Rapid Report

Cooperative membrane insertion of magainin correlated with its cytolytic activity

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Abstract

Using oriented circular dichroism, we have found that magainin adopts an α-helical conformation with two distinct orientations when interacting with a lipid bilayer. At low concentrations, magainin is adsorbed parallel to the membrane surface. However, at high concentrations, magainin is inserted into the membrane. This transition occurs at roughly the same critical concentration required for cytolytic activity, implying that the membrane insertion is responsible for magainin's cell-lysing activity.

Key words: Magainin; Alamethicin; Lipid bilayer; Protein–lipid interaction; Cytolysis

Magainins and a variety of other small peptides are antimicrobials that have recently been found widely distributed in the animal kingdom as a universal means for defense against bacterial infections [1,2]. Unlike other membrane-active proteins, these peptides have been shown to exert their activity directly through the lipid bilayer of the cellular membrane rather than interacting with specific protein targets [3,4]. Recent discussions have focused on identifying the structural mechanism for magainin's cytolytic activity [5,6]. At low concentrations magainin forms occasional individual ion channels in cellular membranes, and at high concentrations causes cell lysis [7–9]. Since membrane-associated magainin forms an amphipathic α-helix, the most obvious structure for a magainin ion channel is an oligomer with several membrane inserted helices forming a water-filled pore. At high concentrations, channels of this form could expand in number or size to cause cell lysis (a sufficient number of channels would effectively cause solubilization of the membrane), making this model very attractive. However, recent NMR results found membrane-associated magainin to be aligned parallel to the membrane surface [10,11], casting doubt on the validity of this model. In response, a

variety of unique new structures for magainin channels have been proposed [5,6]. We have been using the method of oriented circular dichroism (OCD) [12,13] to study the orientation of helical peptides in lipid bilayers. Our experiments provide evidence supporting the original membrane-inserted structure without contradicting the results of the NMR experiments.

Magainin 1 was synthesized by Multiple Peptide Systems (San Diego, CA); its purity was $> 97\%$ as shown by HPLC. Dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) were purchased from Avanti Polar Lipids (Alabaster, AL). The 3:1 DMPC/DMPG mixture used in these experiments was shown to be in the L_α (smectic A liquid crystalline) phase at room temperature ($\sim 25°C$) with and without magainin (as proven by X-ray diffraction and polarizing microscopy, using the methods of Huang and Wu [14]). The lipid and magainin in various molar ratios were codissolved in trifluoroethanol, the solvent was evaporated, then the mixture was partially redissolved in chloroform for transfer to a fused silica slide. The chloroform was evaporated, first under dry N_2, then in vacuum for several hours. After drying, the slide was placed in a chamber at 100% relative humidity at room temperature. After hydrating for ~ 1 h, the sample was covered with a second slide and mechanically perturbed until homeotropically (lipid bilayers parallel to the substrate surfaces) aligned multilayers

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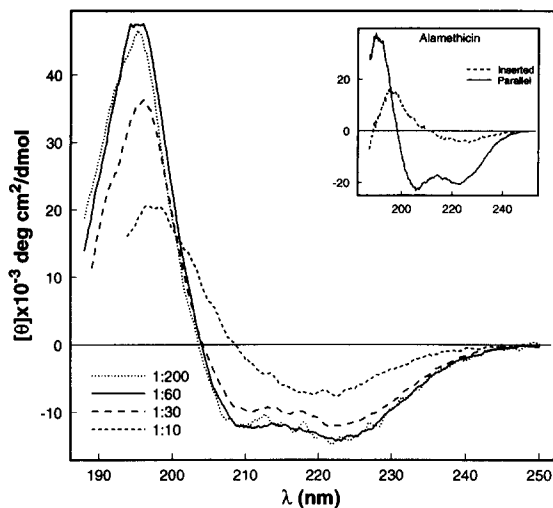* Corresponding author. Fax: +1 (713) 5279033.

Fig. 1. OCD spectra of magainin in DMPC/DMPG (3:1) multilayers at various concentrations, and (inset) OCD spectra of the inserted and membrane parallel states of alamethicin in DPhPC multilayers. At 1:10 magainin is in the inserted state, and at 1:60 and below magainin is in the membrane parallel state. 1:30 is a superposition of the two states. The primary features indicating a shift from the membrane parallel to the inserted state are the disappearance of the negative peak at ~ 208 nm, the red-shift of the positive peak near 195 nm, and an overall reduction in amplitude [12]. All three features are clearly observed in both the alamethicin and magainin spectra. We have observed variations in relative CD band amplitudes between various small peptides with the same secondary structure. For small peptides the effects of protein composition and length are more pronounced than for proteins and long peptides [28]. This effect causes noticeable differences between the OCD spectra of magainin and alamethicin. The shape of our magainin spectra were consistently reproduced to within 2%. Spectra were normalized using the phenylalanine absorption near 260 nm; however, due to the small amount of peptide in each sample, uncertainties in absorption amplitude cause a ~ 30% uncertainty in overall scaling.

had formed. The sample was then allowed to hydrate for at least a week before final measurements were taken. Sample alignment was monitored by observing oily streaks in the sample with a polarizing microscope as described in [15]. Additionally, the alignment of samples prepared in a similar fashion was monitored using X-ray diffraction [14]. Both methods indicate that samples prepared in this fashion form well aligned liquid-crystalline multilayers.

The method of OCD has been described [12]. For a complete analysis of peptide orientation, CD spectra are measured with light incident at several angles relative to the normal of the multilayers. For the present discussion, it is sufficient to show only the OCD at normal incidence. The spectra of magainin in DMPC/DMPG multilayers are presented in Fig. 1. At peptide concentrations from 1:200 to 1:60 (peptide to lipid molar ratio) the spectra are essentially identical and correspond to α helices oriented parallel to the membrane surface. At 1:10 the spectrum has the characteristics of α helices inserted perpendicular to the mem-

brane surface *. At 1:30 the spectrum is a superposition of the parallel and inserted states indicating that ~ 20% of the magainin is inserted in the membrane.

At first sight, spectra of helices oriented in a plane perpendicular to the light are almost the same as the well-known solution CD of helices, but in fact they are clearly distinguishable if both spectra are shown for the same peptide; their peak positions and relative amplitudes are different [12]. (The aqueous form of magainin is random coil, so vesicle CD spectra are not purely α-helical.) These features also vary somewhat with the sidechains, peptide length and solvent (based on experiments (Huang, H.W., unpublished results) with alamethicin, melittin, and a number of helical synthetic peptides: M2δ, 17; des-Aib-Leu-des-Pheol-Phe-alamethicin, 18; (LSSLLSL)₃, 19). In larger proteins the side chain effects are averaged out, but they can be quite significant in short peptides. Therefore, generally speaking, it is not possible to determine from one spectrum alone whether the helices are oriented in a plane perpendicular to the light or isotropically as in a solution. In our case the membranes are all aligned parallel to the substrate surfaces; therefore it is unlikely, if not impossible, for the helices to orient isotropically.

Thus our OCD results show that magainin associated with lipid bilayers adopts an α helical form **. In low concentrations magainin does, indeed, lie parallel to the membrane surface, as shown by solid-state NMR [10,11]. However, once the concentration exceeds a membrane-specific critical concentration (~ 1:30 for DMPC/DMPG), magainin undergoes a transition to a membrane-inserted state. A critical concentration was also found in liposome leakage and cytotoxicity experiments. Below this critical concentration magainin causes only slight leakage, but at higher concentrations magainin causes widespread lysis [7–9,22–25]. The critical concentrations found in liposome leakage experiments are also ~ 1:30 [22,23,25], providing a strong link between the insertion transition of magainin and its antibiotic activity. This is strong evidence for the hypothesis that the inserted state of magainin is responsible for disruption of the membrane structure. Furthermore, the transition between the surface state and the inserted state occurs over a small range of concentration implying that it is a cooperative phenomenon, similar to a phase transition. Indeed many

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* This is most easily seen by the absence of 208 nm band. This band is polarized parallel to the axis of the helix, therefore it disappears if the helices are oriented in the direction of the incident light [16], see below for comparison with alamethicin.

** Magainin has been observed in a primarily α-helical form (with a small random coil component) in trifluoroethanol solution [20,21] and in a suspension of negatively-charged lipid vesicles [22–24].

investigators have observed sigmoidal concentration dependence that characterizes the cooperative nature of magainin's activity [6,7,9,26].

We have found that this concentration dependent transition is not unique to magainin. Our original OCD experiments were performed with alamethicin, a 20-residue amphipathic helical peptide. Alamethicin also exhibited a concentration-dependent change in orientation [13]. OCD spectra of membrane parallel and inserted alamethicin are shown for comparison in the inset of Fig. 1. The primary features indicating a shift from the membrane parallel to the inserted state are the disappearance of the negative peak at ~ 208 nm, the red-shift of the positive peak near 195 nm, and an overall reduction in amplitude [12]. While variations in peptide length and sidechains cause slight differences in relative peak magnitudes, the primary characteristics indicitave of a change in orientation are present in both magainin and alamethicin spectra *. We have also seen this concentration-dependent behavior in a number of other synthetic and natural amphipathic helical peptides with lengths between 20 and 40 residues (Huang, H.W., unpublished material). This leads us to believe that the orientational transition could be a common mechanism for the interaction of amphipathic helices with membranes.

The concentration for this transition is highly peptide and lipid dependent. The lipid dependence of alamethicin's transition has been discussed previously [13]. For example, the critical concentration is 1:120 in diphytanoylphosphatidylcholine (PhPC) and 1:300 in dioleoylphosphatidylcholine. In the magainin-induced liposome leakage experiments, two distinct lipid-dependent effects were observed [6,22–24]. First, the initial step of peptide binding to the liposomes is much stronger for negatively charged lipids. This is expected because magainins carry a net positive charge of +4. Second, the critical concentration (the number of membrane-bound peptide per lipid molecule) for leakage also varies with lipid. All such lipid-dependent effects provide a plausible explanation for the cell-type selectivity exhibited by magainins. Without the recognition of protein targets [3,4] (peptides with D amino acids are equally active as the natural L enantiomers), magainins have to rely on membrane specificity to avoid host cell destruction. In vitro, the peptides have been shown to be active against a broad range of microorganisms including Gram-negative and Gram-positive bacteria, fungi, and protozoa at concentrations that exhibit little toxicity for peripheral blood lymphocytes [9,20,21].

It is well known that amphipathic helical peptides form discrete ion channels in black lipid membranes (or in a membrane patch). In such measurements, the peptide concentrations are low; therefore, most of the peptide molecules would be adsorbed on the surface, with occasional insertions, caused by thermal fluctuations, forming ion channels. Such single channels are unstable; their size and number fluctuate (the peptides pop in and out of the membrane). Magainin has been shown to exhibit this sort of single-channel behavior in patch-clamp experiments [27]. This is consistent with the observation that magainin at sublethal, noncytotoxic concentrations causes membrane potential shifts in target cells, apparently due to channel formation [7–9]. Once the critical concentration is exceeded, however, magainin rapidly causes lysis. In this case, the majority of peptide molecules are inserted in the membrane, causing a drastic change in the membrane permeability and consequently cytolysis.

1. References

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* The orientation of alamethicin was rigorously analyzed by OCD measured with light incident at various angles with respect to the multilayers [12].

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