Orientation of Cecropin A Helices in Phospholipid Bilayers Determined by Solid-State NMR Spectroscopy

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ABSTRACT The orientation of the insect antibiotic peptide cecropin A (CecA) in the phospholipid bilayer membrane was determined using ^{15}N solid-state NMR spectroscopy. Two peptide samples, each specifically labeled with ^{15}N at Val^{11} or Ala^{27}, were synthesized by solid phase techniques. The peptides were incorporated into phospholipid bilayers, prepared from a mixture of dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol, and oriented on glass slides. The ^{15}N chemical shift solid-state NMR spectra from these uniaxially oriented samples display a single ^{15}N chemical shift frequency for each labeled residue. Both frequencies are near the upfield end of the ^{15}N chemical shift powder pattern, as expected for an α-helix with its long axis in the plane of the membrane and the NH bonds perpendicular to the direction of the magnetic field. These results support a mechanism of action in which CecA binds to and covers the membrane surface, thereby causing a general destabilization and leakiness of the lipid bilayer membrane. The data are discussed in relation to a proposed mechanism of membrane lysis and bacterial killing via an ion channel activity of CecA.

INTRODUCTION

The cecropins (Hultmark et al., 1980) are a family of 31- to 39-residue peptides that function as antibiotics. They contribute to an essential part of the innate immunity of insects and mammals (Boman, 1995). Their primary chemical and structural features are common to other peptide antibiotics like the magainins, isolated from frog skin (Moore et al., 1991), and the defensins (Lehrer et al., 1993). All of these peptides cause the rapid lysis of bacteria in a concentration-dependent manner, display a high degree of primary sequence homology within individual families, contain highly positively charged amphipathic structures, and have a specificity for bacterial cells and acidic phospholipids. Importantly, the D-enantiomer of cecropin A (CecA), from the *Hyalophora cecropia* moth, is equally potent as the naturally occurring L-enantiomer, suggesting that the peptide mechanism of action is via self-aggregation of the peptide within the bacterial membrane, and not specific binding to chiral membrane components such as receptors or enzymes (Wade et al., 1990).

The structures of CecA (Holak et al., 1988) and of cecropin P1 (CecP1) (Sipos et al., 1992), its mammalian counterpart from pig small intestine, have been characterized in water/hexafluoropropanol (HFP) solvent mixtures by solution NMR spectroscopy. Both peptides have a long N-terminal, basic, amphipathic α-helix followed by a shorter, more hydrophobic C-terminal helix. The two helices are linked by a Gly-Pro sequence, corresponding to residues 23–24 in CecA, or by a Glu-Gly sequence corresponding to residues 20–21 in CecP1, and while the structure of CecA forms a hinge at the linker region, CecP1 is α-helical along the full length of the peptide.

Cecropin and some of its analogs, when present at the lethal concentration, bind to bacteria of different sizes in an amount equivalent to a surface monolayer (Steiner et al., 1988). This was presumed to destabilize the membrane and lead to lysis. A membrane surface arrangement was also observed for the binding of magainin (Ramamoorthy et al., 1995), and later for the binding of CecP1 to phospholipid bilayers (Gazit et al., 1996). In both cases the long axes of these helical peptides are parallel to the lipid bilayer surface. Again, the surface binding to a cell was thought to destabilize the membrane and lead to leakage of the cell contents, disruption of the electrical potential, and ultimately cell death. Other mechanisms of lipid bilayer lysis and bacterial cell killing have been suggested in which the cecropin peptide is thought to self-aggregate and form pores that span the lipid bilayer and cause electrical conductance (Christensen et al., 1988).

To further investigate the manner in which these antibacterial peptides function, we have employed ^{15}N solid-state NMR spectroscopy to determine the orientation of CecA in phospholipid bilayer membranes. Our approach, based on the orientational dependence of NMR frequencies (Cross and Opella, 1994; Opella, 1997; Marassi and Opella, 1998), can be used to establish the orientation of a peptide or protein domain within the membrane when its secondary structure has been determined independently (Kovacs and Cross, 1997; Kim et al., 1998). With additional frequency measurements, it can be used as a method to determine the complete three-dimensional structures of membrane proteins in bilayers (Ketchem et al., 1993; Opella et al., 1998).

Received for publication 13 May 1999 and in final form 24 August 1999.
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0006-3495/99/12/3152/04 $2.00

MATERIALS AND METHODS

Sample preparation

The samples consisted of synthetic ^{15}N-labeled CecA peptides incorporated into phospholipid bilayers. The solid-phase synthesis of CecA has been described (Merrifield et al., 1982). Two peptide samples with the same amino acid sequence were separately synthesized, each ^{15}N-labeled at one amide backbone site, either Val^{11} or Ala^{27}. The oriented lipid bilayer samples were prepared by cosolubilizing 10 mg of each peptide with dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) (Avanti Polar lipids, Alabaster, AL) in chloroform with trace amounts of HFP, so as to obtain final molar ratios of CecA:DMPC:DMPG equal to 2.5:80:20. Lipid bilayers prepared with this concentration of the acidic phospholipid DMPG approximate the electrostatic composition of the *Escherichia coli* membrane to which CecA binds avidly. For the oriented samples, the peptide-lipid solution was spread on the surface of 20 18 × 18 mm glass slides (#0; Erie Scientific, Portsmouth, NH). After air drying, residual solvents were removed under vacuum for 2 h, and the glass slides were stacked and placed in a sealed chamber containing a saturated solution of ammonium phosphate, which provides a 93% relative humidity atmosphere at room temperature. Oriented bilayers formed after the samples were equilibrated in this chamber at 45°C for 12 h. Before insertion in the rf coil of the NMR probe, the stacked sample was wrapped in thin layers of parafilm and teflon and sealed in a plastic film. The unoriented samples were prepared by evaporating the organic solvents and vortexing the peptide-lipid mixture in water. The resulting homogeneous bilayer dispersions were transferred to 7 × 20 mm cylindrical glass tubes (Wilmad, Buena, NY).

Solid-state NMR spectroscopy

The NMR experiments were performed on a Chemagnetics-Otsuka Electronics (Ft. Collins, CO) CMX 400 spectrometer with a wide-bore Oxford Instruments 400/89 magnet (Oxford, UK). The home-built single-coil probes were double-tuned to the resonance frequencies of ^{1}H (400.3 MHz) and ^{15}N (40.6 MHz). The stacked oriented samples were placed in a square four-turn coil with inner dimensions of 18 × 18 × 5 mm and oriented so that the bilayer planes were perpendicular to the direction of the magnetic field. The unoriented multilamellar vesicle dispersions were placed in a solenoidal coil with a diameter of 7 mm. The temperature, monitored with a thermocouple situated near the sample, was maintained at 30°C with heated, flowing nitrogen gas. The ^{15}N chemical shift spectra were obtained with single-contact 1 ms CPMOIST (cross-polarization with mismatch-optimized IS transfer), under continuous ^{1}H irradiation with an rf field strength equivalent to 1.7 mT, to decouple the ^{1}H-^{15}N dipolar interaction (Pines et al., 1973; Levitt et al., 1986). The ^{15}N chemical shifts were referenced to external liquid ammonia at 0 ppm. The NMR data were processed using the program FELIX (Biosym Technology, San Diego, CA) on a Silicon Graphics computer workstation (Mountain View, CA).

RESULTS AND DISCUSSION

Solid-state NMR spectra obtained on the specifically ^{15}N-labeled CecA peptides in phospholipid bilayers are shown in Fig. 1. The spectra in Fig. 1, *B* and *D*, are from unoriented lipid bilayer samples of CecA ^{15}N labeled at Val^{11} or Ala^{27}, respectively. Both are characteristic amide ^{15}N chemical shift powder patterns that span the range between 220 and 60 ppm. Motional averaging significantly alters solid-state NMR powder patterns; for example, mobile unstructured sites result in narrow resonance intensity centered at the isotropic frequency near 115 ppm (Opella, 1985). The absence of such intensity from both these spectra indicates that

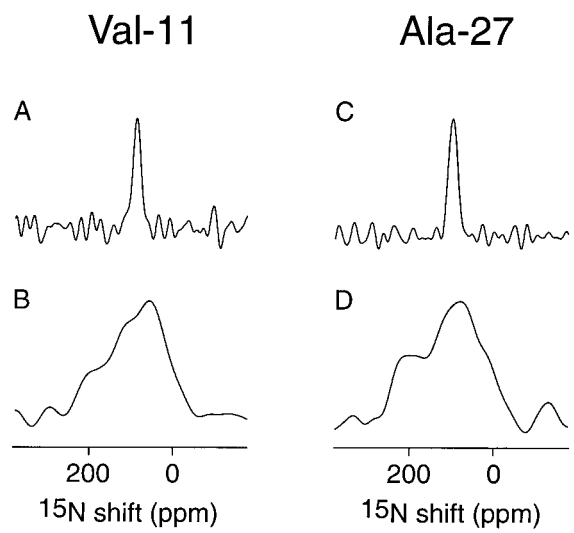

FIGURE 1 One-dimensional solid-state ^{15}N NMR spectra of specifically ^{15}N-labeled CecA in oriented (*A, C*) and unoriented (*B, D*) phospholipid bilayers. The spectra in *A* and *B* were obtained from ^{15}N-Val^{11}-labeled CecA. The spectra in *C* and *D* were obtained from ^{15}N-Ala^{27}-labeled CecA. The ^{15}N chemical shifts measured from the oriented samples and referenced to external liquid ammonia at 0 ppm were 87.6 ppm and 94.7 ppm for ^{15}N-Val^{11} and ^{15}N-Ala^{27}, respectively.

the ^{15}N-labeled residues are structured and immobile on the time scale of the ^{15}N chemical shift interaction at this field strength (10 kHz).

Rotations of the peptide about its long helix axis, which are faster than this time scale, would also alter the appearance of the powder patterns and reduce their frequency breadth. The magnitudes and orientation of the principal elements of the amide ^{15}N chemical shift tensor have been measured in model peptides (Harbison et al., 1984; Hartzell et al., 1987; Oas et al., 1987; Teng and Cross, 1989; Wu et al., 1995). The magnitudes of the principal elements (σ_{11} = 64 ppm; σ_{22} = 77 ppm; σ_{33} = 217 ppm; Wu et al., 1995) can vary by as much as 20 ppm from site to site in a polypeptide (Mai et al., 1993). On the other hand, similar values have been determined for the amide ^{15}N chemical shift tensor orientation: σ_{33} is in the peptide plane and makes an angle of 17° with the NH bond (Wu et al., 1995).

Both of the powder patterns from ^{15}N-labeled CecA span the full frequency range of the ^{15}N chemical shift tensor and show no signs of motional averaging. This confirms that CecA is structured and immobilized by virtue of its interaction with the surface of the lipid bilayer, even though it is not a transmembrane peptide. Similar results have been noted for the surface-bound peptide magainin 2 (Ramamoorthy et al., 1995; Marassi and Opella, 1998) and for a truncated polypeptide corresponding to the cytoplasmic domain of Vpu from the HIV-1 genome (Marassi et al., 1999). Indeed, not all proteins tightly associated with membranes have transmembrane helices (for a review see Biggin and Sansom, 1999).

The spectra obtained from oriented samples of CecA in lipid bilayers are markedly different from those of the

unoriented samples (Fig. 1, *A* and *C*). Each spectrum displays a single ^{15}N resonance arising from a single labeled amide nitrogen in the helical peptide, with a frequency (87.6 ppm for ^{15}N-Val^{11} and 94.6 ppm for ^{15}N-Ala^{27}) that reflects the orientation of the corresponding NH bond relative to the lipid bilayer (Opella et al., 1987). Because both ^{15}N resonances are observed in the upfield (σ_{11}, σ_{22}) region of the amide ^{15}N chemical shift powder pattern, we conclude that the NH bonds from the Val^{11} and Ala^{27} amide sites orient approximately parallel to the bilayer surface and perpendicular to the direction of the applied magnetic field.

The amino acid sequence of CecA is N-KWKLF KKIEK VGQNI RDGII KA GPA VAVVG QATQI AK-CONH2, where the Gly-Pro hinge region linking the N- and C-terminal helices is underlined. The results from the two CecA peptides, each specifically ^{15}N labeled at either Val^{11} or Ala^{27}, are sufficient to determine the respective orientations of the N- and C-terminal helices. Because the secondary structure of CecA is known to be α-helical from solution NMR spectroscopy (Holak et al., 1988), a single measurement of the ^{15}N chemical shift from each of these ^{15}N-labeled amide sites restricts the orientation of its corresponding NH bond relative to the membrane surface. In an α-helix the amide NH bonds align approximately parallel to the long helix axis. Thus the finding that the NH bonds of Val^{11} and Ala^{27} orient parallel to the bilayer plane means that the axes of both N- and C-terminal helices lie parallel to the membrane surface. This arrangement of CecA in a bilayer membrane is shown schematically in Fig. 2.

Although the initial one-dimensional solid-state NMR spectra of oriented samples provide valuable information about the architecture of helical proteins associated with membrane bilayers, an exact analysis of the orientation of CecA in the lipid bilayer is precluded by the lack of additional NMR frequency data. Complete structure determination requires the resolution and assignment of all resonances of uniformly labeled samples, with multidimensional solid-state NMR experiments (Marassi et al., 1997, 1999c; Opella et al., 1998; Tan et al., 1999).

The proposed sequence of events for the action of CecA (Christensen et al., 1988) and for melittin (Juvvadi et al., 1996) in lipid bilayers was the following: 1) the peptide is electrostatically bound to the negatively charged phospholipid bilayer; 2) the helical amphipathic peptide is oriented such that the hydrophobic groups are put in contact with the lipid hydrocarbon chains; 3) under an applied voltage gradient the helical peptides aggregate and form a pore that spans the bilayer. Pore formation accounts for the observed electrical conductivity. These solid-state NMR data make it clear that that the bulk of the peptide is oriented with its helices parallel to the membrane surface, and this is assumed to be the case for the bacterial membrane. The important observation that the membrane potential of a bacterium could be reduced to ~50% by treatment with an antibacterial peptide and would stabilize without complete disruption of the cell membrane or cell death was interpreted to arise from the closing of channels when the membrane potential was below 100 mV (Cociancich et al., 1993). The permeability changes were proposed to reflect the formation of channels in the cytoplasmic membrane.

Whatever the molecular mechanism for membrane lysis, bilayer conductivity, and bacterial killing turns out to be, it must incorporate all of the data obtained from various methods on different systems. Based on the present NMR data and the earlier body of work discussed in this paper, we propose that the CecA peptide binds electrostatically to the surface of lipid membranes in a parallel orientation as shown in Fig. 2. The parallel peptide would then be in equilibrium with a small fraction that self-aggregates and inserts across the lipid bilayer to form pores when the peptide concentration is near the monolayer level. Such an equilibrium would lie far to the side of the parallel surface orientation, and the small amount of perpendicular helices would be undetectable by solid-state NMR or fluorescence measurements. This mechanism also accounts for the specificity observed for different peptides that bind to a similar extent but have very different lethal concentrations.

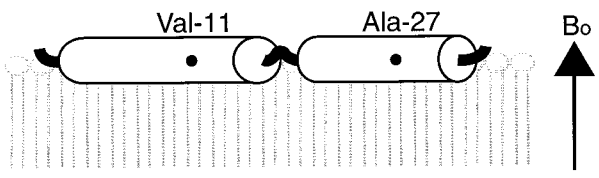

FIGURE 2 (*A*) In-plane orientations of the two helical segments of CecA in lipid bilayers determined by the solid-state ^{15}N chemical shift NMR data in Fig. 1.

This research was supported by grants from the National Institutes of Health (GM29754 to SJO) and from the U.S. Public Health Service (AM 01260 to RBM). It utilized the Resource for Solid-State NMR of Proteins at the University of Pennsylvania, supported by grant P41RR09731 from the Biomedical Research Technology Program, National Center for Research Resources, National Institutes of Health. FMM was supported by postdoctoral fellowships from the Natural Sciences and Engineering Research Council of Canada (153304-1994) and the Medical Research Council of Canada (9304FEN-1004-43344).

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