Interaction of the Mammalian Antibacterial Peptide Cecropin P1 with Phospholipid Vesicles†

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ABSTRACT: Cecropins are positively charged antibacterial polypeptides that were originally isolated from insects. Later on a mammalian homologue, cecropin P1 (CecP), was isolated from pig intestines. While insect cecropins are highly potent against both Gram-negative and Gram-positive bacteria, CecP is as active as insect cecropins against Gram-negative but has reduced activity against Gram-positive bacteria. To gain insight into the mechanism of action of CecP and the molecular basis of its antibacterial specificity, the peptide and its proline incorporated analogue (at the conserved position found in insect cecropins), P^{22}-CecP, were synthesized and labeled on their N-terminal amino-acids with fluorescent probes, without significantly affecting their antibacterial activities. Fluorescence studies indicated that the N-terminal of CecP is located on the surface of phospholipid membranes. Binding experiments revealed that CecP binds acidic phosphatidylserine/phosphatidylcholine (PS/PC) vesicles better than zwitterionic PC vesicles, which correlates with its ability to permeate the former better than the latter. The shape of the binding isotherms suggest that CecP, like insect cecropin, binds phospholipids in a simple, noncooperative manner. However, resonance energy transfer (RET) measurements revealed that, unlike insect cecropins, CecP does not aggregate in the membrane even at relatively high peptide to lipid ratios. The stoichiometry of CecP binding to vesicles suggests that amount of CecP sufficient to form a monolayer causes vesicle permeation. In spite of the incorporation of the conserved proline in P^{22}-CecP, the analogue has reduced antibacterial activity, which correlates with its reduced α-helical structure and its lower partitioning and membrane permeating activity with phospholipid vesicles. Taken together, our results support a mechanism in which CecP disrupts the structure of the bacterial membrane by (i) binding of peptide monomers to the acidic surface of the bacterial membrane and (ii) disintegrating the bacterial membrane by disrupting the lipid packing in the bilayers. These results, combined with data reported for other antibacterial polypeptides, suggest that the organization of peptide monomers within phospholipid membranes contributes to Gram-positive/Gram-negative antibacterial specificity.

The cecropins are a family of 31−39 amino acid residues, strongly basic polypeptides with very potent antibacterial activity, that were originally isolated from immune hemolymph of the *Hyalophora cecropia* moth (Hultmark et al., 1980; Steiner et al., 1981) and subsequently from a wide range of other bacteria injected Lepidopteran and Dipterian insects [for review, see Boman et al. (1991) and Hultmark (1993)]. Later, an antibacterial peptide, with 33% homology to insect cecropins, was isolated from porcine small intestines (Lee et al., 1989). This 31 amino acid peptide, designated as Cecropin P1 (CecP),[1] is as potent as insect cecropins in the inhibition of growth of Gram-negative bacteria. However, it has reduced activity against Gram-positive bacteria. The isolation of CecP from a mammalian source and the isolation of cecropin from insect cuticular matrix (Brey et al., 1993) suggest that cecropins are widely distributed in the animal kingdom and are active in various tissues, serving as mediators of innate immunity.

The structure of CecP in solution, as was determined by 2D NMR, is an elongated amphiphilic α-helix over nearly the whole length of the molecule (Sipos et al., 1992). This structure is remarkably different from the helix−hinge−helix structure of insect cecropins (Holak et al., 1988; Iwai et al., 1993). The hinge in the structure of insect cecropin is due to a conserved proline residue in this family of polypeptides (see Table 1). A similar hinge is common to several other membrane permeating toxins such as melittin, alamethicin, and pardaxin. Proline has been shown to have an important role in the antibacterial activity of insect cecropins (Fink et al., 1989). However, that the same proline is absent in CecP implies that the hinge is not essential for the antibacterial activity of CecP, at least not for Gram-negative bacteria

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[1] Abbreviations: BOC, butyloxycarbonyl; CecP, Cecropin P1; DCC, dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; DMSO, dimethyl sulfoxide; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HOBT, 1-hydroxybenzotriazole; HF, hydrogen fluoride; NBD-F, 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole; Rho, tetramethylrhodamine; NMR, nuclear magnetic resonance; Pam, (phenylacetamido)methyl; PC, egg phosphatidylcholine; PS, phosphatidylserine; RP-HPLC, reverse-phase high-performance liquid chromatography; SUV, small unilamellar vesicles; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol.

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Table 1: Sequence Alignment of the Porcine and Some of the Insect Cecropins[a]

		1 10 20 30
CecA	Hyalophora	KW--KLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK-NH_2
CecB	Hyalophora	KW--KIFKKIEKVGRNIRNGIIKAGPAIEVLGEAKAL-NH_2
CecB	Bombyx mori	RW--KIFKKIEKMGRNIRDGIVKAGPAVAVLGSAKAI-NH_2
CecD	Hyalophora	W--NPFKELERAGQRVRDAIISAGPAVATVAQATALAK-NH_2
CecIA	Sacrophaga	GWLKKIGKKIERVGQHTRDATI-QGLGIAQQAANVAATAR-NH_2
CecP1	Porcine	SWLSKTAKKLENSAK-KR---ISEGIAIAIQGGPR-OH

[a] Underlined amino acids are identical in all or most cecropins.

(Sipos et al., 1992). It was argued that the Ser-Glu-Gly amino acids of CecP may serve as a potential flexible region of the peptide. However, when the NMR constraints are used to construct possible three-dimensional structures, there is no structural flexibility near this region as in insect cecropins (Sipos et al., 1992). Another difference between insect cecropins and CecP is that the former have amidated C-termini, which are important for their activities, while CecP has a carboxyl C-terminus. Amidation of the carboxy terminus of CecP did not have a critical effect on its antibacterial activity (Lee et al., 1989).

The destruction of the energy gradient across the membrane has been suggested to be the mode of action of insect cecropins (Steiner et al., 1981, 1988; Okada & Natori, 1985; Gazit et al., 1994; Mchaourab et al., 1994). This mechanism has also been proposed for several other amphipathic α-helical antibacterial peptides such as magainin (Westerhoff et al., 1989) and dermaseptins (Mor et al., 1991; Pouny et al., 1992; Strahilevitz et al., 1994). A common feature of all of these peptides is that they are highly positively charged, and they interact preferentially with acidic phospholipid membranes. Recent studies aimed at elucidating the mechanism by which insect cecropins permeate phospholipid membranes suggested a process whereby peptide monomers form a layer on the surface of the membrane, leading to the disruption of the bilayer packing. However, some extent of aggregation of insect cecropins within phospholipid membranes did occur at relatively high peptide to lipid ratios (Mchaourab et al., 1994; Gazit et al., 1994). This limited degree of aggregation can explain the formation of ion channels by insect cecropins in planar lipid bilayers (Christensen et al., 1988) that have been modeled as a bundle of transmembrane α-helices (Durell et al., 1992).

The lethal action (lysis) of CecP on *Escherichia coli* was found to be too fast to permit an analysis of its mechanism of action (Boman et al., 1993). Here we have used spectroscopic methods to determine the mode of interaction of CecP with phospholipid membranes. Furthermore, an analogue containing a proline at a similar position as that in insect cecropins (P^{22}-CecP) was also synthesized and investigated. The peptides were labeled selectively on their N-terminal amino acids with either NBD (7-nitrobenz-2-oxa-1,3-diazole-4-yl), to facilitate binding experiments and to serve as an energy transfer donor, or with rhodamine, to serve as energy transfer acceptor. Binding experiments revealed that both peptides bind phospholipid membranes in a simple adhesive process and with higher partition coefficients with acidic than with zwitterionic membranes. However, resonance energy transfer experiments between donor-/acceptor-labeled CecP revealed that, unlike cecropin isolated from the silk worm *Bombyx mori* (Bm-CecB) and dermaseptin, which aggregate in membranes at relatively high peptide to lipid ratios (Pouny et al., 1992; Gazit et al., 1994), CecP does not aggregate. The introduction of proline reduced both the membrane permeating activity and the antibacterial activity of the resulting P^{22}-CecP, which may be explained by the reduction of both its α-helical content and partition coefficients with vesicles. The results, combined with previous data reported for other antibacterial peptides, are discussed in line of the correlation between the organization within membranes of antibacterial peptides and Gram-positive/Gram-negative antibacterial specificity.

EXPERIMENTAL PROCEDURES

Materials. t-Boc-Arg(Tos)-OCH_2 PAM resin was purchased from Applied Biosystems (Foster City, CA), and butyloxycarbonyl (Boc) amino acids were obtained from Peninsula Laboratories (Belmont, CA). Other reagents used for peptide synthesis included trifluoroacetic acid (TFA, Sigma), *N,N*-diisopropylethylamine (DIEA, Aldrich, distilled over ninhydrin), dicyclohexylcarbodiimide (DCC, Fluka), 1-hydroxybenzotriazole (HOBT, Pierce), and dimethylformamide (peptide synthesis grade, Biolab). Egg phosphatidylcholine (PC) and phosphatidylserine (PS) from bovine spinal cord (sodium salt-grade I), were purchased from Lipid Products (South Nutfield, U.K.). Cholesterol (extra pure) was supplied by Merck (Darmstadt, Germany) and recrystallized twice from ethanol. 3,3′-Diethylthiodicarbocyanine iodide [diS-C_2-5] and 5-(and-6)-tetramethylrhodamine succinimidyl ester were obtained from Molecular Probes (Eugene, OR). 4-Fluoro-7-nitrobenz-2-oxa-1,3-diazole (NBD-F) was obtained from Sigma. All other reagents were of analytical grade. Buffers were prepared in double glass-distilled water.

Peptide Synthesis, Fluorescent Labeling, and Purification. The CecP peptides were synthesized by a solid-phase method on t-Boc-Arg(Tos)-OCH_2 PAM resin (Merrifield et al., 1982). Labeling of the N-terminus of the CecP peptides with fluorescent probes was achieved by labeling the resin-bound peptides as previously described (Rapaport & Shai, 1991). Briefly, 10−30 mg of resin-bound peptides was treated with TFA [50% (v/v) in methylene chloride], in order to remove

the BOC protecting group from the N-terminal amino groups of the linked peptides. The resin-bound peptides were then reacted with either (i) carboxy-tetramethylrhodamine succinimidyl ester (3−4 equivv) in dry dimethylformamide containing 5% (v/v) diisopropylethylamine or (ii) NBD-F in dry dimethylformamide. The resin-bound peptides were cleaved from the resins by hydrogen fluoride (HF) and finally extracted with dry ether after HF evaporation. The synthesized peptides were purified by RP-HPLC on a C_4 reverse-phase Vydac semipreoperative column. The column was eluted in 40 min, using a linear gradient of 15−80% acetonitrile in water both containing 0.1% TFA (v/v), at a flow rate of 1.8 mL/min. The purified peptides were shown to be homogeneous (~99%) by analytical HPLC. The peptides were subjected to amino acid analysis.

Antibacterial Activity of CecP and Analogues. The antibacterial activity of synthetic CecP peptides and fluorescently labeled derivatives was assessed using an inhibition zone assay on thin agarose plates seeded with bacteria (Hultmark et al., 1982). *E. coli* D21, *Bacillus megaterium* Bm11, *Acinetobacter calcoaceticus* Ac11, and *Pseudomonas aeruginosa* OT97 were used as test bacteria. Wells were loaded with 3 μL of serial dilutions of a known amount of the synthetic CecP, Rho-CecP, NBD-CecP, or P^{22}-CecP. After overnight incubation at 30 °C, zone diameters were read with the aid of a magnifying glass. Lethal concentrations (LC values) were calculated for each bacteria according to the formula derived by Hultmark et al. (1982).

Preparation of Liposomes. Small unilamellar vesicles (SUV) were prepared by sonication of PC or PC/PS (1:1 w/w) as has been described previously in detail (Shai et al., 1990, 1991). The lipid concentrations of the resulting preparations were determined by phosphorus analysis (Bartlett, 1959). Vesicles were visualized using a JEOL JEM 100B electron microscope (Japan Electron Optics Laboratory Co., Tokyo, Japan) as follows. A drop of vesicles was deposited on a carbon-coated grid and negatively stained with uranyl acetate. Examination of the grids demonstrated that the vesicles were unilamellar with an average diameter of 20−50 nm (Papahadjopoulos & Miller, 1967).

CD Spectroscopy. CD spectroscopy was used to determine the secondary structure of the peptides (Greenfield & Fasman, 1969). The CD spectra of the peptides were measured with a Jasco J-500A spectropolarimeter. The spectra were scanned at room temperature in a capped, quartz optical cell with a 0.5 mm path length. Spectra were obtained at wavelengths of 250−200 nm. Seven scans were taken at a scan rate of 20 nm/min for each peptide. The peptides were scanned at concentrations of 2.0×10^{-5} M in 40% TFE and in buffer (50 mM Na_2SO_4, 25 mM HEPES-sulfate, pH 6.8).

NBD Fluorescence Measurements and Binding Experiments. NBD-labeled peptides (0.2 nmol) were added to 2 mL of buffer (50 mM Na_2SO_4, 25 mM HEPES-SO_4^{2-}, pH 6.8) containing 800 nmol of PC or PS/PC SUV, to establish a lipid/peptide ratio (1:4000) in which all the peptide was bound to lipids. After a 2 min incubation, the emission spectrum of the NBD group was recorded (in three separate experiments) using a Perkin-Elmer LS-50B spectrofluorometer, with the excitation set at 470 nm (10 nm slit). Binding experiments were conducted as previously described in detail (Rapaport & Shai, 1991). We found that the manner in which the lipid component was added, i.e., either in a single dose or in several sequential doses, had no effect on the observed signal magnitude. The concentration of peptides was low enough not to significantly disrupt the bilayer structure.

The binding isotherms were analyzed as a partition equilibrium (Schwarz et al., 1986, 1987; Rizzo et al., 1987; Beschiaschvili & Seelig, 1990; Rapaport & Shai, 1991), using the following formula:

$$X_b = K_p C_f$$

where X_b is defined as the molar ratio of bound peptide per total lipid, K_p corresponds to the partition coefficient, and C_f represents the equilibrium concentration of free peptide in the solution. To calculate X_b, F_∞ (the fluorescence signal obtained when all the peptide is bound to lipid) was extrapolated from a double-reciprocal plot of F (total peptide fluorescence) versus C_L (total concentration of lipids) (Schwarz et al., 1986). Knowing the fluorescence intensities of unbound peptide, F_0, as well as bound peptide, F, the fraction of membrane bound peptide, f_b, could be calculated using the formula

$$f_b = (F - F_0)/(F_\infty - F_0)$$

Having calculated the value of f_b, it is then possible to calculate C_f, as well as the extent of peptide binding, X_b. In practice, it was assumed that the peptides were initially partitioned only over the outer leaflet of the SUV (60% of the total lipid), as had been previously suggested (Beschiaschvili & Seelig, 1990). Therefore, values of X_b were corrected as such:

$$X_b^* = X_b/0.6$$

The curve resulting from plotting X_b^* versus free peptide, C_f, is referred to as the conventional binding isotherm. Surface partition coefficients were calculated from the initial slopes of the conventional binding isotherms. Our intention was to determine the binding isotherm of CecP and P-CecP with phospholipid vesicles with different composition (PC vs PS/PC) and to compare them with other amphipathic α-helical peptides that were determined under the same experimental conditions (Frey & Tamm, 1990; Rapaport & Shai, 1991; Pouny et al., 1992; Strahilevitz et al., 1994; Gazit et al., 1994, 1993a,b). We therefore should stress that the calculated values of K_p are valid for the particular conditions applied.

Resonance Energy Transfer (RET) Measurements. Fluorescence spectra were obtained at room temperature in a Perkin-Elmer LS-50B spectrofluorometer, with the excitation monochromator set at 460 nm, to minimize the excitation of tetramethylrhodamine, with a 5-nm slit width. Measurements were performed in a 1 cm path length glass cuvette and a final reaction volume of 2 mL. In a typical experiment, donor- (NBD-labeled) peptide at a final concentration of 0.1 μM was added to a dispersion of PS/PC SUV (100 μM) in buffer (50 mM Na_2SO_4, 25 mM HEPES-SO_4^{2-}, pH 6.8), followed by the addition of acceptor- (Rho-labeled) peptide in several sequential doses. Fluorescence spectra were obtained before and after the addition of the acceptor peptide. Changes in the fluorescence intensity of the donor due to processes other than energy transfer to the acceptor were determined by substituting unlabeled peptide for the acceptor

Table 2: Sequences and Designations of the Peptides Investigated

no.	designation	sequence
1	Cec-P (**X**=H)	**X**-HN- S W L S K T A K K L E N S A K K R I S E G I S I A I Q G G P R-COOH
2	NBD-Cec-P (**X**=NBD)	
3	Rho-Cec-P (**X**=Rho)	
4	P^{22}-Cec-P (**X**=H)	**X**-HN- S W L S K T A K K L E N S A K K R I S E G **P** S I A I Q G G P R-COOH
5	NBD-P^{22}-Cec-P (**X**=NBD)	

and by measuring the emission spectrum of the acceptor alone in the presence of vesicles.

The efficiency of energy transfer (E) was determined by measuring the decrease in the quantum yield of the donor as a result of the addition of acceptor. E was determined experimentally from the ratio of the fluorescence intensities of the donor in the presence (I_{da}) and in the absence (I_d) of the acceptor at the donor's emission wavelength, after correcting for membrane light scattering and the contribution of acceptor emission. The percentage of transfer efficiency (E), is defined as

$$E = (1 - I_{da}/I_d) \times 100$$

The correction for light scattering was made by subtracting the signal obtained when unlabeled analogues were added to vesicles containing the donor molecule. Correction for the contribution of acceptor emission was made by subtracting the signal produced by the acceptor-labeled analogue alone.

Membrane Permeation Induced by the Peptides. Membrane permeation was assessed utilizing the diffusion potential assay (Loew et al., 1983, Sims et al., 1974) as previously described in detail for the pore-forming neurotoxin pardaxin (Shai et al., 1990, 1991) and the antibacterial dermaseptin (Pouny et al., 1992).

Calculation of Hydrophobic Moment. Hydrophobic moments $\langle \mu H \rangle$ (Eisenberg et al., 1982) were calculated as a function of the degree of rotation and sequence location using the MOMENT program (GCG) (the Genetic Computer Group, Inc.) running on a Silicon Graphics work station.

RESULTS

To study the molecular mechanism by which the mammalian CecP disrupt the structure of phospholipid membranes, and to gain insight into factors which might affect antibacterial specificity, synthetic peptides with sequences corresponding to those of CecP and its proline-containing analogue were synthesized and fluorescently labeled, and their interaction and organization within phospholipid membranes were investigated. The peptides and their designations are listed in Table 2.

CD Spectroscopy. The extent of α-helical secondary structures in CecP and P^{22}-CecP was estimated from their CD spectra in 40% TFE (Figure 1). Interestingly, CecP exhibited a mean residual ellipticity $[\Theta]_{222}$ of −44 520 deg·cm²/dmol, a value that is higher than that suggested for a value of 100% fractional helicity (Wu et al., 1981). Nevertheless, this high value is in agreement with the NMR data (Sipos et al., 1992) which revealed 100% α-helical structure for CecP. The introduction of proline (P^{22}-CecP)

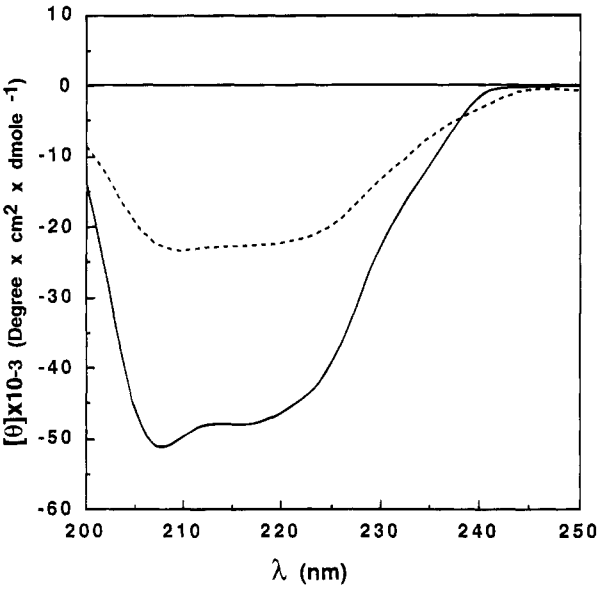

FIGURE 1: CD spectra of CecP peptides. Spectra were taken in 40% TFE as described in Experimental Procedures. Continuous line, CecP; dashed line, P^{22}-CecP.

resulted in a more than 50% decrease in the mean residual ellipticity $[\Theta]_{222}$ (−21 600 deg·cm²/dmol), corresponding to 65% α-helical structure [if 30 000 is taken as 100% (Wu et al., 1981)] or 44% α-helical structure (if 44 520 is taken as 100%). This value is slightly smaller than those obtained for CecA and Bm-CecB insect cecropins which revealed 70−80% α-helical structure (Steiner, 1982; Andreu et al., 1985; Holak et al., 1988; Gazit et al., 1994). This is probably due to the elongated helical structure of CecP compared to the helix−bend−helix of insect cecropin. This is in line of what has been shown recently that a bend along an α-helix causes a significant reduction in its mean residual ellipticity $[\Theta]_{222}$ (Hirst & Brooks, 1994).

Antibacterial Activity of CecP and Its Analogues. The antibacterial activities of CecP, its proline analogue, and the fluorescently labeled derivatives were assessed utilizing inhibition zone assays. Both Gram-negative and Gram-positive bacteria were used. The results are summarized in Table 3. The data reveal that the antibacterial activity of CecP is similar to what has been reported previously (Lee et al., 1989). It is also evident that the fluorescent modification did not significantly alter the antibacterial activity of CecP. Furthermore, P^{22}-CecP has significantly reduced antibacterial activity as compared to CecP, although proline was introduced in a position that is conserved in insect cecropins that are highly potent antibacterial polypeptides.

Localization of the Environment of the NBD Moiety. Due to the sensitivity of the fluorescence of NBD to the polarity

Table 3: Lethal Concentrations (μM) of CecP and Its Fluoroscentelly Labeled Derivatives

bacterium	CecP	NBD-CecP	Rho-CecP	P^{22}-CecP
E. coli D21	0.30	0.32	0.73	7.9
B. megaterium Bm11	4.7	4.9	3.6	11.3
A. calcoaceticus Ac11	0.42	0.57	0.99	21.6
P. aeruginosa OT97	16.9	11.2	15.9	

of its environment, it has been used for polarity and binding studies (Kenner & Aboderin, 1971; Frey & Tamm, 1990; Rapaport & Shai, 1991; Gazit & Shai, 1993a,b). To monitor the environment of the N-termini of the peptides, to which the NBD moiety was attached, the fluorescence emission spectra of NBD-labeled peptides and NBD-aminoethanol (a control) were monitored in aqueous solutions and in the presence of vesicles composed of either PC or PC/PS at pH 6.8. In these fluorometric studies, SUVs were used to minimize differential light scattering effects (Mao & Wallace, 1984), and the lipid/peptide molar ratio was elevated (>3000: 1) so that the spectral contributions of free peptide would be negligible. In buffer, NBD-CecP and NBD-P^{22}-CecP exhibited a maximum of fluorescence emission at 547 ± 1 nm (data not shown), which reflects a hydrophilic environment for the NBD moiety (Rajarathnam et al., 1989). However, when vesicles were added to the aqueous solutions containing NBD-CecP or NBD-P^{22}-CecP, a blue-shift in the emission maximum (toward 533 ± 1 nm) and an increase in the fluorescence intensity of the NBD group were observed for both peptides in the presence of PC/PS. However, in the presence of PC vesicles a similar blue-shift and increase in the fluorescence of NBD occurred only with NBD-CecP (data not shown). The change in the spectrum of the NBD group reflects its relocation to a more hydrophobic environment (Chattopadhyay & London, 1987). This blue-shift is similar to that observed for an NBD group located at or near the surface of the membrane (emission maximum of ~530 nm) (Chattopadhyay & London, 1987; Rajarathnam et al., 1989; Pouny et al., 1992) and is slightly higher than obtained for Bm-CecB [529 ± 1 nm (Gazit et al., 1994)]. No shift was observed with the control, NBD-aminoethanol.

Characterization of Binding Isotherms and Determination of Partition Constants. The enhancement of the NBD fluorescence upon binding to phospholipid membranes was utilized for the generation of binding isotherms for CecP peptides, from which partition coefficients could be calculated, as previously described (Rapaport & Shai, 1991). First, a fixed concentration (0.2 μM) of NBD-labeled peptide was titrated with the desired vesicles (PC or PS/PC with NBD-CecP and PS/PC with NBD-P^{22}-CecP). Plotting of the resulting increases in the fluorescence intensities of NBD-labeled peptides as a function of lipid/peptide molar ratios yielded conventional binding curves (Figure 2A). The curves obtained by plotting X_b* (the molar ratio of bound peptide per 60% of the total lipid) versus C_f (the equilibrium concentration of free peptide in the solution) are referred to as the conventional binding isotherms. The experimental binding isotherms of the interaction of NBD-CecP with PS/PC or with PC SUV and of NBD-P^{22}-CecP with PS/PC SUV were determined (Figure 2B). The surface partition coefficients were estimated by extrapolating the initial slopes of the curves to C_f values of zero. The estimated surface partition coefficients, K_p*, of NBD-CecP are 3.1 ± 0.6 × 10^4 M^{-1} and 1.2 ± 0.3 × 10^5 M^{-1} with PC and PC/PS

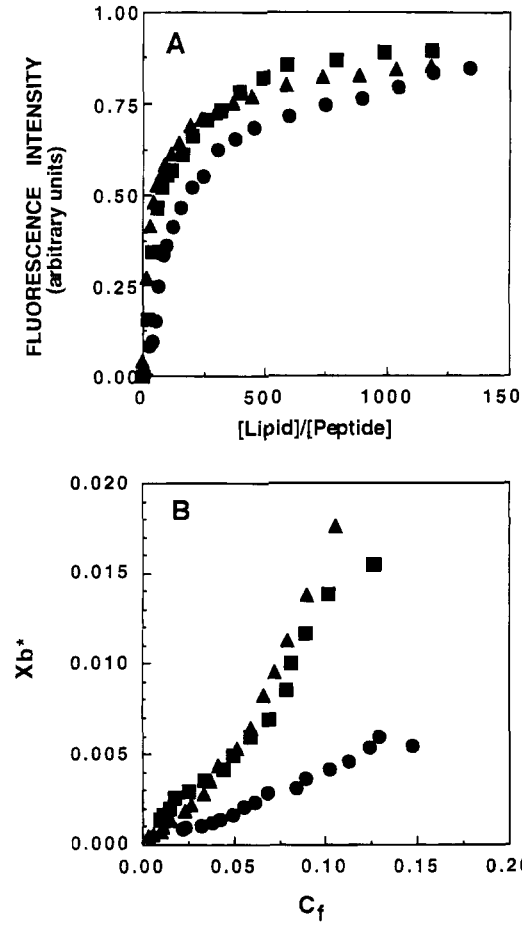

FIGURE 2: Increase in the fluorescence of NBD-CecP upon titration with SUV (A) and the resulting binding isotherm (B). (A) NBD-labeled peptide (0.2 μM) in 50 mM Na_2SO_4 and 25 mM HEPES-SO_4^{2-} was titrated with SUV vesicles at room temperature; the excitation wavelength was 467 nm, and emission was monitored at 530 nm. (B) Binding isotherm derived from panel A by plotting X_b* (molar ratio of bound peptide per 60% of lipid) versus C_f (equilibrium concentration of free peptide in the solution). Designations: squares, NBD-CecP with PS/PC; circles, NBD-CecP with PC; triangles, NBD-P^{22}-CecP with PS/PC.

vesicles, and that of NBD-P^{22}-CecP is 7.2 ± 0.8 × 10^4 M^{-1} (three measurements each). Higher values for K_p* with PS/PC as compared to PC vesicles were observed also with dermaseptins (Pouny et al., 1992; Strahilevitz et al., 1994) and Bm-CecB (Gazit et al., 1994). This difference probably results from electrostatic interactions between the acidic head group of PS and the positive charges of CecP, thus suggesting that electrostatic forces play important role in the interaction of CecP with phospholipid membranes. Similar differences in K_p* values obtained with acidic as compared to zwitterionic phospholipids were not observed with polypeptides assumed to form organized pores within the membrane such as the α5 helix of δ-endotoxin (Gazit & Shai, 1993a) and helix 2 of *Bacillus thuringiensis* var. *israelensis* cytolytic toxin (Gazit & Shai, 1993b) or the highly positively charged S4 segment of Na^+ channel (Rapaport et al., 1992).

Whether a particular polypeptide can form aggregates in its membrane bound state can be elucidated from the shape of its binding isotherm (Schwarz et al., 1986, 1987). The binding isotherms of both NBD-CecP and NBD-P^{22}-NBD in either PC/PS or PC vesicles are practically straight lines, which indicate simple adhesion processes. Similar binding isotherms were obtained also with the antimicrobial peptides

dermaseptins (Pouny et al., 1992; Strahilevitz et al., 1994) and Bm-CecB (Gazit et al., 1994). These binding isotherms are different from those obtained with several pore-forming polypeptides such as the antimicrobial peptide alamethicin (Rizzo et al., 1987), the neurotoxin pardaxin and its analogues (Rapaport & Shai, 1991), the α-5 segment of *B. thuringiensis* CryIIIA δ-endotoxin (Gazit & Shai, 1993a), and helix-2 of *B. thuringiensis* var. *israelensis* cytolytic toxin (Gazit & Shai, 1993b). The isotherms of the latter pore forming peptides display an initial "lag", i.e., initially the curves are flat, but then their slopes rise sharply (about 100-fold) once a threshold concentration is achieved. This is consistent with a process whereby peptides first incorporate into the membrane and, once inside the membrane, then aggregate to form a pore (Schwarz et al., 1986, 1987). There is some difference between the binding isotherms of Bm-CecB and CecP. The former reveals a slight cooperativity at elevated peptide/lipid molar ratios (Gazit et al., 1994).

Resonance Energy Transfer (RET) Experiments. We further studied the organizational state of CecP within the more sensitive acidic phospholipid membranes using RET experiments. In these experiments NBD-labeled peptides were used as energy donors and Rho-labeled peptides as energy acceptors as described previously (Rapaport & Shai, 1992; Gazit & Shai, 1993b). Addition of Rho-labeled CecP (final concentrations of 0.02−0.5 μM) to NBD-labeled peptides (0.05 μM) in the presence of PS/PC phospholipid vesicles (100 μM) quenched the donor's emission and increased the acceptor's emission, which is consistent with energy transfer. In control experiments, no change in the emission spectrum of NBD was observed when equal amounts of unlabeled peptides rather than Rho-labeled peptides were added (data not shown). The resulting curve of the experimentally derived percentage of energy transfer versus the various molar ratios of "bound-acceptor (C_b)" to lipid are depicted in Figure 3. The amounts of lipid-bound acceptors (Rho-CecP), C_b, at the various Rho-CecP concentrations were calculated from the binding isotherm, as previously described (Pouny et al., 1992). First, the fractions of bound acceptor, f_b, were calculated for the various acceptor to lipid molar ratios tested. Next it was possible to calculate the fraction bound, C_b (C_b = [μM acceptor] × f_b). A curve corresponding to a random distribution of monomers assuming R_0 of 51.1 Å, as calculated for the NBD/Rho pair (Gazit & Shai, 1993b), is also depicted.

The results obtained in the RET experiments reveal that CecP monomers are randomly distributed in their membrane bound state. A similar organizational state was observed with Bm-CecB and dermaseptins S and b (Pouny et al., 1992; Strahilevitz et al., 1994). However, while some aggregation occurred with Bm-CecB at a high peptide to lipid molar ratio (Mchaourab et al., 1994; Gazit et al., 1994), no such aggregation was observed with CecP under similar conditions.

Membrane Permeability Induced by the Peptides. CecP, P^{22}-CecP and their fluorescent derivatives were examined for their efficacy in perturbing the lipid packing and causing leakage of vesicular contents, by utilizing the dissipation of diffusion potential assay. Increasing concentrations of CecP or its analogues were mixed either with PC or with PS/PC SUV that had been pretreated with the fluorescent, potential-sensitive dye (diS-C_2-5) and valinomycin. Recovery of fluorescence was monitored as a function of time and usually occurred within 10−20 min. Maximal activity of CecP and its analogues was plotted versus peptide to lipid molar ratios. A high perturbing activity was observed with CecP and its fluorescent derivatives with PS/PC vesicles (upper curve in Figure 4), in contrast to a low level observed with PC vesicles (lower curve in Figure 4), which correlates with the lower partition coefficient of the peptide with PC as compared to PS/PC vesicles. A similar preference in permeation of PS/PC as compared to PC vesicles was obtained with the following antibacterial polypeptides: insect cecropins (Mchaourab et al., 1994; Gazit et al., 1994), magainin (Matsuzaki et al., 1991), and dermaseptin (Pouny et al., 1992; Strahilevitz et al., 1994). All the fluorescently labeled analogues exhibited the same activity as their parent molecules. It should be noted that although P^{22}-CecP has a partition coefficient with PS/PC very close to that calculated for CecP ($7.2 \pm 0.8 \times 10^4$ M^{-1} and $1.2 \pm 0.3 \times 10^5$ M^{-1} respectively), P^{22}-CecP has only 3% activity under the same peptide to lipid molar ratio in which CecP has 100% activity. P^{22}CecP was not active with PC vesicles (Figure 4), which is in agreement with its inability to bind PC vesicles (see section on the binding experiments).

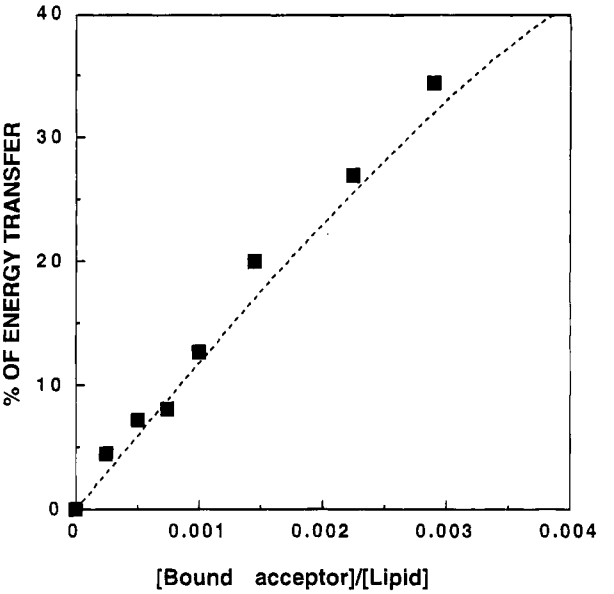

FIGURE 3: Theoretically and experimentally derived percentages of energy transfer versus bound-acceptor/lipid molar ratio, using PS/PC vesicles. The spectrum of NBD-CecP (0.05 μM), the donor peptide, was determined in the presence or absence of various concentrations of the acceptor peptide, Rho-CecP. Each spectrum was recorded in the presence of PS/PC vesicles (100 μM) in 50 mM Na_2SO_4 and 25 mM HEPES-SO_4^{2-}, at pH 6.8. The excitation wavelength was set at 470 nm; emission was scanned from 500 to 600 nm. The amount of lipid-bound acceptor (Rho-peptides), C_b, at various acceptor concentrations was calculated from the binding isotherms as described in Experimental Procedures. Designations are as follows: Filled squares, NBD-CecP/Rho-CecP; dashed line, random distribution of the monomers (Fung & Stryer, 1978), assuming an R_0 of 51.1 Å (Gazit & Shai, 1993b).

Hydrophobic Moments of the Peptides. The Moment program was utilized to determine the hydrophobic moments of Bm-CecB and CecP in various areas and various degrees of rotation of the amino acids of the peptide (Eisenberg et al., 1982) (Figure 5B,C). The figure demonstrates that both Bm-CecB and CecP have hydrophobic moments with maxima at 90−110° (100° is characteristic of an ideal α-helical structure taking 3.6 residues per turn). However, the hydrophobic moment $\langle \mu H \rangle$ of Bm-CecB in its maximal

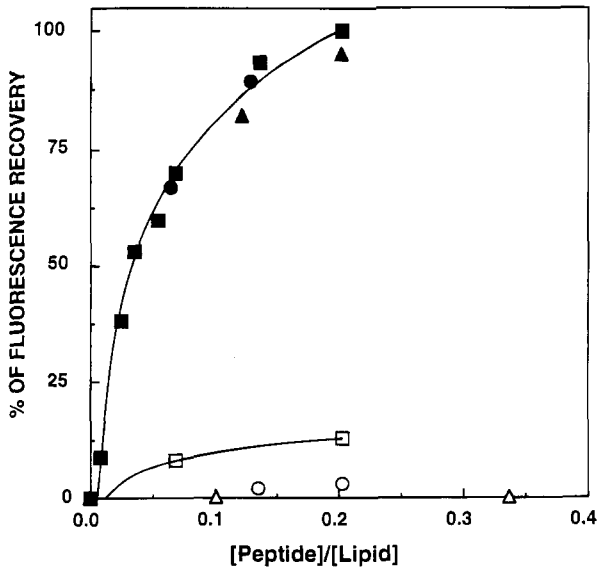

FIGURE 4: Maximal dissipation of the diffusion potential in vesicles induced by the peptides. The peptides were added to isotonic K^+ free buffer containing SUV, preequilibrated with the fluorescent dye diS-C_2-5 and valinomycin. Fluorescence recovery, measured 10−20 min after mixing the peptides with the vesicles is depicted. Designations are as follows: Filled squares, CecP with PC/PS SUV; filled triangles, Rho-CecP with PC/PS SUV; filled circles, NBD-CecP with PC/PS SUV; empty squares, CecP with PC SUV; empty circles, P^{22}-CecP with PC/PS; empty triangles, P^{22}-CecP with PC.

value (0.77) is higher than that calculated for CecP (0.67). This difference correlates with the reduced area of the hydrophobic face of CecP as compared to Bm-CecB as evident from their helical wheel (Figure 5A).

DISCUSSION

Antibacterial peptides are produced by a wide range of species from different classes including mammals, amphibians, and insects (Boman, 1991, 1995). These peptides appear to play an important role in the innate immune response to invasion by pathogenic microorganisms (Zasloff, 1992). Nevertheless, the precise mode of action of these antibacterial peptide is not yet fully understood. CecP is the first and only antibacterial peptide homologous to the insect cecropins to be isolated from a mammalian source (Lee et al., 1989). Previous studies on the *in vivo* activity of CecP (Boman et al., 1993) suggested that CecP like other antibacterial polypeptides (magainins, insect cecropins, and dermaseptins) lyse bacteria in a process that is dose dependent, and that the target is probably the bacterial membrane (Steiner et al., 1988; Westerhoff et al., 1989; Boman et al., 1993; Matsuzaki et al., 1994). Furthermore, the activity of the antibacterial polypeptides appears to be due to their permeation of membranes via peptide−lipid interaction, rather than to receptor-mediated recognition (Wade et al., 1990; Bessalle et al., 1990). Therefore, phospholipid membranes have been used in several cases as a simple model system to elucidate the mechanism by which these polypeptides cause damage of the bacterial membrane. A major difference between CecP and insect cecropins is their three-dimensional structures. Insect cecropins have a helix−hinge−helix structure due to the conserved proline residue in the hinge region (Holak et al., 1988; Iwai et al., 1993), whereas CecP is composed of an elongated α-helix along nearly the full length of the peptide (Sipos et al., 1992). Furthermore, while insect cecropins are highly potent against both Gram-negative and Gram-positive bacteria, CecP is highly potent against Gram-negative bacteria but has a reduced activity against Gram-positive bacteria. Compared to the significant work that has been performed about the mode of action of insect cecropins, little is known on the mechanism by which the mammalian CecP permeates phospholipid membranes.

In this study CecP was synthesized, fluorescently labeled, tested for antibacterial activity, and investigated for its mode of action using model phospholipid membranes. Furthermore, similar studies were performed with an analogue of CecP, P^{22}-CecP, in which proline was introduced in a position similar to that found in insect cecropins.

The data are consistent with a nonpore mechanism as was suggested for the antibacterial peptide dermaseptin ("carpet-like"; Pouny et al., 1992) as an initial stage in which CecP disrupts the structure of phospholipid membranes. The four steps possibly involved in this model and the supportive data are discussed in the following paragraph: (i) preferential binding of peptide monomers to the negatively charged phospholipids, (ii) laying of amphipathic α-helical monomers on the surface of the membrane such that the positive charges of the basic amino acids interact with the negatively charged phospholipid headgroups or water molecules, (iii) rotation of the molecule leading to reorientation of the hydrophobic residues toward the hydrophobic core of the membrane, and (iv) disintegrating the membrane by disrupting the lipid packing in the bilayer structure. Figure 6 shows a schematic representation of such organization. An initial step before the collapse of the membrane packing may include transient holes in the membrane. Holes like these may enable the passage of low molecular weight molecules prior to complete membranal lysis. That a "carpet-like" mechanism is involved in the interaction of CecP with phospholipid membranes is based on the following observations: (i) the noncooperativity in the binding isotherms of CecP (Figure 2); (ii) its inability to self-associate within phospholipid membranes even at elevated peptide to lipid molar ratios as revealed in the RET experiments (Figure 3); (iii) the surface localization of its N-terminus; and (iv) on the basis of the analysis of hydrophobicity $\langle H \rangle$ versus hydrophobic moment $\langle \mu H \rangle$, Eisenberg (1984) has classified proteins into surface, globular, and transmembrane localization. Using these criteria it was predicted that insect cecropins have surface localization (Eisenberg, 1984). The same criteria hold for CecP. This nonpore mechanism is different from the transmembrane pore formation mechanism proposed for alamethicin (Fox & Richards, 1982; Schwarz et al., 1986, 1987; Rizzo et al., 1987) or pardaxin [Rapaport & Shai, 1992; see review Shai (1994)].

By using a variety of biophysical methods (e.g., fluorescence spectroscopy, solid-state NMR, ESR), a nonpore mechanism also was suggested as a possible mode of action of the following antibacterial polypeptides: magainins (Matsuzaki et al., 1991, 1994; Bechinger et al., 1992, 1993), dermaseptin (Pouny et al., 1992; Strahilevitz et al., 1994), and insect cecropins (Steiner et al., 1988; Gazit et al., 1994). Further support of the nonpore mechanism as the mode of action of several other antibacterial peptides comes from the finding that cecropin−melittin hybrids as short as 15 amino acids (Andreu et al., 1992), or a 12 amino acid N-terminal analogue of dermaseptin (Mor et al., 1994), had antibacterial

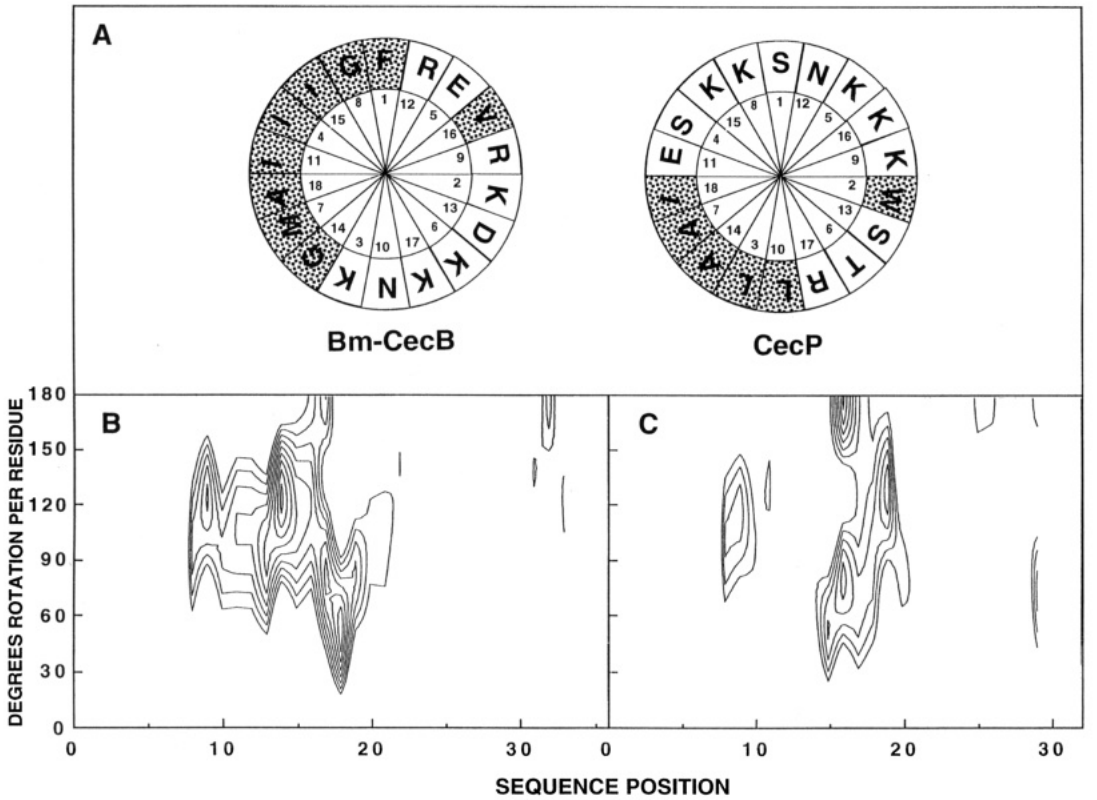

FIGURE 5: (A) Schiffer−Edmundson wheel projection of Bm-CecB and CecP. Number 1 in Bm-CecB represents residue 5. Shaded areas indicate hydrophobic amino acids. (B and C) Hydrophobic moments of Bm-CecB and CecP, respectively. The hydrophobic moments were derived using the MOMENT program of the CGC software package. Contours at 0.35, 0.4, 0.45, 0.5, 0.6, 0.65, 0.7, 0.75, 0.8, and 0.85, window size 5.

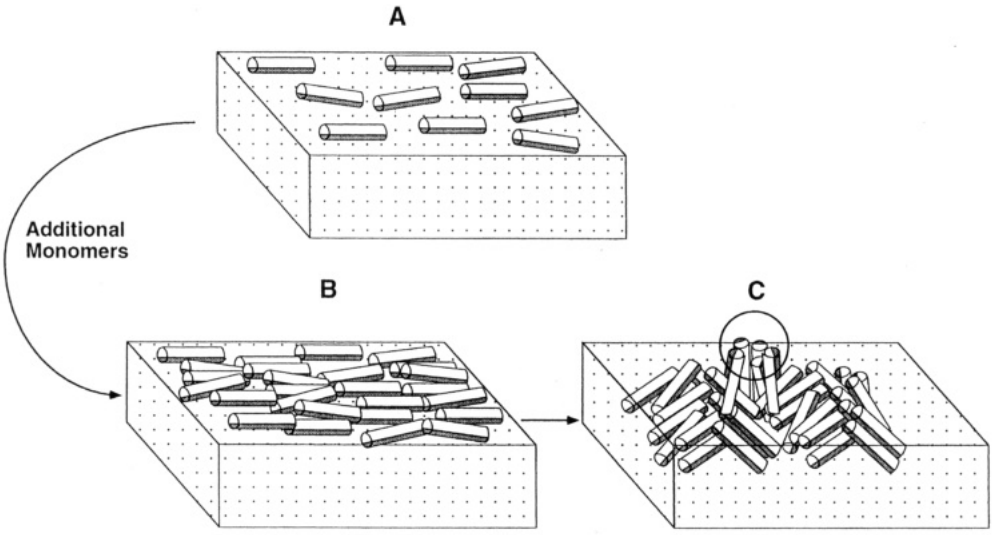

FIGURE 6: Cartoon illustrating the action of CecP on phospholipid membranes. (A) The peptides are bound randomly to the surface of the membrane with their hydrophobic surfaces (shaded area) facing the membrane and their hydrophilic surfaces (unshaded area) facing the solvent. (B) additional monomers are adsorbed onto the surface of the membrane to form a "carpet-like" layer. (C) When a threshold concentration of CecP monomers is bound to the membrane, transient holes are formed leading to complete membranal lysis.

activity similar to that of their intact parent molecules. Assuming these peptides are monomers, the lengths of 15 and 12 amino acid long α-helices are about 22.5 or 18 Å, respectively (1.5 Å per residue) and are therefore too short to span the ~30 Å thick phospholipid bilayers membrane and form a conducting pore. Furthermore, according to the dissipation of diffusion potential experiments, the maximal membrane permeating activity of CecP occurs at a lipid to peptide molar ratio of ~5:1 (Figure 4). Under these conditions the molar ratio between the lipid on the outer surface (~60% of total lipid) and the bound peptide is ~7:1. Since under these conditions not all the peptide molecules are bound to the membrane, the amount of lipid-bound CecP was calculated from to the binding isotherms (Figure 2) as described in the RET experiments section. Assuming a surface area of 70 $Å^2$ for each phospholipid molecule, the area available for each CecP monomer is ~500 $Å^2$. Since the surface area of a 31 amino acids long α-helical peptide is ~520 $Å^2$ (Steiner et al., 1988), it is evident that this amount of bound peptide monomers is sufficient to form a monolayer

Table 4: Membrane Interaction and Spectrum of Activity of Antibacterial Peptides

peptide	source	mode of interaction with membranes	antibacterial specificity	reference
alamethicin	fungi	cooperative binding, aggregation at low [peptide]/[lipid]	$Gram^+$	Meyer and Reusser (1967), Rizzo et al. (1987)
cecropin B	silk worm	noncooperative binding, aggregation at high [peptide]/[lipid]	$Gram^+/Gram^-$	Boman and Hultmark (1987), Gazit et al. (1994)
cecropin P	pig	noncooperative binding, no aggregation	$Gram^-/Gram^+$ (low)	Lee et al. (1989), the present study
dermaseptin S	frog	noncooperative binding, aggregation at high [peptide]/[lipid]	$Gram^-/Gram^+$	Mor et al. (1991), Pouny et al. (1992)
magainin	frog	noncooperative binding, aggregation at high [peptide]/[lipid]	$Gram^-/Gram^+$	Zasloff (1987), Matsuzaki et al. (1989, 1994)
polymyxin B	bacteria	noncooperative binding, no aggregation	$Gram^-$	Storm et al. (1977)

that completely cover the surface on the vesicles. However, we cannot rule out the possibility that a small fraction of CecP will form transmembrane pores as envisioned in Figure 6.

Insect cecropins aggregate within phospholipid membranes at high peptide to lipid molar ratios (Mchaourab et al., 1994; Gazit et al., 1994), while CecP does not (Figures 2 and 3). An explanation for this difference may be obtained by analyzing the Schiffer–Edmundson (1967) wheel projections of both of them (Figure 5A), which reveal that CecP has a larger area of hydrophilic face (83.3%/300°) than Bm-CecB (55.6%/200°). Furthermore, CecP has a lower hydrophobic moment than Bm-CecB. Both properties have been shown to affect the ability of an amphipathic α-helix to form a bundle of monomers that in several cases could form transmembrane pores. A functional difference between CecP and insect cecropins is the significantly reduced antibacterial activity of CecP against Gram-positive bacteria as compared to insect cecropins. Since both cecropins have similar partition coefficients with phospholipid membranes (Gazit et al., 1994; Figure 2), it is reasonable to speculate that the ability of insect cecropins to efficiently lyse Gram-positive bacteria is attributed partially to their ability to assemble in the membrane at high peptide to lipid molar ratio (Mchaourab et al., 1994; Gazit et al., 1994). Support for this hypothesis can be seen in Table 4, which summarizes reported data on the organizational state of various antibacterial polypeptides versus their antibacterial specificity.

In summary, the results discussed herein suggest a nonpore mechanism as an initial stage in the interaction of the mammalian CecP with phospholipid membranes and further demonstrate that a nonpore mechanism seems to be a common initial stage characteristics of several other positively charged antibacterial amphipathic polypeptides. Better understanding of the molecular basis of antibacterial activity may assist in the future design of more specific and potent antibacterial agents.

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