{208}$ between 1:100 and 1:20 (alamethicin:DOPC; M:M), indicating that the peptide was largely associated at the lower concentration of peptide in the lipid (1:100; M:M). The smaller variation in the ratio of $\epsilon_{224}/\epsilon_{208}$ compared with those of the spectra presented by Woolley and Wallace (1993) over a similar range of peptide:lipid ratios results from the very high peptide and lipid concentrations used in the present study [0.25-mM alamethicin, 5–25-mM DOPC, compared with 4–16-μM alamethicin, <0.7-mM DOPC in the experiments described by Woolley and Wallace (1993)], so that the equilibrium between membrane-bound and aqueous peptide is pushed well to the side of membrane-bound peptide.

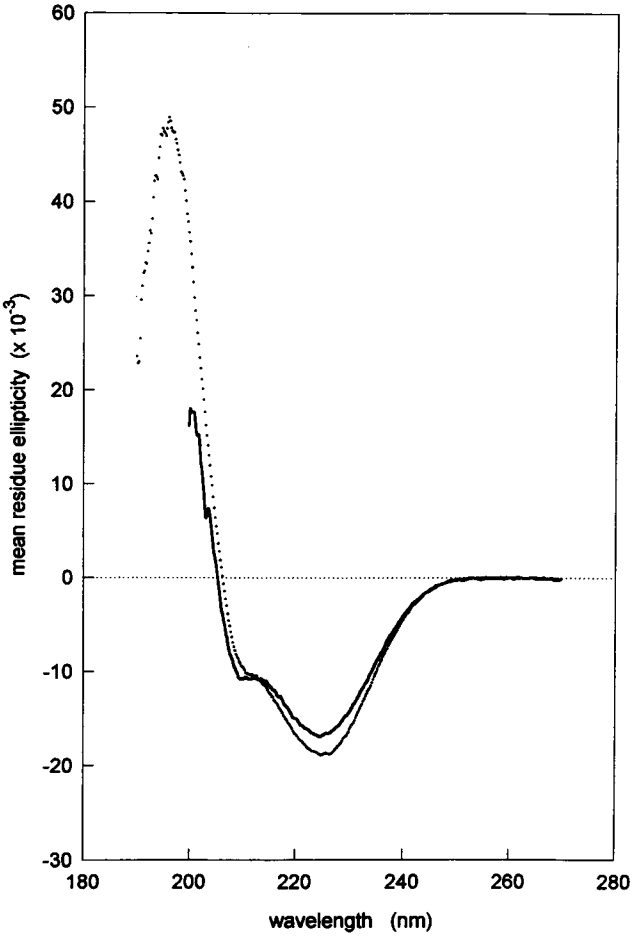

FIGURE 4 Circular dichroism spectra of alamethicin reconstituted in sonicated DOPC vesicles suspended in 30-mM sodium acetate buffer, pH 4.0, by diffusion from a dried film as described in Materials and Methods. Peptide:lipid ratios were 1:20 (alamethicin:DOPC, M:M) (dotted spectrum) and 1:100 (solid spectrum). The alamethicin concentration was always 0.25 mM. Data for the 1:100 sample were unreliable at wavelengths below 200 nm because of light scattering from the high vesicle concentration. Spectra are averages of three scans obtained at 25°C in 0.1-mm path-length demountable cuvettes, baseline corrected by zeroing at 260 nm and plotted without smoothing.

Amide exchange from DOPC-bound alamethicin with excess bulk water

The time course of amide hydrogen–deuterium exchange from membrane-reconstituted alamethicin is shown in Fig. 5 for two conditions. Fig. 5 *A* illustrates amide exchange from alamethicin reconstituted in DOPC at a peptide:lipid molar ratio of 1:100, at pH* 4.0, initiating exchange with a 10-fold excess of 20-mM sodium acetate buffer in D_2O, pH* 4.0 ([DOPC] = 9 mM; [alamethicin] = 90 μM final concentrations; exchange using Method 1). Fig. 5 *B* illustrates amide exchange from a 1:20, M:M (alamethicin:DOPC) sample initiated by direct resuspension of lyophilized reconstituted vesicles in a small volume (50 μl) of 20-mM sodium maleate buffer in D_2O, pH* 6.0 ([DOPC] = 100 mM; [alamethicin] = 5 mM, final concentrations; exchange using Method 2). As well as could be determined from a limited number of exchange time points, the loss of amide signal intensity with time by exchange with deuterium in the membrane-reconstituted state followed a single exponential, which is indicative of a single uniform population of peptide. Examples of the fit of the experimental data to curves defining first-order decay are shown in Fig. 6 for the pH* 6.0 data.

Similar exchange experiments were made under a variety of conditions. To test the effects of varying the molar peptide:lipid ratio, amide exchange was measured from reconstituted vesicles at alamethicin:DOPC molar ratios of 1:20 and 1:100 at pH* 5.0 and at 1:20 and 1:40 at pH* 6.0. Initial experiments indicated that amide-exchange rates at constant peptide:lipid ratios were influenced by the volume of aqueous exchange buffer in which the vesicles were suspended, and we tested this by measuring exchange at constant peptide:lipid ratio (1:20 M:M) in 2.0-, 0.5-, and 0.05-ml buffer made in D_2O. The exchange data from these experiments are summarized in Fig. 7. Within the accuracy of the exchange-rate determinations, there was no significant difference in the exchange-protection factors at peptide:lipid molar ratios of 1:20 and 1:100 (M:M) measured at pH* 5.0. Similarly, the exchange-protection factors measured at pH 6.0* for peptide:lipid molar ratios of 1:20 and 1:40 were not significantly different. Greatly increased exchange-protection factors were measured for exchange at constant peptide:lipid ratio in vesicles suspended in 50-μl aqueous volume compared with similar samples in which the vesicles were suspended in 2.0 ml (Fig. 7). In a similar experiment, increased protection factors (by 3–4-fold) were found for 1:20 (alamethicin:DOPC; M:M) samples suspended in 0.5-ml pH* 5.0 buffer, compared with 2.0-ml pH* 5.0 buffer (not shown).

Amide exchange from alamethicin in water

The observation of a dependence of the exchange-protection factor on the volume of aqueous solution hydrating the membranes for amide exchange in reconstituted alamethicin indicates that exchange under these conditions occurs from an aqueous state resulting from partitioning of the peptide between the membrane and aqueous solution. If this is the case the profile of exchange-protection factors among the backbone amides of the peptide should reflect variations in

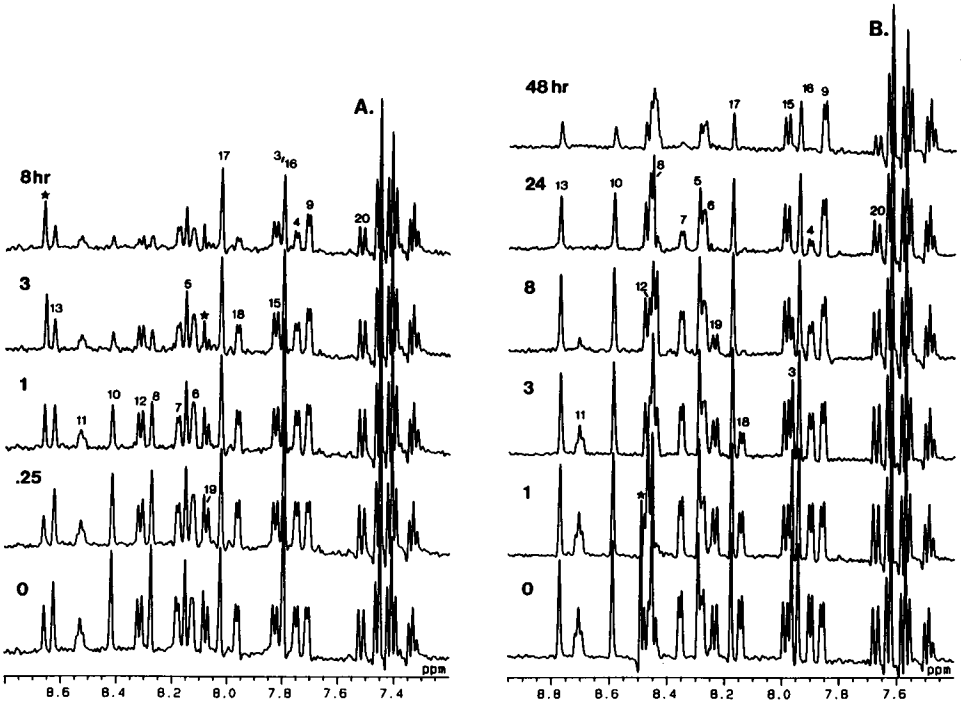

FIGURE 5 *A*, Time dependence of amide signal intensities in the ^1H NMR spectrum of alamethicin in CD_3OD obtained after exchange trapping and lyophilization of alamethicin-reconstituted DOPC vesicles (1:100, M:M alamethicin:DOPC) obtained throughout an exchange time course at pH* 4.0 in 2 ml of 30 mM sodium acetate buffer in D_2O. *B*, Similar data for an exchange-trapping experiment with a 1:20 (alamethicin:DOPC) reconstituted vesicle preparation after increasing periods of amide exchange in 0.05 ml of 30 mM sodium maleate buffer, pH* 6.0. Signals marked with stars are impurities.

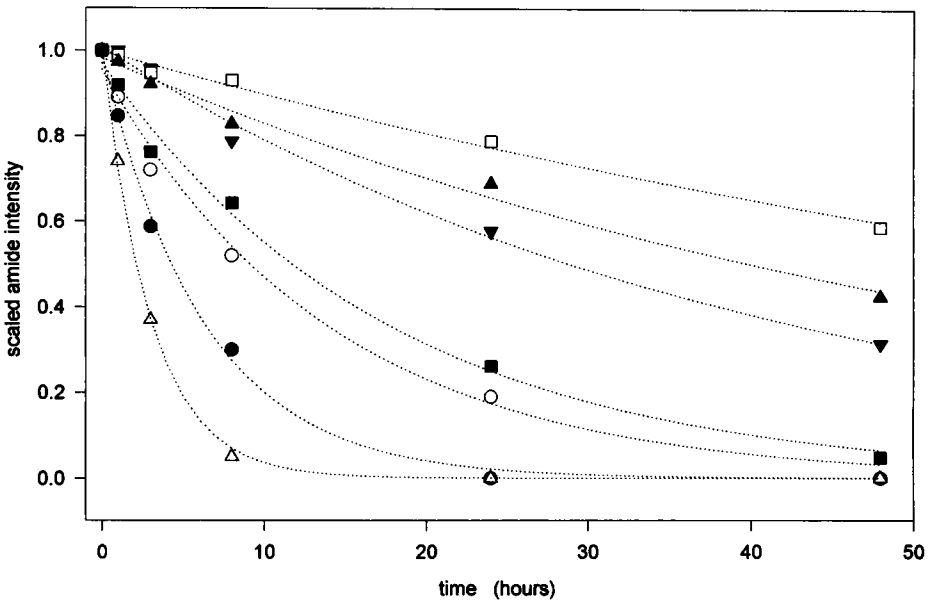

FIGURE 6 ^{1}H NMR amide signal intensities, scaled relative to zero time intensities, fitted to curves defining first-order decay for representative amides in alamethicin in DOPC vesicles obtained during an exchange-trapping experiment at pH* 6.0 in 30-mM sodium maleate buffer (1:20, M:M, alamethicin:DOPC). U3 (●), A4 (○), Q7 (■), V15 (□), U16 (▲), Q18 (△), Phol20 (▼).

exchange-protection factors in aqueous alamethicin. Because of the low aqueous solubility of alamethicin, exchange rates from the aqueous peptide could not be directly measured by NMR from peptide dissolved in D_2O buffer. Exchange rates for aqueous alamethicin were measured by exchange trapping of samples diluted (by 50-fold) from methanolic solution into 20-mM acetate buffer in D_2O, pH* 4.0, to a concentration of 50 μM. After exchange trapping by freezing and lyophilization, dried samples obtained throughout an exchange time course were dissolved in 0.5-ml deuteromethanol (0.2 mM), and we recorded NMR spectra to assay the extent of exchange of each amide at each exchange time point (Fig. 8). Amide-exchange-protection factors for the measurable amides of alamethicin in D_2O, pH* 4.0 are compared in Fig. 9 with the protection factors for alamethicin amides reconstituted in DOPC (1:20, M:M; 0.625-mM alamethicin:12.5-mM DOPC) at pH* 4.0. The profile of exchange-protection factors in aqueous alamethicin is similar to that for the membrane-reconstituted peptide, but the water values are 70-fold reduced on average. The enhancement of exchange-protection factors by ~70-fold in the membrane-reconstituted state relative to the aqueous peptide indicates a partitioning of peptide between the aqueous and membrane states with an equilibrium constant (moles of membrane-bound peptide divided by moles of aqueous peptide) of 70 under these conditions.

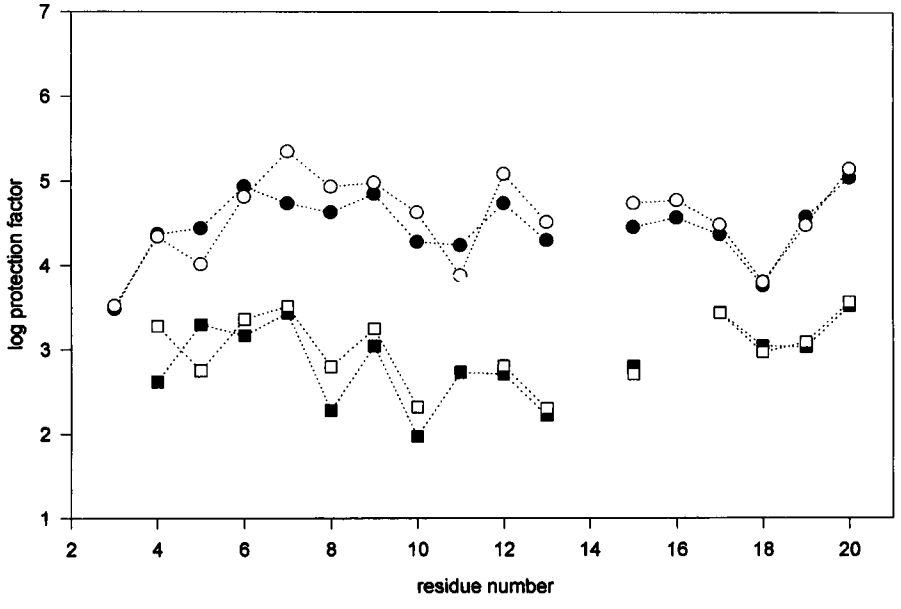

FIGURE 7 Logarithms of the amide-exchange protection factors for alamethicin amides as a function of the amino acid sequence obtained in four separate exchange-trapping experiments: pH* 4.0, 1:100 alamethicin:DOPC in 2.0-ml exchange buffer (□); pH* 5.0, 1:20 alamethicin:DOPC in 2.0-ml exchange buffer (■); pH* 6.0, 1:20 alamethicin:DOPC in 0.05-ml exchange buffer (●); and pH* 6.0, 1:40 alamethicin:DOPC in 0.05-ml exchange buffer (○). Dotted lines are included to guide the eye.

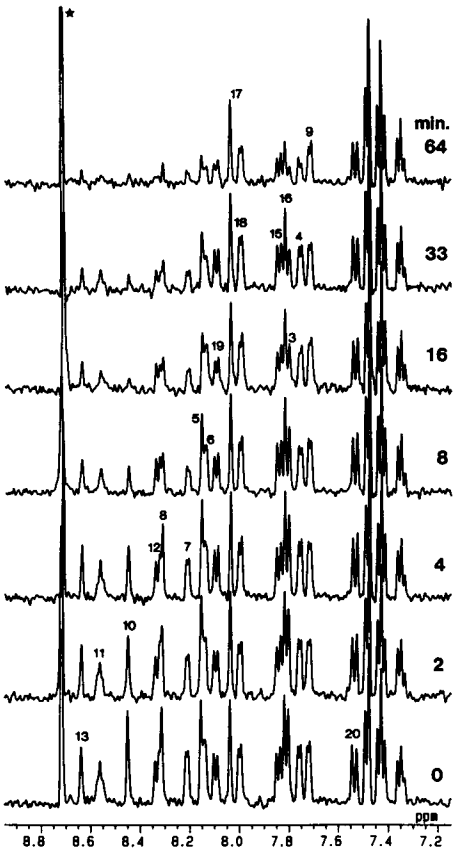

FIGURE 8 Time dependence of amide signal intensities in CD_3OD for alamethicin after increasing periods of exchange in 30-mM sodium acetate buffer (pH* 4.0). The peptide concentration was 50 μM during exchange in D_2O buffer and 200 μM after exchange trapping and dissolving in CD_3OD for spectral accumulation.

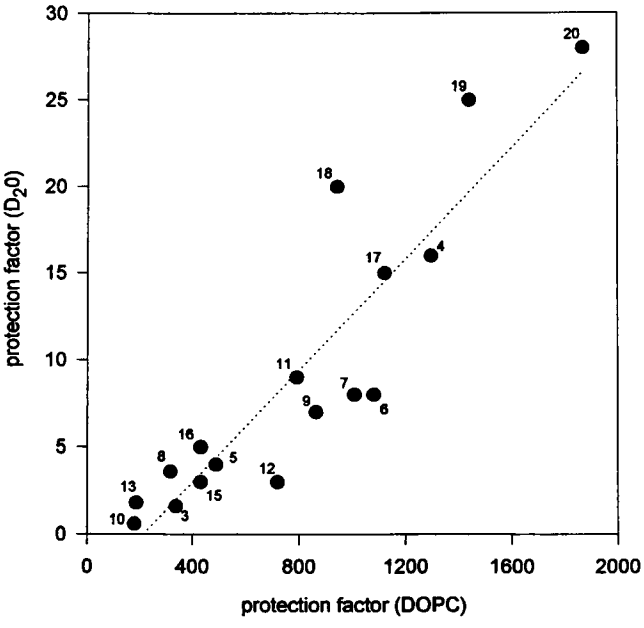

FIGURE 9 Comparison of amide exchange protection factors, annotated with residue numbers, for alamethicin (50 μM) in 30-mM acetate buffer, pH* 4.0, and alamethicin reconstituted in DOPC vesicles (0.625-mM alamethicin: 12.5 mM DOPC) in the same buffer. The dotted line is a least-squares fit of the data to a linear plot.

Amide exchange from DOPC-bound alamethicin with limiting bulk water

Under conditions of limiting bulk water, partitioning of alamethicin into the aqueous phase is expected to be suppressed to the extent that the contribution of exchange-limiting backbone fluctuations in the membrane-bound state might exceed partitioning of the peptide into the aqueous phase as the limiting mechanism for amide exchange (see Discussion, below). Under such circumstances, more detailed information on the relative stabilities of individual hydrogen bonds in the membrane-bound state can be achieved (e.g., Dempsey and Butler, 1992). Fig. 10 illustrates the time dependence of amide exchange from alamethicin in DOPC (1:20, M:M) in which exchange was initiated from freeze-dried membranes prepared in water by hydration with a limiting volume of 50-mM sodium phosphate buffer, pH* 7.0, at 26°C (D_2O:DOPC:alamethicin = 4400:20:1, M:M:M; Method 2). The residue-specific exchange-protection factors are illustrated in Fig. 11. Very similar profiles of protection factors were obtained in related experiments run at 35°C (above T_m for DMPC) in which the peptide:lipid ratio was DOPC:alamethicin, 40:1 or DMPC:alamethicin, 40:1 (M:M) (not shown).

The exchange-protection factors in the experiments at low water content partition with respect to the x-ray structure of alamethicin crystallized from methanol. This is illustrated in Fig. 12, which indicates that amides with large exchange-protection factors in DOPC lie on the nonpolar face of the peptide helix whereas the relatively poorly protected amides lie on the polar surface. This periodicity in exchange-protection factors is observed throughout the peptide, including the C-terminal sequence up to Phol20.

DISCUSSION

What makes amide exchange slow in membrane-reconstituted alamethicin?

Amide exchange from membrane-reconstituted alamethicin is heavily suppressed under a number of conditions of pH, peptide:lipid ratio, and water content. The helical periodicity in exchange-protection factors observed under conditions in which partitioning of the peptide into aqueous solution is suppressed supports helical hydrogen bonding as a major factor limiting amide exchange in these samples (Figs. 11 and 12). It is useful to consider the general case of exchange suppression in reconstituted alamethicin by analogy with exchange in soluble proteins in which amide exchange protection is interpreted in terms of hydrogen bonding. Limited access of water (and exchange catalyst; OH^- in the context of most studies) is important in suppressing exchange of interior amides in proteins, but this

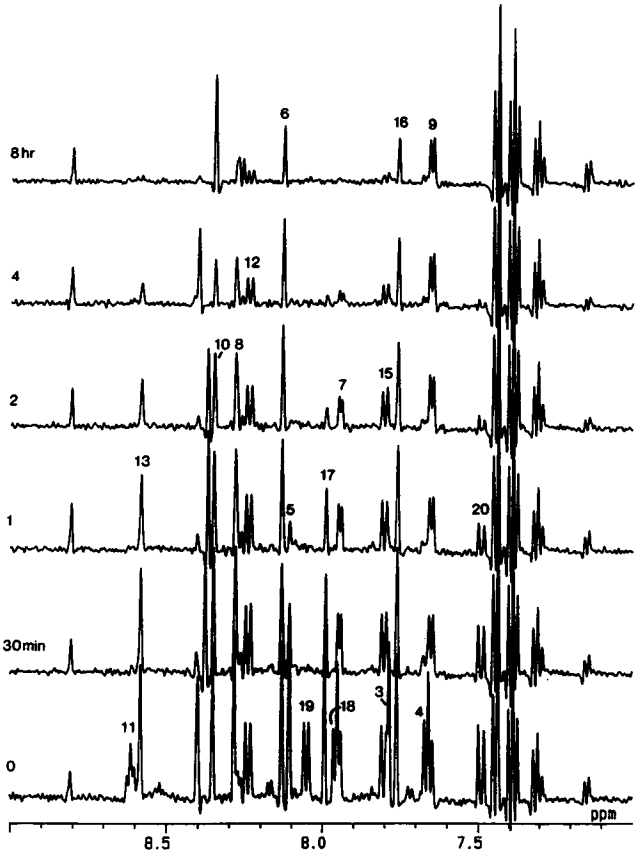

FIGURE 10 Amide signals in the ^{1}H NMR spectrum of alamethicin in CD_3OD obtained after increasing periods of amide exchange in DOPC vesicles. Amide exchange was initiated by hydration of lyophilized vesicles with a small volume (20 μl) of D_2O buffer (50-mM Na phosphate, pH* 7.0 at 26°C), and exchange was trapped after the time indicated as described in the text. The composition of the hydrated vesicles was alamethicin:DOPC:D_2O (1:20:4400; M:M:M).

factor is effectively included in exchange suppression by hydrogen bonding inasmuch as it is rare for nonsolvated backbone amides in the interior of proteins to be without hydrogen-bonding partners. It is accepted that backbone fluctuations involving transient hydrogen-bond "opening" are required for exchange to occur (Englander and Kallenbach, 1984). Similarly, for alamethicin in membranes, significant (see below) suppression of amide exchange is interpreted in the following sections in terms of hydrogen bonding. Although diffusion of water and exchange catalyst into the nonpolar membrane interior is highly unfavorable, so is the likelihood of nonsolvated backbone amides without hydrogen-bonding partners, although the balance of individual free energy contributions to membrane binding may allow this to some extent. Non-hydrogen-bonded amides of melittin reconstituted in membranes (Dempsey and Butler, 1992) and M13 coat protein in detergent micelles (Henry and Sykes, 1990) have negligible exchange-protection factors (less than 2–3-fold), demonstrating that the membrane (or micelle) per se has no major effect on amide-exchange chemistry. Spyracopoulos and O'Neil (1994) have made a systematic study of the effect of the (sodium dodecylsulfate) micellar environment on exchange chemistry in micellized hydrophobic monomeric secondary amides. They found a maximum suppression (of k_{min}, the exchange-rate constant at the minimum of the experimental pH-dependent exchange curve) of ~4-fold and ~25-fold for the two most hydrophobic amides, and these values are probably overestimates because the steric effects of hydrophobic substituents on suppressing amide exchange (Bai et al., 1993) were not fully characterized for these branched-chain hydrophobic amides. Exchange-protection factors should be interpreted with some caution, and, in line with the maximum exchange suppression observed by Spyracopoulos and O'Neil for non-hydrogen-bonded hydrophobic amides in sodium dodecylsulfate micelles, we consider only protection factors of greater than 25-fold in fully hydrated samples to be characteristic of exchange protection by hydrogen bonding. The low water content of the samples prepared by Method 2 may make *absolute* protection factors unreliable because of possible lower diffusion of water and exchange catalyst in these thick suspensions. [Low water content does not indicate low membrane hydration. The hydration levels in these samples are ~10 times greater than the maximal hydration levels achieved in planar bilayer experiments at maximal humidity, for example, those utilizing membrane-reconstituted alamethicin described by Vogel (1987) and Huang and Wu (1991), and yield fully hydrated multilamellar vesicles (not shown).] However, the purpose of using these samples was to identify *relative* protection factors among the alamethicin amides under conditions in which exchange is dominated by backbone fluctuations in the membrane rather than by diffusion into the aqueous phase.

Analysis of amide-exchange-protection factors in a membrane–water partitioning peptide

Under conditions in which amide exchange is dominated by transient opening of hydrogen bonds freeing the amide for exchange with solvent, the exchange-protection factor (PF), corrected for sequence- and conformation-dependent contributions to exchange (Bai et al., 1993), is a measure of the equilibrium constant (K_{op}) defining the exchange-limiting fluctuation (PF $\equiv 1/K_{op}$) and can thus be used to assign local thermodynamic stabilities of secondary structure in terms of stability with respect to hydrogen-bond-breaking backbone fluctuations (Englander and Kallenbach, 1984; Englander et al., 1992; Dempsey, 1992; Rohl and Baldwin, 1994). Under conditions in which amide exchange from membrane-reconstituted alamethicin is dominated by exchange from the poorly structured aqueous state resulting from partitioning between the membrane and water, only a lower limit to the exchange-protection factor in the membrane can be determined. Hydrogen-bond stabilities in membrane-bound polypeptides are accessible, however, under conditions in which the equilibrium is pushed toward the

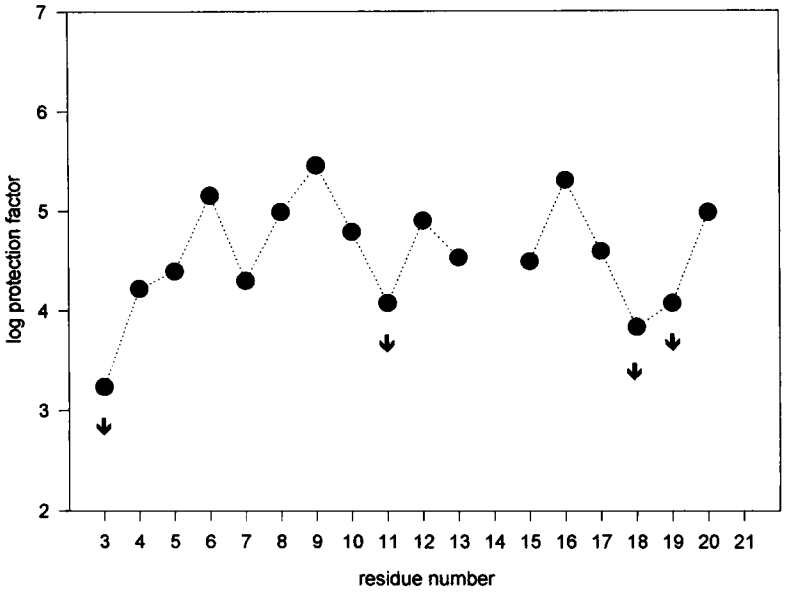

FIGURE 11 Profile of amide-exchange protection factors for alamethicin amides obtained from the exchange-trapping experiment illustrated in Fig. 10. A vertical arrow below a the data point indicates that the value of the protection factor is an upper limit.

membrane-bound state to an extent that K_{op} becomes greater than K_{par}, the equilibrium constant defining the ratio of peptide in the aqueous and membrane states (see the following sections). In the general case, one can model the exchange-protection factor in terms of the contribution to exchange from each state by developing a simple theory taking into account fast partitioning of the peptide between the membrane and water. Equation 2 extends the usual scheme in which amide exchange is limited by hydrogen-bond-breaking backbone fluctuations, characterized by equilibrium constants K_{op} (Hvidt and Neilsen, 1966), to include contributions to exchange from an aqueous state connected to the membrane state by the partition equilibrium constant K_{par}:

$$[\mathrm{ND_o} \xleftarrow{k_1} \mathrm{NH_o} \underset{}{\overset{K_{op}m}{\rightleftharpoons}} \mathrm{NH_c}]_m \overset{K_{par}}{\rightleftharpoons} [\mathrm{NH_c} \underset{}{\overset{K_{op}a}{\rightleftharpoons}} \mathrm{NH_o} \xrightarrow{k_1} \mathrm{ND_o}]_a. \quad (2)$$

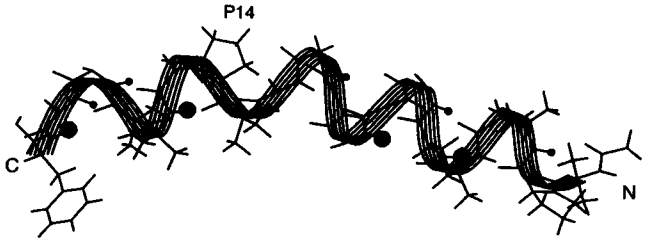

FIGURE 12 Structure of alamethicin obtained from the x-ray crystal structure coordinates (Fox and Richards, 1982) in which backbone peptide amides are marked according to the exchange-protection factors obtained from the experiment illustrated in Figs. 10 and 11 (alamethicin:DOPC:D_2O buffer; 1:20:4400; M:M:M; pH* 7.0). Larger circles indicate amides with PF $\geq 2 \times 10^5$; smaller circles are amides with PF $\leq 10^4$. Other amides have intermediate PF. Some amino acid side chains have been removed for clarity.

The experimental exchange-rate constant k_{ex} is then the sum of exchange contributions from the two states (Eq. 3), where k_1, the exchange-rate constant for a free

$$k_{ex} = k_1 K_{op} m + k_1 K_{op} a\, K_{par} \quad (3)$$

amide (k_{PDLA}) at the pH of interest is assumed to be the same for exchange from the membrane and solution and K_{op}m and K_{op}a define the backbone fluctuations limiting exchange in the membrane-bound state and the aqueous state, respectively. (A similar assumption, i.e., that k_1(protein) is equivalent to k_1(random coil), is made in analyzing exchange-protection factors from amides in proteins. Because k_1(membrane) might differ from k_1(aqueous) (it is likely to be suppressed), only protection factors greater than 25-fold are taken to indicate protection by hydrogen bonding in line with the maximum suppression (by 25-fold) of amide exchange from non-hydrogen-bonded hydrophobic amides in sodium dodecylsulfate micelles measured by Spyracopoulos and O'Neil (1994) as described above). The exchange-protection factor (PF = k_1/k_{ex}) is given by Eq. 4, rearranged in Eq. 5 for convenience of fitting to theoretical values:

$$\mathrm{PF} = k_1/k_{ex} = 1/(K_{op}m + K_{op}a\, K_{par}), \quad (4)$$

$$\log \mathrm{PF} = -\log[K_{op}m + K_{op}a \times 10^{(\log K_{par})}]. \quad (5)$$

Limits on hydrogen-bond stabilities in the membrane can be estimated by reducing K_{par} [by enhancing membrane binding (Dempsey and Butler, 1992) or by reducing the volume of aqueous solution, as described in the following section] so that K_{op}m approaches K_{par}. This is illustrated graphically in Fig. 13, which shows that the experimental protection factor is a function of the relative values of $K_{op}m$, K_{par}, and $K_{op}a$.

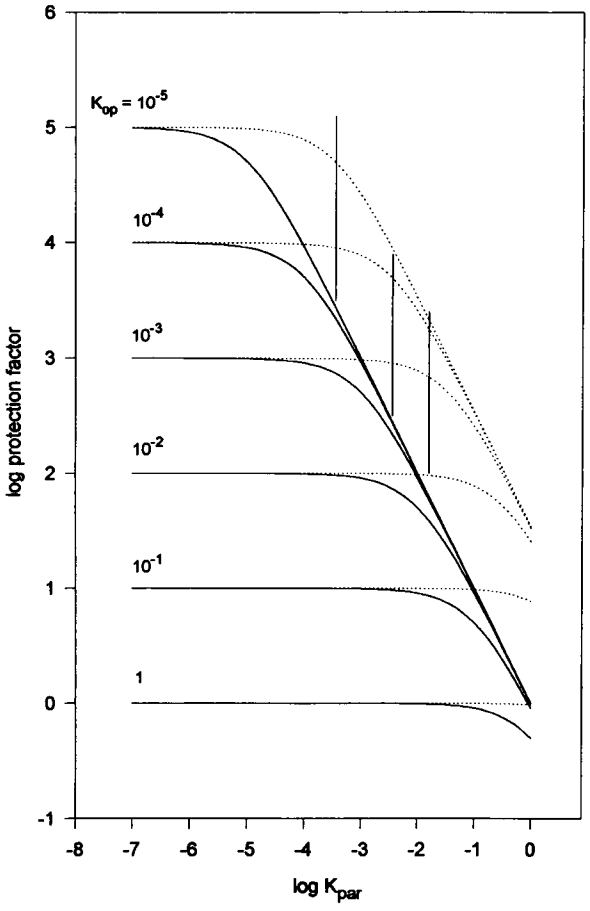

FIGURE 13 Theoretical curves defining amide-exchange protection factors for a polypeptide partitioning between membrane and aqueous phases, obtained by fitting to Eq. 5 (see text). The solid and dotted curves are fits with $K_{op}a$ set to 1 and 0.033, respectively, corresponding to the range of experimental values obtained for exchange in water (Fig. 9). Data were calculated for $K_{op}m$ values between 10^{-5} and 1 in 10-fold intervals (top to bottom). The range of experimental protection factors for alamethicin reconstituted in DOPC vesicles (0.5-mg alamethicin, 3.9-mg DOPC) is shown as vertical bars for exchange in (bottom to top) 2.0-ml buffer (K_{par} = 0.014 as determined in Fig. 9), 0.5-ml buffer (K_{par} = 0.0035), and 0.05-ml buffer (K_{par} = 0.00035 estimated by extrapolation; see text).

Also shown in Fig. 13 (as vertical lines) are the ranges of experimentally determined exchange-protection factors for alamethicin amides under three conditions (1:20 alamethicin:DOPC suspended in 2.0-, 0.5-, and 0.05-ml exchange buffer, respectively) in which K_{par} is determined to be 0.014 (determined from the ratio of exchange-protection factors in water and in the membrane; Fig. 9), 0.0035, and (by extrapolation) 0.00035 (bottom to top, respectively). Much of the variation in protection factors in at least the lower two groups of protection factors results from differences in $K_{op}a$, which fall roughly in the range 1–0.03 (PF $\approx$ 0–33) (Fig. 9). The increase in protection factors on reducing K_{par} is expected under conditions in which exchange results largely from partitioning into the aqueous phase. Exchange from the highly concentrated vesicle population in 50-μl D_2O buffer (K_{par} = 0.00035) indicates that hydrogen-bond stabilities in membrane-bound alamethicin are represented by $K_{op}m$ values equal to or greater than 10^4 and 10^5 (Fig. 7, topmost set of data, and Fig. 13, top group of protection factors). This may be an overestimation because the high membrane concentration in these samples (100–200-mM DOPC) produces thick vesicle suspensions that may limit diffusion of exchange catalyst within the samples and perturb the partitioning of peptide into the aqueous phase. The exchange-protection factors of ~1000 measured for alamethicin in vesicles suspended in 2-ml exchange buffer are expected to extrapolate to protection factors of $\sim 2 \times 10^4$ under similar conditions in 50-μl exchange buffer, and many of the alamethicin amides have protection factors larger than this (Figs. 7 and 13). Nevertheless the data from these samples emphasize the high exchange stabilities of amides in membrane-bound alamethicin.

The determination of an equilibrium constant of ~70-fold (Fig. 7) for the partitioning of alamethicin between membrane and aqueous phases in the 1:20 sample at pH* 4.0 in 2.0-ml exchange buffer (0.625-mM alamethicin; 12.5-mM DOPC) is compatible with previous experimental measurements of membrane partitioning of the peptide (Schwarz et al., 1986). Using Eq. 6, which relates the partition coefficient Γ to the concentrations of lipid (c_L), membrane-associated peptide (c_a), and free aqueous peptide (c_f) gives a value for the partition coefficient Γ of $70 \times 1/0.0125 = 6000\ M^{-1}$. This is larger (by fivefold) than the value determined by Schwarz et al. (1986),

$$\Gamma = c_a/(c_L\, c_f) \qquad (6)$$

but neglects the activity coefficient used by those authors to correct for peptide–peptide association in the membrane. The significant exchange-protection factors observed for the peptide dissolved in water without lipids (Fig. 9) is also consistent with evidence from circular dichroism that aqueous alamethicin is partly structured (Rizzo et al., 1987; Woolley and Wallace, 1993). The nature of structure in the aqueous peptide is not known, although the significant exchange-protection factors for the C-terminal amides (Figs. 8 and 9) suggest that this region of the peptide is structured or involved in intermolecular interactions.

Amide exchange from DOPC-reconstituted alamethicin at low water content

Reducing the aqueous volume solvating the reconstituted membranes reduces the partitioning of alamethicin into solution, and at some point amide exchange will become limited by hydrogen-bond- breaking fluctuations in the membrane-bound state (i.e., in Eq. 3 $K_{op}m > K_{op}aK_{par}$). Within the scheme of Eq. 2 the experimental exchange-protection factors will then reflect relative hydrogen-bond stabilities in the membrane-bound state. Under conditions of relatively low water content (220:1, D_2O:DOPC, M:M), the alamethicin amides show a different profile of exchange-protection factors from the profile of protection

factors in water, or in lipid with excess bulk water (compare Figs. 11 and 7). Analysis of the protection factors in relation to the (essentially helical) crystal structure of alamethicin (Fox and Richards, 1982) demonstrates that the protection factors partition with respect to the helix axis with the most stable amides on the nonpolar face of the helix (expected to face the membrane interior) and the least stable amides on the polar face (Fig. 12). The high exchange stabilities of amides near P14 and in the C-terminal region (especially Phol20) support models for membrane-reconstituted alamethicin in which the peptide adopts a conformation similar to the crystal structure. Analysis of single amide exchange from alamethicin in methanol indicates that, under conditions in which intramolecular hydrogen bonding is stabilized, alamethicin adopts a helical conformation with stable hydrogen bonding throughout (Dempsey, 1995). The proline residues (P2 and P14) are accommodated by local overtwisting of helix to allow 3_{10} hydrogen bonds involving U3 and V15 NH, and stable hydrogen bonding occurs through to the C-terminal Phol20. Analysis of atom trajectories in "methanol-solvated" molecular dynamics simulations demonstrates that this hydrogen-bonding arrangement is favored in alamethicin (P. B. Williams et al., submitted for publication).

The exchange data for alamethicin in DOPC bilayers at relatively low water content are consistent with a stable helical structure in which amides exchange more readily from the polar helix face expected to be directed toward aqueous solvent. This model does not distinguish between membrane-surface-lying peptide (helix axis perpendicular to the bilayer normal) and peptide self-associated in a transmembrane array with the polar helix surface facing a solvating aqueous lumen, because the relative accessibilities of helical amides to water (bulk water or water in the "pore" lumen, respectively) is essentially equivalent. At the relatively high peptide–lipid ratios used in the experiments at limiting water content (20:1 to 40:1, M:M), previous studies indicate that alamethicin is highly associated (Woolley and Wallace, 1993) and largely in a transmembrane orientation (Huang and Wu, 1991). It is tempting to ascribe helical periodicity in exchange protection to a pre-pore or pore arrangement of alamethicin in which stable helical hydrogen bonding protects amides to the C terminus of the peptide, although it cannot be discounted that rare migration ($K_{eq} < 10^{-3}$) of transmembrane to membrane surface states may be required for exchange to occur.

CONCLUSIONS

The data demonstrate that amide-exchange trapping combined with high-resolution NMR can be used to measure amide-resolved hydrogen–deuterium exchange rates for alamethicin reconstituted in fully hydrated phospholipid bilayers under a variety of conditions. The large exchange-protection factors support a model for alamethicin in DOPC bilayers at peptide:lipid ratios of 1:100 (M:M) and greater, in which amides near P14 and at the C terminus form stable (helical) hydrogen bonds, consistent with the conformational preferences observed in the crystal (Fox and Richards, 1982) and methanol solution (Esposito et al., 1987; Yee and O'Neil, 1992; Dempsey, 1995) structures and in solvated molecular dynamics simulations (P. B. Williams et al., submitted for publication). These observations support models for voltage-dependent activation of "pre-pore" states of alamethicin to conducting pores in which stable helices undergo reorientation in the membrane (surface to transmembrane or antiparallel transmembrane to parallel transmembrane states) through interaction of the helix dipole charges with the transmembrane potential (Boheim et al., 1983; Huang and Wu, 1991; Kelsh et al., 1992). It remains uncertain whether the transmembrane associated state of alamethicin in DOPC bilayers at concentrations of 1:100 (peptide:lipid) and greater should be considered a "pre-pore" state or a porelike state equivalent to the conducting state characterized in ion-conductance measurements. The high exchange stabilities of amides near P14 and at the C terminus indicate that if it is a "pre-pore" state this differs from the "pre-pore" model suggested by Fox and Richards (1982) and from any other model involving significant voltage-dependent recruitment of disordered structure into helix as a voltage sensor.

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