Structural Organization of α-Helical Peptide Antibiotic Alamethicin at the Air/Water Interface

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The structural organization of Langmuir monolayers of the α-helical peptide alamethicin, which forms voltage-gated ion channels in lipid membranes, is studied by means of synchrotron X-ray diffraction, surface potential technique, surface pressure/area isotherms, and atomic force microscopy (AFM). Alamethicin adsorbs and adopts a parallel orientation of its helix axis at interfaces, forms 2D crystalline aggregates, and phase-separates from lipids. The structural results obtained correlate with the novel AFM images of alamethicin helices. The effects of hydrogen-bond promoters, pH, salt content of the aqueous subphase, and lipid−peptide interactions were analyzed. The modification of the intrapeptide hydrogen-bond strength, by H_2O_2 hydrogen-bond promoter, affects the helix structural parameters and the density of the crystal structure.

Introduction

The amphiphilic α-helical peptide antibiotics are active at the membrane interface, interact with cell membrane lipids, form ion channels, or may contribute to total membrane disruption.[1] They may adsorb at phospholipid/water and air/water interfaces, and their primary action is to interact with the phospholipid leaflets of the biological membranes. Langmuir monolayer technique[2−8] appears to be a suitable method for modeling these interactions and determining the structural organization of peptides[9] and proteins[10] at the interface.

The natural α-helical peptide antibiotic alamethicin (Figure 1A) forms voltage-gated ion channels in lipid membranes. The structural model of the channel is still under discussion.[9,11,12] Different variants of the "barrel stave model"[13−16] have been used in the literature for the alamethicin channel. All of these suppose a perpendicular orientation of the alamethicin helices at the interfaces. We have recently proposed[9,17] a new lipid-covered ring (LCR) model for this type of ion channels, which is in better agreement with the reported experimental results.[12] According to this model, the alamethicin helices adopt stable planar orientation at the interfaces, form aggregates that possess a ringlike hole and insert in one side (cis side) of the lipid monolayers of the membrane (Figure 2).

In this paper, we present for the first time atomic force microscopy (AFM) images of the alamethicin helices, direct structural evidence of the amphiphilic nature and organization of the alamethicin helices at interfaces, as well as the effect of the hydrogen-bond promoter H_2O_2 on the alamethicin monolayer.

Materials and Methods

Alamethicin (Sigma, MW 1959.9) was spread at the air/water interface from chloroform/methanol (20/1) solution or injected from ethanol solution into the aqueous subphase. Deionized Milli-Q pure water of resistivity 18 MΩ cm, 10% hydrogen peroxide (Fluka) water solution, and 0.1 M NaCl, pH 7.0 (adjusted by means of 1×10^{-3} M phosphate buffer (Na_2HPO_4/NaH_2PO_4 of 1/1 molar ratio) aqueous solution were used. High-purity octadecylamine (ODA) was supplied from Avanti Polar Lipids (Alabaster, AL).

Adsorption experiments were performed by injection of alamethicin solution into the pure water subphase accompanied by homogenization and measurement of the surface pressure by the Wilhelmy plate method. The statistical error of these experiments was about 5%. A Langmuir trough was used to vary the surface pressure of the lipid monolayers. Langmuir−Blodgett (LB) technique was applied to transfer monolayers on purified glass substrates. The surface potential technique was applied to determine the surface potential of the monolayer (see a recent review[8]).

Synchrotron X-ray diffraction from Langmuir monolayers was performed on D41 beamline at LURE, Orsay, France. The experimental setup is described in ref 18.

AFM images were obtained by using the tapping mode of a Nanoscope 3 apparatus.

Results and Discussion

Alamethicin Adsorption. Alamethicin peptide shows important surface activity due to its amphiphilic nature. The surface pressure (Figure 1A) rises sharply from 1 to 29 mN/m with the variation of alamethicin concentration from 2×10^{-8} to 1×10^{-7} M and finally reaches its maximal value of about 30 mN/m at a concentration of 1×10^{-6} M. By use of the concentration dependence of the surface tension, and the Gibbs equation, the approximate surface area of alamethicin could be determined. The Gibbs adsorption isotherm[19] for our noncharged peptide can be written as

$$\frac{1}{A} = \frac{1}{kT}\frac{\mathrm{d}\pi}{\mathrm{d}\ln C}$$

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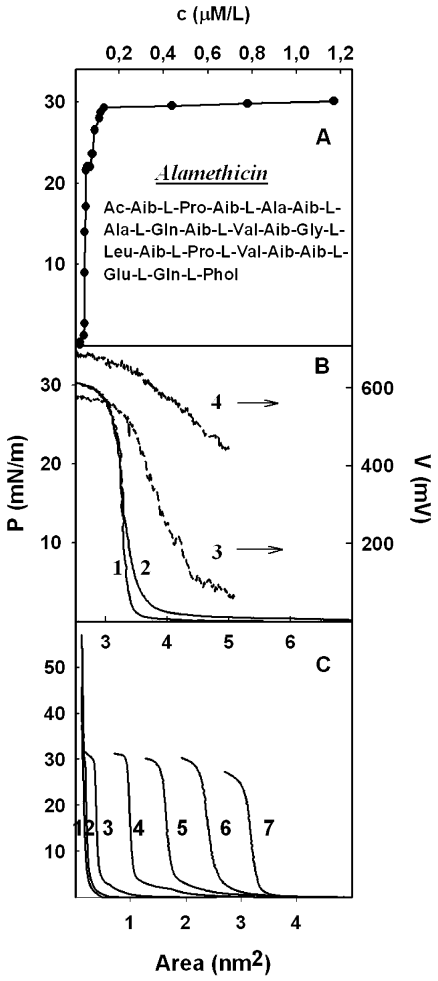

Figure 1. (A) Surface pressure versus alamethicin concentration dependence dissolved from ethanol solution in 0.1 M NaCl aqueous subphase of pH 7.0 at 22 °C. (B) Typical surface pressure/area isotherm of an alamethicin monolayer spread on water surface at 22 °C (curve 1) and in the presence of 10% hydrogen peroxide in the aqueous subphase (curve 2) and surface potential dependencies of the monolayer on pure water surface (curve 3) and in the presence of H_2O_2 (curve 4). The curves are calibrated by respect to the pure aqueous surface. (C) Surface pressure/area isotherms of mixed monolayers at 22 °C on water surface at different peptide/lipid molar ratios of mixtures of alamethicin (curve 7) and octadecylamine (ODA) (curve 1), (2) 1/20, (3) 1/10, (4) 1/3, (5) 1/2, (6) 1/1, (7) 2/1, and (8) 3/1.

where A is the surface area, k is the Boltzmann constant, T is the absolute temperature, π is the surface pressure, and C is the bulk concentration. The sigmoidal character of the adsorption isotherm gives an approximate alamethicin area of about 3.4 nm^2 at the air/water interface.

The experiment demonstrates that at an alamethicin concentration region higher than 1×10^{-7} M, which is usually used to study single ion channels of alamethicin, a dense alamethicin film is formed at the interface. In an additional experiment, which will be reported separately, using grazing X-ray reflectivity measurements of monolayers we have verified that such an alamethicin film forms under the lipid monolayers as well. This alamethicin surface activity at the membrane/water interface should be considered when one develops the model of an alamethicin ion channel. The presence of such a dense peptide monolayer correlates with our LCR model as discussed below.

Asymmetrical LCR Model. Alamethicin is an amphiphilic peptide since its polar hydrophilic groups are either at the C-terminus or lie along a narrow hydrophilic strip parallel to the helix axis (Figure 2A). The other parts of the helix, including the N-terminus, are hydrophobic in nature. Injected on one side of the membrane (cis side, Figure 2B), alamethicin accumulates at the membrane interface beneath densely packed lipid monolayer of a lateral pressure of about 30−50 mN/m. The higher the interfacial concentration, the higher the probability of alamethicin to form two-dimensional aggregates and to penetrate into the cis-side membrane lipid monolayer by two mechanisms: (i) collision of the alamethicin aggregates present beneath the cis monolayers or (ii) local fluctuations of the lateral pressure of the cis membrane monolayers to values less than 30 mN/m. The alamethicin monolayer below the membrane monolayer could be formed by staking the helices two by two by their hydrophobic parts and thus exposing toward the environment their hydrophilic parts (Figure 2B). Upon penetration in the cis membrane monolayer, the helices expose their hydrophobic part toward the membrane interior by simple rotation around their axis. The polar surface of the peptide helix is oriented toward the water phase. The C- and N-terminal groups make energetically favorable contacts with water and hydrophobic regions of the membrane, respectively.

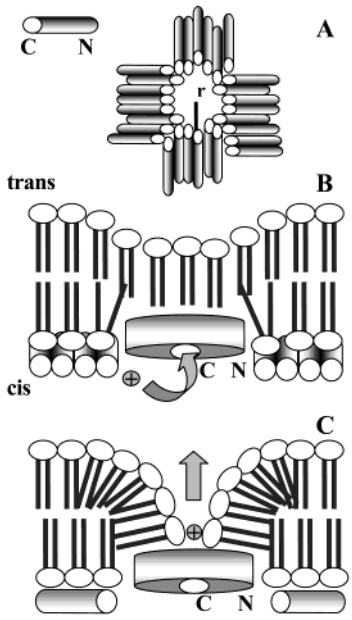

Figure 2. Asymmetrical lipid covered ring (LCR) model of the alamethicin ion channel. (A) The cylinder represents the alamethicin helix with hydrophilic (white) and hydrophobic (dark) regions. The hydrophilic pore of radius r is formed by the two-dimensional aggregation of alamethicin helices. (B) Side view of the pore ring immersed in the "cis"-side membrane monolayer and covered by the "trans" lipid monolayer. (C) Pore opening and cation passage upon application of electrical field.

The asymmetrical lipid-covered ring (LCR) model for the ion channel of alamethicin includes a platelike peptide ring covered with a lipid monolayer (Figure 2B). The peptide aggregate possesses a central hydrophilic ringlike cavity (Figure 2A,B) formed by the polar hydrophilic C-terminal amino acid groups. The positional fluctuations of the helices along the helix axis facilitate the creation of the cavity in the two-dimensional aggregates. The radius, r, of the pore formed by aggregation of n helices could vary discretely by addition or subtraction of helical monomers.

The asymmetry of the ion-channel structure implies an asymmetry of corresponding ion-current characteristics. The formation of a peptide aggregate possessing a pore in the cis-side membrane monolayer reduces the potential barrier due to the lower membrane thickness (Figure 2C). The partial negative

charge of the cavity creates an additional potential well for cations and a potential barrier for anions. Therefore, the current characteristics will be asymmetric as experimentally observed.[11−13,16] If one applies a positive electric field to the cis side of the membrane, the hydrophilic cations will enter within the potential well of the cavity and will disturb the trans-side phospholipid monolayer. In the presence of a strong electrical field, the cation energy may become sufficiently high to open the channel and the cation will pass through the membrane (Figure 2C).

Alamethicin Monolayers: *A. Pure Alamethicin Monolayers.* Alamethicin may form stable monolayers at the air/water interface as shown in Figure 1B (curve 1). Similar isotherms were obtained for pure alamethicin monolayers spread on pure water and on aqueous subphase (not shown) containing up to 1 M NaCl, $N_2C_2O_4$, $CaCl_2$, and pH varying from 4 to 9.4. The steep rise of the isotherm and the compressibility coefficient of 2.9 m/N (at $\pi = 20$ mN/m) indicate a solidlike structural organization of the alamethicin monolayer. The molecular area of 3.2 nm^2, determined at a surface pressure of 20 mN/m, indicates that the peptide molecules are oriented with their α-helix axes parallel to the air/water interface. This value correlates with the area determined from the adsorption measurements. The "collapse" of the monolayer occurred at a reproducible π value of 29 mN/m and the plateau of monolayer "collapse" continues up to zero molecular areas.

The monolayer surface potential values measured at 3.2 nm^2 (Figure 1B, curve 3) determine an apparent perpendicular molecular dipole moment component $\mu_\perp/\epsilon$ (where ϵ is the dielectric constant) of about 3.2 D on pure water subphase.

X-ray diffraction spectra at grazing angles of pure alamethicin monolayers on pure water subphase (Figure 3A) show diffraction peaks indicating well-defined one-dimensional crystalline structure with correlation length of about 75 nm. The positional fluctuations along the alamethicin helix axis could transform the orthorhombic two-dimensional crystals observed previously[9] into one-dimensional crystalline monolayers. Such fluctuations are consistent with our LCR model for alamethicin ion channel as discussed above. These fluctuations result in strong disturbance of the two-dimensional orthorhombic unit cell and limit the number of peaks observed to two with wavevectors $Q_{10} = 6.473$ nm^{-1} and $Q_{11} = 6.660$ nm^{-1} at a surface pressure of 10 mN/m. For pure water subphase the lattice parameter (corresponding wavevector Q_{10}) vary from 0.9702 nm up to 0.9419 nm (±0.0004 nm) as the pressure increases from 10 to 25 mN/m.

One alamethicin monolayer was transferred from pure water subphase on a hydrophilic glass substrate via the LB technique at surface pressure of 20 mN/m. The transfer ratio was 1 and the withdrawal speed was 40 μm/s. AFM images (Figure 3C) of alamethicin monolayers are obtained for the first time and they confirm the one-dimensional crystalline structure of the monolayers. The monolayer periodicity corresponds to the lattice parameters obtained by X-ray diffraction. The diameter of the alamethicin α-helix is about 1 nm and the helix length is about 3.2 nm. The observed kink up to 35° could be explained by the presence of proline at position 14. The images were reproducible on three different samples and on five different surface areas of each sample. The total film thickness of 1 nm measured corresponds to the thickness of one alamethicin monolayer. At the present state, the glass substrates were the best for Langmuir−Blodgett (LB) monolayer deposition because of the highest transfer ratio obtained. This ensures the quality and reproducibility of the monolayer structure deposited. However,

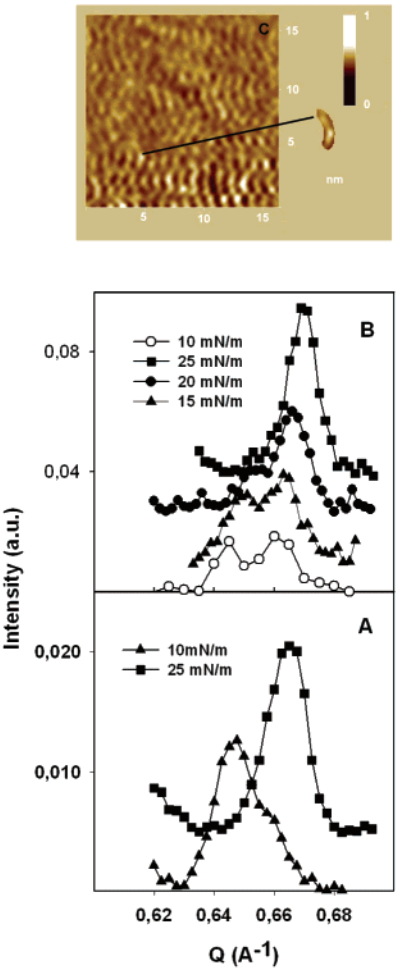

Figure 3. Surface pressure dependence of the X-ray peak of the short in-plane spacing of a pure alamethicin monolayer at 23 °C on pure water surface (A) and in the presence of 10% hydrogen peroxide in the aqueous subphase (B). The wavevector is $Q = 2\pi/d$, where d is the Bragg spacing. The experimental data were fitted by use of a Lorenzian function. The detector noise and the baseline were subtracted for all spectra. (C, top panel) AFM image of alamethicin monolayer on glass substrate. The inset shows one helix.

the large-scale glass surface fluctuations limit the quality of AFM image. The surface smooth substrates, like silicium plates and mica, resulted in partial transfer of the monolayer. It is well-known[3−5,7] that incomplete LB transfer modifies the structural organization of the deposited film. Detailed analysis of the AFM study will be presented elsewhere.

B. Mixed Alamethicin Monolayers. Figure 1C presents surface pressure/area isotherms of mixed monolayers of the peptide alamethicin with the cationic lipid octadecylamine (ODA). Analysis of the two-dimensional miscibility agrees with our previous conclusions[9] for uncharged phospholipids that alamethicin does not mix with lipid molecules in the binary monolayers. The observed linear variation of the mean molecular area with the molar fraction of alamethicin in the monolayers, together with the registration of two distinct monolayer "collapse" pressures, independent of the monolayer composition, indicate two-dimensional immiscibility[4−7] of alamethicin with lipids. At higher pH value, the ionization of the cationic ODA headgroups does not influence the isotherm. Comparison with our previous study[9] shows the existence of a small shift to higher area at pressures higher than 30 mN/m for alamethicin mixtures with phospholipids. Such a shift is not observed in the case of mixture of alamethicin with ODA or fatty acid monolayers. At

molar peptide/lipid ratios of about 1/20, the observed shift can be well explained by parallel orientation of the alamethicin helix to the interface. This shows that a small portion of the alamethicin aggregates could be blocked within the headgroup region of the phospholipid monolayers due to the local fluctuations of the surface pressure, free space available in the headgroup region, and matching of the alamethicin helix diameter of about 1 nm with the phospholipid headgroup dimension. The tendency of alamethicin to insert and interact with the lipid headgroup region at higher lipid monolayer pressures was previously[12,17] discussed in relation to the LCR model and phase transitions in lipid systems. Additional evidence obtained by an X-ray reflectivity study of alamethicin/lipid monolayers at the air/water interface will be published separately.

The weak X-ray diffraction peak observed (not shown) at about 0.9483 nm from mixed alamthicin/DOPE (1/8) monolayers compressed up to 20 mN/m indicates that alamethicin aggregates preserve their crystalline structure in these monolayers.

Effect of the Hydrogen-Bond Promoter H_2O_2 on the Alamethicin Monolayers. The important role of the hydrogen bonds for the structural organization of alamethicin is evidenced by the well-verified modification of the isotherm at surface pressure below 12 mN/m in the presence of hydrogen-bond promoter (Figure 1B, curve 2). The shift toward lower surface densities at low surface pressure may be related to molecular reorganization or structural modification of the monolayer as discussed below. The two isotherms merge at higher pressures where alamethicin monolayers adopt their stable configurations. One may determine that the work applied by lateral surface forces to overcome the disturbance hydrogen-bond effect of H_2O_2 and to bring the alamethicin helix to its unextended state (see below) at about 12 mN/m is about 3×10^{-21} J/molecule.

The apparent perpendicular molecular dipole moment component value of about 5.1 D measured at 3.2 nm^2 (Figure 1B, curves 3 and 4) in the presence of H_2O_2 in the aqueous subphase is different from the one measured on pure water subphase. In the presence of H_2O_2, the surface potential curve is modified. At an extended monolayer state (at about 5 nm^2), the surface potential value of the alamethicin monolayer is about 400 mV higher than the one on pure water subphase. At the monolayer collapse, both surface potential values reach the region 600−650 mV. This indicates spontaneous molecular dipole orientation of the noncompressed monolayer due to hydrogen-bond formation between the monolayer and the subphase[8] and is related to the extension of the alamethicin helix induced by the presence of the hydrogen-bond promoter H_2O_2. The promoter helps to extend the hydrogen-bond network to larger areas per molecule, which is the reason the surface potential is so much higher at large areas than for the monolayers on pure water.

X-ray diffraction spectra at grazing angles of pure alamethicin monolayers in the presence of hydrogen peroxide in the aqueous subphase (Figure 3B) show well-defined diffraction peaks. The lattice parameter (corresponding to the wavevector Q_{10}) changes from 0.9500 to 0.9359 nm as the pressure increases from 10 to 25 mN/m. In this case one observes a splitting of the peak at low surface pressure, which could be explained by the distortion of the rectangular lattice and distinction of the peaks with wavevectors Q_{10} and Q_{11}. The peak with wavevector Q_{01} in the region at about 0.18 nm^{-1} was not observed because of the lower signal-to-noise ratio in this region and the helix length fluctuations due to the hydrogen-bond promoter effect. Hydrogen-bond promoter leads to more dense crystalline structure, which demonstrates the important role of the hydrogen bonds for the structural organization of the alamethicin at interfaces. The increase of the peptide−water hydrogen-bond strength influences the strength of the intramolecular hydrogen bonds, responsible for the peptide helical structure. At low surface pressure, the helix length extends and the helix diameter has a lower value as detected by X-ray diffraction.

The observed higher surface area and surface potential (Figure 1B, curves 2 and 4) at low surface pressure correlate with the smaller crystalline parameters determined, in the case where hydrogen peroxide is present in the water subphase. The hydrogen-bond promoter strengthens the hydrogen bonds of the molecule with the aqueous subphase and influences on the hydrogen-bond network responsible for the helicoidal structure of alamethicin. It causes elongation of the helix and reduction of its diameter at low surface pressure of the monolayer. Therefore, the molecular area increases, but the short lattice parameter has a smaller value.

We have shown that alamethicin forms dense monolayers at interfaces within the alamethicin concentration range typically used for ion current experiments; the peptide forms aggregates where the helices adopt a planar orientation. The structural organization of the peptide at the air/water interface is dominated by the hydropobic−hydrophilic molecular balance and depends on the hydrogen-bond strength. The alamethicin helix remains stable and its structure is influenced at low surface pressures only by the presence of hydrogen-bond promoters.

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