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Comparison of the Conformation and Orientation of Alamethicin and Melittin in Lipid Membranes

Horst Vogel
Department of Biophysical Chemistry, Biocenter, University of Basel, CH-4056 Basel, Switzerland
Received December 15, 1986; Revised Manuscript Received March 5, 1987

ABSTRACT: The secondary structure of alamethicin in lipid membranes below and above the lipid phase transition temperature T_t is determined by Raman spectroscopy and circular dichroism (CD) measurements. In both cases structural data are obtained by fitting the experimental spectra by a superposition of the spectra of 15 reference proteins of known three-dimensional structure. According to the Raman experiments, in a lipid bilayer above T_t alamethicin is helical from residue 1 to 12, whereas below T_t the helix extends from residue 1 to 16. The remaining C-terminal part is nonhelical up to the end residue 20 both above and below T_t. A considerable lower helix content is derived from CD, namely, 38% and 46% above and below T_t, respectively, in agreement with several reported values for CD in the literature. It is shown that the commonly used set of CD spectra of water-soluble reference proteins is unsuitable to describe the CD spectra of alamethicin correctly. Therefore the secondary structure of alamethicin as derived from CD measurements is at the present state of analysis unreliable. In contrast to the case of alamethicin, the CD spectra of melittin in lipid membranes are correctly described by the reference protein spectra. The helix content of melittin is determined thereby to be 72% in lipid membranes above T_t and 75% below T_t. The data are in accord with a structure where the hydrophobic part of melittin adopts a bent helix as determined recently by Raman spectroscopy [Vogel, H., & Jähnig, F. (1986) *Biophys. J. 50*, 573]. The orientational order parameters of the helical parts of alamethicin and of melittin in a lipid membrane are deduced from the difference between a corresponding CD spectrum of a polypeptide in planar multibilayers and that in lipid vesicles. The presented method for determining helix order parameters is new and may be generally applicable to other membrane proteins. The orientation of the helical part of both polypeptides depends on the physical state of the lipid bilayer at maximal membrane hydration and in the ordered lipid state furthermore on the degree of membrane hydration. Under conditions where alamethicin and melittin are incorporated in an aggregated form in a fluid lipid membrane at maximal water content the helical segments are oriented preferentially parallel to the membrane normal. Cooling such lipid membranes to a temperature below T_t changes the orientation of the helical part of alamethicin as well as melittin toward the membrane plane. On the contrary in dried planar membranes at 2% (w/w) water content both polypeptide molecules are oriented with their helical parts preferentially parallel to the membrane normal, similar to the case of fluid lipid membranes.

Several polypeptides exhibit similar properties to certain naturally channel-forming membrane proteins in the sense that after incorporation into lipid membranes they increase the membrane permeability depending on the transbilayer electrical potential. One of the most thoroughly investigated model compound in this context is the polypeptide alamethicin. Its channel properties have been characterized by a variety of electrical conductance measurements on planar bilayer membranes [for a review see Hall et al. (1984)]. From such experiments it was concluded that the alamethicin ion channel is composed of several polypeptide molecules. However, the structure of this channel is unknown.

The structure of alamethicin in crystals grown in a solvent mixture of methanol-acetonitrile has been determined. The molecule adopts a largely α-helical conformation with a bend in the helix axis near an internal proline residue (Fox & Richards, 1982). According to CD[1] measurements alamethicin is only about 40% helical when dissolved in apolar organic

[1] Abbreviations: CD, circular dichroism; NMR, nuclear magnetic resonance; IR, infrared; FA, fluorescence anisotropy; UV, ultraviolet; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DTPC, ditetradecylphosphatidylcholine; NRMSD, normalized standard deviation.

0006-2960/87/0426-4562$01.50/0

solvents or incorporated in lipid membranes (McMullen et al., 1971; Jung et al., 1975; Cascio & Wallace, 1985). The reported values of the helix content depend somewhat on the nature of the solvent and more critically on the method for analyzing the CD spectra. NMR measurements indicate that the conformation of alamethicin in methanol is α-helical over nine residues in the N-terminal part, while the C-terminus seems to form a β-structure (Banerjee et al., 1983). From IR spectra of alamethicin in planar multilayer membranes it was concluded that in the absence of water the polypeptide molecule traverses the lipid bilayer as an α-helix with the helix axis mainly parallel to the membrane normal, whereas upon addition of water alamethicin changes its conformation to a membrane-spanning β-structure (Fringeli & Fringeli, 1979). Taken together the data indicate that the conformation of alamethicin in organic solvents is considerably different from that in the crystal form. However, the relevance of these various conformations with respect to the alamethicin channel in membranes is open for speculation. In view of the CD and IR data the conformation of alamethicin in lipid membranes remains unresolved.

The present work is concerned with the conformation and orientation of alamethicin in lipid membranes in the absence of a transbilayer electric field. We determined the secondary structure of alamethicin in methanol and in membranes of two different lipids by Raman spectroscopy and by CD measurements. Structural data derived by both techniques are compared and discussed in view of the reliability. Concerning the orientation of alamethicin in lipid membranes, we measured the orientational order parameter of the α-helical part of the polypeptide by its CD spectrum in glass-supported planar multilayer membranes.

The method of determining the orientational order parameter of an α-helix in lipid membranes by CD is new and may be generally applicable for helical membrane proteins. For this method a calibration is made by measuring the CD spectrum of a helical polypeptide in planar bilayers where the helix order parameter is already known from other experiments. The polypeptide melittin was chosen for this purpose because the conformation and orientation of melittin in lipid membranes was elucidated recently (Vogel & Jähnig, 1986; Vogel et al., 1983).

It is quite interesting to compare the structure of alamethicin and melittin in the context of the present work because they share many similar properties. As does alamethicin, melittin forms voltage-gated pores in lipid bilayers (Tosteson & Tosteson, 1981). The sequence of alamethicin is AcAib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Gln-Gln-Phol (Aib = α-aminoisobutyric acid, Phol = phenylalaninol) and that of melittin is H_2NGly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-GlnCONH_2. Both molecules are amphipathic in the sense that they are composed of a predominantly hydrophobic N-terminal part of 17 and 20 amino acid residues, respectively, and a polar C-terminal segment. At position 14 both polypeptides possess a Pro residue, responsible for the bent helical structure found in the crystals (Fox & Richards, 1982; Terwilliger et al., 1982). The crystallographic data show that even the amphipathy of the two helices is preserved each with one hydrophobic and one hydrophilic side.

Up to now the secondary structure of melittin was determined by Raman spectroscopy in aqueous membrane dispersions only below the lipid phase transition temperature T_t; the orientational order parameter of the helical part of the polypeptide was determined by polarized IR measurements only in dry lipid multilayers (Vogel & Jähnig, 1976; Vogel et al., 1983). In the present work the structure of melittin in lipid membranes was characterized further by CD measurements in order to study (i) the influence of the lipid phase transition on the polypeptide conformation and (ii) the orientational order parameter of the helical part of melittin in hydrated multilayers.

Materials and Methods

Chemicals. DMPC from Fluka (Buchs, Switzerland) was used without further purification. DTPC was a gift from Dr. J. Stümpel (Max-Planck-Institut für Biophysikalische Chemie, Göttingen, FRG); its synthesis is described elsewhere (Stümpel, 1982). Melittin from Mack (Illertissen, FRG) was purified as described by Vogel and Jähnig (1986). The alamethicin R_f 50 component used in the present work was a gift of Dr. V. Rizzo. It was isolated and purified according to Rizzo et al. (1987).

Sample Preparation for Raman Measurements. A saturated solution of alamethicin in methanol was prepared by mixing 1.3 mg of the polypeptide with 3.7 μL of methanol. Nondissolved material was removed by centrifugation of the mixture at 20000g for 10 min at 14 °C, and the supernatant was filled into a glass capillary for Raman measurements. Membrane-incorporated alamethicin was prepared as follows: 1 mg of alamethicin and either 7 mg of DMPC or 6.7 mg of DTPC were dissolved in 2.5 mL of chloroform/methanol = 1/1 (v/v), corresponding to a lipid/alamethicin molar ratio of 20/1. After evaporation of the solvent, the samples were dried in a vacuum of 10^{-3} Torr for 3 h. To each of the dried samples was added 0.5 mL of water, and the samples were incubated for 2 h at 35 or 40 °C in the case of DMPC or DTPC, respectively. Subsequently, samples were freeze-dried. Water was added to yield a lipid/water weight ratio of 1/2, followed by incubation for 2 h at 35 and 40 °C in the case of DMPC and DTPC, respectively. The membrane preparations were transferred to glass capillaries and centrifuged at 50000g for 30 min at 14 °C. Pure lipid membranes were prepared identically without using alamethicin.

Raman Measurements and Analysis of Data. These were performed as described previously (Vogel & Jähnig, 1986). The conditions for the Raman measurements were 514.5-nm excitation wavelength, 50-mW light power at the sample, and 5-cm^{-1} slit width at the monochromator. Spectra of multiple scans were summed and stored by a microcomputer. Only these data were used for analysis for which the first 20 and last 20 scans agreed within experimental error. A protein amide I band was analyzed in terms of secondary structure as described by Vogel and Jähnig (1986) with a minor modification. The sum of the percentages of all structure classes as obtained from the fit of the experimental spectrum with the spectra of 15 reference proteins is not normalized to 100%. The followign structure classes are distinguished: ordered (O) and disordered helix (D), antiparallel (A) and parallel β-strand (P), turn (T) and undefined (U). The ordered helix corresponds to an α-helix, whereas all other helical structures that deviate from an α-helix, including two residues at each end of an α-helix, are counted as a disordered helix (Williams, 1983). The maximal error in the percentages of secondary structure classes was $\pm$4.

Sample Preparation for CD Measurements. A solution of 10 mg of DTPC in 1 mL of methanol was mixed with 196 μL of a solution of 1 mg of alamethicin in 1 mL of methanol. The concentration of alamethicin was determined with an uncertainty of about $\pm$5% from measurement of the ellipticity at

222 nm by using a value of −12 750 deg cm^2 dmol^{-1} for the mean residue ellipticity of alamethicin in methanol at 20 °C (Schwarz et al., 1986). After evaporation of the organic solvent, the samples were dried in vacuo for 3 h. Water (1 mL) was added, and the samples were incubated for 2 h at 40 °C and freeze-dried. Subsequently the sample was mixed with 750 μL of water and sonicated for 5 min in a bath-type sonifier up to optical clearance. A part of this vesicle dispersion was diluted with water to yield a concentration of 80 μg/mL alamethicin and filled into a cylindrical, thermostatable quartz cuvette for CD measurements. To obtain oriented lipid membranes 200 μL of either the undiluted vesicle dispersion or 2 and 4 times water-diluted ones was spread on a l-mm-thick quartz disk of 22-mm diameter (Hellma, FRG). Samples were dried in a vacuum desiccator at 200 Torr over silica gel at room temperature. Thereby in the case of the undiluted dispersion a stack of about 1500 lipid bilayer membranes with 2% (w/w) water content was produced, oriented parallel to the glass surface. The quartz glass plate with the membranes was mounted in a thermostatable metal block and sealed with a second quartz glass plate held at a 5-mm distance from the membranes by a Teflon ring. A relative humidity of 97% can be maintained in this cuvette by a drop of a solution of 1 M NaCl. Under these conditions the maximal possible water content of 30% (w/w) is reached in the lipid sample, which corresponds to 22–24 water molecules per lipid molecule. The polypeptide concentration of these samples, which is needed for calculation of the mean residue ellipticity, was determined from the macroscopic area of the multibilayers and the amount of of the membrane-inserted peptide by using a value of 0.71 mL/mg for its partial specific volume.

Lipid membranes containing melittin were prepared by mixing appropriate amounts of a pure lipid vesicle dispersion and an aqueous melittin solution. After sonification, the dispersion was used for CD measurements or for preparing oriented membranes as described in the case of alamethicin. The concentration of melittin in vesicles was determined with an uncertainty of about ±1% by measuring the optical density at 280 nm and using a value of $\epsilon = 5500$ L·mol^{-1}·cm^{-1} for the molar extinction coefficient. Pure lipid membranes were prepared as described above but without polypeptides.

CD Measurements. A modified Cary 61 circular dichrograph was used (Bächinger et al., 1979). The instrument had been calibrated with *d*-10-camphorsulfonic acid. For organic solutions and membrane vesicle dispersion spectra were recorded by using quartz cuvettes with a path length of either 1 or 0.12 mm. The optical density of the samples did not exceed 1.0 between 180 and 250 nm. In the case of planar membranes the direction of the incoming light was parallel to the normal of the membrane-supporting glass plate. Spectra of such oriented samples did not show any differences after rotation in 45° steps about the glass plate normal, demonstrating that the CD of the samples is free of noticeable linear dichroism and birefringence artifacts. In all cases the intensity of the CD signal varied linearly with film thickness of 3–20 μm. CD spectra remained constant during variation of the distance between sample and photomultiplier for both vesicles and planar membranes, showing the absence of light scattering artifacts.

Evaluation of Protein Secondary Structure from CD Spectra. The far UV CD spectrum of polypeptides and proteins in solution or in lipid vesicle dispersions is sensitive to their secondary structure. The CD spectrum, therefore, can be used to determine the protein secondary structure. For this analysis we employed a modified method of Hennessey and Johnson (1981): 33 data points were used to describe the measured CD spectrum between 186 and 250 nm. These points were fitted by a superposition of the spectra of 15 reference proteins of known three-dimensional structure.[2] Spectra of reference proteins are those of Hennessey and Johnson (1981); however, that of polyglutamic acid is omitted. The fit determines the weight of the individual reference proteins in the measured spectrum. Knowing the mean secondary structure of the reference proteins, we can derive the mean secondary structure of the protein under study. Four structure classes are distinguished: α-helix (H), β-stand (S), β-turn (T), and undefined (U), which are assigned from the X-ray data according to Levitt and Greer (1977); these assignments differ from those of Hennesey and Johnson (1981) but are in agreement with the Raman analysis (Vogel & Jähnig, 1986). The fit procedure was performed without constraints; therefore, any positive or negative number can be obtained for the percentage of a structure class as well as for the sum of all structure classes. The maximal error in the percentage of helix content and the other structure classes was ±2 and ±4, respectively.

The method for analyzing the CD is in principle identical with that for analyzing the Raman amide I band (Vogel & Jähnig, 1986). Although different sets of reference proteins are used in the analysis of CD and Raman spectra, the results should be unaffected as long as all relevant structures are represented by both sets of reference proteins.

Evaluation of Helix Order Parameter from CD Spectra. Let us consider a helical polypeptide crossing a lipid bilayer. The helix performs orientational fluctuations around its equilibrium position that are characterized by the orientational order parameter

$$S_h = \langle 3 \cos^2 \beta - 1 \rangle / 2 \qquad (1)$$

In an ensemble of helices in a lipid bilayer the angular brackets denote the average over the distribution of the actual angles β between the helix axes and the membrane normal. Only angular distributions with cylindrical symmetry around the membrane normal are considered; furthermore, the helix itself is assumed to be symmetric around its axis. S_h can be determined by the difference between the CD spectra of the polypeptide in planar bilayers and in lipid vesicles as follows. Under our experimental conditions circularly polarized light passes a planar bilayer parallel to the membrane normal. Two Cartesian coordinate systems are defined, a molecule-fixed one (x, y, z) with the z axis oriented parallel to the polypeptide helix axis and a laboratory-fixed coordinate system (X, Y, Z) with the Z axis parallel to the propagation of light. A unit vector $\vec{k}$ is defined, representing the direction of propagation of light in the laboratory-fixed coordinate system. The CD of an ensemble of polypeptide helices in an oriented membrane (o), i.e., a planar bilayer, under the above conditions is

$$[\theta]'_o = \vec{k}'\mathbf{O}' \begin{pmatrix} [\theta]_\perp & 0 & 0 \\ 0 & [\theta]_\perp & 0 \\ 0 & 0 & [\theta]_\parallel \end{pmatrix} \mathbf{O}\vec{k} \qquad (2)$$

$[\theta]'_0$ is the mean residue ellipticity, $\vec{k}'$ is the transpose of $\vec{k}$, $\mathbf{O}$ is the transformation matrix which transforms the laboratory-fixed coordinate system to the molecule-fixed one, and $\mathbf{O}'$ is the transpose of $\mathbf{O}$. $[\theta]_\parallel$ and $[\theta]_\perp$ are the mean residue ellipticities when light traverses parallel or perpendicular, respectively, to the helix axes where all molecules have the same orientation. From eq 2 we obtain after averaging over

[2] A new set of five orthogonal basis CD spectra differing from that of Hennesey and Johnson (1981) has been calculated according to the 15 reference proteins and the spectral range from 186 to 250 nm.

all possible helix orientations around the Z axis

$$[\theta]'_o = [\theta]_\perp + ([\theta]_\parallel - [\theta]_\perp)\langle\cos^2\beta\rangle \quad (3)$$

The corresponding CD of an unoriented sample, in our case polypeptide helices inserted in lipid vesicles (v), is given as

$$[\theta]_v = ([\theta]_\parallel + 2[\theta]_\perp)/3 \quad (4)$$

from which the difference of the CD between the oriented and unoriented membrane sample is obtained

$$[\theta]'_o - [\theta]_v = \tfrac{2}{3}([\theta]_\parallel - [\theta]_\perp)S_h \quad (5)$$

In actuality, however, the experimentally measured difference CD spectrum between planar multibilayers and membrane vesicles is given by the relation

$$[\theta]_o - [\theta]_v = \tfrac{2}{3}f_h([\theta]_\parallel - [\theta]_\perp)S \quad (6)$$

f_h is the helix content of the polypeptide as determined from an unconstrained fit of the corresponding CD spectrum, and the other parameter S is the product of two order parameters, $S = S_h S_m$, where S_m takes into account the mosaic spread of the multibilayers (Rothschild & Clark, 1979). A value of $S_m = 0.9 \pm 0.05$ is used in the present work as in a former study (Vogel et al., 1983) according to the experimental observations of others (Büldt et al., 1979). The latter authors have measured neutron diffraction of DPPC planar membranes prepared similarly to those in our case; the first-order lamellar reflections of such membranes showed a full half-width at half-height between 15 and 30°, corresponding to a S_m of 0.85–0.95. Relations analogous to eq 6 have been derived for CD spectra of molecules in solution oriented by a shear gradient in a streaming apparatus (Hofrichter & Schellmann, 1973).[3] Equation 6 holds for a polypeptide with $f_h < 1$ only if the nonhelical peptide parts show no preferred orientation with respect to the membrane normal. In this case the isotropoic nonhelical contribution of the CD spectrum is identical in oriented membranes and in membrane vesicles and therefore cancels in eq 6, leaving only the CD difference spectrum of the helical part. The difference between the parallel and perpendicular CD component $\Delta = ([\theta]_\parallel - [\theta]_\perp)$ of a polypeptide helix is not known at present but it can be measured by using a helical polypeptide with known S. Once determined, Δ values within the whole spectral range of interest can be applied to calculate S_h for any helical polypeptide from the corresponding difference spectrum ($[\theta]_o - [\theta]_v$). Here we used melittin in planar lipid membranes in order to calibrate Δ. The average orientational order parameter of the helical part of melittin in lipid membranes of DTPC at low water content has been determined to be $S = 0.62$ (Vogel et al., 1983). Recent spectroscopic experiments indicate that the N-terminal segment from residue 1–21 of melittin is incorporated in the lipid bilayer and forms a helix with a bend between Thr-11 and Gly-12, whereas the nonhelical positively charged C-terminal part from residue 22–26 is located in the water phase (Vogel & Jähnig, 1986). According to this model it is reasonable to assume that the nonhelical part of melittin adopts no preferred orientation with respect to the bilayer. Therefore eq 6 is applicable to the case of melittin.

Results

Raman Measurements of Alamethicin

The Raman spectra in the range of 50–900 and 600–1800 cm^{-1} of alamethicin in methanol are shown in Figures 1A and 2A, respectively. After the spectral components of the solvent are subtracted, the pure polypeptide spectra are obtained and are represented in Figures 1C and 2C. The observed bands can be assigned to molecular vibrational modes by comparison with a normal mode calculation of poly(L-alanine) and poly(α-aminoisobutyric acid) (Dwivedi & Krimm, 1984, 1985).

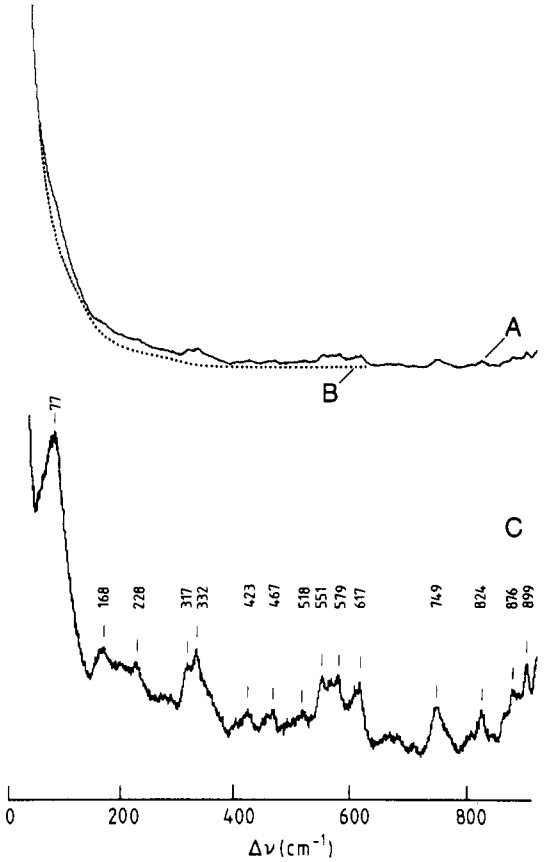

FIGURE 1: Raman spectra between 50 and 900 cm^{-1} of (A) alamethicin in methanol, (B) methanol, and (C) difference spectrum (A – B). Each of the spectra in A and B represents the sum of 40 scans at 15 °C.

The amide I mode appears at 1660 cm^{-1} with shoulders at 1648 and 1639 cm^{-1}. Bands at 1303, 1294, 1281, and 1269 cm^{-1} were assigned to amide III modes; those at 670, 659, and 617 cm^{-1} are due to amide V modes. The wave numbers of these bands together with the skeletal mode at 898 cm^{-1}, the CO out-of-plane bend mode at 768 cm^{-1}, and the skeletal bending modes at 708 and 687 cm^{-1} suggest the presence of α-helical structures as well as helical conformations different from the typical α-helix. According to the normal mode analysis of Dwivedi and Krimm (1984, 1985) conformations such as the α_{II}- and the 3_{10}-helix are in accord with several observed Raman bands in the alamethicin spectrum. In an α-helix the plane of the peptide group is essentially parallel to the helix axis, whereas in α_{II} it is tilted with the N–H bond pointing inward to the axis. The helix rise per residue and the number of residues per helix turn are identical in the α- and α_{II}-helices but are different in the 3_{10}-helix. α_{II}-Helices were suggested to be present in bacteriorhodopsin on the basis of the analysis of its IR spectrum. Short 3_{10}-helical regions are also present in the predominantly α-helical crystal structure of alamethicin (Fox & Richards, 1982). In the low-wavenumber range of the alamethicin spectrum in Figure 1C a strong broad band appears at 77 cm^{-1}, which is also present in the spectrum of solid alamethicin (not shown); the latter preparation, obtained by freeze-drying a solution of alamethicin in tertiary butyl alcohol, shows a helix content similar

[3] Equation 14 in the paper of Hofrichter and Schellmann (1973) is in error. The correct expression in the formalism of these authors is $C = 3\langle\cos^2 Zz\rangle - 1$.

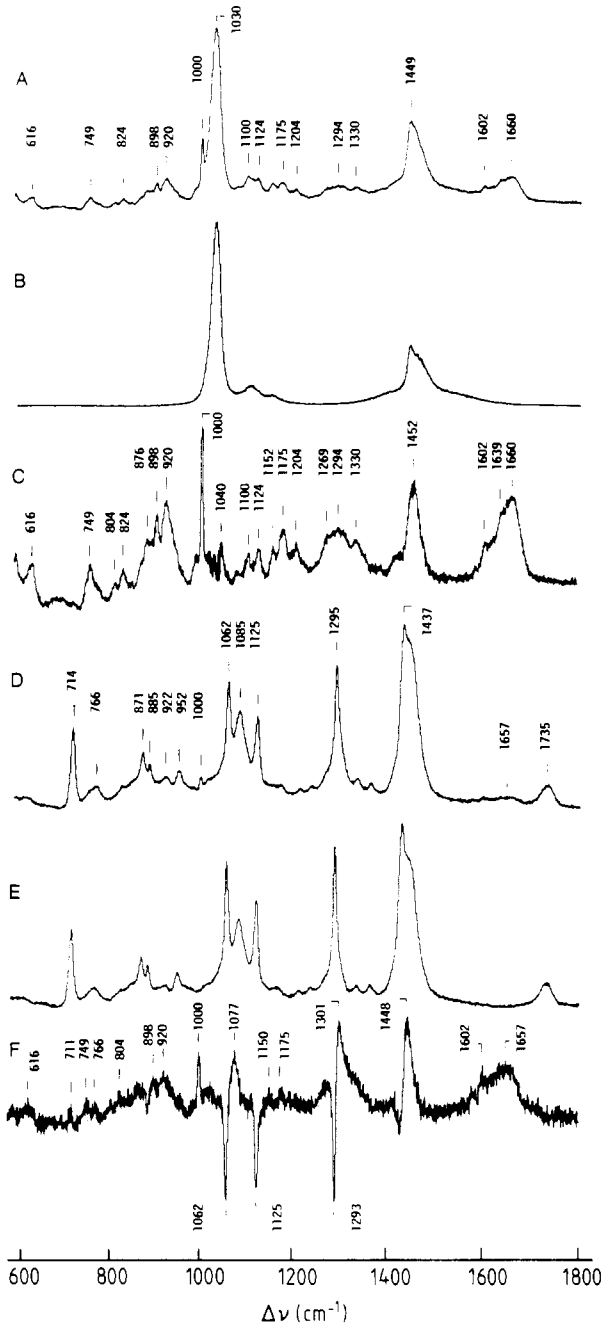

FIGURE 2: Raman spectra between 600 and 1800 cm^{-1} of (A) alamethicin in methanol, (B) methanol, (C) difference spectrum (A – B), (D) alamethicin in lipid membranes of DTPC at a molar lipid/peptide ratio of 20, (E) lipid membranes of DTPC, and (F) difference spectrum (D – E). Each of the spectra in A and B represents the sum of 50 scans, in D and E the sum of 100 scans at 15 °C.

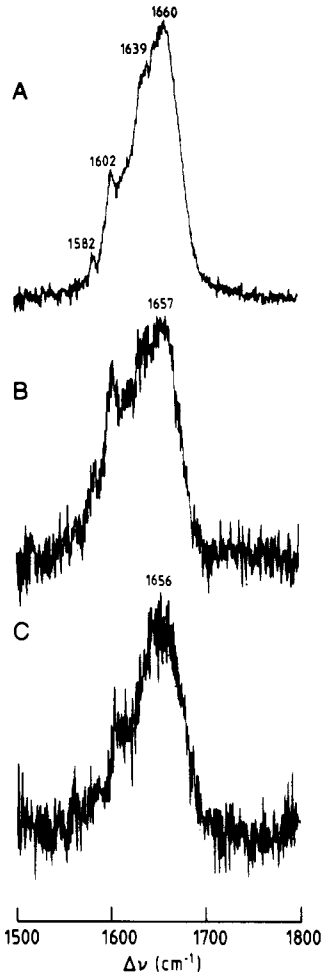

FIGURE 3: Raman spectra between 1500 and 1800 cm^{-1} of (A) alamethicin in methanol, (B) alamethicin in DMPC at a molar lipid/peptide ratio r = 20, and (C) alamethicin in DTPC at r = 20. Spectra represent the sum of 170 (A) and 300 scans (B, C) at 15 °C.

to that of alamethicin in methyl alcohol (see below). The band is assigned to the acoustical mode of an α-helix.

The Raman spectrum of alamethicin in lipid bilayers of DMPC in the ordered membrane state at 15 °C is shown in Figure 2D and that of the pure lipid membranes in Figure 2E. In the difference spectrum in Figure 2F most of the strong alamethicin bands appear to be unchanged from those in Figure 2C. The difference spectrum furthermore shows that upon incorporating alamethicin into the lipid bilayers the strong lipid bands at 1062, 1125, and 1293 cm^{-1}, characteristic of stretched (trans conformation) hydrocarbon chains, decrease in intensity, while new bands appear at 1077, 1301, and 1448 cm^{-1}, characteristic of disordered (gauche conformation) hydrocarbon chains. We conclude that alamethicin decreases the conformational order of the lipid hydrocarbon chains at a temperature below the lipid phase transition. A similar effect was observed with melittin in DMPC membranes (Jähnig et al., 1982).

From the amide I band of alamethicin in methanol and in aqueous dispersions of vesicles of DMPC and DTPC in Figure 3A–C, the secondary structure of the polypeptide was deduced and is summarized in Table I. In methanol alamethicin is 61% helical, 15% is composed by β-strands, turns contribute 16%, and the remaining 8% is designated to the undefined structure class. The secondary structure of alamethicin in DMPC above the lipid phase transition temperature T_t is very similar to that found in methanol. Upon interaction with lipid membranes of DMPC and DTPC below T_t the helix content of alamethicin is increased to 71%, mainly at the cost of β-strands, which now contribute only 6%, whereas the content of T and U structures is similar to that found in fluid membranes of DMPC. Interestingly, the amount of ordered helix structure remains constant at 6–7 amino acid residues in all cases, whereas the number of amino acids with disordered helix structure increases from 5 in DMPC below T_t to 7–8 in lipid membranes above T_t. Table I shows that the unconstrained fit to the amide I bands, allowing any negative or positive number for the calculated amount of the secondary structure classes, is excellent in three respects: (i) No large negative numbers appear as an amount of secondary structure classes. (ii) The sum of the six structure classes is either 100%, as in the case of DTPC/alamethicin, or deviates only slightly from

Table I: Secondary Structure of Alamethicin and Melittin As Determined from Raman Amide I Bands

	O[a]	D	A	P	T	U	H	S	T + U	sum	NRMSD
alamethicin in methanol (15 °C)											
%	31	29	5	10	16	8	60	15	24	99	0.040
R	6	6	1	2	3	2	12	3	5		
DMPC/alamethicin = 20 (15 °C)											
%	34	40	−2	8	17	7	74	6	24	104	0.082
R	7	7	0	2	3	1	14	1	5		
(33 °C)											
%	31	27	19	3	17	8	58	22	25	105	0.065
R	6	5	4	0	3	2	11	4	5		
DTPC/alamethicin = 20 (15 °C)											
%	35	36	1	7	14	7	71	8	21	100	0.049
R	7	7	0	1	3	1	14	2	4		
DTPC/melittin = 100 (15 °C)[b]											
%	58	18	8	−1	8	9	76	7	17	100	0.041
R	15	5	2	0	2	2	20	2	4		

[a] Abbreviations: O, ordered helix; D, disordered helix; A, antiparallel strand; P, parallel strand; T, turn; U, undefined; H, O + D; S, A + P; sum, H + S + T + U; NRMSD, normalized standard deviation = $[\sum_n(I^{exptl} - I^{calcd})^2/\sum_n(I^{exptl})^2]^{1/2}$, where I^{exptl} and I^{calcd} are the experimental and calculated Raman intensities and n is the number of data points; R, number of amino acid residues (normalized to a sum of 20 for alamethicin). [b] From Vogel and Jähnig (1986).

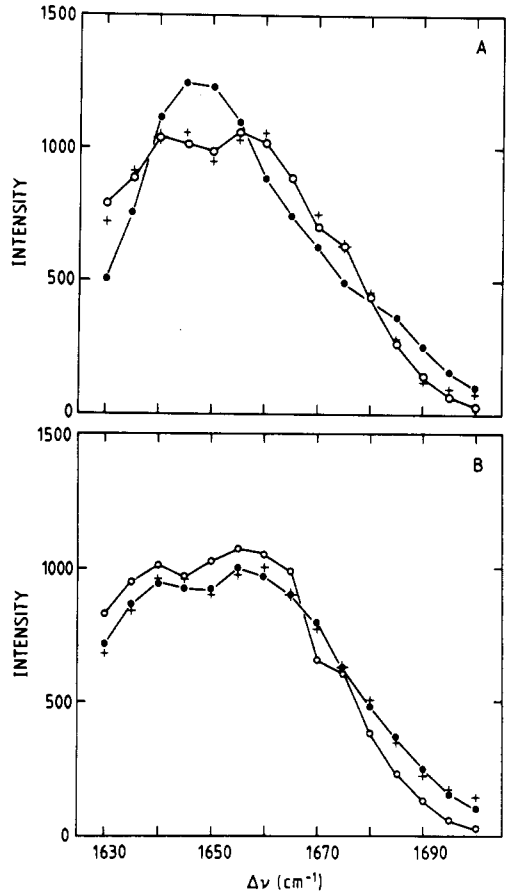

FIGURE 4: (A) Fifteen-point representation of the experimental (O) and fit (+) data of the amide I band of alamethicin in DTPC at a molar lipid/peptide ratio r = 20 at 15 °C. For comparison the experimental data of melittin in DTPC with r = 100 at 15 °C are shown (●). (B) Experimental Raman spectra of alamethicin in DMPC membranes with r = 20 at 33 °C (●) and at 15 °C (O). The fit for the spectrum at 33 °C is included (+).

this value. (iii) The agreement between calculated and experimental spectral data is good as reflected by the values of NRMSD. This is also demonstrated in Figure 4 where for the case of alamethicin in DTPC and DMPC membranes 15-point representations of the experimental and calculated spectra are shown together. In view of these criteria, the structure of alamethicin in methanol and in lipid membranes seems to be very well represented within the structures of reference proteins used for the fit of the Raman spectra. The structural data, listed in Table I, are therefore reliable. The two polypeptides alamethicin and melittin in lipid membranes of DTPC below T_t adopt structures with similar helix content. They differ, however, in the content of disordered helical residues, which is considerably higher in the case of alamethicin. Interestingly, this structural difference is clearly reflected in the amide I bands of the two polypeptides, as shown in Figure 4A.

Table II: Secondary Structure of Alamethicin and Melittin As Determined from CD

	H[a]	S	T	U	sum	NRMSD
alamethicin in methanol	42	24	12	7	85	0.063
DTPC/alamethicin = 100						
15 °C	46	24	8	7	85	0.052
35 °C	38	21	10	7	76	0.063
DTPC/melittin = 120						
15 °C	74	5	8	7	94	0.041
35 °C	72	8	9	7	96	0.035

[a] Abbreviations: % of H, helix; S, strand; T, turn; U, undefined; sum, H + S + T + U. NRMSD = $[\sum_n(\theta^{exptl} - \theta^{calcd})^2/\sum_n(\theta^{exptl})^2]^{1/2}$, where θ^{exptl} and θ^{calcd} are the experimental and calculated mean residue ellipticities and n is the number of data points used.

CD Measurements in Solution and Lipid Vesicle Dispersions

During the course of the CD measurements DTPC has been used exclusively as a lipid component. In DTPC the saturated hydrocarbon chains are connected via ether bonds to the glycerol moiety. Therefore the optical absorption in the far UV down to 186 nm is minimized for a phospholipid molecule. Furthermore DTPC forms planar multilayers of uniaxial symmetry below and above T_t as shown by conoscopy (unpublished) and the lack of linear dichroism effects.

Alamethicin. CD measurements have been performed with alamethicin in methanol and in lipid vesicles of DTPC below and above T_t (Figure 7) as well as with melittin in lipid vesicles of DTPC below and above T_t (Figure 5). The structural data as obtained from the unconstrained fit of the spectra by a superposition of the spectra of 15 reference proteins are listed in Table II. The calculated data points of the fit describe the experimental CD spectra of alamethicin quite well, as reflected by the low values of NRMSD. However, in all of our alamethicin CD spectra the sum of the calculated secondary structure classes is considerably below 100%. In principle an incorrectly determined polypeptide concentration will affect the value of the sum by the same factor as the experimentally determined concentration deviates from the actual one. In our

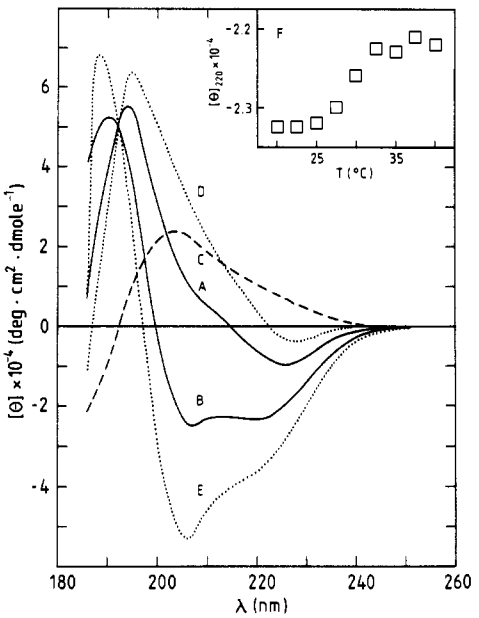

FIGURE 5: CD spectra of melittin in dry planar multibilayer membranes (A) and in an aqueous dispersion of vesicles (B) of DTPC at a molar lipid/peptide ratio of 120 at 15 °C. (C) Difference spectrum (A − B). For comparison, spectra of oriented multibilayers were calculated according to eq 6 with $f_h = 0.74$ and an order parameter of $S = 1$ (D), and $S = -0.5$ (E). Insert (F) shows the temperature dependence of $[\theta]_{220}$, the mean residue ellipticity at 220 nm of melittin in DTPC vesicles.

case the concentration of alamethicin has been determined with an error of about ±5%. The large deviation of the sum values from 100% therefore indicate that the actual structure of alamethicin under our conditions of investigation is not correctly represented by the structures of the reference proteins. Therefore, the percentages of the different secondary structure classes for alamethicin in Table II are much less confident than those in Table I obtained from the Raman analysis. Although the absolute values of the structural data of alamethicin in Table II should be taken with care, one would expect that the differences determined between the polypeptide secondary structure in the ordered membrane state of DTPC at 15 °C and the disordered state at 35 °C reflect at least relative structural changes correctly. In the CD spectrum of alamethicin in DTPC vesicles the minima at 208 and 222 nm in Figure 7, typical of helical polypeptides, change their intensities by about 20% at the phase transition temperature of DTPC at 30 °C, as seen in Figure 7C. The spectral change is reflected in Table II by a change of the helical content, again by about 20%. This result is in accord with similar structural changes of alamethicin in DMPC membranes as determined by Raman spectroscopy (Table I).

Melittin. Uncertainties such as those seen in the analysis of the CD spectra of alamethicin are absent in the case of melittin in DTPC vesicles. According to the low NRMSD values the fits are good. Furthermore, the sum of the different secondary structure classes of 94% at 15 °C and 96% at 35 °C deviates from 100% within experimental error. The structural data of melittin in Table II are therefore reliable. Interestingly, the secondary structure of melittin in DTPC below T_t as determined from CD is nearly quantitatively identical with the secondary structure derived from Raman spectroscopy in Table I. For comparison melittin in DTPC membranes shows similar but much smaller changes in the CD spectrum and in the secondary structure than alamethicin when a phase transition from the ordered to the fluid lipid membrane state is performed. The differenence of $[\theta]_{220}$, the mean residue ellipticity at 220 nm, and the helix content between the ordered and fluid lipid membrane is only about 5% as shown in Figure 5F and Table II.

CD Measurements on Planar Multibilayer Membranes

Melittin. Figure 5A shows a CD spectrum of melittin in planar lipid multilayers of DTPC at about 2% water content and 15 °C. The spectrum resembles that of dried purple membranes of halobacteria (Muccio & Cassim, 1979). On the basis of present theories of protein CD spectra these authors interpreted such spectra as an indication of protein helices oriented preferentially parallel to the membrane normal. The isotropic counterpart of the spectrum in Figure 5A would be a CD spectrum of melittin in dried, isotropically distributed, small membrane fragments. Unfortunately, such samples are very turbid and therefore are unsuited for CD measurements. However, we assume that the CD spectrum of melittin in an aqueous vesicle dispersion of DTPC in the ordered membrane state, shown in Figure 5B, is a good representation of a corresponding spectrum of dried nonoriented membranes. Our assumption is supported by Raman measurements performed with melittin in an aqueous dispersion of DTPC membranes in the ordered membrane state and a corresponding freeze-dried membrane preparation. Within experimental error, no difference was detectable in the amide I band of the polypeptide in the two systems, showing that the conformation of melittin in ordered lipid membranes is almost identical in the presence and absence of water.

As already pointed out in Materials and Methods, the helix order parameters can be determined from the CD spectra of helical polypeptides in planar bilayers according to eq 6. However, in order to do that the difference spectrum $\Delta = ([\theta]_{\parallel} - [\theta]_{\perp})$ in eq 6 must be known first. The helix order parameter of melittin in dried planar bilayers has been determined already elsewhere by IR spectroscopy to be $S = 0.62 \pm 0.08$ (Vogel et al., 1983), and the helix content of melittin as obtained from the analysis of the CD spectrum in Figure 5B is $f_h = 0.74 \pm 0.02$ (Table II). With the difference spectrum $([\theta]_o - [\theta]_v)$ in Figure 5C, one finds according to eq 6 $\Delta = 3.27([\theta]_o - [\theta]_v)$. Due to the uncertainties contained in S and f_h and that of ±5% in $([\theta]_o - [\theta]_v)$ the total uncertainty within Δ is ±20%. These values of Δ are now generally applicable to evaluate helix order parameters with eq 6 from the difference of the CD spectrum of a protein in planar bilayers to that of the protein in membrane dispersions. CD spectra of melittin in planar membranes were calculated with eq 6 and the measured CD spectrum in lipid vesicles for a helical order parameter of $S = 1$ and $S = -0.5$, i.e., for cases where the helix (without a bend) is oriented perfectly parallel either to the membrane normal or to the membrane surface. The calculated spectra are shown in parts D and E of Figure 5 for comparison.

In the following section this method is used to investigate how the helical order parameter of melittin is influenced by the lipid phase transition. Parts A and B of Figure 6 show CD spectra of melittin in fully hydrated (97% relative humidity) planar bilayers in the fluid lipid state at 35 °C and the ordered lipid state at 15 °C. The CD spectrum at 35 °C is very similar to that of dry lipid membranes (Figure 5A). However, the CD spectrum is changed drastically at 15 °C, where it resembles the spectrum of melittin in vesicles as shown in Figure 5B. The main spectral change occurs around the lipid phase transition at 30 °C, as monitored by the temperature dependence of $[\theta]_{208}$, the mean residue ellipticity at 208 nm (Figure 6C). The CD spectra of melittin in lipid vesicles at 15 °C (Figure 5A) and at 35 °C (not shown) and the corresponding f_h values in Table II were used to calculate fits

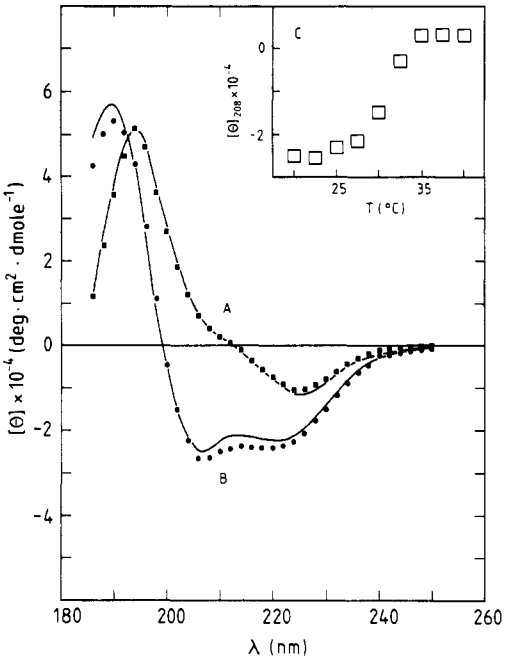

FIGURE 6: CD spectra of melittin in planar multibilayers of DTPC at a molar lipid/peptide ratio of 120 and 97% relative humidity (A) above the lipid phase transition temperature T_t at 35 °C and (B) below T_t at 15 °C. Continuous curves represent experimental spectra. Data points at 2-nm intervals are the fit of the spectra according to eq 6 with $f_h = 0.72$, $S = 0.56$ (■) and $f_h = 0.74$, $S = -0.03$ (●). Insert (C) shows the temperature dependence of $[\theta]_{208}$, the mean residue ellipticity at 208 nm of melittin in the planar multibilayers.

Table III: Helix Order Parameters of Melittin and Alamethicin in Lipid Membranes of DTPC As Determined by the CD Difference Spectrum ($[\theta]_o - [\theta]_v$) according to Eq 6

	dry lipid membranes	lipid membranes at 97% relative humidity	
	15 °C ($T < T_t$)	15 °C ($T < T_t$)	35 °C ($T < T_t$)
DTPC/melittin			
S	0.62 ± 0.08[a]	−0.03 ± 0.01	0.56 ± 0.16
S_h[b]	0.69 ± 0.13	−0.03 ± 0.01	0.62 ± 0.20
DTPC/alamethicin			
S	0.69 ± 0.20[c]	−0.26 ± 0.07	0.79 ± 0.22[d]
S_h	0.77 ± 0.26	−0.29 ± 0.10	0.88 ± 0.29[d]

[a] Adopted from Vogel et al (1983). [b] $S_h = S/0.9$. [c] CD spectrum of corresponding planar membranes not shown. [d] One is the maximal value in the physical range.

to the spectra in Figure 6A,B by using eq 6, and the results are listed in Table III. The fits included in Figure 6 were obtained with the order parameters $S = -0.03$ below and $S = 0.56$ above the phase transition temperature. Taking a mosaic spread of $S_m = 0.9$ into account, the actual values of the helix order parameters are calculated to be $S_h = -0.03/0.9 = -0.03$ and $0.56/0.9 = 0.62$.

Alamethicin. Analogous measurements as for melittin were performed with alamethicin in planar lipid bilayers in the dry state (spectrum not shown) and at maximal water content both below and above T_t (Figure 7A,B). The spectral changes in Figure 7 induced by the lipid phase transition are qualitatively similar to those observed for melittin in Figure 6. In Table III the average helix order parameters for alamethicin and melittin, as derived from CD measurements, are compared. For alamethicin in dry lipid membranes a value of $S_h = 0.77$ was obtained. At 97% relative humidity in fluid lipid membranes the helix order parameter of alamethicin adopts a value of $S_h = 0.88$ and drops drastically at the phase transition

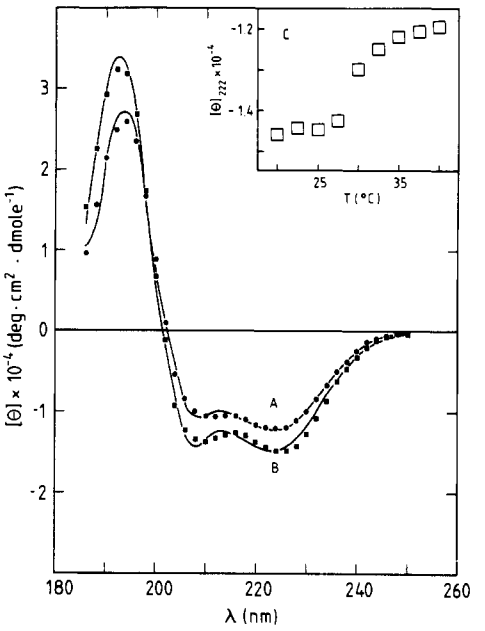

FIGURE 7: CD spectra of alamethicin in lipid vesicles of DTPC at a molar lipid/peptide ratio of 100 (A) at 35 °C and (B) at 15 °C. Continuous curves represent experimental spectra. Data points (■, ●) at 2-nm intervals are the fit of the spectra by a superposition of the spectra of 15 reference proteins. Insert (C) shows the temperature dependence of $[\theta]_{222}$, the mean residue ellipticity at 222 nm of alamethicin in the lipid vesicles.

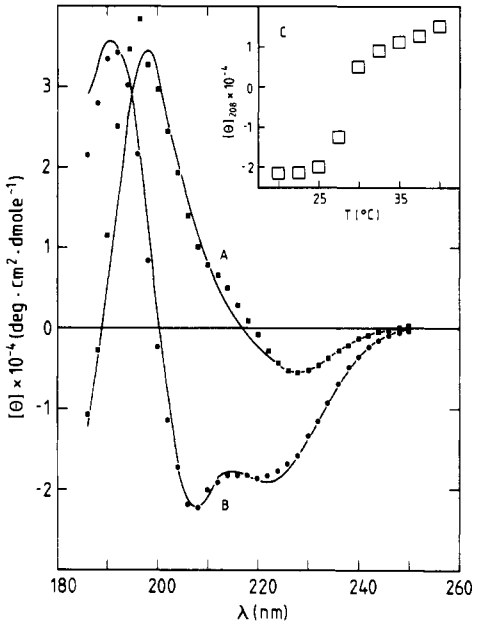

FIGURE 8: CD spectra of alamethicin in planar multibilayers of DTPC at a molar lipid/peptide ratio of 100 and 97% relative humidity at (A) 35 °C and (B) 15 °C. Continuous curves represent experimental spectra. Data points at 2-nm intervals are the fit of the spectra according to eq 6 with $f_h = 0.38$, $S = 0.79$ (■) and $f_h = 0.46$, $S = -0.26$ (●). Insert (C) shows the temperature dependence of $[\theta]_{208}$, the mean residue ellipticity at 208 nm of alamethicin in the planar multibilayers.

(Figure 7C) to $S_h = -0.29$ in the ordered state at 15 °C.

Discussion

According to our Raman and CD measurements the conformation of alamethicin in methanol and in lipid membranes is predominantly helical. However, the helix content as determined by Raman spectroscopy is in all cases considerably higher than that derived from CD measurements. Probably the main reason for this difference is due to the uncertainty

of the CD structural data, as mentioned before, although slight conformational changes due to different sample preparations in Raman and CD spectroscopy cannot be ruled out. Concerning the secondary structure of alamethicin, the structural data obtained from Raman spectroscopy seem to the present stage more reliable than those from CD experiments.

How are the different secondary structure elements distributed within the amino acid sequence of alamethicin? If alamethicin in the crystal form adopts a very stable molecular conformation which will be preserved as far as possible also in more disordered polypeptide structures, then the following scheme represents the most likely conformation for alamethicin in lipid membranes below T_t:

αPαAα	AQαVα	GLαPV	$\alpha\alpha$QQF-OH
DUDOO	OOOOD	DDDUD	D

The first row shows the alamethicin sequence in one-letter code (α = Aib). In the second row secondary structure classes are assigned to amino acid residue 1–16, assuming that the polypeptide conformation as found in the crystal form (Fox & Richards, 1982) is preserved in this part of the molecule while the remaining four residues at the C-terminus are nonhelical. According to Williams (1983) the two Pro residues are counted in class U, although actually within the helix. The proposed model is in excellent agreement with the corresponding Raman structural data in Table I. In a fluid lipid membrane of DMPC only six ordered and five disordered helical amino acid residues were observed (Table I). It is tempting to assign the first 12 N-terminal residues to a helix with identical conformation as that of the corresponding molecular segment in the ordered lipid membrane. The similarity of the alamethicin structure in fluid lipid membranes and in methanol solution suggests a relatively stable N-terminal helical segment. On the other hand a conformational transition from helix to β-structures seems to occur in the C-terminal segment due to changes of the physical state of the lipid membrane. Our model is to some extent in agreement with NMR measurements of alamethicin in methanol by Bannerjee et al. (1983). These authors deduced a solution conformation where the first nine or ten residues from the amino end form a stable α-helix, and the ten residues at the C-terminus form a parallel β-sheet.

In comparison the helical conformation of melittin is only slightly affected by the lipid phase transition, indicating a more stable structure of the bent helix than in alamethicin. According to recent Raman spectroscopic measurements melittin in lipid membranes below T_t is helical from amino acid 1 to 21 (Vogel & Jähnig, 1986). The CD measurements of the present work have shown that the helix content of melittin in a fluid lipid membrane is decreased by about 3%. Therefore in a fluid lipid membrane the helical part extends from amino acid 1 to 20.

From the wavenumber of the acoustical mode in the Raman spectrum of alamethicin in methanol (Figure 1C), the length of the helical segment was calculated as five amino acid residues according to Peticolas (1979). This is much shorter than the 13 residue long helical region in the model discussed above and probably reflects the presence of helical structures that are different from pure α-helices at the helix ends.

The orientation of the alamethicin helix in a membrane strongly depends on the physical state of the lipid bilayer and in the ordered lipid state furthermore on the state of membrane hydration. In a fluid lipid membrane the orientational order parameter of the alamethicin helix was determined by CD measurements to be $S_h = 0.88$, indicating that the helical segment from amino acid 1 to 12 is preferentially oriented parallel to the membrane normal. In the ordered lipid membrane at maximal water content S_h is calculated to be -0.29, indicating that the helical part from residue 1 to 16 is now preferentially oriented parallel to the membrane surface. In contrast to that case the orientation of the alamethicin helix in an ordered lipid membrane at 2% water content is very similar to that in a fluid membrane at maximal water content, according to CD measurments (spectrum not shown).

The conformation of alamethicin in dipalmitoylphosphatidylcholine multilayers was investigated by means of IR-attenuated total reflection spectroscopy by Fringeli and Fringeli (1979). In dry lipid membranes alamethicin was found to be incorporated into the lipid membrane as an α-helix oriented along the membrane normal, in agreement with the results of the present work. After addition of water, considerable changes were observed in the IR spectrum of alamethicin in the lipid multilayers at 25 °C ($T < T_t$). The authors interpreted their results as a conformational change of alamethicin from an α-helix in the dry membrane to an extended chain in hydrated membranes. However, as pointed out by the same authors, the spectral changes are in principle also in accord with a change of the orientation of an α-helix that is parallel along the membrane normal in the dry state and perpendicular to the membrane normal in the presence of water. The latter explanation is likely from the results of the present work.

The orientation of melittin in lipid membranes parallels that of alamethicin. In a fluid lipid membrane the order parameter of the helical part of melittin is $S_h = 0.62$, indicating that this part of the molecule is preferentially oriented parallel to the membrane normal. As mentioned above, in a fluid membane the helical part of melittin extends from amino acid 1 to 20. It is composed of two helix segments with a bend between Thr-11 and Gly-12 (Vogel & Jähnig, 1986). S_h, therefore, is an average value of the order parameter S_{h1} of helix segment h1 and S_{h2} of helix segment h2 according to $S_h = (11S_{h1} + 9S_{h2})/20$. Recently the orientational fluctuations of melittin in lipid vesicles and planar bilayers have been studied by measuring the time-resolved FA of the single tryptophan in the polypeptide (Vogel & Rigler, 1987). For the order parameter of the tryptophan-containing segment h2, a value of $S_{h2} = 0.38$ was determined. Together with the present result of $S_h = 0.62$, we obtain for the order parameter of segment h1 a value of $S_{h1} = 0.82$. On the basis of time-resolved FA measurements the difference between S_{h1} and S_{h2} indicates a highly flexible structure of the helical part of melittin in a fluid lipid membrane where the amplitudes of the fluctuations of h2 are higher than those of h1 (Vogel & Rigler, 1987). If internal motions within h1 and h2 are neglected, the bent region between the helical segments must act as a flexible element mediating the rigid body motions of h1 and h2.

In a lipid membrane below T_t at maximal water content S_h drops to a value of -0.03. This can be explained either by an isotropic distribution of the melittin helices in the bilayer or by an orientation of the bent helix with the helix segment h2 preferentially parallel to the membrane surface ($S_{h2} \approx -0.5$), whereas h1 dips into the bilayer ($S_{h1} \approx 0.4$). An isotropic distribution of the polypeptide helices in the planar multilayers can be excluded on the basis of time-resolved FA measurements[4] (Vogel & Rigler, 1987). Recently Dufourc et al.

[4] The time course $r(t)$ of the Trp FA of melittin in planar bilayers at $T < T_t$ and maximal water content reaches at time $t \rightarrow \infty$ a residual value of $r(\infty) = -0.16$ under a certain geometry of experimental arrangement. An isotropic distribution of melittin molecules in the sample would be compatible only with a value of $0.25 \geq r(\infty) \geq 0$.

(1986) observed a reversible disk ↔ vesicle transition in aqueous dispersions of DPPC–melittin complexes triggered by the ordered ↔ fluid lipid phase transition. It is not clear if in our planar multilayer membranes similar morphological changes occur. If, however, in our case discoidal structures are present at temperatures $T < T_t$ and maximal hydration, then they are not isotropically distributed as shown by FA experiments of Vogel and Rigler (1987). In dry lipid membranes the helical part of melittin is oriented preferentially along the membrane normal ($S_h = 0.69$), again analogous to alamethicin under corresponding conditions.

Interestingly the N-terminal helical segment of alamethicin (residue 1–12) in fluid membranes shows an orientational order parameter $S_h = 0.88$, which is very similar as the corresponding value $S_{h1} = 0.82$ of the N-terminal segment (residue 1–11) of melittin. The question of whether alamethicin or melittin traverses the membrane or whether instead the N-terminal helix segments end within the lipid bilayer cannot be answered at present. At least in the case of alamethicin, the 12 residue containing helix segment is too short to span the bilayer by itself. Upon insertion of either alamethicin or melittin helices into lipid membranes free energy is gained, mainly due to the hydrophobic effect. At the same time the lipid structure is disturbed by the incorporated polypeptides, decreasing the above gain of free energy (Jähnig, 1983). In the case of melittin and alamethicin these opposing effects seem to be finely balanced. In a fluid lipid membrane the hydrophobic effect dominates, whereas in an ordered membrane in the presence of water it is the lipid perturbation effect. In the latter case the polypeptide is excluded largely from the interior of the lipid bilayer and now lies preferentially on the membrane surface. In this way lipid perturbation is reduced while the hydrophobic sides of the amphipathic helices are still in contact with the lipid membrane, although to a lesser extent than in the fluid membrane.

On the other hand in ordered lipid membranes at 2% water content the polypeptide helices adopt an orientation very similar to that in fluid lipid membranes at maximal water content. The dried multilayers contain about one water molecule per lipid molecule. In such samples there is practically no aqueous layer between adjacent membranes, giving a reasonable exlanation why the polypeptide molecules are pressed back into the lipid bilayers as much as possible.

The Raman and CD measurements of the present work have been performed at polypeptide concentrations where both alamethicin as well as melittin are practically totally incorporated into the lipid membrane in an aggregated form as was shown elsewhere (Rizzo et al., 1987; Vogel & Jähnig, 1986). Therefore the structural model proposed above, where both polypeptides are inserted in a fluid lipid membrane with their N-terminal helix pedominantly parallel to the membrane normal, holds for the aggregated polypeptides. Electrical conductance measurements on planar lipid bilayers which have shown that alamethicin and melittin form voltage-gated membrane channels were performed, however, at nanomolar polypeptide concentrations (Hall et al., 1984; Tosteson & Tosteson, 1981). Under these conditions a considerable fraction of the membrane-associated polypeptide molecules, alamethicin as well as melittin, is monomeric (Schwarz et al., 1986; Rizzo et al., 1987; Vogel & Jähnig, 1986). The conformation and orientation of corresponding polypeptide molecules may be different in the monomeric and aggregated form. It is therefore still an unresolved question whether the structure of the aggregated polypeptide molecules in the fluid lipid membrane as discussed in this work is the structure of an open or a closed channel. If our model represents an already open channel, then the conductance increase of planar bilayers induced by melittin or alamethicin in the presence of a transbilayer electrical potential can be explained by an increased partitioning of the polypeptide molecules in the lipid bilayer as discussed recently by Rizzo et al. (1986). If on the other hand our model represents a still closed channel, then the role of the transbilayer electrical potential would be to induce a structural change of the polypeptide in order to open the channel as proposed by Fox and Richards (1982). The question of which one of the proposed channel-gating models is correct or if a combination of both is true should be answered by further studies.

Acknowledgments

I am grateful to V. Rizzo for providing the alamethicin and to F. Jähnig for the use of the Raman spectrometer in his laboratory. I thank R. Ford and S. Stankowski for critically reading the manuscript and Prof. G. Schwarz for his interest and support during this work.

Registry No. DMPC, 18194-24-6; DTPC, 81303-73-3; methanol, 67-56-1; alamethicin, 27061-78-5; melittin, 20449-79-0.

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Different Types of Microheterogeneity of Human Thyroxine-Binding Prealbumin†

Tom Pettersson,* Anders Carlström, and Hans Jörnvall

Department of Clinical Chemistry, Karolinska Institutet, Danderyd Hospital, S-182 88 Danderyd, Sweden, and Department of Chemistry I, Karolinska Institutet, S-104 01 Stockholm, Sweden

Received June 17, 1986; Revised Manuscript Received December 9, 1986

ABSTRACT: Human thyroxine-binding prealbumin or transthyretin has been isolated by three different methods. In all cases, the isolated material is microheterogeneous in an isoelectric focusing system, revealing a pattern of at least 10 bands. These subforms represent tetramers. Dissociation of the protein in serum samples and dissociation in the presence of urea or sodium dodecyl sulfate (SDS) reveal eight other forms, differing in isoelectric point and concluded to be monomers. Two different sets of dissociated forms are identified, one from urea treatment and the other from SDS treatment. The latter set is apparently also present in serum. Interpretations are complicated by multiplicities of all forms, atypical electrophoretic migrations, nonidentical effects of urea and SDS, and the absence of dimer under most conditions. However, reassociations of monomers and formation of interspecies hybrid molecules identify dimers and clarify interpretations of the dissociated sets. In at least five of the forms likely to represent monomers, the difference is dependent on the nature of the SH group at Cys-10, which can be oxidized, can be reduced, or can be in a mixed disulfide, probably with glutathione and its degradation products. Amino acid sequence analysis reveals an additional N-terminal heterogeneity (with start at positions 1, 2, and 3) of the three most abundant monomers, but this does not explain the observed differences among monomeric forms. It is concluded that prealbumin exhibits different types of microheterogeneity, the major pattern of which is explained by the status of the subunit SH group and the ability of prealbumin to dissociate into monomers. A relation appears to exist between SH group status on the one hand, and retinol-binding protein interaction, the ability to dissociate into monomers, and possibly also binding of thyroxine on the other. These interactions suggest complex functional relationships for prealbumin in serum.

Three different binding proteins for the thyroid hormone thyroxine occur in blood, thyroxine-binding globulin, thyroxine-binding prealbumin, and albumin. Of these three proteins, thyroxine-binding globulin has long been considered to be the most important thyroxine carrier (Woeber & Ingbar, 1968; Gordon & Coutsoftides, 1969; Pages et al., 1973; Wosilait, 1977; Cheng et al., 1977; Andrea et al., 1980; Yamamoto et al., 1980). However, it is now known that, under physiological conditions, prealbumin and thyroxine-binding globulin are of almost equal importance for thyroxine transport; 40–45% of the total thyroxine is bound to prealbumin, 45–50% to thyroxine-binding globulin, and 5–10% to albumin (Pettersson & Carlström, 1979). This suggests a higher affinity of prealbumin for thyroxine than has been anticipated from in vitro studies. The discrepancy calls for further studies of prealbumin and its interactions.

The prealbumin tetramer consists of four identical subunits (Blake et al., 1978). The amino acid sequence of the 127-residue subunit is known (Kanda et al., 1974). Two genetic variants involving amino acid substitutions (Val-30 → Met, Gly-49 → Thr) have been reported in patients suffering from familial amyloidotic polyneuropathy (Dwulet & Benson, 1983; Pras et al., 1983). An electrophoretic microheterogeneity of human prealbumin has also been reported (Altland et al., 1981; Felding & Fex, 1984). X-ray chrystallographic studies show that thyroxine is bound in a channel passing through the tetramer (Blake et al., 1978) and suggest the presence of two identical binding sites per tetramer, one at each end of the channel. However, thyroxine is known to bind to the prealbumin tetramer in a molar ratio of 1:1, suggesting a negative cooperativity (of unknown nature) between the two sites (Ferguson et al., 1975). In this respect, the complete identity of the subunits could be questioned. This is further substan-

† This work was supported by the Swedish Medical Research Council (Projects 03X-6231 and 03X-3532) and the Swedish Cancer Society (Project 1806).

* Address correspondence to this author at the Department of Clinical Chemistry, Danderyd Hospital.

0006-2960/87/0426-4572$01.50/0