Temperature Dependence of the Interaction of Alamethicin Helices in Membranes†

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ABSTRACT: The interaction of the voltage-dependent channel-forming peptide alamethicin with dioleoylphosphatidylcholine (DOPC) small unilamellar vesicles (SUV) has been studied using circular dichroism spectroscopy over a range of wavelengths and temperatures. Evidence is presented for the existence of two distinct membrane-bound states of the peptide which reflect different extents of peptide–peptide interaction. An elevated temperature is found to diminish the apparent peptide–peptide interaction. These results provide insight into the general problem of helix–helix interaction in membranes and provide experimental support for the proposal [Popot, J. L., & Engelman, D. M. (1990) *Biochemistry 29*, 4031–4037] that these interactions can be enthalpically favorable.

The peptide antibiotic alamethicin (Figure 1) forms voltage-dependent ion channels in lipid membranes. It has served as an important model for understanding the structure and function of α-helical membrane proteins generally and particularly those which form voltage-gated ion channels [for a review see Woolley and Wallace (1992)]. Alamethicin is composed of just 20 residues and contains no charged groups (in the R_f50 version used here). Extensive data on the conformation of alamethicin in organic solutions is available (Jung et al., 1975; Esposito et al., 1987; Cascio & Wallace, 1988; Chandrasekhar et al., 1988; Yee & O'Neil, 1992), and the structure of alamethicin in crystals grown from acetonitrile/methanol has been solved by X-ray crystallography (Fox & Richards, 1982). A picture emerges of a predominantly α-helical peptide with a kink induced by the proline in position 14. Some flexibility in the C-terminal region has been observed (Fox & Richards, 1982), and infrared (IR)[1] and Raman data have suggested that alamethicin may contain elements of 3_{10}-helical structure and other, nonstandard, geometries in this region (Vogel, 1987; Haris & Chapman, 1988). Structural data on the peptide in membrane environments are more limited, but IR, Raman, and circular dichroism (CD) studies are consistent with a secondary structure broadly similar, although not identical, to that described for alamethicin in organic solutions (Cascio & Wallace, 1985; Vogel, 1987; Cascio & Wallace, 1988; Haris & Chapman, 1988). At a length of 20 residues with predominantly helical conformation, alamethicin resembles a transmembrane (TM) domain of an α-helical membrane protein. Due to the presence of proline in the sequence, alamethicin more closely resembles a TM domain in a protein with multiple membrane-spanning helices (such proteins often have a transport or channel function) than it does a TM domain in a protein which crosses the membrane only once (Deber et al., 1990; Woolfson et al., 1991).

Ac-Aib-L-Pro-Aib-L-Ala-Aib-L-Ala-L-Gln-Aib-L-Val-Aib-Gly-L-Leu-Aib-L-Pro-L-Val-Aib-Aib-L-Gln-L-Gln-L-Pheol

FIGURE 1: R_f50 version of alamethicin. The short form Aib represents α-amino-isobutyric acid and Pheol represents phenylalaninol. The N-terminus of alamethicin is acetylated (Ac).

The nature of self-association of alamethicin in membranes is of particular interest both for the elucidation of the mechanism of alamethicin channel formation and for the information it may provide on the factors governing helix–helix association in membrane proteins. Alamethicin aggregates have been described in every model of the active channel thus far proposed [for a review see (Woolley and Wallace (1992)]. Aggregation provides a ready explanation for the observation that the conductance induced by alamethicin across lipid membranes is highly dependent on alamethicin concentration. From the point of view of molecular structure, it is easy to envisage an ion channel formed by a bundle of helices with a central lumen whereas an alamethicin monomer provides no obvious pathway for ion movement. The lateral association of α-helices in membranes is thought to be a critical aspect of the folding and overall stability of many membrane proteins [for reviews see Popot and Engelman (1990) and Lemmon et al. (1992)]. It may also be functionally important in the action of certain receptor proteins (Bormann & Engelman, 1992; Cao et al., 1992).

Physical data on the association state of alamethicin in membranes is difficult to obtain and has been rather equivocal. Examination of alamethicin/dipalmitoylphosphatidylcholine (DPPC) mixtures using freeze fracture electron microscopy (EM) revealed the presence of distinct rows of particles with a spacing between rows between 60 and 70 Å (McIntosh et al., 1982). Such a long-range order would seem to require some sort of interaction among peptides in the membrane. X-ray diffraction of oriented multibilayers of alamethicin in DOPC at relatively high peptide:lipid ratios gave reflections indicating some ordering of the peptide/lipid sample in a direction perpendicular to the bilayer normal, again suggesting peptide–peptide interaction (P. E. Fraser, personal communication). The long-range order found using these techniques probably reflects larger-scale aggregation than would occur under the normal conditions of channel formation by alamethicin. Interestingly, however, long-lived high-conductance

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[1] Abbreviations: CD, circular dichroism; DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; egg PC, egg phosphatidylcholine; EM, electron microscopy; ESR, electron spin resonance; FAB, fast atom bombardment; HPLC, high-performance liquid chromatography; IR, infrared; MRE, mean residue ellipticity; NMR, nuclear magnetic resonance; OCD, oriented circular dichroism; SUV, small unilamellar vesicle; TM, transmembrane.

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states of alamethicin have occasionally been observed in bilayer conductance studies where a lateral segregation of the peptide is thought to occur (Boheim et al., 1983; Molle et al., 1988) and have been suggested to be due to the formation of large sheets of alamethicin.

Circular dichroism studies of alamethicin in bilayer vesicles (Cascio & Wallace, 1988) and oriented multibilayers (Huang & Wu, 1991) having shown a dependence of the CD signal on the peptide:lipid ratio, again suggesting some sort of interaction between peptides. Schwarz and colleagues (Schwarz et al., 1986; Rizzo et al., 1987) published a detailed study of the binding of alamethicin to DOPC lipid vesicles as monitored by CD spectroscopy. They reported a change in the mean residue ellipticity (MRE) of alamethicin at 224 nm as DOPC vesicles were added to solutions of the peptide. They made the important observation (confirmed here) that the extent of the change of MRE at 224 nm depended on the total concentration of alamethicin. Taking the MRE at 224 nm to reflect the extent of binding of alamethicin to lipid (*i.e.*, the ratio of bound peptide to free peptide), Schwarz and colleagues developed a detailed model of the interaction of alamethicin with membranes. In this model, the observed dependence of the MRE change at 224 nm on the total peptide concentration is a direct result of aggregation of alamethicin in the membrane phase (Schwarz et al., 1986; Rizzo et al., 1987).

We have examined the binding of alamethicin to DOPC vesicles using CD spectroscopy over a range of wavelengths and temperatures and have been able to identify *three* spectroscopically distinct states—one corresponding to peptide in the aqueous phase and *two* membrane-bound states. While the data provide evidence of peptide–peptide interaction as Schwarz et al. (1986) proposed, it appears that use of the mean residue ellipticity at 224 nm to characterize the extent of binding in a *two*-state model is not justified. Changes in temperature are found to alter the relative proportions of the two membrane-bound states we observe. Specifically, higher temperatures reverse the spectral changes which occur upon peptide–peptide interaction. The simplest explanation for the observed effects is to postulate a direct, temperature-dependent self-association of alamethicin in membranes. Other possible explanations are also considered. These results are discussed in light of current proposals regarding the nature of helix–helix association in membranes (Popot & Engelman, 1990; Wang & Pullman, 1991).

MATERIALS AND METHODS

Alamethicin was obtained from Sigma Chemical Co. (St. Louis, MO). This is the R_f50 version of alamethicin with Gln at position 18 in place of Glu (Figure 1). The sequence is heterogeneous with respect to position 6; Ala and Aib (α-aminoisobutyric acid) occur in approximately equal proportions as confirmed using HPLC and FAB mass spectrometry. Dioleoylphosphatidylcholine (DOPC) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Aliquots of stock solutions of alamethicin (0.5 or 5 mM in HPLC grade methanol) were initially added to a 5 mM sodium phosphate, pH 7.0, buffer solution. Methanol concentrations in the samples for CD analysis never exceeded 5%, and equivalent concentrations of methanol were also added to control samples. The CD spectrum of alamethicin in water is little affected by additions of methanol up to almost 20%. Stock solutions of small unilamellar vesicles (SUV) at a known concentration were made routinely by sonication of lipid suspensions as described previously (Cascio & Wallace, 1988; Woolley & Deber, 1988). Aliquots of these stock solutions were then added to the alamethicin samples to obtain final lipid concentrations as indicated in the figure legends. No time dependence was observed for any of the reported CD spectra.

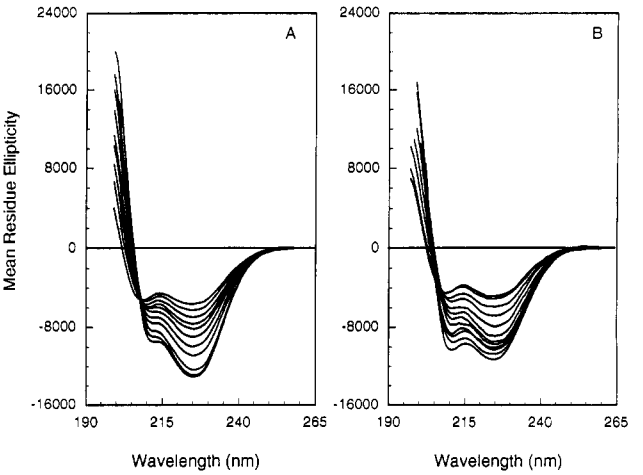

FIGURE 2: Circular dichroism spectra showing the titration of alamethicin with DOPC vesicles. (A) Alamethicin, 16 μM; DOPC concentrations (mM): 0 (uppermost spectrum), 0.0045, 0.009, 0.0133, 0.018, 0.022, 0.027, 0.045, 0.089, 0.178, 0.395 (lowermost spectrum). (B) Alamethicin, 4 μM; DOPC concentrations (mM): 0 (uppermost spectrum), 0.0014, 0.0046, 0.0092, 0.018, 0.045, 0.089, 0.177, 0.31, 0.43, 0.56, 0.68 (lowermost spectrum). Three scans were obtained at each concentration, the baseline was zeroed at 260 nm, and the scans were averaged and smoothed. Equivalent spectra of samples without alamethicin were subtracted to correct for lipid contributions to the spectra.

CD measurements were made in quartz cells (1–0.01 cm path lengths) using an AVIV 62DS spectropolarimeter. The measurement of optical rotation was calibrated using (+)-10-camphorsulfonic acid, and the wavelength scale was calibrated using benzene vapor. The temperature was controlled to ±0.2 °C using a thermoelectric sample holder. The photomultiplier was placed as close to the sample compartment as possible in order to maximize the detection of any scattered light although this proved not to be a problem in the current experiments. For each CD spectrum reported at least three separate scans were taken. The data were then imported into a graphical spreadsheet (Deltagraph, Deltapoint, Inc.) where a correction for baseline drift at 260 nm was made and the scans were averaged and smoothed. Direct comparisons of spectra to linear combinations of basis spectra could be done readily using this spreadsheet. Details of specific experimental conditions are found in the figure legends.

RESULTS

The titration of alamethicin with DOPC vesicles results in large changes in the CD spectrum over the whole wavelength range from 260 to 205 nm. Below about 205 nm the absorbance of the solutions is too large to reliably permit measurement of the spectra under these conditions (Figure 2). Samples with two different concentrations of the peptide (16 and 4 μM) are shown (Figure 2A,B, respectively). While the double minima (the $n\pi^*$ transition near 224 nm and the $\pi\pi^*\|$ transition at 208 nm) characteristic of helical conformations are observed in each case, the families of spectra for the two different peptide concentrations have distinctly different shapes. The titration of 16 μM sample (Figure 2A) shows a smooth progression of curves where the minima move from 224 to 225 nm and from 208 to 211 nm over the course of the titration. The minimum near 224 nm is always more pronounced than that near 210 nm, and there is an isodichroic

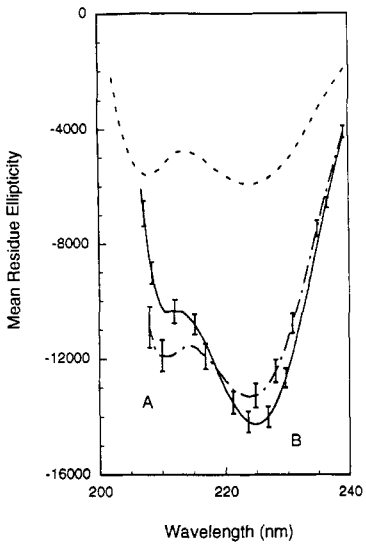

FIGURE 3: Circular dichroism spectra of 16 μM alamethicin in 5 mM phosphate buffer, pH 7.0 (---), and of alamethicin in the presence of DOPC vesicles (0.64 mM lipid). The curve labeled A (– – –) was obtained with an alamethicin concentration of 4 μM and curve B (—) with an alamethicin concentration of 16 μM in 1-cm quartz cells at 25 °C. Five separate preparations were averaged, and standard deviations are represented by the error bars.

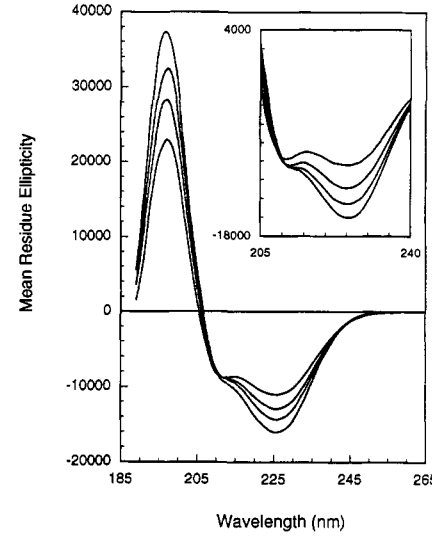

FIGURE 4: Effects of temperature on the CD spectra of alamethicin (500 μM) bound to DOPC vesicles (6.4 mM). The inset shows the spectra of alamethicin (100 μM) bound to DOPC (6.4 mM) recorded using a 0.05-cm-path-length cell. Spectra were recorded at 4 (lowermost spectrum), 25, 45, and 75 °C (uppermost spectrum).

point at 208 nm. Each spectrum can be represented by a linear combination of the spectrum of aqueous alamethicin and the fully-bound spectrum. In contrast, the titration of the 4 μM sample (Figure 2B) shows biphasic behavior. Additions of small amounts of DOPC vesicles cause changes similar to those seen with the 16 μM sample with a pronounced change in negative ellipticity near 224 nm and red shifts of the 208 and 224 nm minima. However, as the lipid concentration is increased, the shape of the spectrum changes as the minimum at 210 nm becomes more pronounced and undergoes a small blue shift. In this case the family of spectra cannot be represented as linear combinations of free and bound forms.

Figure 3 shows the spectra of 4 and 16 mM alamethicin in the presence of 0.64 mM DOPC vesicles from 240 to 205 nm as well as in the absence of vesicles for comparison. This concentration of DOPC is enough to ensure that the CD changes observed on binding are essentially complete; *i.e.*, the plateau of the binding curve described by Schwarz et al. (1986) has been reached in each case. These samples are therefore identical except for a 4-fold difference in the total peptide concentration (and therefore peptide:lipid ratio). Clearly the shapes of the spectra are different, that for the 16 μM concentration having a more pronounced negative band at 224 nm but also a much less pronounced band at 210 nm. The previous observation that the MRE at 224 nm was less for lower concentrations of alamethicin (*i.e.*, the 4 μM sample) was interpreted as meaning that the peptide was incompletely bound at these concentrations (Schwarz et al., 1986; Rizzo et al., 1987). However, if the ellipticity at 210 nm had been used instead to characterize the extent of binding, the opposite conclusion might have been arrived at. In contrast, the full CD spectra suggest the presence of at least two distinct membrane-bound states for alamethicin (and three distinct states if we include peptide in the aqueous phase). Thus, the state assumed in the membrane-bound form is apparently determined by the peptide-to-lipid ratio.

CD measurements of membrane systems are prone to artifacts arising from differential scattering and absorption flattening (Gordon & Holzwarth, 1971; Wallace & Mao, 1984; Bustamante & Maestre, 1988). These effects are minimized (Mao & Wallace, 1984) in the present experiments since SUVs with relatively high lipid-to-peptide ratios (>40- to 160-fold for the comparison in Figure 3) have been used. The spectra were found to be insensitive to the position of the detector, indicating that differential scattering artifacts are minimal (Wallace & Mao, 1984). Absorption flattening is expected to diminish the observed ellipticity (both positive and negative) at shorter wavelengths (Cascio & Wallace, 1988). Thus, while a diminished signal for the 16 μM sample at 210 nm could be explained by an absorption flattening effect, the increased signal at 224 nm is inconsistent with this interpretation. Finally the observation of an isodichroic point in the titration of alamethicin with DOPC vesicles (Figure 2B) would not be expected if a significant membrane artifact was contributing to the spectra. Thus, the spectra in Figure 3 reflect a real difference in the interaction of alamethicin with circularly polarized light as a function of the peptide's concentration in membranes.

The effect of temperature on the spectrum of alamethicin in DOPC vesicles was then investigated. This lipid has a gel-to-liquid crystalline phase transition temperature of –19 °C (Marsh, 1990) so that it is in the liquid crystalline state for all the experiments reported here. Changes in temperature affect the ratio of the ellipticities of the 210 and 224 nm bands (Figure 4) just as do changes in the membrane-bound peptide: lipid ratio, and a clear parallel is evident between these data and the data on the effect of the peptide:lipid ratio (Figure 3). As the temperature is lowered, the ellipticity at 224 nm becomes progressively more negative while that at 210 nm is much less affected. The use of higher peptide concentrations in this experiment enabled the use of a shorter path length cell which permitted the additional measurement of the low wavelength 195 nm ($\pi\pi^* \perp$) helix peak. This peak mirrors the behavior of the 224 nm peak: lower temperatures result in more pronounced ellipticity. As with all the spectra in this study, no time dependence of CD parameters was observed. Effects of temperature on the CD spectra were rapid (they occurred within the several minutes needed to change the temperature of the sample) and reversible.

In order to directly compare the role of temperature and the peptide:lipid ratio, we have examined the effect of

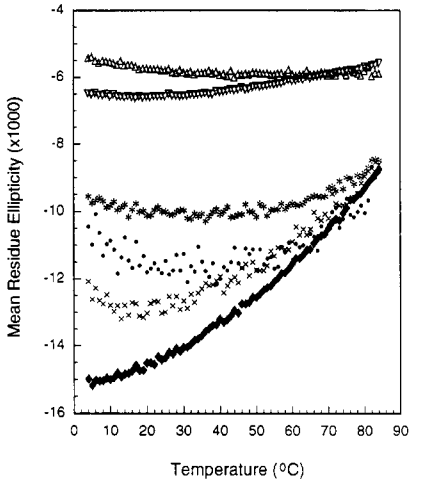

FIGURE 5: Temperature dependence of the mean residue ellipticity (MRE) of alamethicin in aqueous solution and bound to DOPC vesicles: For each point the signal was averaged for 30 s at 1 °C intervals after a 0.3-min equilibration period in 1-cm quartz cells. The MRE was corrected for baseline drift by monitoring the signal at 260 nm. Replicate measurements in the absence of peptide were performed to correct for the contribution of lipid to the signal. Alamethicin at 16 μM in 5 mM phosphate buffer, pH 7.0, at 210 nm (△) and 224 nm (▽); in the presence of 0.64 mM DOPC, alamethicin at 16 μM at 210 nm (*) and 224 nm (♦) and alamethicin at 4 μM at 210 nm (●) and 224 nm (×).

temperature on CD parameters under exactly the same conditions used to obtain the spectra in Figure 3. In Figure 5 the MRE at 210 and 224 nm is plotted as a function of temperature for three different samples. The two lower sets of data points correspond to the conditions of Figure 3 with alamethicin at 4 and 16 μM in DOPC vesicles (0.5 mM). The upper set of data points is for alamethicin in water (16 μM). Importantly, at high temperatures, differences due to the different peptide concentration in the membrane-bound samples disappear, and indeed the spectra for alamethicin at 4 and 16 μM when recorded at 70 °C are indistinguishable.

Although an increase in temperature might cause dissociation of alamethicin from vesicles and thereby explain the coalescence of the two sets of data points at high temperatures, we believe this is not occurring for the following reasons: (1) Even at high temperatures the spectra do not correspond to those in water and in fact show considerably larger mean residue ellipticity. (2) Schwarz and colleagues (Schwarz et al., 1986; Rizzo et al., 1987) report little effect of temperature on the association reaction. Although the use of mean residue ellipticity at 224 nm to quantify the extent of binding is inaccurate, a large effect of temperature on the affinity of alamethicin for DOPC would nevertheless have been evident in such an analysis. That is, more lipid would have been required to effect a maximal change in the ellipticity at higher temperatures. (3) The changes in the shape of the spectra with temperature (Figures 4 and 5) do not correspond to the changes in the shape of the spectra as alamethicin binds lipid (Figure 2). The changes seen must therefore reflect some effect of temperature on the membrane-bound state. Considering the similarity between the effects of temperature and the effects of the peptide:lipid ratio, it seems reasonable to conclude that the CD changes at elevated temperatures reflect, at least in part, a diminished interaction of membrane-bound alamethicin helices.

DISCUSSION

Evidence for the Interaction of Alamethicin Helices. We began our titrations of alamethicin with DOPC vesicles using two concentrations of alamethicin (4 and 16 μM) which correspond to the minimum and maximum concentrations reported by Schwarz and colleagues (Rizzo et al., 1987) in order to be able to compare our results with theirs. Rather than monitor the mean residue ellipticity at one wavelength, we have recorded the amide-region CD over all experimentally accessible wavelengths. In keeping with previous observations (Schwarz et al., 1987), we found that the interaction of alamethicin with membranes was a rapid and reversible process. The present study has identified two spectroscopically distinct (and therefore likely structurally distinct) populations of alamethicin bound to DOPC membrane vesicles, the relative proportions of which depend on the peptide-to-lipid ratio. We have obtained a qualitatively similar result with DMPC vesicles (Cascio & Wallace, 1988) and egg PC vesicles (not shown). Our observations at 224 nm are consistent with those of Schwarz and colleagues (Schwarz et al., 1986; Rizzo et al., 1987), and although we disagree with the details of their analysis, the essential conclusion of peptide-peptide interactions reached by them is inescapable: It is difficult to imagine any situation which could produce the observed changes in the CD spectra without some interaction, even if indirect, between peptide molecules in the membrane.

At present there is insufficient data to permit us to describe conclusively the molecular nature of peptide–peptide interactions in the alamethicin system. The simplest type of interaction would be self-association of alamethicin peptides in the membrane. We have reviewed the evidence for such an interaction above. A further indirect piece of evidence that self-association occurs is that pore formation by alamethicin in membranes in the absence of a transmembrane potential is found to begin at approximately the concentration where the CD changes occur (Woolley & Deber, 1988; Woolley & Deber, 1989; Schwarz & Robert, 1990).

Archer et al. (1991) have reported that the ESR signal of a spin-labeled alamethicin derivative in egg PC membranes [where we have observed spectra similar to those reported here for DOPC (not shown)] is insensitive to the addition of unlabeled alamethicin up to peptide:lipid ratios as high as 1:5. We suggest that this might occur if the motion of the nitroxide label itself at the alamethicin C-terminus is rather insensitive to peptide–peptide association. Wille et al. (1989), using the same spin-labeled derivative, report that the ESR spectrum reflects primarily segmental motion of the nitroxide label. The absence of spin–spin interactions between alamethicin peptides at low concentrations, however (Archer et al., 1991), demonstrates that a monomeric membrane-bound form of the peptide can exist. A systematic analysis, with spectral simulations, of the dependence of spin–spin interactions on the peptide concentration and peptide:lipid ratio, such as that described in detail by Gordon and Curtain (1988), might reveal to what extent a simple partitioning/diffusion model involving only monomeric alamethicin helices [as suggested by Archer et al. (1991)] could fully describe the association of alamethicin with membranes.

Origin of the CD Spectral Changes. There are several possible reasons for the appearance of the spectra in Figure 3 corresponding to the two distinct membrane-bound states. These include (1) secondary structure changes caused by self-association, (2) association of helices without secondary structure changes, (3) a change in the average environment (*e.g.*, membrane location) of the chromophores which in turn affects the secondary structure, and (4) a change in the average environment of the chromophores which affects the rotatory strengths of certain transitions. These possibilities are not all

mutually exclusive, but we distinguish between them for clarity of discussion. Although it is not possible at present to decide conclusively among these possibilities, we will consider the evidence for and against each of them.

The simplest explanation for the differences between the spectra labeled A and B in Figure 3 would be to postulate that alamethicin helices actually self-associate in the membrane and that spectra A and B in Figure 3 correspond to monomeric and self-associated helices, respectively. Such an explanation was offered many years ago for the differences between the CD spectra of alamethicin in organic solutions and an organic nematic phase (Jung et al., 1975). Clearly, a monomeric form of membrane-bound alamethicin can exist under conditions approximately similar to those of Figure 3A as the ESR data (Archer et al., 1991) demonstrate, and furthermore, self-association might well entail secondary structure changes. The overall strength of the CD bands in Figure 3 is not much different, indicating that large-scale secondary structure changes are unlikely. However, small changes in α-helical backbone torsion angles can result in altered chromophore interactions and changes in the CD spectrum without significantly altering the characteristic double-minimum spectrum (Manning et al., 1988). Small changes in the nonstandard regions of alamethicin secondary structure could also produce the observed chiroptical changes.

The spectra reported in Figure 3 bear a strong resemblance to those reported for peptides which undergo monomer to two-stranded coiled-coil transitions (Lau et al., 1984; Zhou et al., 1992). Cooper and Woody (1990) have calculated the CD changes to be expected upon parallel two-stranded α-helical coiled-coil formation. The close apposition of two rigid α-helices was predicted to result in a decrease in the $\pi\pi^*\|$ band (210 nm) intensity. Distortion of the α-helices to a coiled-coil conformation through systematic changes in the backbone torsion angles produces further changes in the calculated spectra, including a red shiftt of the $\pi\pi^*\|$ minimum and an increase in the strength of the $n\pi^*$ (224 nm) band. These calculations as well as experimental spectra (Lau et al., 1984; Zhou et al., 1992) are in keeping with the differences observed between the CD spectra of putatively monomeric and interacting helices in Figure 3. A further prediction of a decrease in the $\pi\pi^*\perp$ (195 nm) band could not be measured under the conditions used. Movement of the $\pi\pi^*\|$ minimum is particularly evident in Figure 2B where it is seen to be red shifted with respect to the starting state (alamethicin in aqueous solution) when small amounts of lipid are added but then moves to shorter wavelengths (blue shifts) in excess lipid.

Alamethicin does not have an obvious 7-fold hydrophobic repeat in the primary sequence—something which typifies standard coiled-coils—but the overall hydrophobicity of the alamethicin sequence might obscure such a feature (Cohen & Parry, 1990). The presence of a proline residue (Pro^{14}) is also not typical of coiled-coil-forming sequences so that alamethicin coiled-coils, if they exist, would have to be somewhat distorted. Moreover, alamethicin aggregates would need to contain more than two monomers if they were to provide a pathway for ion movement although two-stranded coils might occur as intermediates in the aggregation process. Higher order coiled-coils and parallel and antiparallel coiled-coils have been discussed, and suggestions about their occurrence in membrane proteins have been made (Dunker & Jones, 1977; DeGrado et al., 1989; Cohen & Parry, 1990). Membrane proteins consisting of multiple membrane spanning α-helices, and particularly those with proline-containing helices, could engage in helix–helix interactions which might resemble coiled-coils but with certain nonstandard geometries. For instance, evidence for helix–helix association in a right-handed supercoil where one of the helices has a pronounced kink has recently been found in the electron crystallographic analysis of the plant light-harvesting complex (Kuhlbrandt & Wang, 1991). To what extent the CD spectra of such structures would resemble those of canonical coiled-coils is at present not clear.

Interestingly, a simple 21-residue polypeptide containing only leucine and serine residues has been designed which potentially could form two-stranded as well as higher order (*e.g.*, four-stranded) coiled-coils. This peptide has been found to form ion channels with several features in common with alamethicin channels (Lear et al., 1988). A recent study using a fluorescently-labeled version of this peptide has identified a dimeric form of the peptide as a major component of the membrane-bound equilibrium (Chung et al., 1992). It would be interesting then to examine the CD spectrum of this peptide as a function of membrane-bound peptide concentration for signs of coiled-coil formation.

An unusual α-helical CD spectrum has been reported for fd bacteriophage which shows an intense band near 222 nm similar to that observed with alamethicin at high concentrations in membranes (Figure 3, curve B). This system has been studied extensively by Dunker and colleagues (Arnold et al., 1992) who have attributed the unusual spectrum to a specific interaction of aromatic residues in the structure which causes a coupling of a tryptophan side-chain transition with a phenylalanine transition in close proximity. The only aromatic chromophore in alamethicin is the phenyl ring of the C-terminal phenylalaninyl residue (Figure 1). Although the formation of an alamethicin aggregate might result in clusters of phenylalaninyl residues oriented in specific ways with respect to one another and the peptide backbone, the intensity of phenyl transitions is comparatively weak (for wavelengths $\geq$220 nm) so that effects on the overall CD spectrum would likely not be so dramatic as with other aromatic groups (Strickland, 1974; Wetlaufer, 1962). Note that this type of process could result in CD changes with little change in the secondary structure of alamethicin. It should also be pointed out that part of the CD changes calculated to occur upon coiled-coil formation do not involve a secondary structure change but only the close apposition of two helices such that their transition dipoles interact (Cooper & Woody, 1990).

Association without a significant secondary structure change would help to explain the observation that the IR spectrum of alamethicin at high concentrations in vesicles is similar to that of the peptide in methanol (Haris & Chapman, 1988) where it is known to exist as a monomer (Cascio & Wallace, 1988). Unfortunately, the low peptide concentrations involved make it difficult to detect monomeric membrane-bound alamethicin directly using IR spectroscopy, so a direct comparison with the CD data reported here is not possible. Interestingly though, the CD spectrum of alamethicin in methanol is more like curve A than curve B in Figure 3 (Cascio & Wallace, 1988).

The CD spectral changes observed need not necessarily be a direct result of the physical self-association of alamethicin helices. For instance, the spectra in Figure 3 might reflect alamethicin in two different types of interaction with lipid resulting from different orientations with respect to the bilayer, for instance surface-associated and transmembrane-inserted. This might result in small secondary structure changes and/or different effective solvation of ground and excited states

which would result in changes in the relative rotary strengths of the $\pi\pi^*\|$ and $n\pi^*$ bands. The wavelength shifts of the bands noted above might be a consequence of the more hydrophobic environment of the membrane interior with respect to the surface and with respect to the aqueous solution (Cascio & Wallace, 1988). ESR spectra obtained with labeled alamethicin in oriented multibilayers (Archer et al., 1991) have suggested the presence of more than one distinct average orientation of the membrane-bound peptide. Using the technique of oriented circular dichroism (OCD), Huang and Wu (1991) have reported a change in the orientation of alamethicin which depends on the peptide:lipid ratio. The differences in the CD spectra reported in Figure 3 (curves A and B) are sufficiently small that they would be apparent in the OCD experiment which explains why Huang and Wu (1991) did not report a change in spectral shape accompanying insertion. These researchers have suggested that reorientation might not require the actual self-association of the peptides, at least not on a large scale; inserted peptides might interact over relatively long distances through a process of bilayer deformation.

Fasman and colleagues have recently reported an extensive study of the CD spectra of a variety of membrane proteins (Park et al., 1992). This collection of spectra was deconvoluted to a basis set of five component spectra in a process which is independent of any structural knowledge of the proteins. Interestingly two distinct α-helical spectra were found, one with approximately equal minima at 208 and 222 nm and one with a more pronounced minimum at 222 nm. These two components were assigned to "soluble" (*i.e.* soluble-protein-like) and transmembrane helices, respectively. The shape of the transmembrane CD spectrum was attributed to a possible environmental effect on transition dipoles and/or the difference in the average length of soluble and transmembrane helices.

As pointed out earlier, the various possible origins of the CD spectral changes just discussed are not mutually exclusive. For instance, a transition from a monomeric surface-associated helix to a transmembrane coiled-coil-like structure is quite possible.

Effects of Temperature. We have presented evidence above that the effects of temperature on the CD spectrum of the alamethicin/DOPC system do not reflect dissociation of the peptide from the membrane and instead reflect changes in the membrane-bound states. CD changes with temperature appear to be in part due to temperature-dependent changes in the secondary structure of the membrane-bound alamethicin monomer since the mean residue ellipticities at 210 and 224 nm of the 4 μM alamethicin sample (putatively monomeric) are also affected by temperature. We interpret this to be a result of fraying at the helix ends, particularly the C-terminal end, since NMR studies (Esposito et al., 1987; Yee & O'Neil, 1992) and molecular dynamics simulations (Fraternali, 1990) have suggested this to be the most flexible region of the molecule.

Aside from effects of temperature on the secondary structure of alamethicin, there appears to be a clear effect of temperature on the peptide–peptide interaction described above. As the temperature is raised, the effects of differing peptide:lipid ratios disappears, and indeed at high enough temperatures the ellipticities of the 4 and 16 μM samples become equal (Figure 5). This suggests the presence of a common stable core structure of the peptide, probably the regular N-terminal α-helix identified by NMR studies of alamethicin in solution (Esposito et al., 1987) and X-ray structural analysis of alamethicin crystals (Fox & Richards, 1982). The presence

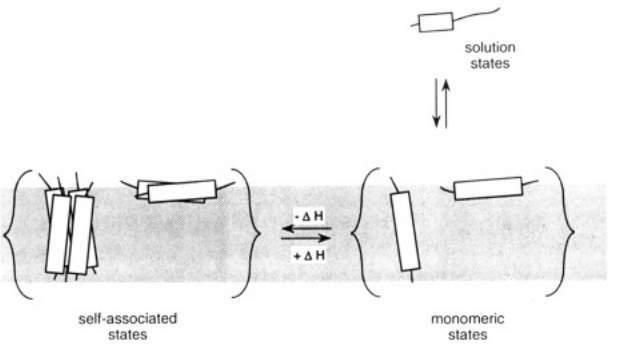

FIGURE 6: A schematic illustration of proposed alamethicin–membrane interactions. The peptide in solution has a small percentage of helical content as indicated by the short box. When alamethicin associates with the membrane, its helical content increases. Within the membrane, self-association can occur and channel activity is believed to arise from aggregated forms of the peptide. A number of orientations with respect to the membrane are possible, although the relative populations of these species are not completely defined. Self-association is enthalpically favorable; higher temperatures favor monomeric species.

of several Aib residues in this region probably accounts for the enhanced stability of the N-terminal helix. The effect of temperature on the CD spectrum of alamethicin at relatively high concentrations [500 and 100 μM (inset)] in DOPC vesicles is shown in Figure 4. Lower temperatures cause changes, particularly in the 224 nm band, which are similar to those seen with increasing peptide:lipid ratios. We expect that the behavior of the higher peptide concentration samples (Figure 4) should approximately parallel that of the 16 μM sample (Figure 5) since both report the behavior of interacting peptide species. While this is true, the sample with the highest peptide:lipid ratio (main curves in Figure 4) still shows a pronounced 224 nm band even at 75 °C (compare with Figure 4 inset and Figure 5). This indicates that the effectiveness of increased temperature in diminishing the interaction of peptides in the membrane is in turn dependent on the membrane-bound peptide concentration, as would be expected.

In support of the proposal that the CD spectrum of alamethicin at high concentrations in membranes reflects an interaction between peptides which share features with a coiled-coil structure, the effect of temperature on the CD spectrum of this system (Figure 4) is very similar to the temperature-induced denaturation of coiled-coils in solution (Engel et al., 1991). Denaturation is not so complete in the alamethicin system, however, presumably since membrane association and the presence of Aib residues stabilize the secondary structure. It should be pointed out that the decrease in the intensity of the 195 nm band as the temperature is raised is not in keeping with what is predicted by the calculations of Cooper and Woody (1990) but temperature-induced changes in the secondary structure complicate the interpretation of changes in the intensity of this band.

A schematic diagram illustrating the interaction of alamethicin with membranes is presented in Figure 6. Although the precise structures involved are presently unknown, the two types of membrane-association states which can be distinguished by CD are indicated. The equilibrium between these states is affected by temperature such that higher temperatures promote dissociated states.

Popot and Engleman (1990) have proposed a general model for the folding of α-helical membrane proteins which involves the insertion of monomeric transmembrane helices followed by the association of these helices into bundles. The thermodynamics of such a two-stage process are largely unknown

although recent microcalorimetric measurements with related systems have suggested that insertion may be either enthalpy or entropy driven and depends on the membrane curvature (Beschiaschvili & Seelig, 1992). *Prima facie*, entropy would seem to favor helix dispersion in the membrane. However, the possible restriction of lipid conformations in the vicinity of a helix may give rise to some entropic preference of lipid molecules to be next to other lipids (Popot & Engleman, 1990). Wang and Pullman (1991) have recently performed an energy calculation which suggests that helices in membranes prefer to form bundles rather than remain dispersed in the lipid phase due to a favorable enthalpy of helix–helix interaction. This enthalpy is calculated to be due mainly to favorable van der Waals contacts between helices. Although the simultaneous effects of temperature on the secondary structure and peptide–peptide interaction make it difficult to provide a quantitative estimate of the enthalpy of alamethicin self-interaction, and the uncertainty about the detailed structure and orientation of the monomeric and interacting states complicates the interpretation of this enthalpy in molecular terms, it is clear that the interaction of alamethicin helices is enthalpically favorable. Furthermore, the rapid kinetics and the reversibility of the observed changes make the alamethicin system a suitable one for detailed study of the thermodynamics of helix–helix interactions. Current work is now focused on understanding the energetics of alamethicin membrane interactions in molecular terms.

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