Electrostatic Binding of Proteins to Membranes. Theoretical Predictions and Experimental Results with Charybdotoxin and Phospholipid Vesicles

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ABSTRACT We previously applied the Poisson-Boltzmann equation to atomic models of phospholipid bilayers and basic peptides to calculate their electrostatic interactions from first principles (Ben-Tal, N., B. Honig, R. M. Peitzsch, G. Denisov, and S. McLaughlan. 1996. Binding of small basic peptides to membranes containing acidic lipids. Theoretical models and experimental results. *Biophys. J.* 71:561–575). Specifically, we calculated the molar partition coefficient, K (the reciprocal of the lipid concentration at which ½ the peptide is bound), of simple basic peptides (e.g., pentalysine) with phospholipid vesicles. The theoretical predictions agreed well with experimental measurements of the binding, but the agreement could have been fortuitous because the structure(s) of these flexible peptides is not known. Here we use the same theoretical approach to calculate the membrane binding of two small proteins of known structure: charybdotoxin (CTx) and iberiotoxin (IbTx); we also measure the binding of these proteins to phospholipid vesicles. The theoretical model describes accurately the dependence of K on the ionic strength and mol % acidic lipid in the membrane for both CTx (net charge +4) and IbTx (net charge +2). For example, the theory correctly predicts that the value of K for the binding of CTx to a membrane containing 33% acidic lipid should decrease by a factor of 10^5 when the salt concentration increases from 10 to 200 mM. We discuss the limitations of the theoretical approach and also consider a simple extension of the theory that incorporates nonpolar interactions.

INTRODUCTION

Many important peripheral proteins contain clusters of basic residues that interact electrostatically with acidic lipids in membranes. Examples include cytochrome C (Pinheiro, 1994; Pinheiro and Watts, 1994a, b; Heimburg and Marsh, 1995, 1996), myelin basic protein (MacNaughtan et al., 1985), protein kinase C (Newton, 1995), phospholipases (Roberts, 1996), Src (Resh, 1993, 1994; Buser et al., 1994; Sigal et al., 1994), myristoylated alanine-rich C-kinase substrate (MARCKS) (Aderem, 1992; Blackshear, 1993; McLaughlin and Aderem, 1995), HIV matrix protein (Zhou et al., 1994; Massiah et al., 1994; Hill et al., 1996), K-Ras (Hancock et al., 1990; Cadwallader et al., 1994), and human carbonic anhydrase IV (Stams et al., 1996).

Several groups have attempted to calculate these interactions using classical electrostatics, i.e., by solving the Poisson-Boltzmann (PB) equation (Yoon and Lenhoff, 1992; Roush et al., 1994; Roth and Lenhoff, 1993; Ben-Tal et al., 1996). In the mapping of molecular systems into the parameters of the PB equation the solvent, water, is treated implicitly and is represented as a structureless region with high dielectric constant. The solutes, e.g., the protein/peptide and the bilayer, are assigned a low dielectric constant. The description of the solutes ranges from highly simplistic geometric approximations, such as a ball and a plane (Roth and Lenhoff, 1993) to representations with atomic detail (Ben-Tal et al., 1996). In these treatments the molar partition coefficient, K, (the reciprocal of the lipid concentration at which half the protein/peptide is adsorbed) has been calculated by evaluating the Gibbs surface excess of protein/peptide adjacent to the membrane. The Gibbs surface excess (e.g., Bockris and Kahn, 1993; Fig. 1 of Ben-Tal et al., 1996) is the integral of the difference between the concentration of protein/peptide near the membrane and its bulk concentration. The Boltzmann equation describes the dependence of the protein/peptide concentration on its free energy of interaction with the membrane. The electrostatic component of this free energy can be calculated by solving the PB equation.

We have studied the membrane binding of basic peptides such as pentalysine and compared the theoretical predictions of the molar partition coefficient with the experimental results. The theory predicted that the binding should depend on the ionic strength of the aqueous solution, the mol % of acidic lipids in the membrane, and the number of basic residues in the peptide; these predictions agreed well with our experimental measurements. The agreement could, however, have been fortuitous. These small basic peptides are flexible, whereas the model assumes that they have only one (extended) conformation. We wanted to test our theoretical approach with proteins that interact electrostatically with membranes, and chose to study two basic toxins of known structure.

We measured the membrane binding of two basic miniglobular proteins of the α-K-toxin family: the radioactively labeled α-KTx1.1 or charybdotoxin (CTx) has a net charge

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of +4, and the labeled α-KTx1.3 or iberiotoxin (IbTx) has net charge of +2. These 37-residue toxins are found in the venom of scorpions and have been used to probe the activity of potassium channels (Miller, 1995). Their amino acid sequences are ~70% identical and their structure, determined from multidimensional nuclear magnetic resonance (Bontems et al., 1991a, b; 1992; Johnson and Sugg, 1992), reveals that they share the same fold: a two-turn α-helix affixed by three disulfide bonds to a three-stranded β-sheet. We chose to use these toxins because they are positively charged hydrophilic molecules with a well defined structure. They each have three disulfide bonds and are therefore unlikely to change their conformation when they adsorb through nonspecific interactions to the surface of a bilayer membrane.

We have extended our model by introducing nonpolar contributions to the binding. Following an approach used to study stability and binding in proteins (e.g., Honig et al., 1993; Froloff et al., 1997), the nonpolar component is assumed to be proportional to the reduction of the water-accessible surface area of the protein and the membrane; these surfaces decrease as the protein approaches the membrane.

We compare the molar partition coefficient predicted by the theoretical model with our measurements. We also discuss the limitations of the model, which assumes the lipid bilayer is a rigid surface. Our main conclusion is that the theoretical approach works well when the distance between the protein and the bilayer is large and the precise structural information is not very important; it describes adequately the effect of the Coulomb attraction on the binding. At short distances, however, water molecules no longer separate the toxin and the bilayer, and desolvation phenomena may occur. These give rise to an electrostatic Born repulsion and a nonpolar attraction between the proteins and the "soft" surface of the membrane. Our model and theoretical treatment of these phenomena is highly oversimplified.

MATERIALS AND METHODS

Materials

Lipids

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (PS) were obtained as solutions in $CHCl_3$ from Avanti Polar Lipids (Alabaster, AL). 1,2-Di[1-^{14}C]oleoyl-L-3-phosphocholine ([^{14}C]PC) was purchased from Amersham (Arlington Heights, IL).

Proteins

Both CTx and IbTx were tritium-labeled according to the "spinster-cysteine" strategy described previously (Shimony et al., 1994). Briefly, synthetic genes encoding these peptides were expressed in *Escherichia coli* (Park et al., 1991), except that a unique reduced cysteine residue was substituted at position 19. The purified, cysteine-substituted protein was then reacted with 3[H]-*N*-ethylmaleimide (20-40 Ci/mmol), and the resulting labeled protein was purified to homogeneity by C18 reversed-phase HPLC. Tritiated CTx and IbTx were dissolved to final concentrations >1 nM in 100 mM NaCl/10 mM MOPS, pH 7.0, and stored in aliquots at −20°C.

Methods

Preparation of vesicles

We determined the concentrations of PC and PS in $CHCl_3$ by measuring the weight of a dried sample on a Cahn electrobalance, a method that gives the same results as phosphate analysis (Kim et al., 1991; Peitzsch and McLaughlin, 1993). A mixture of PC and PS in a $CHCl_3$ solution was dried under vacuum in a rotary evaporator and resuspended in a sucrose solution (e.g., 176 mM sucrose, 1 mM MOPS, pH 7.0). The method of Hope et al. (1985) was used to produce large unilamellar vesicles (LUVs): lipid dispersions were taken through 5 cycles of freezing in liquid N_2 and thawing in a 40°C water bath followed by 10 cycles of extrusion through a stack of two polycarbonate filters (100-nm diameter pore size) in a Lipex Biomembranes Extruder (Vancouver, BC, Canada). The resulting vesicles are unilamellar and have a diameter of ~100 nm (Mui et al., 1993). The sucrose solution on the outside of the vesicles was removed by mixing the LUVs with an isosmotic salt solution (e.g., 100 mM KCl, 1 mM MOPS, pH 7.0) and centrifuging the solution [1 h, 100,000 *g*, 25°C; for details see Rebecchi et al. (1992), Buser et al. (1994), Buser and McLaughlin (1997)]. We used vesicles in the resuspended pellet for the binding measurements, and monitored the lipid concentration by incorporating a trace amount of [^{14}C]PC into the lipid mixture.

Binding measurements

Under our conditions ([toxin] ≪ [lipid]) the toxins bind only a small fraction of the acidic lipid in the vesicles. The toxins were equilibrated with sucrose-loaded LUVs for 15 min at room temperature (22°C), then separated by centrifugation (1 h, 100,000 *g*, 25°C). The supernatant was separated from the pellet immediately, and the concentrations of toxin in both the supernatant and pellet were determined using a Beckman LS3801 Liquid Scintillation System. Separate channels were used to measure the ^{3}H of the toxin and the ^{14}C of the lipid. We corrected the percent bound toxin for the 1–5% of the lipid that remained in the supernatant. Details of the experimental technique, which is formally equivalent to an equilibrium dialysis measurement, are discussed in Buser and McLaughlin (1997).

Determining the binding constant, *K*, and the binding free energy, Δ*G*, from experimental measurements

As discussed in Peitzsch and McLaughlin (1993; see their Eq. 1), the binding of proteins to lipid bilayers can be described by defining a molar partition coefficient, K, that does not require any assumptions about the adsorption mechanism. K is the proportionality constant between the mole fraction of protein bound to the membrane, $\chi = [P]_m/([P]_m + [L])$, and the molar concentration of protein in the bulk aqueous phase, $[P]$. The molar concentration of lipid accessible to the toxin, $[L]$, is much greater than the molar concentration of toxin bound to the membrane, $[P]_m$, under our conditions. If $[L] \gg [P]_m$, $\chi \approx [P]_m/[L]$ and

$$[P]_m = K[P][L] \tag{1}$$

The hydrophilic toxins we studied should not permeate the LUVs. Thus they bind only to the outer surface of the vesicles and $[L] = [L]_{tot}/2$, where $[L]_{tot}$ is the total molar concentration of lipid in the solution.

The total concentration of toxin in the solution, $[P]_{tot}$, is the sum of the bound and free concentrations: $[P]_{tot} = [P] + [P]_m$. Substituting this expression in Eq. 1 we obtain

$$\frac{[P]_m}{[P]_{tot}} = \frac{K[L]}{1 + K[L]}. \tag{2}$$

We determine the value of K by measuring the fraction of bound toxin, $[P]_m/[P]_{tot}$, as a function of $[L]$. We plot $[P]_m/[P]_{tot}$ vs. $[L]$ and obtain the value of K from a least squares fit of Eq. 2 to the data. Note that K is the reciprocal of the lipid concentration that binds half the toxin.

We stress that when we calculate the molar partition coefficient by fitting Eq. 2 to the experimental data, we are not assuming the peptide forms a 1:1 complex with a lipid even though Eq. 2 has the same form as a conventional Langmuir adsorption isotherm or mass action treatment that does assumes 1:1 complexes are formed. White et al. (1997) discuss the importance of using partition coefficients (rather than assuming 1:1 complexes are formed) when dealing with the nonspecific hydrophobic or electrostatic adsorption of peptides to membranes; Peitzsch and McLaughlin (1993) give conversion factors between the different commonly used partition coefficients. Of course all adsorption isotherms (e.g., Langmuir, Volmer) reduce to Henry's law when the peptide concentration is low and thus all have the same form as simple partition equations under our experimental conditions. When we refer to a peptide such as pentalysine or CTx as "bound" or "adsorbed" to the membrane we refer to the Gibbs surface excess of the peptide, which is illustrated in Fig. 1 of Ben-Tal et al. (1996) and discussed in standard texts (e.g., Bockris and Khan, 1993). Specifically, a CTx molecule accumulated in the diffuse double layer will be measured as a bound peptide even though it does not physically contact the membrane.

We determine the value of K experimentally (Eq. 2) and calculate its value theoretically (Eq. 4 below). In theoretical studies, however, it is conventional to represent binding in energy units rather than in the units of a partition coefficient. If we assume (incorrectly) that the toxin forms a 1:1 complex with a lipid, the molar partition coefficient is related to the standard Gibbs free energy by

$$\Delta G = -NkT \ln(K), \tag{3}$$

where N is Avogadro's number, k is Boltzmann's constant, and T is the temperature. Thus we use Eq. 3 merely as a formalism to convert both our experimental and theoretical values of K into energy units. We refer below to ΔG as the experimentally determined Gibbs free energy, or simply the binding free energy.

THEORETICAL MODEL

We represented the toxin molecule and the lipid bilayer in atomic detail and the solvent as a homogeneous medium of constant dielectric. Both the Debye length and the dimensions of CTx and IbTx are much smaller than the radius of the vesicles so we can assume the toxin molecules interact with a planar surface. The toxin molecules do not interact with each other so we can analyze the binding of a single toxin molecule to the surface. The model is similar to the one we used to analyze the membrane binding of basic peptides such as pentalysine (Ben-Tal et al., 1996); it also resembles continuum solvent models used to study stability and binding in proteins (Honig et al., 1993; Honig and Nicholls, 1995).

The structure of CTx was taken from Bontems et al. (1991a) and that of IbTx from Johnson and Sugg (1992). PDB files were kindly provided by Drs. F. Toma and B. Johnson. Residue 19 of each toxin (Arg in CTx and Asp in IbTx) was replaced with a Cys residue using the Insight/Biopolymers molecular modeling package (MSI). The Cys residue is bound covalently to *N*-ethylmaleimide (NEM). In the text below, we refer to the NEM-labeled CTx(R19C) and IbTx(D19C) as CTx and IbTx because these analogs were used for both the modeling and experimental studies. We construct lipid bilayers as described previously (Ben-Tal et al., 1996) and assume their structures do not change when the toxins bind. The zwitterionic PC and negatively charged PS lipids in each leaflet are uniformly distributed in a hexagonal lattice.

We built atomic models with the toxin molecules in different configurations relative to the lipid bilayers; one of them is shown in Fig. 1. Each configuration is determined by six coordinates: three cartesian coordinates (x, y, z) define the location of the geometrical center of the toxin molecule relative to the center of the membrane section, and three angular coordinates (θ, α, η) define its orientation with respect to the bilayer. The rotational angles were measured relative to the orientation of Fig. 1. θ denotes rotations of the toxin molecule around z, the normal to the membrane surface, α denotes rotation of the toxin molecule around x, an axis parallel to the membrane surface in the plane of the figure, and η denotes rotation of the toxin around y, an axis parallel to the membrane surface and perpendicular to the plane of the figure.

We define R as the minimal distance between the van der Waals surfaces of the toxin molecule and the lipid bilayer along the z-axis, with $R = 0$ at van der Waals contact. Denoting the free energy of interaction

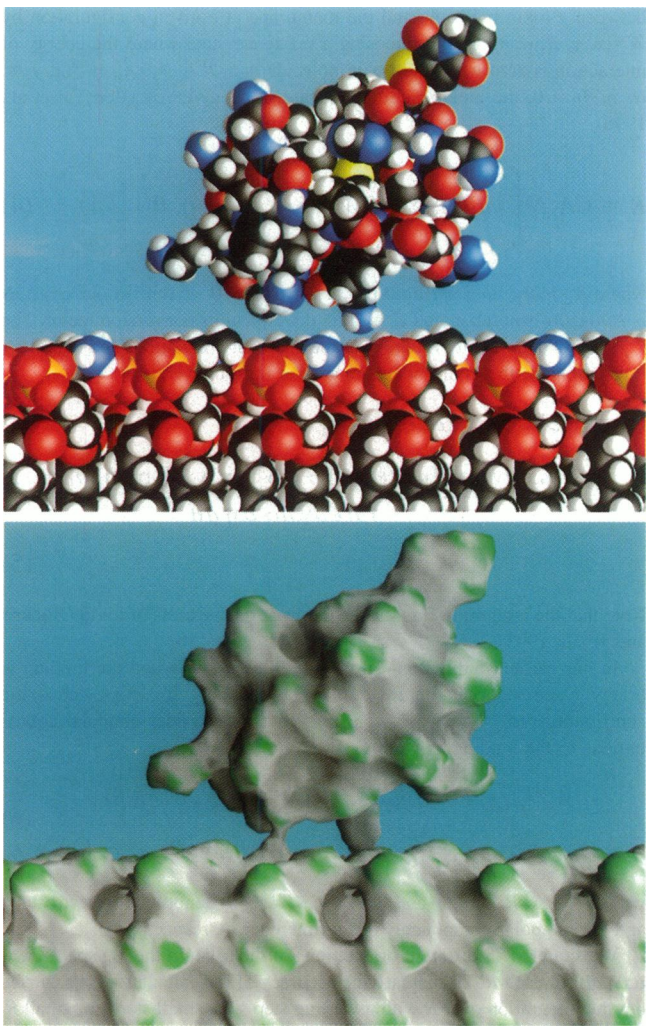

FIGURE 1 Molecular model of a portion of a PC/PS (3:1) bilayer membrane and labeled CTx. (*upper*) The van der Waals surfaces. The PS lipids in the membrane can be recognized by their blue nitrogen atoms. The oxygen atoms are red, the phosphorus atoms orange, sulphur atoms yellow, the carbon atoms grey, and the hydrogen atoms white. The toxin molecule is oriented with Cys-19 and its covalently bound NEM facing away from the surface of the lipid bilayer and four positively charged residues facing this surface. The membrane is oriented with the normal to its surface along the z-axis. The minimal distance between the van der Waals surfaces of the toxin and the membrane in this configuration is $R = 2$ Å. (*lower*) The molecular surface created by rolling a sphere with the radius of a water molecule, 1.4 Å, on the atomic model above. The surface is colored in grey and green to emphasize the curvature. Notice that, where the van der Waals surfaces of CTx and the lipid bilayer are <2.8 Å from each other in this configuration, their molecular surfaces are united. The figure was drawn using GRASP (Nicholls et al., 1991).

between a toxin molecule and the membrane at a given configuration by $W^{int}(x, y, R, \alpha, \eta, \theta)$ and choosing a reference state where the energy of interaction is zero when the toxin molecule is at $R = \infty$, i.e., $W^{int}(x, y, \infty, \alpha, \eta, \theta) = 0$, the molar binding constant, K, is given by (Ben-Tal et al., 1996)

$$K = CA_L N \int_0^\infty dR \langle \exp[-\beta W^{int}(x, y, R, \alpha, \eta, \theta)] - 1 \rangle \qquad (4)$$

where A_L is the area per lipid (68 Å^2 = 68 × 10^{-18} dm^2 in our case), N is Avogadro's number, and $C = 10^{-9}$ dm/Å, a units conversion factor. The $\langle\ \rangle$ symbol denotes an average over the different orientations and lateral x y translations of the toxin molecule and the lipid bilayer:

$$\langle \exp[-\beta W^{int}(x, y, R, \alpha, \eta, \theta)] - 1 \rangle = \frac{\int dx\, dy\, d\alpha\, d\eta\, d\theta [\exp[-\beta W^{int}(x, y, R, \alpha, \eta, \theta)] - 1]}{\int dx\, dy\, d\alpha\, d\eta\, d\theta} \qquad (5)$$

Note that the integral in Eq. 4 is the Gibbs surface excess of toxin (Bockris and Khan, 1993).

In our earlier study (Ben-Tal et al., 1996) we assumed the binding is purely electrostatic in nature and accounted only for the electrostatic contributions to $W^{int}(x, y, R, \alpha, \eta, \theta)$. Here we consider nonpolar contributions to the binding as well:

$$W^{int}(x, y, R, \alpha, \eta, \theta) = W^{elc}(x, y, R, \alpha, \eta, \theta) + W^{np}(x, y, R, \alpha, \eta, \theta) \qquad (6)$$

with $W^{elc}(x, y, R, \alpha, \eta, \theta)$ and $W^{np}(x, y, R, \alpha, \eta, \theta)$ denoting the electrostatic and the nonpolar components of the free energy, respectively.

We sampled 78 different orientations of the toxin in the 5D space (x, y, α, η, θ) for each R. To increase the accuracy of the calculations we carried out the integration over R with a varying step size; small steps for small R, where the toxin concentration depends strongly on R, and larger steps for large R, where the toxin concentration does not depend strongly on R (see below).

Electrostatic contributions

We calculated $W^{elc}(x, y, R, \alpha, \eta, \theta)$ for each of the 78 configurations by solving the (nonlinear) Poisson-Boltzmann equation following the procedure described in Ben-Tal et al. (1996).

We assigned each of the atoms of the toxin molecule and lipid bilayer a radius and a charge, using the same set of atom charges and radii reported previously; the charges and radii of the amino acids were derived from the CHARMM22 forcefield (Brooks et al., 1983), and those for the lipids are from Peitzsch et al. (1995). The toxin molecule and the membrane were mapped onto a cubic lattice of 129^3 grid points. The molecular surfaces of the toxin and the membrane (Fig. 1 *lower*) are defined as the point of contact between a spherical probe with the radius of a water molecule (1.4 Å) and the van der Waals surface (Fig. 1 *upper*). The spaces enclosed by the molecular surfaces, or interior regions, were assigned a low dielectric constant of 2. The space outside the molecular surfaces, or the exterior region, was assigned a high dielectric constant of 80. Salt ions were excluded from a 2 Å-wide region adjacent to the van der Waals surface of the toxin or the membrane.

We solved the (nonlinear) Poisson-Boltzmann equation (Eq. 21 of Ben-Tal et al., 1996) for each configuration of the toxin and the membrane, and calculated $W^{elc}(x, y, R, \alpha, \eta, \theta)$ at this configuration from the spatial distribution of the charges and the electrostatic potential [using equations 17–20 of Ben-Tal et al. (1996)]. We tested the convergence of the results with respect to the lattice size and scale: increasing the grid box from 129^3 to 193^3 points (at a constant scale of 2 grids/Å) alters the electrostatic free energy of interaction of CTx with a 2:1 PC/PS membrane in 100 mM monovalent salt by <0.3 kcal/mol. The convergence with respect to lattice scale depends on the distance between the peptide and the membrane and on their relative orientation. The results obtained using a grid box of 129^3 points and scales of 0.5, 1.0, and 2.0 grids/Å differed by <0.3 kcal/mol when at least one water molecule fit between the van der Waals surfaces of the membrane and the peptide; the depth of the minimum in the electrostatic free energy, which dominates the electrostatic component of the binding free energy (see Eq. 4), changed by <0.2 kcal/mol. We estimate that the electrostatic contribution to the free energy of binding deduced from these calculations is accurate to at least 0.5 kcal/mol.

Nonpolar contributions

Work from many laboratories has demonstrated the importance of nonpolar contributions, $W^{np}(x, y, R, \alpha, \eta, \theta)$, to the free energy even for the interaction between polar and/or charged surfaces, and suggested that $W^{np}(x, y, R, \alpha, \eta, \theta)$ can be estimated using a surface tension coefficient (Nozaki and Tanford, 1971; Hermann, 1972; Chothia, 1976). We calculated $W^{np}(x, y, R, \alpha, \eta, \theta)$ for each of the 78 configurations by assuming it is proportional to the water-accessible surface area, A, of the toxin-membrane complex

$$W^{np}(x, y, R, \alpha, \eta, \theta) = \gamma(A - A^{max}) \qquad (7)$$

where the surface tension coefficient γ = 28 cal/mol/Å^2, a value derived from the partitioning of alkanes between water and liquid alkanes (Sitkoff et al., 1996). We calculated the water accessible area, A, using a modified Shrake and Rupley (1973) algorithm (Sridharan et al., 1992). $A = A^{max}$, its maximal value for configurations where at least one layer of water molecules fits between the toxin and the lipid bilayer; for these configurations $W^{np}(x, y, R, \alpha, \eta, \theta) = 0$. In configurations where the distance between the van der Waals surfaces of the toxin molecules and the lipid bilayer are <2.8 Å at various points along the z-axis (e.g., Fig. 1), water molecules are excluded from these regions, and the water-accessible area decreases. This gives rise to attractive nonpolar interactions.

The accuracy of this approach depends both on knowing the structure of the surfaces of the toxin and the lipid bilayer that are desolvated, and on having a set of atomic charges and radii that describe desolvation phenomena for these molecules. We are, therefore, more confident of our treatment of the long-range Coulombic attraction than of the short-range desolvation phenomena.

RESULTS

Experimental measurements

Fig. 2 illustrates the binding of labeled CTx to vesicles containing 33% acidic lipid (2:1 PC/PS). The experimental results are described well by Eq. 2 (*curves* in Fig. 2). A least squares fit of Eq. 2 to the data in Fig. 2 gives the molar partition coefficients for CTx illustrated in Fig. 3 *A*.

Fig. 3 *A* illustrates how the molar partition coefficients, K, of CTx (*open circles*) and IbTx (*filled circles*) vary with the ionic strength of the solutions. Note that a 10-fold increase in the ionic strength, from 10 to 100 mM, produces about a 3000-fold decrease in the binding constant for CTx (valence = +4) and a 400-fold decrease in binding constant for IbTx (valence = +2). At salt concentrations >100 mM for IbTx or 200 mM for CTx, the affinities of the toxins for 2:1 PC/PS vesicles become too weak to measure experimentally using this approach. The results shown in Fig. 3 *A* are consistent with our working hypothesis that these toxins

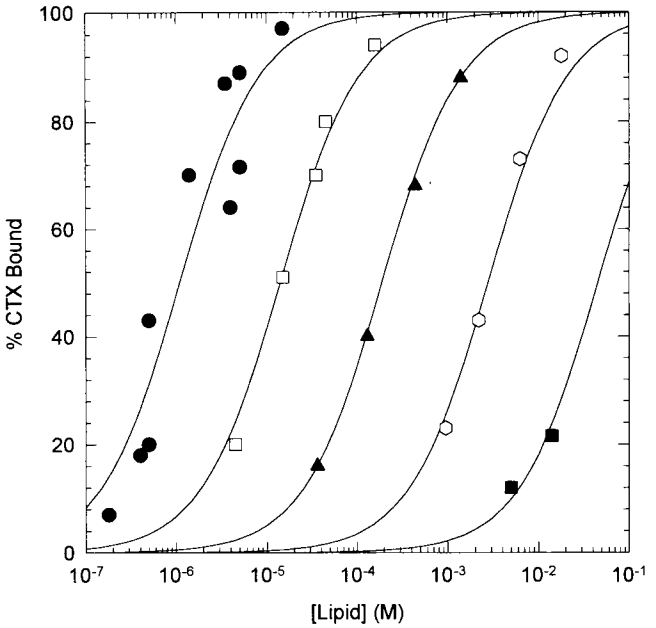

FIGURE 2 Binding of labeled charybdotoxin (CTx) to large unilamellar vesicles (LUVs) formed from a 2:1 PC/PS mixture. The solutions bathing the vesicles contained different concentrations of KCl: 10 (*filled circles*), 25 (*open squares*), 50 (*filled triangles*), 100 (*open hexagons*), or 200 (*filled squares*) mM KCl plus 1 mM MOPS, pH = 7.0, T = 25°C. The percent CTx bound to the sucrose-loaded vesicles is plotted against the concentration of lipid accessible to CTx. Fifty percent of the total concentration of lipid is in the outer leaflet of the vesicle. The curves are drawn according to Eq. 2. The molar partition coefficient, K, is the reciprocal of the accessible lipid concentration that binds 50% of the toxin. There is significant scatter in the data obtained in the 10 mM KCl solution: because the value of K is high, the concentrations of lipid required are low, and lipid can be lost onto the pipette tips and centrifuge tubes used for the experiment. Limited data are available for the experiments in 200 mM KCl: for technical reasons it is difficult to perform experiments with [lipid] $> 10^{-2}$ M.

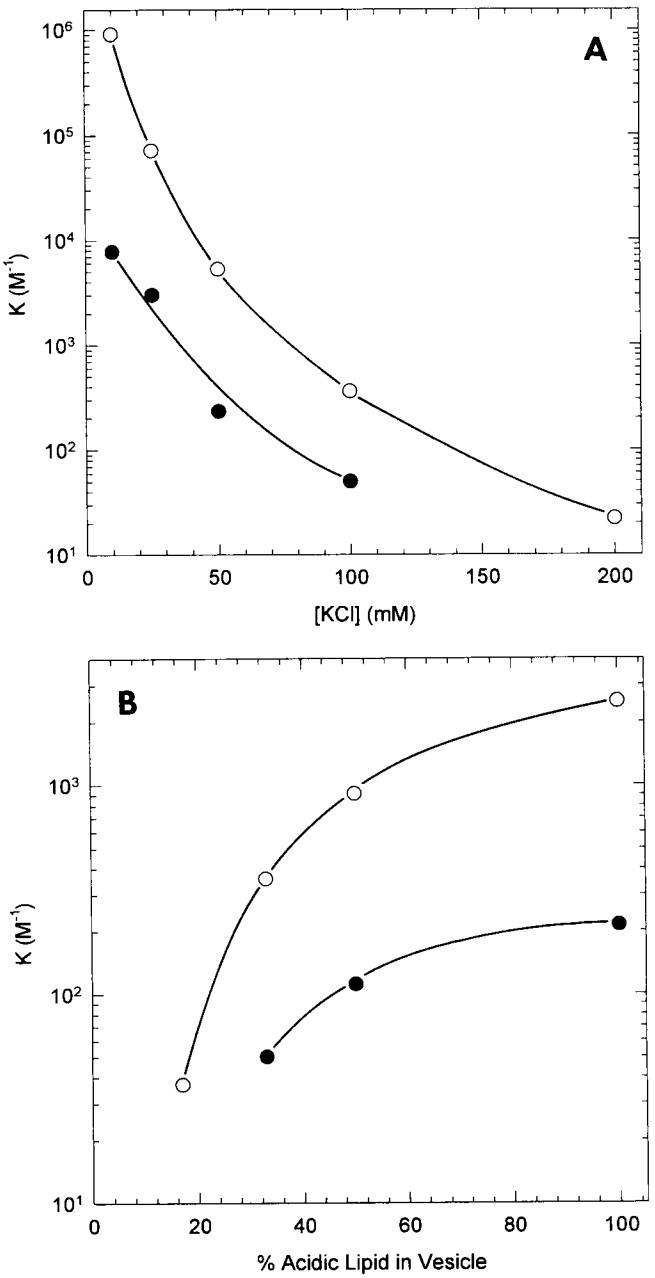

FIGURE 3 (*A*) The molar partition coefficient, K, of CTx (*open circles*) and IbTx (*filled circles*) with 2:1 PC/PS vesicles plotted as a function of [KCl]. The open circles were obtained from a fit of Eq. 2 to the data illustrated in Fig. 2. The filled circles were obtained from a fit of Eq. 2 to similar data obtained with IbTx (not shown). The lines through the points in *A* and *B* are drawn to guide the eye: they have no theoretical significance. (*B*) The molar partition coefficient of CTx (*open circles*) and IbTx (*filled circles*) plotted as a function of the mol % acidic lipid in the sucrose-loaded PC/PS vesicles. The solution bathing the vesicles contained 100 mM KCl, 1 mM MOPS, pH = 7.0, T = 25°C. These values of K were obtained from a fit of Eq. 2 to data similar to the open hexagons in Fig. 2, which illustrate the binding of CTx to vesicles containing 33% acidic lipid.

bind to phospholipid vesicles mainly via a simple electrostatic interaction. Increasing the ionic strength should decrease the membrane affinity of the CTx and IbTx for two reasons. First, the magnitude of the negative surface potential adjacent to the vesicles decreases at higher ionic strengths: e.g., the zeta potential [the potential at the hydrodynamic plane of shear, which is 0.2 nm from the surface (McLaughlin, 1989)] of vesicles containing 33% acidic lipid decreases from ~ -100 to -50 mV when the monovalent salt concentration increases from 10 to 100 mM (McLaughlin, 1989). Second, increasing the ionic strength from 10 to 100 mM decreases the Debye length (from ~3 to 1 nm), which implies that many of the positive charges on the toxin molecules experience less than the full value of the surface potential.

Fig. 3 *B* illustrates how the molar partition coefficient for the membrane binding of CTx (*open circles*) and IbTx (*filled circles*) depends on the mol % acidic lipid in the vesicle. The affinity of the toxins for electrically neutral PC vesicles is too weak to measure using this technique (data not shown), an observation consistent with our working hypothesis that these toxins interact with phospholipid membranes mainly through electrostatic interactions. The affinity of the toxins for the vesicles increases as the mole fraction of acidic lipid in the vesicle increases. Larger effects are seen for CTx (valence = +4) than for IbTx

(valence = +2). In summary, the experimental results we have obtained with the two toxins (Figs. 2 and 3) are consistent with our working hypothesis that binding is due mainly to a long-range Coulombic interaction between the positively charged residues on the toxin and the negatively charged lipids in the membrane.

Theoretical model

The most attractive orientation

We start by estimating the orientation where the toxin molecule is attracted most strongly to the membrane, i.e., the orientation of minimal (most negative) $W^{int}(\alpha, \eta, \theta)$. We sample around this orientation, so it is not obligatory to start at the precise minimum, but starting near the minimum reduces the number of sampled orientations needed for convergence.

The most attractive orientation should have the maximum number of positive charges facing the lipid bilayer because the binding is driven mainly by electrostatic interactions. Fig. 1 shows this orientation for CTx. Fig. 1 *upper* illustrates that four positively charged (two Lys and two Arg) and no negatively charged residues face the negatively charged lipid bilayer. IbTx has a similar orientation; three basic (one Arg and two Lys) and no acidic residues face the negatively charged membrane. Fig. 4 shows that the region of CTx with the most positive electrostatic potential is oriented toward the lipid bilayer in this configuration. (Note that Cys-19 with its covalently bound *N*-ethylmaleimide faces away from the surface.) Our sampling procedure includes changing the lateral (x, y) location of the toxin on the membrane. However, the calculations indicate a very weak dependence of the electrostatic component of the free energy of interaction of the toxins with the membranes, as long as R is larger than the diameter of a water molecule.

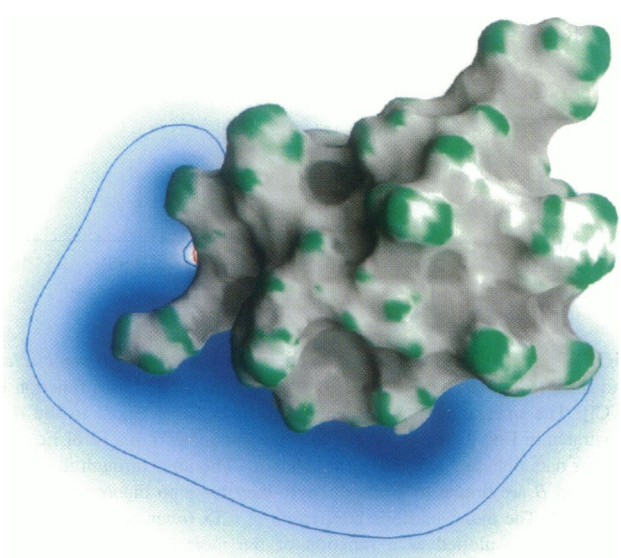

FIGURE 4 The electrostatic potential around CTx oriented as in Fig. 1, with its molecular surface colored in grey and green to emphasize the curvature. A color-code map shows the electrostatic potential in a cross section through the midplane of the molecule. The potential was calculated for CTx in 100 mM salt solution. Potentials more negative than −5 kT/e are deep red, potentials more positive than +5 kT/e are deep blue, and neutral potentials (0 kT/e) are white. The red and blue contour lines show the potential at −1 kT/e and +1 kT/e in the molecular midplane. The figure was drawn using GRASP (Nicholls et al., 1991).

The free energy as a function of R

Fig. 5 plots the electrostatic, nonpolar, and total free energy of interaction of CTx with a 2:1 PC/PS bilayer in 100 mM salt as a function of the distance R. The toxin molecule is oriented as in Fig. 1, i.e., with its most positively charged surface facing the lipid bilayer. The electrostatic free energy curve reflects only the Coulombic attraction between the positively charged toxin molecule and the negatively charged membrane for $R > 2.8$ Å, i.e., the minimum distance between the van der Waals surfaces is larger than the 2.8 Å diameter of a water molecule. For $R \leq 2.8$ Å a portion of the region between the toxin molecule and the membrane is no longer accessible to water and is assigned a low dielectric constant. When CTx approaches the membrane surface the charges on the protein and the membrane in this region are transferred to a low dielectric region, which gives rise to Born repulsion (see, for example, Parsegian, 1969; Andersen, 1978; Torrie et al., 1982; Ben-Tal, 1995).

The nonpolar interaction between CTx and the lipid bilayer is zero for $R > 2.8$ Å, i.e., when there is a layer of water molecules between CTx and the lipid bilayer, but becomes negative for $R \leq 2.8$ Å. Thus, the total free energy is identical to the electrostatic contribution for $R > 2.8$ Å, but becomes more negative as R decreases to smaller values.

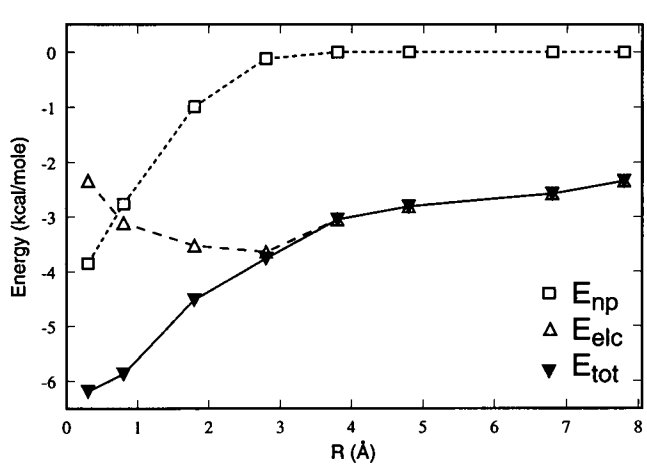

FIGURE 5 Calculated free energy curves. The electrostatic (*open triangles*), nonpolar (*open squares*), and total (*filled triangles*) free energy of interaction between CTx and a 2:1 PC/PS lipid bilayer in 100 mM monovalent salt as a function of the distance R. The toxin molecule is oriented as in Fig. 1 with respect to the lipid bilayer. See text for details.

Exact binding constant

A binding constant of 1200 M^{-1}, which corresponds to a binding free energy of −4.2 kcal/mol, was calculated for CTx binding to 2:1 PC/PS membrane in 100 mM salt using Eq. 4 when both $W^{elc}(x, y, R, \alpha, \eta, \theta)$ and $W^{np}(x, y, R, \alpha, \eta, \theta)$ were taken into account. This binding free energy (*open triangle*, Fig. 6 *A*) agrees well with the measured value of −3.7 kcal/mol.

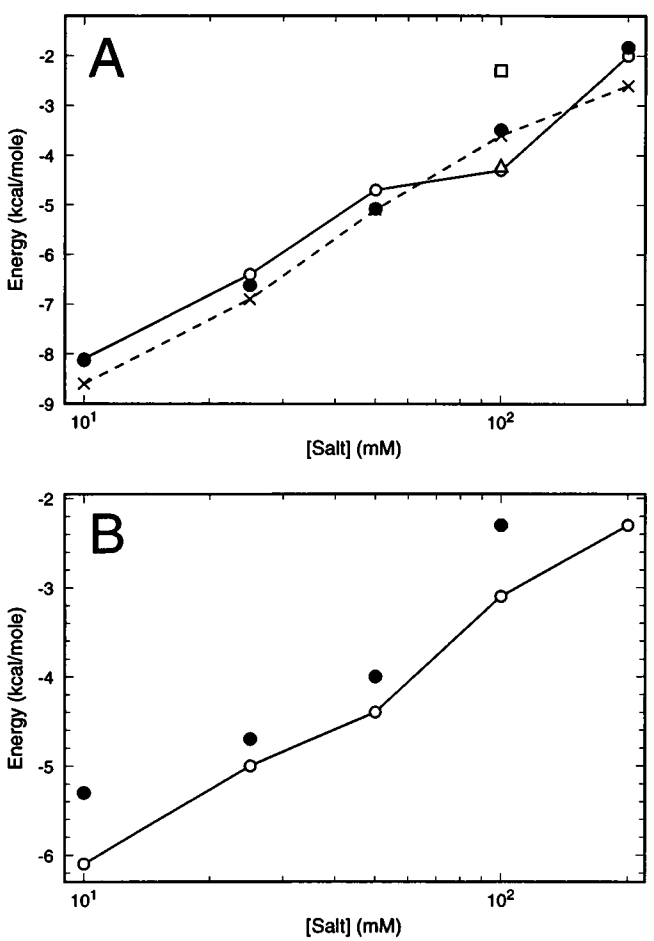

FIGURE 6 Binding energy of CTx and IbTx to bilayer membranes as a function of the ionic strength. The filled circles represent the experimentally determined standard Gibbs free energies of binding of CTx (*A*) and IbTx (*B*) to 2:1 PC/PS bilayers, taken from Fig. 3 *A*. (*A*) The potential energy of CTx in the minimal electrostatic free energy orientation is represented as crosses connected by a dashed line; the orientation is illustrated in Fig. 1. As discussed in the text, this is not the binding energy of the toxin expected theoretically, although the energies fortuitously agree well with the experimental values. The open circles connected by a solid line depict the approximate theoretical predictions based on integrating over a single free energy curve, e.g., the filled triangles in Fig. 5 (Eq. 8). The open triangle represents the theoretical prediction made by sampling 78 orientations for each R (Eq. 4), and the open square represents the prediction made by sampling 78 orientations for each R (Eq. 4) accounting only for the electrostatic component of the binding. (*B*) The open circles connected by a solid line show the approximate free energy values for binding of IbTx to 2:1 PC/PS bilayers calculated by integrating over a single free energy curve (Eq. 8).

When only $W^{elc}(x, y, R, \alpha, \eta, \theta)$ is considered, we calculate a smaller binding constant of 50 M^{-1} (binding free energy of −2.3 kcal/mol). The binding free energy assuming a purely electrostatic interaction between the toxin and the membrane (*open square*, Fig. 6 *A*) is less negative than the measured value.

Relative binding constant estimated from a single toxin-bilayer configuration

Calculating the binding constants for the toxins to a membrane requires significant computer time because the (nonlinear) Poisson-Boltzmann equation must be solved numerically for many different configurations of the toxin molecule adjacent to the membrane. In the next subsection we suggest an approximate method for estimating the binding constants. Before doing so we deal with a computationally less expensive task—calculating the *relative* binding constant or equivalently the *relative* binding free energy (e.g., the dependence of the binding free energy on the ionic strength and on the mol % of acidic lipids). We use the approach described previously (Ben-Tal et al., 1996), estimating the relative binding free energy from the changes in the depth of the well in the electrostatic free energy curve (Fig. 5, *open triangles*) that was obtained for CTx and IbTx oriented in the most attractive orientation.

Fig. 6 *A* (*crosses*) shows the minimal electrostatic free energy of CTx as a function of the ionic strength. The slope of this curve agrees very well with the slope obtained from the experimental data (*filled circles*). Similar agreement was observed for the dependence of the binding free energy of IbTx on the ionic strength and for the dependence of the binding free energy of each of these toxins on the mol % acidic lipid in the bilayer (not shown). For the particular system studied here, the calculated minimal electrostatic free energy coincides with the experimentally determined Gibbs free energy (e.g., Fig. 6 *A*). Presumably this is fortuitous; it is evident from Eq. (4) that the binding constant, and therefore the binding free energy, depends on $W^{int}(x, y, R, \alpha, \eta, \theta)$ at many different configurations. Here we calculated $W^{elc}(x, y, R, \alpha, \eta, \theta)$ for only a single configuration.

Approximate binding constant

Eq. 4 shows that K depends primarily on the toxin-membrane configurations with the most negative values of $\Delta W^{int}(x, y, R, \alpha, \eta, \theta)$. Recall that the free energy curve (Fig. 5, *filled triangles*) for CTx was calculated with the molecule in an orientation of maximal electrostatic interaction (Fig. 1). In order to reduce the computational effort we estimate K by integrating over this single free energy curve so that

$$K \approx CA_L N \int_0^\infty dR[\exp[-\beta W^{int}(x^0, y^0, R, \alpha^0, \eta^0, \theta^0)] - 1] \quad (8)$$

where $(x^0, y^0, \alpha^0, \eta^0, \theta^0)$ refer to the orientation of Fig. 1. This expression yields a value of 960 M^{-1}, or a binding free energy of −4.1 kcal/mol, for CTx binding to 2:1 PC/PS membrane in 100 mM salt when both $W^{elc}(x, y, R, \alpha, \eta, \theta)$ and $W^{np}(x, y, R, \alpha, \eta, \theta)$ are taken into account.

Dependence of binding on the ionic strength

The open circles in Fig. 6 show the calculated values (based on Eq. 8) of the binding free energy of CTx (Fig. 6 *A*) and IbTx (Fig. 6 *B*) to 2:1 PC/PS lipid bilayers as a function of the ionic strength. The binding gets weaker as the ionic strength increases. The calculated binding free energies agree well with the measured values for CTx (Fig. 6 *A*) and within 1 kcal/mol for IbTx (Fig. 6 *B*).

Dependence of binding on the mol % acidic lipid

The open circles in Fig. 7 show the calculated values (based on Eq. 8) of the binding free energy of CTx (Fig. 7 *A*) and IbTx (Fig. 7 *B*) to lipid bilayers plotted as a function of the mol % acidic lipid in the membrane. The filled circles in Fig. 7 show the experimental data illustrated in Fig. 3 *B*. The theoretical model describes adequately how the magnitude of the binding free energy increases with the percent acidic lipid.

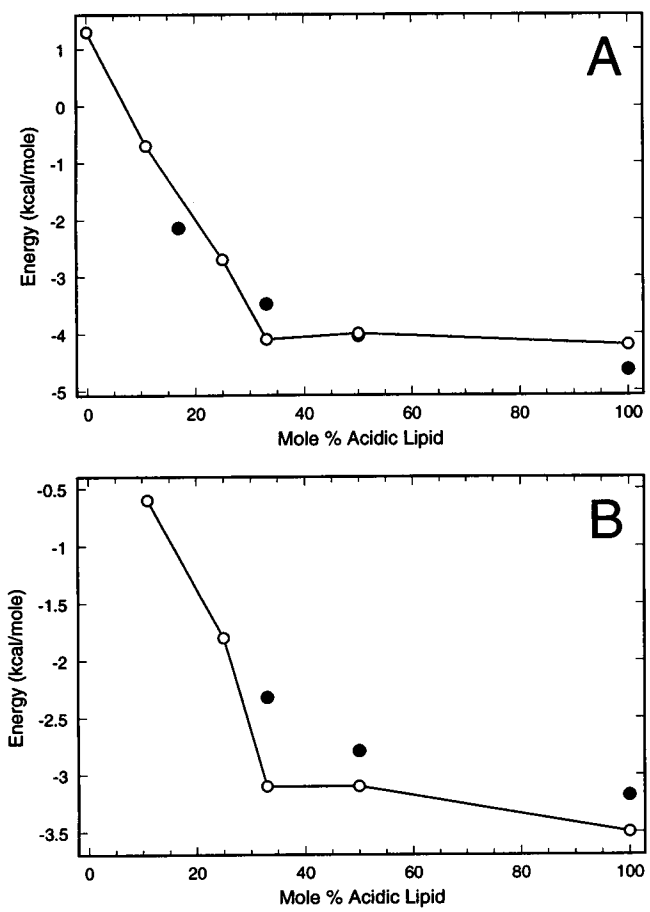

FIGURE 7 Binding energy of CTx (*A*) and IbTx (*B*) to bilayers as a function of the mol % acidic lipid. The filled circles represent the experimentally determined Gibbs free energies for binding of the toxins to PC/PS vesicles in 100 mM KCl (plus 1 mM MOPS, pH 7), taken from Fig. 3 *B*. The open circles connected by a solid line depict the approximated theoretical predictions made by integrating over a single free energy curve (Eq. 8).

DISCUSSION

The studies presented here and in Ben-Tal et al. (1996) introduce a model that describes theoretically the electrostatic binding of peptides and proteins to lipid bilayers. Our most important result is that the calculated dependence of the free energy of binding on the ionic strength of the solution and the mol % acidic lipid in the bilayer agrees well with the measurements for both CTx (valence +4) and IbTx (valence +2). As we discuss below, the success of the theoretical model suggests that our treatment of the long-range Coulomb attraction of these toxins to the membrane is adequate.

When we use a model that assumes purely electrostatic binding, the predicted binding energies for CTx (*open square* in Fig. 6 *A* of this paper) and pentalysine [*filled square* in Fig. 5 of Ben-Tal et al. (1996)], are 1–2 kcal/mol less negative than the measured values. The missing 1–2 kcal/mol could arise from nonpolar contributions to the binding. We incorporated these interactions into the model by assuming they are proportional to the water-accessible surface area of the toxins and the lipid bilayer. The water-accessible surface area decreases as the protein/peptide approaches the bilayer, which gives rise to a short-range nonpolar attraction. This approach is novel in the context of protein-bilayer interactions, but has been used to analyze binding and stability in proteins (e.g., Honig et al., 1993; Froloff et al., 1997). Alternatively, the missing 1–2 kcal/mol could arise from either structural changes in the bilayer that occur on binding or some other deficiency in the model, as we discuss below.

We now consider the assumptions inherent in the model and the limitations of the approach. When a positively charged protein like CTx is far from a negatively charged membrane, it experiences only a long-range Coulomb attraction: the experimental results we reported here and in Ben-Tal et al. (1996) illustrate that our approach can adequately describe this attraction, which provides the main component of the binding energy. When the protein approaches the membrane, however, water molecules must ultimately be removed from the interface between the protein and the bilayer. This gives rise to two opposing short range effects: a Born repulsion and a nonpolar attraction (Fig. 5). Our calculations show that these two effects approximately balance each other. Thus, membrane binding is driven mainly by the net Coulomb attraction, in accord with our working hypothesis.

We showed previously that the long-range Coulomb attraction does not depend significantly on the set of atomic

partial charges and radii used for the calculations (Ben-Tal et al., 1996). Both the nonpolar attraction and the Born repulsion do depend strongly on parameters used for the calculations, i.e., the surface tension coefficient and radii used to calculate the nonpolar interactions and the set of atomic partial charges and radii used in the Born calculations. The surface tension coefficient we used was derived from partitioning of alkanes between water and liquid alkanes (Sitkoff et al., 1996). It has been used successfully to evaluate the nonpolar component of binding and stability in proteins (e.g., Honig and Nicholls, 1995), but its applicability to protein-bilayer systems remains to be determined. Much effort has been devoted to developing sets of atomic charges and radii that describe desolvation phenomena in proteins (e.g., Sitkoff et al., 1994; 1996), but we are not aware of similar work for lipids. In the absence of a better choice, we used the set from our earlier studies (Peitzsch et al., 1995; Ben-Tal et al., 1996). For these reasons, and the reasons discussed below, our calculations of the nonpolar attraction and the Born repulsion remain suspect. [Despite the missing parametrization work, the model does not fail badly in its description of the balance between the Born repulsion and the nonpolar attraction. For example, it predicts that the toxins will not bind to neutral bilayers composed of pure PC lipids (Fig. 7 *A*), in agreement with our experimental observations. The analysis shows that the Born repulsion overcomes the nonpolar attractions.] These Born and nonpolar calculations are not crucial for a theoretical description of the membrane binding of a hydrophilic peptide that does not penetrate the polar head group of the bilayer (e.g., pentalysine). Many interesting peripheral proteins (e.g., the myelin basic protein and the effector domain of MARCKS) and peptides (e.g., melittin and signal sequence peptides) do penetrate the polar head group region of a bilayer. Thus an important future project is the development of new theoretical and experimental approaches for describing these short-range interactions. The experimental determination of partition coefficients for the penetration of amino acids into the polar head group region of bilayers is an important step in this direction (Wimley and White, 1996; Thorgeirsson et al., 1996).

The model has several additional shortcomings. For example, our assumption that the bilayer surface is rigid and does not change its structure when the toxins bind is probably incorrect: molecular dynamics and Monte Carlo simulations indicate that the polar head groups are flexible (e.g., Pastor, 1994) and that interactions with proteins may perturb the structure (e.g., Zhou and Schulten, 1995; Woolf and Roux, 1996; Damodaran and Merz, 1996). The "soft" or nonrigid nature of the bilayer surface may strongly affect the calculations of the short-range Born and nonpolar interactions, but should have a much smaller effect on the calculation of the long-range Coulomb attraction.

Our treatment of the dielectric constant of the polar head group region introduces another potential source of error. There is good experimental evidence that the dielectric constant of the interior of the membrane is 2. There is also good justification for treating the water outside the envelope of the polar head group region as a dielectric continuum and the salt ions at the mean field level (e.g., McLaughlin, 1989; Honig et al., 1993; Honig and Nicholls, 1995). New theoretical approaches, however, may be required to explicitly treat the water and ions in the polar head group region.

A strong feature of our approach is that it explicitly treats the shape and charge distribution of the membrane and the adsorbing protein. When the Gouy-Chapman theory is applied to protein-membrane interactions, the protein is regarded as a point charge which is not a valid assumption: even small proteins like CTx and IbTx are larger than the Debye length. For example, our calculations show that CTx (net charge of +4) is electrostatically repelled from a 2:1 PC/PS bilayer in 100 mM salt when it is oriented with its Cys-19-NEM toward the bilayer (a 180° rotation around the *x*-axis in Fig. 1). In this orientation, the net charge on CTx that is located within a Debye length of the membrane is negative. Increasing the Debye length, by decreasing the salt concentration to 10 mM, turns the repulsion into attraction for this orientation. Many larger proteins such as MARCKS have a net negative charge but use a cluster of basic residues to interact electrostatically with acidic lipids in a membrane (McLaughlin and Aderem, 1995). Realistic molecular models of such proteins are needed to adequately describe their electrostatic interaction with membranes.

The biological function of the toxins used here is to block potassium channels by binding to a receptor site located at the external opening of the ion conduction pathway (Miller, 1995). CTx binding to potassium channels is, of course, mediated by specific structural determinants on both toxin and channel; but a large through-space electrostatic interaction also contributes to binding of CTx to its natural receptor (Anderson et al., 1988; Miller, 1990; Goldstein and Miller, 1993). Binding of CTx to *Shaker*, for instance (Goldstein and Miller, 1993), is weakened ~25-fold by increasing ionic strength from 50 to 150 mM, an effect somewhat milder than that observed in the charged phospholipid membranes used in the system here. The *Shaker* channel carries a net charge of −32 on the 12 externally exposed "loops" of sequence in the functional tetrameric channel. Assuming that these charges are distributed uniformly over the ~7000 $Å^2$ projected area of the *Shaker* channel (Li et al., 1994), this represents a charge density slightly less than that employed in the 2:1 PC/PS membranes used here. Thus, this phospholipid system, in addition to providing a chemically defined testing ground for studying protein-membrane interactions, faithfully mimics the effects of salt on decreasing the binding of CTx to the *Shaker* channel.

CTx and IbTx share the structural motif found in antibacterial insect defensins (Bontems et al., 1991b), which have a net positive charge that facilitates electrostatic interaction with acidic lipids in phospholipid membranes (Wimley et al., 1994; White et al., 1995, Ganz and Lehrer, 1995). The biological importance of these interactions, however, remains to be determined. As mentioned in the introduction,

many important peripheral proteins use electrostatic interactions to bind to biological membranes and our model should be useful for analyzing their interaction with acidic phospholipids in membranes.

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