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Conformation of Alamethicin in Phospholipid Vesicles: Implications for Insertion Models

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ABSTRACT **The secondary structure of alamethicin, a membrane channel-forming polypeptide, has been examined by circular dichroism spectroscopy to determine the relationship of its conformation in organic solution to its conformation in a membrane-bound state. The spectrum of alamethicin in small unilamellar dimyristoyl phosphatidylcholine vesicles is significantly different from its spectrum in 10% methanol/acetonitrile, the solvent from which it was crystallized (Fox and Richards: *Nature* 300:325-330, 1982), as well as its spectrum in methanol, the solvent in which NMR studies have been done (Banerjee and Chan: *Biochemistry* 22:3709-3713, 1983). This suggests that structural models based on studies of the molecule in organic solvents may not be entirely appropriate for the membrane-bound state. To distinguish between different models for channel formation and insertion, two different methods were used to associate the alamethicin with vesicles; in addition, the effect of oligomerization on the conformation of the membrane-bound state was investigated. These studies are consistent with a modified insertion model in which alamethicin monomers, dimers, or trimers associate with the bilayer and then spontaneously oligomerize to form a prechannel with a higher helix content. This aggregate could then "open" upon application of an appropriate gating transmembrane potential.**

Key words: membrane proteins, channels, circular dichroism spectroscopy

INTRODUCTION

Alamethicin is a linear polypeptide antibiotic isolated from the fungus *Trichoderma viride*.[1] Its major component (~70%) has the following sequence:[2]

Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phl

where Aib is aminoisobutyric acid and Phl is phenylalaninol. Minor components contain single or double amino acid substitutions, the most common of which replaces the alanine at position 6 with aminoisobutyric acid.[3] Alamethicin spontaneously inserts into black lipid membranes and produces relatively nonspecific, voltage-gated ion channels such that the conductance varies proportionately with the sixth power of the polypeptide concentration, the transmembrane potential, and the ion concentration.[4] It was suggested that the dependence on polypeptide concentration arises because the oligomeric channel is composed of six monomers. Furthermore, it was found that if the voltage across the membrane is clamped at a particular value, steplike current fluctuations are found, with the number of steps increasing at higher voltages.[5,6] This led to the proposal that each current step arises from an incremental increase or decrease in the number of alamethicin molecules which form the oligomeric channel. The number of alamethicin monomers per channel was estimated to vary between six and twelve.

At least three different general models for the alamethicin channel have been proposed based on conductance studies. One is a "barrel staves" model[7,8] in which monomeric alamethicin adsorbs to the surface of the membrane and, upon application of a transmembrane potential, inserts into the hydrophobic interior of the bilayer. In this model, monomers then diffuse laterally and aggregate to form an open channel. Alternately, the "preaggregate" model[9,10] hypothesizes that both aggregation and incorporation of alamethicin into the bilayer precedes the voltage-dependent opening of the oligomeric channel. In a third model,[11] alamethicin is proposed to aggregate as a two-dimensional micelle on the surface of the membrane, but is not incorporated into the bilayer. The applied field then pushes these aggregates into the membrane.

To establish a physical basis for such models, a number of structural studies, including circular dichroism (CD)[12-15] and NMR spectroscopy,[16-18] have been conducted. These studies examined the conformation of alamethicin in a variety of organic solvent systems. In addition, Fox and Richards[19] have used X-ray crystallography to determine the three-dimensional structure of a trimeric form of the molecule crystallized from 10% methanol/acetonitrile. Consequently, Fox and Richards[19] and Hall and coworkers[20] have proposed two different structural

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models for the open multimeric channel. In both models the channel is a funnel-shaped oligomer; the constricted narrow N-terminal end is composed of a barrel-like arrangement of alpha-helices with the potentially helix-breaking proline residue at position 14 tipping the C-terminal residues of the flanged region away from the channel axis. The models differ in the structure of their C-terminal regions; the former considers the C-terminal end as helical, whereas the latter models this end as a beta-barrel with intermolecular H-bonds between adjacent monomeric units. Additionally, Fox and Richards proposed that the molecule oligomerizes spontaneously to form a solvent-filled channel traversing approximately half the bilayer before the application of any transmembrane potential. A voltage-gated reorientation of the surface-associated C-terminus then facilitates the movement of the oligomer through the remainder of the bilayer so that a pore traversing the whole bilayer is open. This is a more detailed version of the "preaggregate" insertion model.

The structural models, however, were based on data derived from studies of alamethicin in organic solvents. Since the conformations of small peptides tend to be flexible and sensitive to environmental effects, it is unclear whether this data is relevant to the structure of the polypeptide in the more ordered, hydrophobic environment of a phospholipid membrane. The structure adopted by alamethicin has been shown to vary when it is dissolved in different organic solvents.[12,13] Structural polymorphism is also evident in comparisons of the crystal structure of the intact molecule with the structures of synthetic alamethicin fragments. The crystal form of alamethicin is entirely alpha-helical; the only deviations arise due to short (one or two residues) 3_{10} helical regions in the C-terminal domain,[19] while crystals of the amino terminal tetrapeptide of alamethicin[21] exhibit two consecutive beta-turns which form an incipient 3_{10} helix. NMR[22] and infrared spectroscopic studies[23] have also indicated that the 1–6 and the 7–13 synthetic fragments of alamethicin adopt entirely 3_{10} helical conformations. Hence, it is expected that the structure of alamethicin in the more structured hydrophobic environment of a membrane bilayer, which imposes directionality on orientation of monomers, might differ substantially from that in an isotropic and more hydrophilic organic solution.

In order directly to determine the effects of environment on its conformation, alamethicin should be examined in both organic solutions and in phospholipid bilayers by a single physical method. In this study, circular dichroism (CD) spectroscopy has been used to compare the conformation of alamethicin in 10% methanol/acetonitrile (the solvent from which the molecule has been crystallized), in methanol (the solvent in which the NMR studies were conducted), in acetonitrile, and in small unilamellar phospholipid vesicles.

By examining the dependence of conformation on both the polypeptide concentration and the method of incorporation into the vesicles, we may distinguish which model best depicts the insertion process and channel-gating properties of alamethicin. Alamethicin can be incorporated into vesicles either by cosolubilization with lipid or bound by addition to preformed vesicles, analogous to the methods used by Mueller and Rudin[4] in conductance studies in black lipid membranes. Comparison of the conformations of alamethicin in these two preparations may establish whether the structure of alamethicin spontaneously adsorbed to the vesicles is the same as that of alamethicin incorporated into the bilayer. All the proposed models except for the "two-dimensional micelle" model would predict the structure in both types of preparations should be identical. Of the other two models, only in the "barrel-staves" model is the alamethicin conformation independent of its concentration (assuming that the conformation of alamethicin differs between the monomeric and oligomeric states); in this model, oligomerization does not occur before the application of an appropriate transmembrane potential, so at any concentration only monomers will exist in the vesicles. Experiments examining the concentration dependence of alamethicin have been conducted in order to distinguish between these two models.

MATERIALS AND METHODS

Materials

Alamethicin was the generous gift of J.E. Grady of the Upjohn Company (Kalamazoo, MI), and was used without further purification. Dimyristoyl phosphatidylcholine (DMPC) was purchased from Calbiochem-Boehring (La Jolla, CA). All solvents were HPLC grade.

Alamethicin concentrations were determined by quantitative amino acid analysis on representative samples. A factor correlating the concentrations as determined by these analyses with the protein concentrations as determined by the assay of Lowry et al.[24] in the presence of 1% sodium dodecyl sulfate (SDS) or by modified Lowry assay (for samples containing salt) in which the protein was first precipitated with tricarboxylic acid[25] was calculated. This factor was then used to determine the actual alamethicin concentrations in other samples which were analyzed by Lowry assay but not by quantitative amino acid analysis. This method of quantitation, rather than the potentially inaccurate gravimetric or colorimetric analyses typically used in other studies, is crucial for the determination of the lipid-to-alamethicin ratios, which is information necessary for correct interpretation of the spectral data. Blanks containing equivalent quantities of DMPC, but without added alamethicin, were prepared and used in all

protein determinations. Lipid concentrations were determined by a modified Fiske-Subbarow phosphate assay.[26]

Sample Preparation

Solutions

Alamethicin was dissolved in a 10% methanol/acetonitrile solution at concentrations ranging from 0.12 to 10 mg/ml, in methanol (0.2 mg/ml), and in acetonitrile (0.2 mg/ml). All solutions were centrifuged for 6 minutes at 12,800*g* to remove any insoluble material.

Type I vesicles

Alamethicin was cosolubilized with DMPC in chloroform using initial DMPC-to-alamethicin mole ratios ranging from 10:1 to 155:1. The solvent was removed under vacuum, leaving a thin layer on the wall of a pear-shaped flask. This lipid-containing layer was then hydrated by adding a known volume of deionized water. The suspension was heated above the phase transition temperature of the lipid and sonicated to clarity with a Soniprep 150 ultrasonicator microprobe using repeated 8–12-second bursts at ~25°C. The vesicle suspension was centrifuged at 12,800*g* for 6 minutes and the supernatant, which contained the small unilamellar vesicles (SUVs), was retained. Final lipid-to-polypeptide mole ratios ranged from 12:1 to 2,200:1. Samples containing equivalent amounts of DMPC, but no alamethicin, were prepared in the same manner.

Type II vesicles

DMPC vesicles were prepared as above, except that no alamethicin was added during formation. Small aliquots of the alamethicin stock solution were then diluted 80-fold with these vesicle samples. Vesicle samples with final lipid-to-protein mole ratios ranging from 10:1 to 2,200:1 were prepared. In all studies, identical vesicle preparations treated with equivalent amounts of methanol containing no alamethicin were also prepared.

Spectroscopy and Data Analysis

CD spectra were recorded on either a Cary 60 spectropolarimeter with a Model 6001 CD attachment or an Aviv 60DS spectropolarimeter. Both instruments were equipped with a variable position detector. The detector acceptance angle was varied from $\geqslant 90°$ to 2° by translating the end window photomultiplier tube detector along the optic axis and by varying the phototube aperture.[27,28] Unless otherwise noted, the spectra reported here have been obtained with the maximum acceptance angle ($\geqslant 90°$). The wavelength range scanned was from 300 to 190 nm. The optical rotation was calibrated using d-10-camphorsulfonic acid at 192.5 and 290 nm and checked with sperm whale myoglobin. The wavelength was calibrated using benzene vapor.

Measurements of CD spectra were routinely made at ~25°C using a 0.5 or a 1.0 mm pathlength demountable quartz cell (Hellma Kuvetten, Mullheim/Baden, Germany). Three or four reproducible scans were averaged for each sample and smoothed[29]; averaged smoothed base lines of either solvent or vesicles without alamethicin were subtracted to yield the contribution due solely to the polypeptide component. In general, the spectra reported are the average of at least three independent preparations for each type of sample.

Absorption spectra were recorded on either a Cary 15 or a Cary 2200 spectrophotometer with the cell placed adjacent to the photomultiplier. First and second derivative spectra were obtained with the latter instrument.

The CD data were analyzed in the wavelength range from 190 to 240 nm by a linear, unconstrained least-squares curve-fitting procedure as previously described[30] using a reference data set derived from 15 water-soluble proteins.[31] The spectrum of an alpha-helix is dependent on the length of the helix, and this parameter was set to correspond with the calculated helix content in these analyses (e.g., if the fraction of helix was calculated to be 40%, a helix length of 8 was used in our analysis of this 20-residue polypeptide). The results were normalized to 100% by dividing each fraction by the sum of the fractions. For soluble proteins, the helical content calculated by this type of analysis has been shown to correlate strongly with the amount of helical structure as determined by x-ray crystallography.[35] The quality of the computer fit of the calculated to the measured data was evaluated by the normalized root mean standard deviation (NRMSD), as given by the equation

$$\mathrm{NRMSD} = \left[\frac{\sum_{n} (\theta_{exp} - \theta_{cal})^2}{\sum_{n} \theta_{exp}{}^2}\right]^{1/2}$$

where θ_{exp} and θ_{cal} are the experimental and calculated mean residue ellipticities and n is the number of data points.

The spectra of samples in nonaqueous environments (which have polarizabilities significantly different from that of water) may be red- or blue-shifted relative to spectra in aqueous environments. To assay for this, the reference basis sets were deconvoluted to yield the three Gaussian absorption bands which define their characteristic spectra, and these spectra were shifted differentially to optimize the fit of the calculated spectra to the experimental data, as reflected by minimization of the NRMSD parameter.[32]

Statistical analyses were performed to determine both the variation within a data set and also between averaged data sets of independent samples prepared by identical methods. The former values reflect the reproducibility of the instrument, while the latter shows variations between samples.[33] For each sample type, standard deviations were calculated at all wavelengths in order to determine if differences between the averaged spectra were significant.

TABLE I. Calculated Secondary Structures

	Concentration (mg/ml)	Hel. length	Fraction Helix	Fraction Beta structure	Sum	NRMSD
Solvent						
10% Methanol/ acetonitrile	0.12–10	7	0.33	0.41	1.08	0.163
Methanol	0.2	7	0.35	0.37	1.08	0.143
Acetonitrile	0.2	7	0.37	0.37	1.01	0.156
Vesicles						
Type I	2×10^{-3}	10	0.50	0.19	0.85	0.099
Type II	2×10^{-3}	10	0.53	0.20	0.79	0.148
Type I	0.25	12	0.68	0.22	0.70	0.150
Type II	0.25	12	0.62	0.24	0.83	0.153

Electron Microscopy

The vesicle samples were diluted 20-fold with deionized water and placed on formvar-coated copper grids and stained with 1% uranyl acetate for 15–30 seconds. Grids were examined in a JEOL JEM-100S electron microscope fitted with anticontamination device and operating at 80 kV. Both type I and type II vesicles were unilamellar and had average diameters of approximately 230 ±30 Å. These dimensions were independent of the presence of alamethicin.

RESULTS

Analysis of CD Spectra

In examining the optical properties of membrane-associated proteins, potential artifacts may arise due to the particulate nature of the lipid-protein complexes. CD spectra may be distorted by differential light scattering of the incident light outside of the acceptance angle of the detector[27] and absorption flattening effects arising from the nonuniform distribution of chromophores in the path of the beam.[34]

Differential light scattering, which is significant in large unilamellar vesicles, may be reduced by incorporating the protein in SUVs, which are small relative to the wavelength of the incident light. Mao and Wallace[28] have shown that for SUVs, most light is scattered in the near forward direction, and that placement of the sample cell directly adjacent to the detector (thereby increasing the acceptance angle to $\geqslant 90°$), results in negligible distortions of the spectra due to differential light scattering. For each sample type, spectra collected with acceptance angles of $\geqslant 90°$ and 2° were found to be superimposable over the wavelength range from 300 to 190 nm.

Absorption flattening may be minimized by incorporating the protein into small unilamellar vesicles at high lipid-to-protein ratios.[28,35] As the ratio increases and the polypeptide concentration ratio approaches one per vesicle, the absorption flattening goes to zero. Therefore, low alamethicin concentrations were used, which produced vesicles containing from ~4 to 400 molecules per vesicle so the absorption flattening effects would be very small.

The net secondary structural composition of alamethicin was estimated from the CD data using a least-squares algorithm[30] (Table I). Similar values for helix content were also obtained using the singular value deconvolution calculation method of Hennessey and Johnson.[36] For example, for alamethicin in vesicles at high concentrations, the least-squares procedure yields a helix content of 68%, while the other method produced a value of 67%. The high correspondence between results obtained by these two different calculation methods increases confidence in the calculated helix contents. On the other hand, the β-structure (the sum of β-sheet and β-turn components), is not as reproducibly or reliably determined by either method. For the same sample, the former method yields a value of 22%, while the latter method calculates only 5%. However, these methods can be used to provide relative measures of the beta contents in different samples.

The normalized root mean square deviation (NRMSD) parameter provides an indication of the extent of agreement between the calculated structure and the measured CD spectrum. For protein samples, analyses resulting in NRMSD values less than 0.1 generally indicate a close correspondence between the calculated and actual structures. For NRMSD values between 0.1 and 0.2, the calculated structure is characterized as having a secondary structure generally similar to the actual structure.[37] The least-squares analyses of all the alamethicin spectra resulted in NRMSDs $\geqslant$ 0.1 (Table I). Other workers have also found that the reference spectra derived from aqueous-soluble proteins fail to describe adequately the spectra of alamethicin incorporated into small unilamellar vesicles.[15] The poor fits may result from a polypeptide geometry in alamethicin which is not reflected in the reference data, or may reflect effects due to the different dipole moments of the peptide environment (relative to water, the solvent of data base), as described below.

An unconstrained least-squares method of analysis has been used for the secondary structural determinations because it is more reliable when the sum of

TABLE II. Calculated Secondary Structures: Spectral Shift Effects

Solvent	Fraction Helix	Fraction Beta structure	NRMSD	λ-shift G_1*	λ-shift G_2*	λ-shift G_3*
Methanol	0.48	0.26	0.119	4	5	4
Vesicles—type I	0.55	0.05	0.060	−5	0	−3

*G_1, G_2, and G_3 refer to shifts (in wave numbers) of the three Gaussian components which produce the optimal fit (lowest NRMSD).

the fractions does not equal one.[35] The presence of multiple conformations for this relatively small, flexible polypeptide may result in net ellipticities near zero, hence reducing the sum to less than one (Table I). For this reason, algorithms which constrain the sum to unity were not appropriate for these samples.

It has been reported that microbial polypeptides, such as alamethicin, which contain aminoisobutyric acid (Aib) residues have a high propensity to form 3_{10} helices.[38] However, expansion of the reference data set to include a theoretical spectrum for 3_{10} helices [39] did not produce a calculated secondary structure with any 3_{10} helix content for alamethicin in solution; for alamethicin in vesicles, the analysis indicated that a small fraction of 3_{10} helix may be present with a concomitant nearly equivalent decrease in the amount of alpha-helix (i.e., the total helix content remained relatively invariant). The conservation of total helix content is not surprising since the spectral waveforms of the reference alpha-helix and 3_{10} helix are very similar, with the magnitude of the 3_{10} helix being ~50% that of the alpha-helix. The replacement of alpha-helix with 3_{10} helix does, however, increase the sum in the unconstrained analysis, and the presence of this alternative helix form may be another source of the low sums for the vesicle samples in our least squares analyses. However, the differences between the solution and membrane spectra cannot be accounted for by the presence of 3_{10} rather than alpha-helices.

The absorption of light by a molecule occurs when the energy of an impinging photon is equal to the difference in energy between adjacent electronic states of the molecule. Peptide n to π^* and π to π^* transitions occur in the ultraviolet range of the spectrum. The interaction of the peptide electronic states with the local environment may affect these states and consequently raise or lower the transition energy leading to red- or blue-shifting of the spectra. Such a wavelength shift phenomenon is seen in both the unpolarized absorption and CD spectra of the small protein crambin, which was solubilized in a variety of organic solvents of differing dipole moments.[32] This type of shifting of the CD spectra might also be expected for alamethicin; due to its small size, probably few, if any, of its peptide bonds are shielded from their environment. Indeed, in the highly polar solvent methanol, the peaks were all red-shifted (Table II), whereas in the less polar hydrocarbon chains of the lipid, the peaks were blue-shifted (Table II) relative to the reference data set of aqueous-soluble proteins. To examine the wavelength shift effects on the calculated secondary structures derived from these spectra, individual Gaussian bands for each type of secondary structure in the reference data set were shifted prior to the data analysis. The effect of these shifts was to produce an improved fit (lower NRMSD). For the methanol sample, the shifting also results in a calculated secondary structural content which is

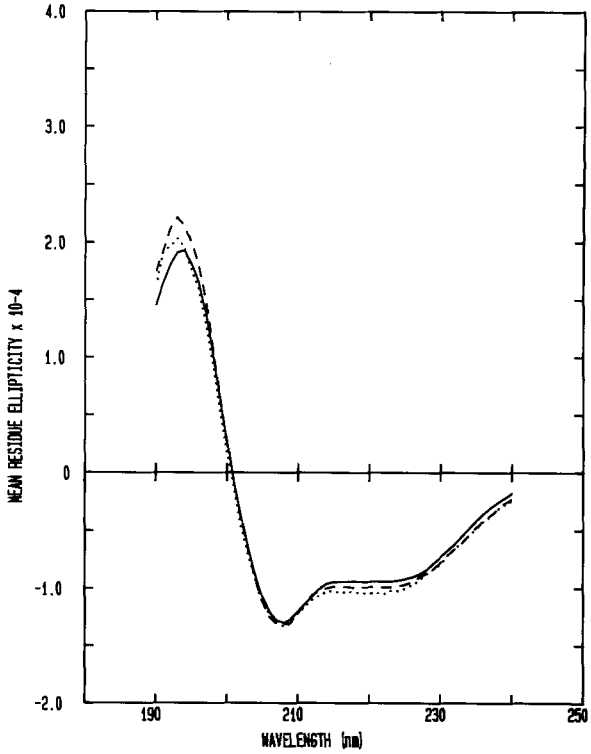

Fig. 1. Circular dichroism spectra of alamethicin in 10% methanol/acetonitrile (————), in methanol (· · ·), and in acetonitrile (– – –).

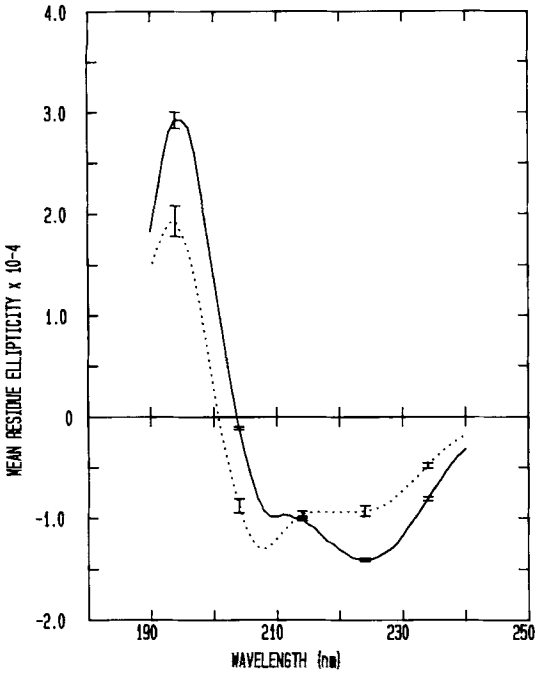

Fig. 2. Comparison of the CD spectra of alamethicin in type II vesicles with low lipid-to-protein mole ratios (50:1) (———) and in 10% methanol/acetonitrile (· · ·). The reproducibility (standard deviations) between independent preparations of the same type of sample is indicated by the error bars.

more consistent with the values estimated by the NMR experiments.[17]

Environmental Effects on Structure

Detailed structural models for alamethicin pores have been proposed based primarily on crystallographic analyses of crystals formed from 10% methanol/acetonitrile and on NMR studies of the polypeptide in methanol solution. To compare the secondary structures of this potentially flexible molecule in an isotropic, hydrophilic organic solvent and in the more ordered, hydrophobic environment of the lipid bilayer, CD spectra were obtained for alamethicin in each of these systems (Figs. 1, 2).

The spectra of alamethicin in the organic solvents, 10% methanol/acetonitrile, acetonitrile, and methanol, were virtually superimposable (Fig. 1), differing at most by ±4% in the lower wavelength regions, where the detector signal-to-noise ratio is lowest due to increased light absorption. The mean residue ellipticities for all three curves exhibit a minima at ~208 nm and a maximum at 193–194 nm. This superimposability strongly suggests that the net conformations of the molecule in these solvents are the same. Since CD measures the net secondary structure, it is possible, but unlikely, that the structures differ in a reciprocal manner in different regions of the molecule.

In contrast, the CD spectrum of alamethicin incorporated in type II vesicles at a final lipid-to-protein mole ratio of ~50 (a ratio which will permit oligomer formation in bilayers—see below) exhibits profound differences in both its shape and in the magnitude of the mean residue ellipticities from that of the polypeptide dissolved in organic solvents (Fig. 2). These large spectral effects cannot be accounted for by differences in solvent polarities. The spectra of alamethicin associated with DMPC vesicles show double-minima at ~209 and 223 nm with the latter being the larger of the two minima, instead of the single minimum exhibited in the spectra of the peptide in organic solution. The peak maxima in the spectra of alamethicin in DMPC vesicles and in organic solvents differ in magnitude by ~1.5-fold. The differences between the spectra of alamethicin in different environments are much larger than the associated reproducibility limits between preparations of the same type of sample (see errors bars, Fig. 2), and suggest that alamethicin adopts a different conformation in the hydrophobic milieu of the bilayer than in any of the organic solvents. The calculated net secondary structures (Tables I and II) derived from these spectra also reflect these differences; with respect to the structure found in organic solvents, the alpha-helix content was increased by ~10% and the β-content decreased when alamethicin was incorporated into vesicles.

Effect of Alamethicin Concentration

Sedimentation studies of alamethicin have indicated that the molecule aggregates in aqueous environments, but that the aggregation state is independent of concentration in a wide variety of organic solvents including ethanol, methanol, 1-butanol, and 1-octanol.[13,40,41] We have examined the CD spectra of alamethicin in 10% methanol/acetonitrile at concentrations ranging from 0.12 to 10 mg/ml. The spectra for all concentrations are superimposable and thus the secondary structure of alamethicin appears to be independent of concentration in 10% methanol/acetonitrile.

Alamethicin has been proposed to form an oligomeric pore in membranes composed of six to twelve monomers. Preliminary equilibrium density centrifugation studies have indicated that the distribution of alamethicin molecules in vesicles is uniform (Cascio and Wallace, unpublished observation). Therefore, vesicles containing an average number of alamethicin molecules per vesicle can be prepared by varying the lipid-to-polypeptide ratios. Vesicles were constructed which contained less than six, or more than twelve, monomers per vesicle, the potential lower and upper limits for oligomerization, to examine whether there exists any conformational concentration dependence. The CD spectra and associated calculated secondary structure composition for type I and II vesicles containing <4 or >75 alamethicin molecules per vesicle were found to differ considerably (Fig. 3; Table

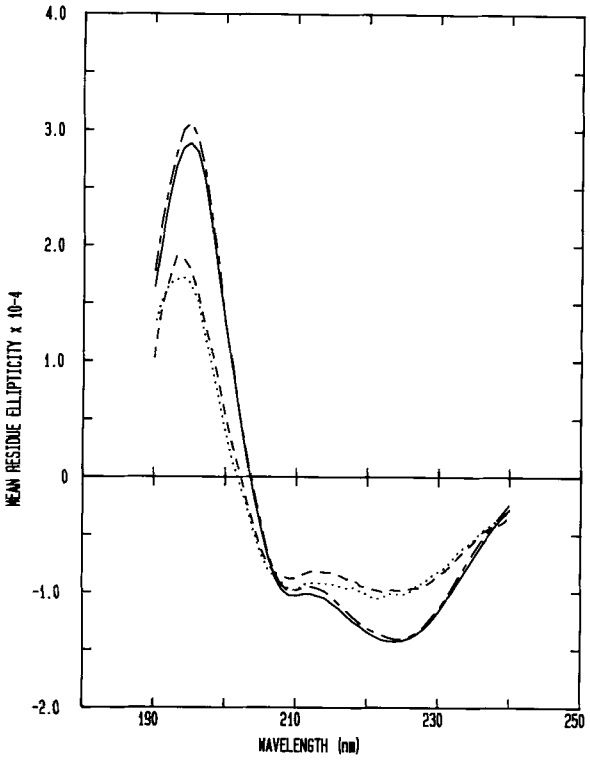

Fig. 3. CD spectra of alamethicin in different types of vesicle preparations at high and low protein-to-lipid mole ratios: type I vesicles at 1:50 (—— · ——) and 1:2,200 (· · ·), and type II vesicles at 1:50 (———) and 1:2,200 (– – –).

I). The spectra of alamethicin at the lower concentration also had the double minima characteristic of the higher concentration sample, but the ratio of the magnitudes of the two peaks was different: the two minima for the low concentration sample were approximately equal in magnitude, whereas at higher concentrations the magnitude of the 223-nm peak was ~1.5-fold that of the 209-nm peak. Also the maxima of the lower concentration sample at 194 nm had a mean residue ellipticity only ~60% that of the higher concentration sample. The differences between the two spectra cannot be attributed to differential extents of absorption flattening. If flattening were responsible, the magnitudes of the 194 and 209 peaks should both be decreased relative to the 223 nm peak in the higher concentration samples and at all wavelengths relative to the low concentration sample, instead of the observed increase in all the wavelength peaks. The differences indicate that at the lower concentrations, the structure has a lower helix content (Table I) than that of the higher concentration sample. Alamethicin incorporated into vesicles at intermediate concentrations (>4 and <75) yielded spectra which were a sum of the CD curves at the low and high concentrations. Thus, the net conformations of alamethicin are different in samples with polypeptide concentrations equivalent to less than one oligomer, or greater than six oligomers, per vesicle, suggesting that the conformation of alamethicin may be dependent on the oligomeric state of the molecule.

At a higher concentration (~400 molecules per vesicle), the CD spectrum of alamethicin is virtually superimposable with the spectrum obtained with ~75 molecules per vesicle; the secondary structure of alamethicin is thus independent of concentration at these elevated levels (>75 molecules per vesicle).

Effects of Incorporation Method

The conductance states of alamethicin have been shown to be identical whether alamethicin is cosolubilized with lipids before formation of black lipid membranes or added to preformed membranes via addition to the cis-positive aqueous chamber of a black lipid membrane apparatus.[4] To determine whether the conformation of alamethicin added to preformed DMPC vesicles (type II) is the same as alamethicin pre-incorporated (by cosolubilization) in DMPC vesicles (type I), the CD spectra and associated calculated net secondary structures of the polypeptide in both type I and II vesicles were compared. The alamethicin concentration was adjusted such that vesicles contained on the average ~75 molecules. The resulting spectra were found to be essentially identical, with the greatest difference between the two curves being ±3% in the low-wavelength region where signal-to-noise is lowest (Fig. 3). These experiments were repeated at a lower concentration (<4 alamethicin molecules per vesicle) and the resulting spectra obtained for type I and II incorporation methods were again nearly superimposable (Fig. 3). The calculated composition were also found to be similar (Table I).

DISCUSSION

Alamethicin is a small flexible hydrophobic polypeptide whose conformation was previously shown to be extremely sensitive to changes in its environment.[12-14] In this study, CD spectroscopy has been used to compare the secondary structures of alamethicin in organic solvents and in the hydrophobic, anisotropic environment of lipid bilayers. Previously published structural studies on alamethicin were carried out in organic solvents. The secondary structure of alamethicin was also examined in a nematic phase (42% 1-octyl sulfate, 4% 1-octanol, 4% Na_2SO_4, 50% H_2O)[13]; however, the conformation of a small molecule in such a detergent environment may not necessarily be equivalent to its structure in bilayers. Indeed, the conformation of alamethicin in this "membrane mimetic" environment is more similar to that in organic solvents than in a true phospholipid membrane.

CD experiments on the susceptibility of alamethicin secondary structure to environment have indi-

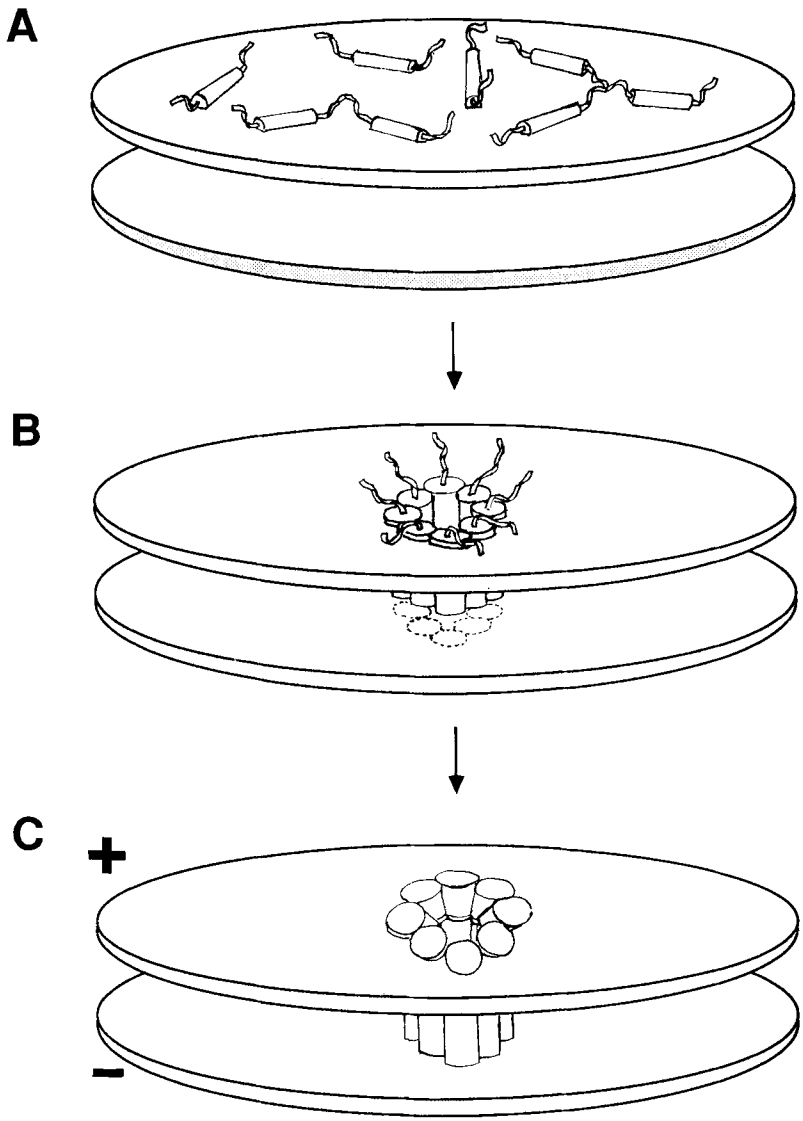

Fig. 4. Proposed model for alamethicin insertion into the bilayer. **A:** Alamethicin associates with the bilayer as monomers, dimers, or trimers in the absence of a transmembrane potential. **B:** These units spontaneously oligomerize to form a "pre-aggregate" with a concommitant change in conformation. The "pre-aggregates" are partially embedded in the bilayer. **C:** Upon application of a cis-positive transmembrane gating potential, the alamethicin channel forms the open pore. The mechanism of this transition is unknown.

cated that the net conformation of this molecule is probably identical in methanol, the solvent in which NMR studies of Banerjee and Chan[17] were conducted, and in 10% methanol/acetonitrile, the crystallization solvent of Fox and Richards.[19] In both solvents the helix content is calculated to be 48% (Table II), a value similar to the ~45% helix estimated by NMR studies, but different from the entirely helical molecule observed in the crystallographic studies.

Not surprisingly, we found that alamethicin adopts a different conformation in phospholipid vesicles. The differences between the spectra of the molecule in organic solutions and in phospholipid vesicles at high concentrations are large (~40% at both 194 and 223 nm and ~25% at about 209 nm). Even though the flexibility of this small polypeptide makes it difficult to determine quantitatively the periodic structure by CD, differences in secondary structure are apparent and correspond to an increased helix content in the membrane-bound form. Interestingly, the helix content of the vesicle form is nearer to that of the crystal structure than that found in solution. It is possible that the packing matrix in the crystal may be more representative of the ordering imposed by phospholipids in a bilayer than of an isotropic solution. However, the crystal structure (in which the asymmetric unit is a trimer with no central pore) is clearly not the same as the structure observed for the oligomers

in SUVs. Thus, these studies demonstrate that the conformation of alamethicin is very sensitive to environmental effects, and these effects must be considered when structural data derived from studies conducted in organic solvents are used for model-building.

Three type of models have been proposed for alamethicin channel formation: the "barrel staves" model of Baumann and Mueller[7] and Boheim,[8] the "pre-aggregate" model of Gordon and Haydon,[9,10] and the two-dimensional micelle model of Hall.[11] These models could potentially be distinguished by the sensitivity of the alamethicin conformation to concentration and the method of polypeptide incorporation. For any given lipid-to-polypeptide ratio, the CD spectra of alamethicin incorporated either by cosolubilization with the phospholipids before vesicle formation (type I) or by addition to a solution containing preformed SUVs (type II) were superimposable. This suggests that the net conformations of alamethicin are identical and independent of the method of incorporation. This result precludes the polypeptide functioning as a two-dimensional micelle associated at the bilayer surface unless the alamethicin in the micellar state is identical in structure to cosolubilized alamethicin at those concentrations tested (i.e., surface-associated and incorporated polypeptide respond in an identical manner to changes in concentration).

To distinguish between the other two models, the effects of concentration on conformation have been examined. Only the "pre-aggregate" model is potentially concentration-dependent. The "barrel-staves" model predicts that the conformation of alamethicin will be independent of concentration—prior to the application of a transmembrane potential all membrane-associated molecules are monomeric. Comparison of the CD spectra of alamethicin associated with phospholipid at a concentration of ~4 molecules per vesicle (too few to form a potential pre-aggregate which is composed of at least six monomers) and at ~75 molecules per vesicle (much greater than the maximum of twelve monomers necessary to form one potential pre-aggregate, if this event indeed takes place), shows a significant difference. Thus, alamethicin seems to exhibit a concentration-dependent conformational change. This implies that prior to the application of any transmembrane potential, alamethicin associates with the bilayer as a monomer (or possibly a dimer or trimer) and that these units may spontaneously aggregate with the concomitant observed conformational change (Fig. 4). This model incorporates elements present in both the Fox and Richards pre-aggregate[19] and the Baumann and Mueller[7] and Boheim[8] barrel-staves models.

The nature of the association of alamethicin with the bilayer (e.g., bound to surface or integral membrane protein) prior to the application of a potential cannot be determined from these experiments. However, cross-linking,[42] infrared attenuated total reflection spectroscopy,[43] and CD studies [44] have shown that the N-terminal region of the polypeptide is at least partially embedded in the membrane. Although the secondary structural characteristics detected in these experiments are not attributable to any specific region of the molecule, one could speculate that alamethicin is entirely helical from its N-terminus to the potentially helix-breaking proline at position 14, which would result in a molecule which is approximately 65% helical. The calculated helix content from our CD studies is reasonably consistent with this value.

In summary, different interpretations of previous conductance studies and structural studies conducted in organic solvents led to proposals for three possible models for alamethicin function. Our studies are consistent with only one of these — a modified "pre-aggregate" model in which monomeric (or possibly dimeric or trimeric of) alamethicin spontaneously associates with the lipid bilayer and then laterally diffuses and oligomerizes to form a pre-aggregate before a transmembrane potential is applied. Upon application of an appropriate potential, the "pre-aggregate" would then form the open membrane-spanning pore.

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