Binding and State of Aggregation of Spin-Labeled Cecropin AD in Phospholipid Bilayers: Effects of Surface Charge and Fatty Acyl Chain Length†

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ABSTRACT: The binding and state of aggregation of cecropin in large unilamellar vesicles of different surface potential and varying acyl chain length were examined using a Cys-33 spin-labeled derivative of cecropin AD (CAD). Association isotherms of the peptide were measured for vesicles of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) containing 5, 15, and 30 mol % 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol (POPG). The isotherms display a concentration-dependent positive cooperativity indicating the possible formation of cecropin aggregates in the lipid phase. The critical aqueous concentration for aggregation was dependent on the fraction of POPG, suggesting the involvement of acidic lipids in the formation and stabilization of the putative aggregate. Our data also indicate that cooperativity depends on the state of side-chain ionization of an acidic residue that titrates between pH 7 and 4.4. The binding of spin-labeled Cys-33 CAD was found to be influenced by the acyl chain length of the host lipid. The association isotherm of the peptide for dilaureoyl-*sn*-glycero-3-phosphatidylcholine vesicles containing 30 mol % dilauroyl-*sn*-glycero-3-phosphatidylglycerol (DLPG) differed significantly from that in POPC/POPG and could be interpreted in terms of a monomer–monomer partitioning between the aqueous and lipid phases. ESR line-shape analysis was consistent with peptide aggregation in dioleoyl-*sn*-glycero-3-phosphatidylglycerol vesicles but not in DLPG vesicles. These results indicate that peptide binding depends on surface potential as well as acyl chain length and provide experimental support for the involvement of conserved residues in the N terminus in facilitating peptide self-association [Durell, S. R., Ragunathan, G., & Guy, R. H. (1992) *Biophys. J. 63,* 1623–1631].

One of the major components of the immune system of a variety of insect species is a class of water-soluble, basic peptides called cecropins (Boman *et al.*, 1991). They are induced in the hemolymph following injection of live bacteria and display a broad spectrum of antibacterial activity. There are three principal homologous types of cecropins, namely cecropin A, B, and D. They are polar molecules with a primary sequence that consists of a strongly basic N terminus followed by a hydrophobic C terminus. All three peptides increase the permeability of liposomes (Steiner *et al.*, 1988) and form voltage-gated ion channels in lipid bilayers (Christensen *et al.*, 1988), indicating direct action on membranes.

Numerous studies have been undertaken to elucidate the molecular basis of membrane activity. Structural studies using CD spectroscopy (Steiner, 1982; Andreu *et al.*, 1985) have shown that the cecropins are in a predominantly random coil conformation in aqueous solution. Titration with 0–15% v/v hexafluoropropanol (HFP)[1] leads to a cooperative increase in helical content, indicating intrinsic helical propensity. The NMR solution structure in 15% HFP (Holak *et al.*, 1988) revealed a helix-bend-helix structural motif that was predicted from sequence analysis. The NMR structure shows an N terminal helix spanning residues 5–21 and a more hydrophobic helix extending from residue 24 to 37.

Functional studies established the role of this structural motif in mediating biological activity. Using cecropin A analogs with varying helical propensity in the N terminal region, Andreu *et al.* (1985) showed that a strongly helical, highly basic N terminus is a prerequisite for antibacterial activity. Further studies using cecropin analogs altered in the bend and the C terminal regions (Fink *et al.*, 1989a) indicated that the structural motif underlying cecropin activity is a basic amphipathic N terminus connected to a hydrophobic helix by a flexible hinge. A chimeric protein, cecropin AD (Fink *et al.*, 1989b), designed to optimize these structural features has increased antibacterial potency, while peptide analogs lacking a central hinge region are inactive. In addition, patch-clamp studies showed that the voltage dependence of cecropin channels also requires a nonhelical hinge region between the N and C terminal segments (Christensen *et al.*, 1988).

Two models for cecropin activity have been proposed. The first (Christensen *et al.*, 1988) is based on structure–function studies in bacteria and planar lipid bilayers. In this model, electrostatic interactions between cecropin oligomers in the aqueous phase and membrane acidic lipids mediate the binding of the peptide to the membrane. This step is followed by the rotation of the N terminal helix around its axis and the insertion of the hydrophobic C terminus into the membrane hydrophobic core. A channel-conducting state is induced when a large transmembrane potential is applied causing the N terminus

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[1] Abbreviations: cecropin AD, CAD; 1-oxyl-2,2,5,5-tetramethylpyrroline-3-(methyl methanethiosulfonate) spin label, MTSSL; cysteine-33 cecropin AD, cys-33 CAD; 1,1,1,3,3,3-hexafluoro-2-propanol, HFP; 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phophatidylcholine, POPC; 1-palmitoyl-2-oleoyl-*sn*-slycero-3-phosphatidylglycerol, POPG; dilaureoyl-*sn*-glycero-3-phosphatidylcholine, DLPC; dilaureoyl-*sn*-glycero-3-phosphatidylglycerol, DLPG; dioleoyl-*sn*-glycero-3-phosphatidylcholine, DOPC; dioleoyl-*sn*-glycero-3-phosphatidylglycerol, DOPG; dipalmitoyl-*sn*-glycero-3-phosphatidylglycerol, DPPG; dimyristoyl-*sn*-glycero-3-phosphatidylglycerol, DMPG; dioleoyl-*sn*-glycero-3-phosphate, DOPA; multilamellar vesicles, MLV.

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to insert vertically across the bilayer. The second model, proposed by Durell *et al.* (1992), while relying largely on the same experimental data, differs in a few important aspects. On the basis of the principle that conserved residues at the N terminus play an important role in peptide–peptide interactions, these authors proposed that cecropins bind to the membrane as antiparallel dimers with each monomer arranged in a helix-bend-helix motif. The dimers were postulated to bind to the membrane with the N terminal helices sunken into the headgroup layer and the COOH terminal helices spanning the hydrophobic core, thus avoiding the energetically unfavorable placement of the C terminal lysine in the hydrophobic core. Cecropin channels of type I are formed when a collection of the membrane-bound dimers aggregate, with the pore formed by C terminal helices. Type I channels were then assembled into a hexagonal lattice to explain the high density of bacterium-bound peptides. Type II channels are formed under the action of a transmembrane voltage. A concerted conformational change of type I channels leads to the formation of the larger type II channels, in which the pore is formed by the N terminal helices. The proposed dimer conformation allows these helices to be inserted into the bilayer without having to expose the charged residues to the hydrophobic core.

While there is little doubt that the primary site of cecropin activity is the membrane (Wade *et al.*, 1990), considerable uncertainty exists as to the actual mechanism of cell killing. Previous efforts directed toward understanding the structural features mediating cecropin activity did not resolve questions concerning the energetics of membrane binding, the conformation and location of the peptide within the membrane, and its aggregation state in either the aqueous or lipid phases. This is partly because previous studies were carried out in systems were quantitation of membrane binding is difficult, as is the deconvolution of the various physical states of the peptide. Resolving these questions is central to the understanding of the molecular mechanism underlying antibacterial activity, as well as the mechanism of channel formation. In a more general context, small-peptide ion channels such as the cecropins provide a powerful tool for studying various aspects of membrane–protein interactions that have been implicated in key cellular processes such as signal transduction, protein translocation across membranes, and voltage-dependent conformational transitions.

This study addresses two fundamental aspects of cecropin interaction with membrane lipids. The first is the energetics of cecropin insertion into the bilayer and the factors that modulate the peptide affinity. From an electrostatic point of view, cecropins are highly charged molecules. The net charge on the AD form is 7, of which 6 are in the N terminal helix. Cecropin AD contains two acidic residues, one of which (Glu 9) is highly conserved among the cecropin superfamily (Boman *et al.*, 1991). Previous studies have demonstrated that cecropin-induced conductance as well as its lysis potential depends on the electrostatic surface potential. Positively charged lipids were found to inhibit liposome lysis (Steiner *et al.*, 1988) and significantly reduced cecropin-induced conductance (Christensen *et al.*, 1988). Furthermore, bacterial potency is highly correlated with a strongly basic N terminus (Fink *et al.*, 1989b). Therefore, one of the main objectives of this study is to evaluate membrane association of cecropin in a quantitative way with explicit account of electrostatic effects.

The second aspect concerns the association state of cecropin bound to the bilayer. It is not known whether a pore-forming aggregate exists in the membrane in the absence of a transmembrane potential. Certainly, the ability of cecropins to induce liposomal membrane lysis would suggest the possible formation of such an aggregate. If cecropin monomers can aggregate in the membrane in the absence of a voltage, then a simple model can account for both activities. This model has been proposed to explain the voltage-gating mechanism of the peptide alamethicin and does not require particular voltage-dependent conformational changes (Rizzo *et al.*, 1987). Instead, it requires the peptide (or segments of the peptide) to be bound to the membrane such that its helical dipole is parallel to the direction of the applied transmembrane electric field. The primary effect of this interaction is an increase in membrane partitioning of the peptide leading to ion-conducting aggregate formation when a sufficient concentration of membrane-bound peptide is reached. The critical distinction here is between a model where the voltage causes a conformational change required for channel formation and a model where the voltage induces conductance through its effects on the equilibrium between membrane-bound conformations (Andrew & Wallace, 1992).

Recently, we have shown that a spin-labeled analog of cecropin AD forms a stable aggregate in 10% HFP and that its self-association is stabilized by ion-pair formation and hydrophobic interactions (Mchaourab *et al.*, 1993). Furthermore, we have shown that the peptide is monomeric in aqueous solution at pH 7, and therefore, aggregation in the aqueous phase can not account for the shallow concentration dependence of peptide-induced conductance. In this paper, the partitioning of cecropins between the aqueous and lipid phases is examined using this spin-labeled cecropin analog. Binding isotherms indicate that interaction of the peptide with the bilayer depends strongly on membrane thickness. This makes it highly unlikely that cecropin is only adsorbed to the bilayer surface. Furthermore, we find that binding isotherms display a characteristic cooperativity in POPC/POPG vesicles, while in DLPC/DLPG vesicles they are hyperbolic. Cooperativity was found to depend on side-chain ionization of the peptide, in a similar fashion to self-association in HFP. The ESR spectrum of the membrane-bound peptide displays a change in line shape at a high protein to lipid ratio in the case of POPG vesicles, while for DLPG vesicles, it is unchanged, further supporting aggregation in the former system. The analysis of our data is consistent with the model proposed by Schwarz (Schwarz, 1989; Stankowski *et al.*, 1989) that interprets cooperativity as arising from peptide aggregation in the lipid phase and supports the existence of peptide aggregates in vesicles of certain thickness.

MATERIALS AND METHODS

Materials. All lipids were purchased from Avanti Polar Lipids (Birmingham, AL). 1-Oxyl-2,2,5,5-tetramethylpyrroline-3-(methyl methanethiosulfonate) spin label (MTSSL) was obtained from Reanal (Budapest, Hungary). Trifluoroacetic acid from Pierce (Rockford, IL) and acetonitrile from Fisher (Pittsburgh, PA) were HPLC grade. Cecropin AD (CAD) and Cys-33 CAD[2] were synthesized in the protein and nucleic acid shared facility of the Medical College of Wisconsin. Cys-33 CAD was spin-labeled and purified as described previously (Mchaourab *et al.*, 1993).

Preparation of Lipid Vesicles. Lipids were dissolved in chloroform, and the solvent was evaporated under a stream

[2] The amino acid sequence of Cys-33 CAD is K-W-K-L-F-K-K-I-E-K-V-G-Q-R-V-R-D-A-V-I-S-A-G-P-A-V-A-T-V-A-Q-A-C-A-L-A-K-NH_2.

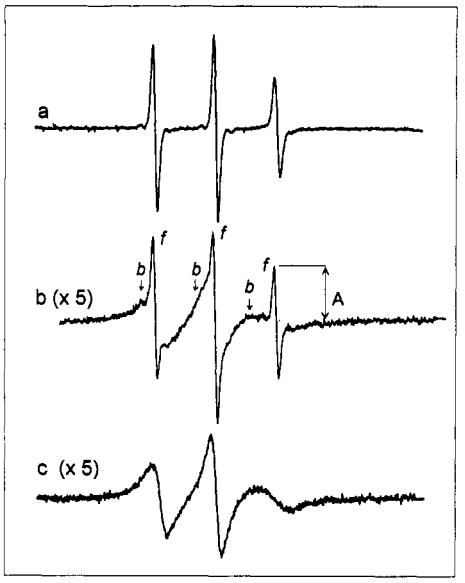

FIGURE 1: ESR spectrum of 40 μM MTSSL-Cys-33 CAD: (a) in aqueous solution at pH 7, (b) in the presence of POPC/POPG (75/25 mol/mol) vesicles, and (c) same as b with addition of 50 mM cromium oxalate.

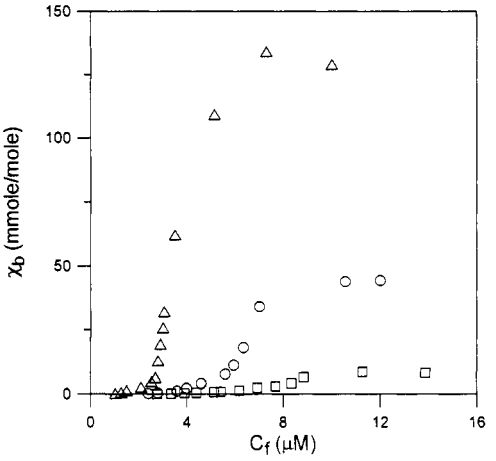

FIGURE 2: Binding of the spin-labeled peptide to POPC/POPG vesicles. The amount of cecropin bound per mole of lipid is plotted versus the equilibrium concentration of cecropin free in solution: (□) POPC/POPG 95/5 (mol/mol), (○) POPC/POPG 85/15 (mol/mol), and (△) POPC/POPG 70/30 (mol/mol). In this experiment, the total concentration of MTSSL-Cys-33 CAD is 18 μM and the buffer contains 100 mM NaCl and 50 mM MOPS, pH 7. The temperature was maintained at 25.5 ± 0.2 °C.

of nitrogen gas. The mixture was further dried overnight in a vacuum dessicator. The lipids were then dispersed in the appropriate buffer by vortex mixing at 50 °C. The vesicle suspension was then freeze–thawed five times and extruded 10 times through 0.1-μm filters using a commercially available instrument (Lipex Biomembranes Inc., Vancouver, BC). Final lipid concentrations were determined using a Bartlett assay (Bartlett, 1959).

Electron Spin Resonance. CW ESR measurements were performed on a Varian E-109 spectrometer (Varian Assoc., Palo Alto, CA) fitted with a two-loop one-gap resonator (Hyde & Froncisz, 1989). Samples of 5 μL were loaded in 0.84-mm o.d. capillaries which were sealed on both ends. Temperature was maintained using a Varian temperature controller.

Multiquantum ESR (MQESR) measurements were performed on a self-built spectrometer (Sczaniecki *et al.*, 1991) fitted with a similar resonator. Samples were loaded in a TPX gas-permeable capillary. The oxygen concentration was maintained by equilibrating the sample with certified O_2/N_2 gas mixtures (Airco).

RESULTS

ESR Spectrum of MTSSL-Cys-33 CAD in the Presence of Lipid Vesicles. Figure 1a shows the ESR spectrum of the spin-labeled peptide in aqueous solution at pH 7. As previously reported, the spectrum is characteristic of fast rotational motion, consistent with a predominantly random coil conformation. Upon addition of unilamellar vesicles containing POPC/POPG, the spectrum shows evidence of two components with different rotational mobility. One component, labeled f, is due to peptide free in the aqueous phase, while the other, which has considerably broader lines and is labeled b, arises from membrane-bound peptide. As shown previously, the quantity A can be used to determine the fraction of free peptide and can therefore provide a direct measurement of the extent of binding (Castle & Hubbell, 1976). If 50 mM chromium oxalate (Crox) is added to the sample in Figure 1b, the spectral component arising from aqueous peptide is completely broadened, while the bound component is essentially unaffected (Figure 1c). Crox is a hydrophilic paramagnetic compound that shortens the electron relaxation time of the spin label through bimolecular collision. The insensitivity of the bound spectral component indicates a lack of interaction between Crox and the labeled site of the bound peptide. This is possibly due to the insertion of the spin-labeled site into the membrane hydrophobic core or to the negative surface potential of the bilayer, which lowers the effective concentration of the negatively charged Crox near the plane of binding (Greenlagh *et al.*, 1991). The location of the spin-labeled site is addressed in more detail later in the paper. In any case, addition of Crox provides a convenient way to examine the line shape of the bound species. Figure 1c shows that the motion of the spin label for the membrane-associated species is considerably more restricted than when the peptide is free in solution, with a rotational correlation time of approximately 2 ns.

Cecropin Binding to POPC/POPG Vesicles: Binding Isotherms. In order to characterize the binding affinity to membrane lipids, spin-labeled peptides at a concentration of 17 μM were titrated with varying concentrations of POPC/POPG vesicles. With this low concentration of peptide, it is assumed that the bilayer structure remains intact. For every ratio of peptide to lipid concentration, the amount of peptide in each phase was determined using the method outlined above. To obtain the association isotherm, the data were plotted as the molar ratio of associated peptide to lipid, χ_b, versus the free peptide concentration C_f. Typical binding isotherms for MTSSL-Cys-33 CAD with 5, 15, and 30% POPG vesicles are shown in Figure 2.

The most striking feature of the binding isotherms in POPG vesicles is their sigmoidal shape, which implies a cooperative effect. They exhibit an initial lag and above a certain aqueous concentration rise sharply. A thermodynamic model presented by Schwarz (1989) interprets this cooperative behavior in terms of peptide self-association in the lipid phase. According to this model, the shape of this isotherm precludes aggregation in the water phase, consistent with our previous data (Mchaourab *et al.*, 1993). Therfore, peptide monomers first incorporate into the membrane and then aggregate.

From the sharp increase in the slopes of these curves, a critical aqueous concentration, C_f^*, for the aggregation of the peptide can be determined. Above C_f^*, which occurs at the origin of the upward bend in the slopes, the concentration of

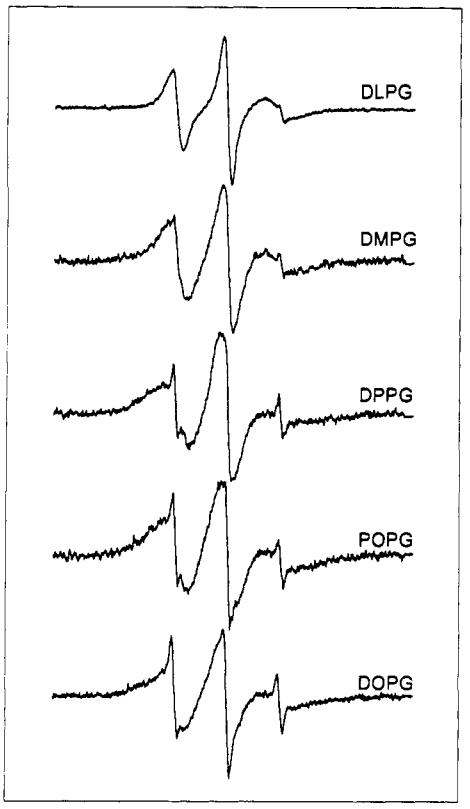

FIGURE 3: ESR spectra of the spin-labeled peptide in the presence of vesicles composed of lipids with different fatty acyl chain length. Each sample contained 50 μM peptide and 10 mM lipids in 100 mM NaCl and 50 mM MOPS, pH 7.

bound cecropin becomes sufficient for peptide self-association, leading to an increase in the apparent partition coefficient.

Two other thermodynamic quantities are needed to describe the association isotherms, namely the monomer partition coefficient, K, and the aggregation constant, K_d (Schwarz, 1989). K describes the affinity of the monomer toward the hydrophobic phase. A rather moderate affinity is evident from the shallow slope of the initial linear region. An accurate estimation of this quantity requires the use of lower aqueous peptide concentrations but may be estimated from our data to be roughly 100 M^{-1}. K_d is determined using the relation between C_f^*, K, and K_d given by

$$C_f^* = \frac{1}{KK_d} \tag{1}$$

For POPC/30% POPG vesicles, C_f^* is around 2.5 μM; therefore, K_d is on the order of 5×10^3. Figure 2 shows that C_f^* depends on the surface potential. A higher fraction of POPG leads to a decrease in C_f^*, indicating a critical role of the lipids in increasing membrane affinity and in promoting self-association.

Cecropin Binding to Acidic Membranes of Different Fatty Acid Composition. We also examined the binding of cecropin to acidic vesicles of different fluidity and lipid acyl chain length. Figure 3 is a qualitative illustration of the differential affinity of the peptide to various model bilayers. In all spectra, 50 μM peptide was added to 10 mM multilamellar vesicles (MLV). The spectra show a clear decrease in the aqueous peptide population as the acyl chain length is reduced. The peptide also associates strongly with gel-phase bilayers (DPPG), the only significant difference relative to fluid phase (POPG, DOPG) being a greater immobilization of the bound component.

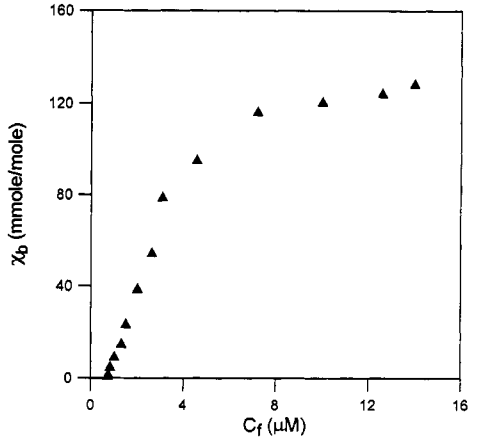

FIGURE 4: Association isotherm of the peptide for DLPC/DLPG 70/30 (mol/mol). Experimental conditions are the same as those in Figure 2.

In order to quantitate the apparent higher binding affinity of the peptide to thinner (*i.e.*, shorter acyl chain length) vesicles, we determined the binding isotherm of the spin-labeled peptide to DLPC/DLPG unilamellar vesicles. Peptides at a concentration of 18 μM were titrated with 30% DLPG vesicles using identical conditions to these for Figure 2. Figure 4 shows the corresponding binding isotherm. The shape of this binding isotherm differs significantly from those in Figure 2.[3] Little change in the slope is detected as the concentration of the free peptide is increased, indicating absence of the cooperativity that characterizes binding to POPC/POPG bilayers. Rather, we observe a pronounced curvature at large C_f values, suggesting the existence of repulsive interactions between the associated peptide when bound to the membrane. If aggregation causes the apparent cooperative binding in POPC/POPG vesicles, the shape of the isotherm in Figure 4 implies a monomer partitioning into DLPC/DLPG vesicles. A hyperbolic binding curve was also obtained for DLPC vesicles containing 5% DLPG (data not shown).

The binding isotherm for DLPC/DLPG 70/30 mol/mol vesicles was analyzed as a monomer partition equilibrium using the method outlined by Beschiaschvili and Seelig (1990). Briefly, the electric surface charge density on the outer leaflet of the bilayer can be described by

$$\sigma = \left(\frac{e_0}{A_l}\right)\frac{(\bar{\chi}_{PG} + \chi_b z_p)}{\left[1 + \chi_b\left(\frac{A_p}{A_l}\right)\right]} \tag{2}$$

where χ_{PG} is the mole fraction of POPG, A_l is the surface ara of a POPG molecule, e_0 is the elementary electric charge, and z_p and A_p are the valency and surface area of the peptide, respectively. The surface electrostatic potential is given by

$$\sigma^2 = k\sum C_i\left[\exp\left(\frac{Z_i F_0 \psi}{RT}\right) - 1\right] \tag{3}$$

where $k = 2000\epsilon_0\epsilon_R RT$ with ϵ_0 being the dielectric constant of water and ϵ_R the permitivity of free space, C_i is the concentration of the ith electrolyte in the bulk aqueous phase,

[3] In all experiments conducted at 25 °C, the binding isotherms did not intersect the peptide concentration axis at zero. A similar observation has been reported by Archer *et al.* (1991) and was attributed to the adsorption of alamethicin to the EPR cell. Using the same peptide and vesicle preparation, we determined binding isotherms in DLPC/DLPG (95/5 mol/mol) at 25 and 45 °C. Neither curve displayed cooperativity, and the binding isotherm at 45 °C intersected the axis at 0.0 (data not shown).

Table 1: Binding of Cecropin to DLPC/DLPG (70/30 mol/mol) Vesicles (0.1 M NaCl and 50 mM MOPS, pH 7)[a]

C_f	χ_b	ψ	σ	C_{dd}	K_p
1.5	23.1	−57.2	−63.0	9.1	2544.9
2.0	38.3	−54.0	−58.4	10.8	3537.8
2.6	53.9	−50.7	−54.0	12.8	4206.2
3.1	78.6	−45.7	−47.6	12.8	6129.3
4.6	94.9	−42.4	−43.6	17.2	5525.1
7.2	118.0	−38.2	−38.8	23.7	4887.5
10.0	120.3	−37.4	−37.9	32.1	3746.7
12.6	124.0	−36.7	−37.1	39.6	3134.9

[a] C_f and C_{dd} are in μM, χ_b is in mmol/mol, ψ is in mV, σ is in mC/m^2, and K_p is in M^{-1}.

F_0 is the Faraday constant, R is the gas constant, T is the temperature, Z_i is the valence of the ith electrolyte, and ψ is the electrostatic potential at the membrane surface. This surface potential leads to an accumulation of the peptide in the diffuse double layer. Therefore, at a given surface potential, the partition coefficient is

$$K_p = \frac{\chi_b}{C_{dd}} \tag{4}$$

where

$$C_{dd} = C_f \exp\left(\frac{Z_p F_0 \psi}{RT}\right) \tag{5}$$

is the concentration of the peptide in the diffuse double layer.

Equation 3 can be solved by numerical methods (*e.g.*, the Newton–Raphson method). An algorithm based on this method (Press *et al.*, 1990) was used to calculate σ, ψ, C_{dd}, and K_p. Table 1 shows the numerical data obtained for the binding isotherm in Figure 4. The surface charge of the peptide was set to 0.8 and was distinctly smaller than the true electric charge of 7. A similar effect was observed in the case of the peptide melittin and was attributed to charge discreetness and displacement of some of the peptide charge away from the interface. The partition coefficient of cecropin to 30% DLPG/DLPC is 4000 ± 1200 M^{-1}, leading to a free energy of binding of 4.9 kcal/mol.

Effect of Surface Potential on Binding Affinity. Figure 5a illustrates the dependence of cecropin binding to POPC/POPG MLV on surface potential. The peptide does not bind significantly to bilayers consisting of zwitterionic lipid alone. This is a distinctive feature as compared to melittin and was verified for a variety of vesicle sizes and concentrations. However, the peptide binds strongly in the presence of negatively charged lipids. The binding displays a fast increase of the bound peptide as the surface potential increases, indicating that peptide binding is mediated by electrostatic interactions. The shape of this curve does not display the apparent cooperativity predicted for peptides with multiple positive charges (Kim *et al.*, 1991; Mosior & McLaughlin, 1992), consistent with an effective charge less than 1.

Figure 5b shows the dependence of χ_b/C_{dd} on the surface potential. This curve displays a sigmoidal increase in this quantity as the surface potential is increased. If the cooperativity detected in the binding isotherm represents the onset of aggregate formation, then negatively charged lipids appear to have a key role in the formation and stabilization of the putative aggregate.

Further evidence of a differential behavior in DLPC/DLPG vesicles is obtained from the dependence of the binding affinity on surface potential in that system. Figure 6a shows the extent of binding as a function of mole fraction of DLPG. If χ_b/C_{dd}

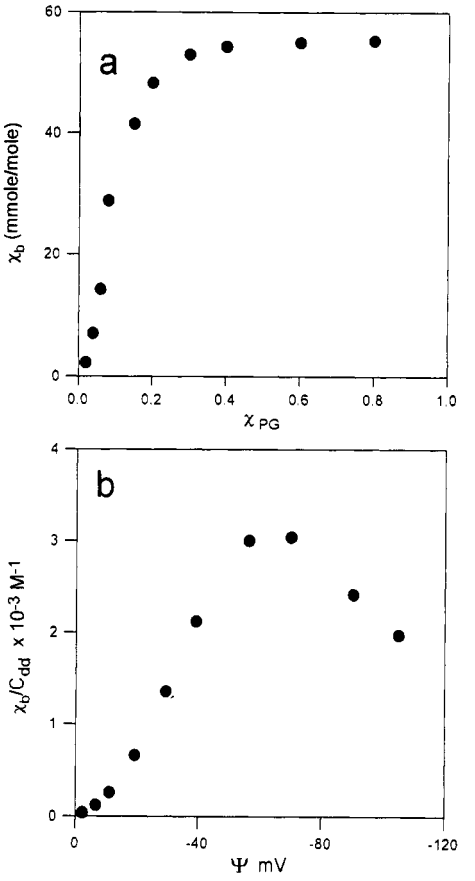

FIGURE 5: Binding of the spin-labeed peptide to POPC vesicles containing varying amounts of POPG: (a) extent of incorporation versus fraction of POPG and (b) apparent partition coefficient versus membrane surface potential. The data in b were calculated on the basis of the Gouy–Chapman theory (for details, see text). Solutions of 58 μM MTSSL-Cys-33 CAD in 100 mM NaCl and 50 mM Mops, pH 7, were added to dried lipids to make a final lipid concentration of 1 mM.

is plotted against the surface potential, a linear increase in binding affinity is obtained (Figure 6b). This trend is quite different from that observed for POPC/POPG vesicles and can be attributed to change in the intrinsic partition coefficient without the need to invoke aggregate formation.

Effect of pH on Binding to POPC/POPG Vesicles. The remarkably low effective charge of the peptide suggests at least a partial role for the acidic residues, namely Glu 9 and Asp 17, in modulating membrane affinity. It was previously proposed that if the C terminal helix is to span the bilayer vertically, the N terminal helix has to sink into the lipid headgroup region (Durell *et al.*, 1992). As a result, there is the possibility of strong repulsion between Glu 9 and the phosphate groups. Residues in the $i \pm 4$ positions relative to Glu 9 are polar but not charged; thus, it is unlikely that this residue is involved in an intramolecular salt bridge. Therefore, it was proposed that membrane-bound cecropin exists as a dimer in an antiparallel conformation such that Glu 9 salt bridges to Arg 16 on the adjacent molecule (Durell *et al.*, 1992). We have proposed that aggregate formation in 10% HFP is stabilized by an ion pair that involves a titratable group with an apparent pK_a in the range of 4–4.5 (most likely Glu 9) (Mchaourab *et al.*, 1993). Therefore, we have measured the association isotherm in POPC/POPG vesicles at pH 4.4[4] with the goal of detecting any significant change in the binding affinity, effective charge, or cooperativity.

Spin-labeled peptides at a concentration of 18 μM were titrated with POPC/POPG (70/30 mol/mol)-extruded vesicles

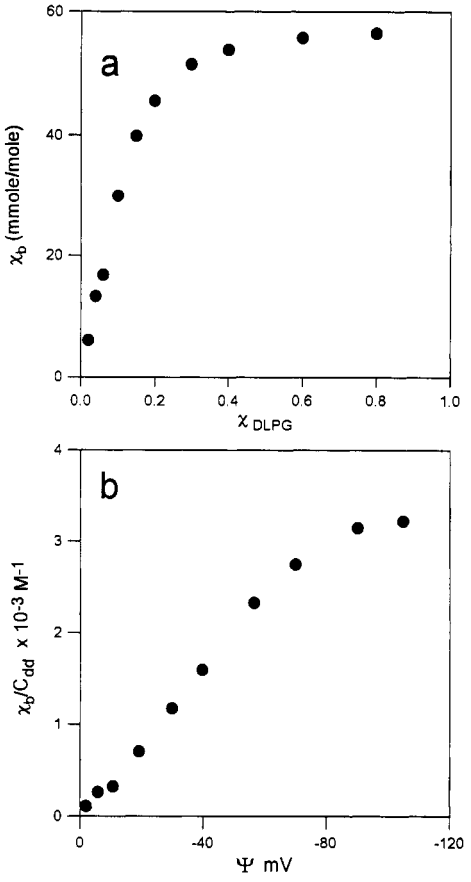

FIGURE 6: Binding of the spin-labeled peptide to DLPC vesicles containing varying amounts of DLPG: (a) extent of incorporation versus fraction of DLPG and (b) apparent partition coefficient versus membrane surface potential. The data in b were calculated on the basis of the Gouy–Chapman theory (for details, see text). Experimental conditions are the same as those in Figure 5.

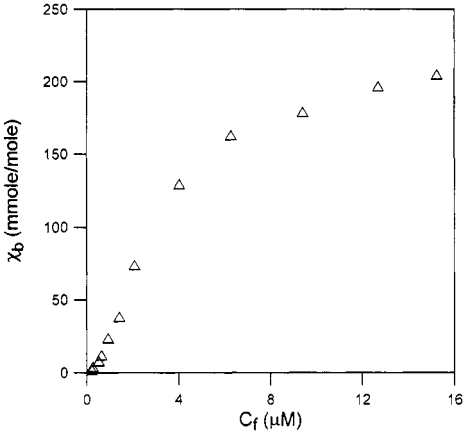

FIGURE 7: Association isotherm of the peptide for POPC/POPG 70/30 (mol/mol) at pH 4.4. In this experiment, the total concentration of MTSSL-Cys-33 CAD is 18 mM and the buffer contains 100 mM NaCl and 50 mM acetate, pH 4.4. The temperature was maintained at 25.5 ± 0.2 °C.

in 50 mM acetate and 100 mM NaCl, pH 4.4. Figure 7 shows the corresponding binding isotherm. The shape of the curve shows no evidence of cooperativity; rather saturation binding is observed. A hyperbolic binding curve was also obtained in vesicles composed of POPC/POPG (95/5 mol/mol) (data not shown). Compared to the corresponding association isotherm at pH 7, this curve is shifted to the left, indicating a large increase in the monomer partition coefficient. A monomer partition coefficient of 8200 ± 1940 M^{-1} was calculated, reprsenting a large increase in binding affinity.

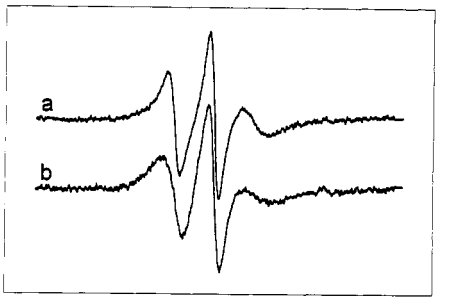

FIGURE 8: (a) ESR spectrum of 55 μM spin-labeled cecropin in DOPG vesicles at a lipid to peptide ratio of 250. (b) ESR spectrum of 55 μM spin-labeled peptide in the presence of 450 μM native CAD to yield a lipid to peptide ratio of 25. For each case, the lipid concentration was 12.5 mM and the buffer contained 150 mM NaCl, 50 mM MOPS, and 50 mM Crox, pH 7. The scan width is 130 G.

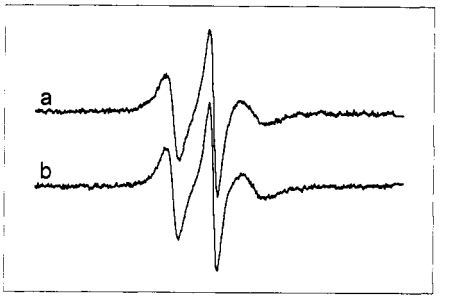

FIGURE 9: (a) ESR spectrum of 55 μM spin-labeled cecropin in DLPG vesicles at a lipid to peptide ratio of 250. (b) ESR spectrum of 55 μM spin-labeled peptide in the presence of 450 μM native CAD to yield a lipid to peptide ratio of 25. For each case, the lipid concentration was 12.5 mM and the buffer is 150 mM NaCl, 50 mM MOPS, and 50 mM Crox, pH 7. The scan width is 130 G.

These data lend support to the dimer model by illustrating the importance of an acidic titratable group, possibly Glu 9, in mediating cooperative behavior.

Aggregation State of Membrane-Bound Spin-Labeled Cecropin. To test the idea that the observed cooperativity in the binding is due to self-association of the peptide, ESR spectra were obtained from peptide bound to acidic vesicles under conditions of high and low peptide to lipid concentrations (Figure 8). Self-association of the peptide should produce a reduction in the rotational correlation time of the spin label, observable as a change in the ESR line shape (Archer *et al.*, 1991). Therefore, the ESR spectrum of the membrane-bound peptide at a peptide to lipid ratio of 1/500 was compared to that at a peptide to lipid ratio of 1/20. This ratio was increased by adding excess native cecropin AD to avoid a contribution to the line width from spin–spin interaction. Evident in Figure 8 is a change in the line shape that reflects an approximately 50% increase in rotational correlation time (from about 2 ns in a to about 3 ns in b). Similar results were observed for POPG bilayers and vesicles composed of brain phosphatidylserine (PS).

If the same experiment is repeated using DLPG vesicles, no significant change in the spectral line shape is detected. Figure 9 shows that the ESR spectra of the bound peptide at high and low peptide to lipid ratios are nearly superimposable. This is consistent with the conclusion based on the analysis of the binding isotherms, that aggregation occurs in vesicles of long acyl chain lipids (palmitoyl or greater) but not in short (laureoyl) phospholipid bilayers.

[4] The intrinsic pK_a of Glu is 4.4. However, due to the increased concentration of H^+ near the membrane–water interface, the apparent pK_a is expected to be larger, and as a result, most of the Glu residues are protonated.

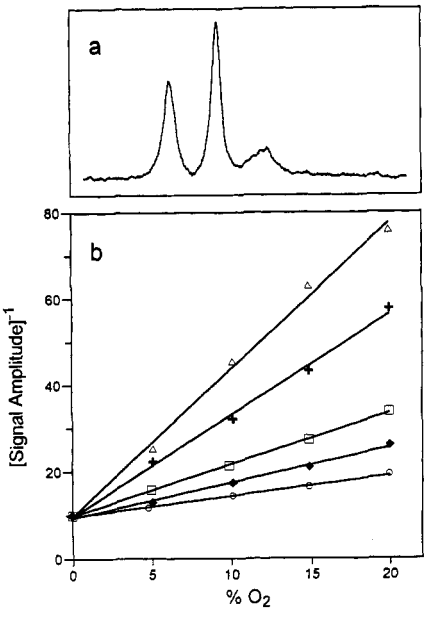

FIGURE 10: (a) 3Q-ESR spectrum of the peptide bound to DOPC/DOPA 60/40 (mol/mol) liposomes. (b) Dependence of the 3Q-ESR signal amplitude on the percent of molecular O_2. The slope of this curve is compared to that obtained for stearic acid spin labels in the same host lipids: (◆) 5-SASL, (+) 9-SASL, (△) 12-SASL, (○) MTSSL-Cys-33 CAD in the aqueous phase, and (□) membrane-bound MTSSL-Cys-33 CAD.

Location of the Spin-Labeled Site. In order to determine whether the spin-labeled site is buried in the membrane or is accessible to the aqueous phase, we measured its exposure to the hydrophobic phase. We used molecular oxygen as a lipophilic probe in conjunction with a T_1 display, MQESR (Mchaourab & Hyde, 1993). Molecular oxygen is a fast relaxing paramagnetic species that is highly soluble in the membrane. It interacts with membrane-incorporated spin labels via Heisenberg exchange and enhances the rate of spin–lattice relaxation. This change can be easily detected using the 3Q-ESR signal. The signal amplitude in this display is directly proportional to T_1, which allows fast and direct measurements of relative changes in that quantity (Mchaourab & Hyde, 1993).

Figure 10a shows the 3Q-ESR spectrum of the peptide bound to dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) containing 40 mol % dioleoyl-*sn*-glycero-3-phosphate (DOPA). This spectrum was obtained by centrifuging the sample briefly so that the aqueous spin label does not contribute to the spectrum. Figure 10b shows the change in the signal amplitude as a function of $[O_2]$. Upon binding, there is a 2-fold increase in the slope of the curve, indicating peptide incorporation well below the lipid headgroup region. Also shown in the same figure is the corresponding exposure of stearic acid spin labels. The slopes of the curves depend on the position of the spin label across the membrane bilayer and increase as the spin label approaches the hydrophobic core. The location of 5-SASL has been shown to be near the carbonyl region of the lipid backbone (Ellena *et al.*, 1989). These data suggest that the spin-labeled site is located slightly below that level.

DISCUSSION

A cysteine-33 analog of the chimeric peptide cecropin AD was synthesized, spin-labeled, and used to examine the energetics of membrane binding and the conformation of cecropins in phospholipid bilayers. The goals of this study were to relate functional properties of cecropins to their structural features by examining the mode of interaction with membrane lipids and to test previously proposed models. Recently, we utilized this peptide to examine the aggregation state of cecropin in solution (Mchaourab *et al.*, 1993). It was demonstrated that cecropins are monomeric in aqueous solution at pH 7, while they self-associate in 5–10% HFP. The relevance of peptide self-association to the mechanism of channel formation is examined in this paper.

Our data show that cecropin binding is mediated to a large extent by electrostatic interactions. A negative membrane surface potential is required for association of the peptide to the bilayer, and there is a strong dependence of the apparent partition coefficient on the surface potential.

An unexpected finding was the dependence of the peptide binding affinity on the fatty acid chain length. For POPC/POPG vesicles, the binding isotherm displayed a characteristic cooperativity suggesting self-association of peptide monomers in the bilayer, while for DLPC/DLPG vesicles, the curve was interpreted as simple monomer partitioning. ESR line-shape studies were consistent with peptide aggregation in the former lipid vesicles but not in the latter.

A rather surprising result was the calculated low effective charge of the peptide. It was obtained indirectly by fitting the DLPC/DLPG binding isotherm using the Gouy–Chapman theory. A similar reduction in the net charge was observed for the peptide melittin (Beschiaschvili & Seelig, 1990). One of the explanations for such a charge reduction is the discreetness of the charge in proteins and lipids. If this is the case for cecropins, then we would expect a larger intrinsic binding affinity for negatively charged lipids than for neutral lipids, consistent with the observed dependence of the monomer binding constant on surface potential. Furthermore, arginine residues seem to have little contribution to the total interfacial charge (Stankowski & Schwarz, 1989; Stankowski *et al.*, 1991). In fact, our data (Table 1) suggest that the curvature of the binding isotherms at large C_f values (Figures 4 and 7) might be due to repulsion among peptide monomers rather than to charging of the bilayer. A high degree of complexity is introduced by the dependence of almost all parameters on the surface potential. A detailed consideration of the energetics of binding requires understanding the various entropic and enthalpic terms involved in the transfer of the peptide from the water to the interface and then from the interface to the bilayer interior. However, on the basis of the interpretation of the results, a tentative model for cecropin–membrane interaction is presented.

The simplest model that is qualitatively consistent with all our results must comprise the following features: (1) A membrane–water equilibrium where aqueous monomers undergo a secondary structure change upon binding. (2) A transbilayer conformation that allows the peptide to sense membrane thickness. (3) Aggregation of the peptide within the membrane and/or increased membrane affinity at large extent of binding that is due to processes other than peptide self-association, *e.g.*, changes in lipid (Archer *et al.*, 1991). Our data do not allow us to distinguish between these two possibilities. Previous results strongly suggest that cecropins self-associate in bacterial membranes (Steiner *et al.*, 1988) and planar lipid bilayers (Chistensen *et al.*, 1988). Therefore, self-association is included in our model, despite the need for further experimental investigation.

A putative first step in membrane insertion involves the accumulation of the unfolded peptide monomers from the aqueous phase into the diffuse double layer, the plane of binding. The electrostatic interaction with negatively charged

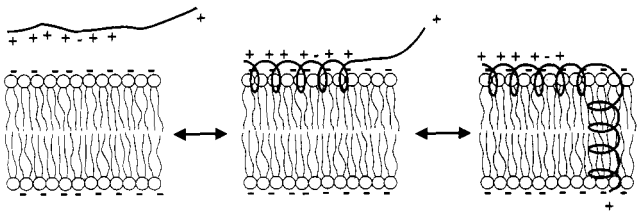

FIGURE 11: Schematic illustration of proposed cecropin–membrane interactions. The peptide in solution is in a largely random coil conformation. When cecropin associates with the membrane, its helical content increases. The helical hairpin structure is proposed to explain the dependence of peptide affinity on membrane thickness. Self-association probably involves the initial formation of membrane-bound dimers from independently stable helices.

lipid headgroups at the bilayer interface lowers the energy barrier for folding by increasing the proximity of amide and carbonyl groups, promoting formation of the N terminal helix (Jacobs & White, 1989). Evidence for this initial step includes the strong dependence on membrane surface potential observed in our studies. These electrostatic interactions will help to offset the unfavorable free-energy contribution from peptide immobilization and folding. Upon binding, the peptide loses two rotational and one translational degree of freedom, leading to an energy cost of 12 kcal/mol (Beschiaschvili & Seelig, 1990). Further conformational restriction due to a particular geometrical assembly (see Figure 11) will involve a larger entropic loss, and hence, this value provides only a lower limit. In that respect, the bilayer interface may act as a catalytic surface that allows the peptide to undergo a rate-limiting change of secondary structure formation that is prerequisite for membrane insertion. Therefore, interaction between anionic lipids and positively charged N terminal residues, along with insertion of Trp 2 into the bilayer, provides a mechanism for anchoring the peptide at the membrane–solution interface (Jacobs & White, 1989).

Following accumulation of peptide at the membrane surface, a further gain in free energy can be achieved by removing the C terminal hydrophobic side chains from the aqueous phase and inserting them into the bilayer as depicted in Figure 11. However, the terminal lysine causes considerable energetic cost if buried in the hydrophobic core (Engleman & Steitz, 1981). Therefore, the C terminal helix most likely must span the entire hydrophobic region of the bilayer. The vertical insertion of this segment is supported by two lines of evidence: the dependence of the mode of binding on the membrane thickness and the location of the spin-labeled site. We propose that the differential affinity toward bilayers of different thickness is related directly or indirectly to the conformation imposed by the requirement that the terminal lysine reach the phosphate group of the opposite leaflet. The hydrophobic core thickness of a POPC bilayer is around 27 Å, while that of a DLPC bilayer is 20 Å (Lewis & Engelman, 1983). Assuming that the C terminal helix extends for 13 residues (Pro 24–Lys 37), the terminal lysine will be 19.5 Å away from Pro 24. Durell *et al.* proposed that due to the length mismatch, the N terminal helix has to be sunken into the lipid headgroup. Since the absence of a cationic residue in a suitable helix position makes it unlikey that the two negatively charged residues are involved in contacts within a single peptide, this conformation brings these residues into close proximity to the phosphate headgroup. Therefore, dimerization that involves the formation of a salt bridge between Arg 16 and Glu 9 of adjacent monomers might be energetically more favorable in POPC/POPG vesicles than in DLPC/DLPG vesicles. Protonation of the acidic residues should eliminate their putative electrostatic repulsion with lipid phosphates, and our data show that at acidic pH, no cooperativity is detected in POPC/POPG vesicles and the monomer binding affinity is increased. This is consistent with a critical role of these acidic residues in binding and self-association of the peptide.

The helical hairpin-like structure, however, is not the only possible conformation of membrane-bound cecropin. It has been suggested that cecropin can be induced to exist in an extended helical conformation for most of its length when bound to the membrane–solution interface (Fink *et al.*, 1989a). Certainly, the changes in the ESR line shapes that were attributed to aggregate formation are not those expected for large aggregates or extensive aggregation. These changes may be due to dimer formation, in which case a transmembrane voltage is needed to induce aggregation. Alternatively, it is possible that the monomeric peptide can exist in an equilibrium between at least two conformations, one of which, we believe, has the C terminal helix spanning the hydrophobic core and thus is compatible with oligomer formation. Therefore, the self-associated form might be only a fraction of the total membrane-bound peptide.

Cooperativity can also arise from lipid–protein interactions that lead to changes in the free energy of binding. Archer *et al.* (1991) reported that despite the apparent cooperative binding curve of the peptide alamethicin, they did not detect any evidence of spin–spin interactions between the labeled monomers. They suggest that the cooperativity can result from changes in lipid morphology that accompany peptide binding. It has previously been shown that binding of the positively charged peptide melittin to phosphatidylcholine vesicles leads to structural alterations in lipid headgroups (Beschiavili & Seelig, 1991). However, in that particular case, the binding curves of melittin did not display any cooperativity.

The oxygen profile of the membrane-bound spin-labeled peptide suggest that residue 33 is located just below the headgroup region. This is consistent with the location of that residue if the C terminal helix is inserted vertically into the hydrophobic core.

CONCLUSION

We have used a spin-labeled cecropin analog to examine the binding affinity of the cecropins to the membrane and to determine the factors involved in modulating their binding constant and conformation. A model for membrane association and insertion that is consistent with our data and encompasses many of the features deduced from functional assays and structural studies is proposed. Important differences between this and previous models are the monomeric state of the aqueous peptide and the formation of peptide aggregates from independently stable helices (Popot & Engelman, 1990), as suggested by the binding isotherms. An interesting finding was the influence of bilayer thickness on peptide self-association, which seems to be a direct consequence of length mismatch between the C terminal helix and the bilayer. An important extension of this work will be to examine conformational and aggregation states in the presence of a transmembrane potential. To further examine the transbilayer configuration of the C terminal helix, one can introduce spin-labeled sites sequentially in that region of the peptide and determine their relative exposure to molecular oxygen.

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REFERENCES

Andreu, D., Merrifield, R. B., Steiner, H., & Boman, H. G. (1985) *Biochemistry 24*, 1683–1688.

Andrew Woolley, G., & Wallace, B. A. (1992) *J. Membr. Biol. 129*, 109–136.

Archer, S.-J., Ellena, J. F., & Cafiso, D. S. (1991) *Biophys. J. 60*, 389–398.

Barlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Beschiaschvili, G., & Seelig, G. (1990) *Biochemistry 29*, 52–58.

Boman, H. G., Faye, I., Gudmundsson, G. H., Lee, J. Y., & Lidholm, D. A. (1991) *Eur. J. Biochem. 201*, 23–31.

Castle, J. D., & Hubbell, W. L. (1976) *Biochemistry 15*, 4818–4831.

Christensen, B., Fink, J., Merrifield, R. B., & Mauzerall, D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5072–5076.

Durell, S. R., Ragunathan, G., & Guy, R. H. (1992) *Biophys. J. 63*, 1623–1631.

Ellena, J. F., Archer, S. J., Dominey, R. N., Hill, B. D., & Cafisco, D. S. (1989) *Biochim. Biophys. Acta 940*, 63–70.

Engelman, D. M., & Steitz, T. A. (1981) *Cell 23*, 411–422.

Fink, J., Boman, A., Boman, H. G. & Merrifield, R. B. (1989a) *Int. J. Pept. Protein Res. 33*, 412–421.

Fink, J., Merrifield, R. B., Boman, A., & Boman, H. G. (1989b) *J. Biol. Chem. 264*, 6260–6267.

Hyde, J. S., & Fronsicz, W. (1989) *Advanced ESR. Applications in Biology and Biochemistry*, Elsevier, Amesterdam.

Grennlagh, D. A., Altenbach, C., Hubbell, W. L., & Khorona, H. G. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8226–8230.

Holak, T. A., Engström, Å., Kraulis, P. J., Lindeberg, G., Bennich, H., Jones, T. A., Groenenborn, A. M., & Clore, G. M. (1988) *Biochemistry 27*, 7260–7269.

Jacobs, R. E., & White, S. H. (1989) *Biochemistry 28*, 3421–3437.

Kim, J., Mosior, M., Chung, L. A., Wu, H., & McLaughlin, S. (1991) *Biophys. J. 60*, 135–148.

Lewis, B. A., & Engelman, D. M. (1983) *J. Mol. Biol. 166*, 211–217.

Mchaourab, H. S., & Hyde, J. S. (1993) *J. Magn. Reson. 101*, 178–184.

Mchaourab, H. S., Hyde, J. S., & Feix, J. B. (1993) *Biochemistry 44*, 11895–11902.

Mosior, M., & Mclaughlin, S. (1992) *Biochim. Biophys. Acta 1105*, 185–187.

Popot, J. L., & Engelman, D. M. (1990) *Biochemistry 29*, 4031–4037.

Press, W. H., Flannery, B. P., Teukolsky, S. A., & Vetterling, W. T. (1990) *Numerical recipes in C*, Cambridge University Press, Cambridge.

Rizzo, V., Stankowski, S., & Schwarz, G. (1987) *Biochemistry 26*, 2751–2759.

Schwarz, G. (1989) *Biochimie 71*, 1–9.

Sczaniecki, P. B., Hyde, J. S., & Froncisz, W. (1991) *J. Chem. Phys. 94*, 5907–5916.

Stankowski, S., & Schwarz, G. (1989) *Biochim. Biophys. Acta 1025*, 164–172.

Stankowski, S., Schwarz, V. D., & Schwarz, G. (1989) *Biochim. Biophys. Acta 941*, 11–18.

Stankowski, S., Wey, J., Kalb, E., & Goundis, D. (1991) *Biochim. Biophys. Acta 1068*, 61–67.

Steiner, H. (1982) *FEBS Lett. 137*, 283–287.

Steiner, H., Andreu, D., & Merrifield, R. B. (1988) *Biochim. Biophys. Acta 939*, 260–266.

Wade, D., Boman, A., Wåhlin, B., Drain, C. M., Andreu, D., Boman, H. G., & Merrifield, R. B. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4761–4765.