Characterization of Electrostatic and Nonelectrostatic Components of Protein−Membrane Binding Interactions†

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ABSTRACT: A general method was developed to determine the thermodynamic parameters for the interaction of proteins with membranes. Protein intrinsic tryptophan fluorescence was quenched by titration with large unilamellar vesicles containing 9,10-dibrominated distearoylphosphatidylcholine (Br_4-DSPC) or a small amount of trinitrophenylphosphatidylethanolamine (TNP-PE). Binding was modeled as a bimolecular reaction of free protein with a unit of "*n*" lipid molecules and a dissociation constant, K_d. The contribution of residual fluorescence and light scattering could be eliminated by using the second derivative of the titration function as the basis for calculations. For the binding of C-terminal channel domain polypeptides (178−190 residues) of the colicin E1 ion channel, $n = 50-60$ and $K_d = 2-3$ nM at pH 4, ionic strength, $I = 0.12$ M, and anionic lipid content = 40% (surface potential, $\psi_o = -30$ mV), conditions for which the protein has high activity. Values of $n = 95$ and 210 for the binding of a C-terminal 293-residue colicin fragment and the 522 residue intact colicin E1 molecule scale qualitatively according to the increase in molecular size. General methods are presented to distinguish the electrostatic (ΔG_{el}) and nonelectrostatic (ΔG_{nel}) components of the total ΔG for binding. Using Br_4-DSPC as the quencher, the binding of the channel polypeptide, P178, was characterized by $\Delta G \approx -9.8$ kcal/mol, $\Delta G_{nel} \approx -7.0$ kcal/mol, and $\Delta G_{el} = -2.8$ kcal/mol ($\psi_o = -30$ mV). Using TNP-PE as the quencher, similar values of $\Delta G \approx -9.3$ to -9.9 kcal/mol were determined, a somewhat smaller value for $\Delta G_{nel} \approx -5.0$ kcal/mol, and a correspondingly larger value for $\Delta G_{el} \approx -4.9$ kcal/mol. The existence of a ΔG_{nel} component of this magnitude may distinguish proteins that have the potential to insert into the membrane.

The import, secretion, and function of many proteins involve interactions with cellular and organelle membranes. The interaction of a peptide or protein with the polar lipid headgroups in the membrane surface is commonly assumed to be electrostatic, while insertion of protein segments into the hydrophobic core of a membrane must largely be nonelectrostatic. These concepts are embodied in a simple equation for the binding energy:

$$\Delta G = z_{eff} F \psi_o - RT \ln K_{nel} \quad (1)$$

where the first term is the electrostatic energy (ΔG_{el})[1] determined by the effective charge on the protein, z_{eff}, and the membrane surface potential, ψ_o ($F = 96\,485$ C/mol, Faraday's constant). The second term is the nonelectrostatic binding energy (ΔG_{nel}), where K_{nel} is the partition coefficient for the protein between membrane and bulk phase at $\psi_o = 0$. Using a suitable membrane binding assay, the contributions of the components of eq 1 can be determined, with obvious implications for the nature and strength of the interaction of a particular protein with a membrane.

In general, membrane binding assays can be grouped into two classes: (i) those that require a physical separation step to prepare the distinct phases or compartments for analysis, such as centrifugation (Heimburg & Marsh, 1995), dialysis, or ultrafiltration (Kim *et al.*, 1991; Mosior & McLaughlin, 1991; Mosior & McLaughlin, 1992), and (ii) those directly measuring the partitioning in the intact system using a particular biophysical property, such as quenching (González-Mañas *et al.*, 1992; Palmer & Merrill, 1994) or enhancement of Trp fluorescence (Bardelle *et al.*, 1993). The latter approach can be more useful because of the relative simplicity and flexibility of fluorescence measurements, although it also has specific background effects for which corrections must be made.

A method was developed in the present study to analyze the binding of proteins to artificial membranes, using fluorescence resonance energy transfer (FRET) from the Trp residues of the protein to TNP conjugated to lipid headgroups in the membrane or dipole−dipole energy transfer to dibrominated fatty acyl chains of the lipids. The technique involves the titration of prospective membrane-interacting proteins in solution with quencher-containing artificial vesicles of defined anionic and neutral lipid composition, through measurement of the decrease in Trp fluorescence. The thermodynamic parameters, dissociation constant, K_d, and lipid/protein stoichiometry, *n*, were determined, using the second derivative of the function describing the dependence of fluorescence quenching on lipid concentration. The second derivative of the fluorescence quenching function eliminates or simplifies interference from nonspecific binding and nonquenched residual fluorescence that are linearly dependent on the concentration of lipid. Binding energies were derived from these parameters and the electrostatic and nonelectrostatic components calculated. The method was applied to the membrane-active bactericidal protein, colicin E1, and polypeptide fragments derived from the colicin that contain (i) the C-terminal channel-forming domain or (ii)

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both the channel and the receptor-binding domains. A preliminary account of these studies has been presented (Zakharov *et al.*, 1995; Zhang *et al.*, 1994).

MATERIALS AND METHODS

Preparation of Colicin E1 and Its Channel Polypeptides. (i) Colicin E1 was purified as previously described (Zhang & Cramer, 1992). (ii) P293, the polypeptide fragment of colicin E1 that contains the receptor-binding and channel domains (Cramer *et al.*, 1995), was expressed from pSKRC and purified as previously described (Zhang, 1993). (iii) The C-terminal channel polypeptide with a ragged N-terminus, 174, 177, or 178 residues in length, P178 (Elkins *et al.*, 1994), was prepared by proteolysis of the intact colicin E1 with thermolysin (Bishop *et al.*, 1985). (iv) The engineered colicin E1 C-terminal channel polypeptide, P190, was expressed from pSKHY(−), a plasmid with the colicin channel domain under control of the colicin E1 promoter (Elkins *et al.*, 1994), and purified as previously described (Zhang & Cramer, 1992). In addition, egg lysozyme (lysozyme C) was obtained from the Sigma Chemical Co. The modified Lowry assay (Peterson, 1977) was used to determine protein concentration.

Preparation of LUV. LUV were prepared by an extrusion procedure (Hope *et al.*, 1985). Aliquots of synthetic DOPG, DOPC (Avanti Polar Lipids, Inc., Alabaster, AL), and TNP-PE (Sigma) (10% w/w ≡ 8.4 mol %) in chloroform were mixed to obtain the desirable molar ratios in a total of 10 mg of lipid. For titrations with brominated lipid as quencher, 60% Br_4-DSPC (Avanti Polar Lipids, Inc., Alabaster, AL) was used in the place of DOPC as neutral lipid, with the remainder DOPG (mol %). The lipids were coated on the glass surface of a test tube by evaporation of the solvent under a stream of nitrogen during vortexing, and traces of solvent were removed by vacuum for 3−4 h. The lipid was resuspended in 1 mL of buffer (0.1 M KCl, 10 mM DMG, pH 5.0) by vortexing under nitrogen and freeze−thawed 10 times. The vesicles were extruded 10 times through a double polycarbonate filter with pore size 0.1 μm (Nuclepore, Costar) to obtain extruded LUV. The Bartlett assay for phosphorus was carried out according to the wet digestion method in New (1990). Accessible lipid, x_a, was measured by derivatization of DOPE lipid (incorporated in LUV in place of TNP-PE) by TNBS (New, 1990).

Fluorescence Measurements. Binding of protein through the quenching of Trp fluorescence by TNP-PE vesicles was assayed at 22 °C with an SLM 8000C photon counting spectrofluorimeter (SLM Instruments, Inc., Urbana, IL) using 1 cm cuvettes with magnetic stirring. Excitation and emission wavelengths were calibrated using the 296.7 and 365.0 nm Hg lines of a low pressure mercury lamp. Emission spectra were acquired with excitation at 280 nm to obtain a complete Trp emission spectrum (which includes excitation of tyrosine residues, although their contribution to the emission spectrum is negligible) and a band-pass of 4 nm for both beams. Kinetic and titration data were recorded through excitation of intrinsic tryptophans, at 293 nm (which minimizes excitation of tyrosine residues) and emission measured at 340 nm, with half-band widths of 4 and 8 nm, respectively. The signal was corrected for the variation of excitation intensity using a reference channel. Because the titration involved measurements of highly quenched low signal levels, polarization optics was not used. However, the existence of polarization artifacts was shown to be insignificant at low levels of quenching by setting the emission beam polarizer 54.7° relative to the vertical polarization axis of the exciting light (Lakowicz, 1983). The R_o value for 50% of fluorescence resonance energy transfer from the Trp residues of the colicin E1 channel domain to TNP was determined to be 27 ± 1 Å by A. Szabo and J. Brennan (personal communication) from the overlap integral (Lakowicz, 1983).

Titration of Protein with Membrane Vesicles. Protein (4 μg/mL ≡ 192 nM for P190; 206 nM for P178; 200 nM for P293; 11 μg/mL ≡ 196 nM for colicin E1) was titrated with small aliquots of LUV suspension (2−8 μL of 0.5−5 mg/mL additions) to a final concentration of 20−500 μM. All titration points were recorded at equilibrium, 5−10 min after each addition of vesicles.

The values of the binding parameters and interaction free energies were determined according to the analysis described in the Results section.

RESULTS

Parameters for the Binding of a Protein to a Membrane. The binding of the protein to a membrane was approached using a bimolecular interaction model (Hille *et al.*, 1981). This approach assumes that free protein, P_f, binds to a membrane, interacting with a unit of multiple (n) lipid molecules, $(L_f)_n$, to form a complex, P_b:

$$P_f + (L_f)_n \overset{K_d}{\rightleftharpoons} P_b$$

[1] Abbreviations and symbols: DMG, dimethylglutaric acid; DOPC, DOPE, DOPG, 1,2-dioleoylphosphatidylcholine, -phosphatidylethanolamine, and -phosphatidylglycerol; Br_4-DSPC, 1,2-distearoyl(9,10-dibromo)phosphatidylcholine; FRET, fluorescence resonance energy transfer; LUV, large unilamellar vesicles; P178, P190, C-terminal colicin E1 channel-forming polypeptides, thermolytic, engineered; P293, C-terminal colicin E1 receptor-binding and channel-forming polypeptide; TNP, 2,4,6-trinitrophenol; TNP-PE, *N*-(trinitrophenyl)phosphatidylethanolamine; Trp, tryptophan. F_o, total fluorescence of free protein in solution; f_f, fraction of free protein; f_x, experimentally determined fluorescence relative to total fluorescence of protein; f_m, fractional fluorescence (f_x) at the point of maximum curvature in the titration function; ΔG, free energy of membrane−protein interaction (kcal/mol); ΔG_{el}, ΔG_{nel}, and ΔG_{ns}, electrostatic, nonelectrostatic, and nonspecific components of binding free energy (kcal/mol); I, ionic strength (M); K_b, coefficient for partitioning of protein between membrane and aqueous phases; K_d, protein−membrane dissociation constant (M); K_{dlo}, lower limit for determination of K_d (M); K_{NaL}, sodium-negative lipid association constant (M^{-1}); K_{nel}, nonelectrostatic binding coefficient; K_q, Stern−Volmer quenching constant for nonelectrostatic membrane binding (M^{-1}); K_s, vesicle light scattering constant (M^{-1}); [L], total lipid concentration (M); [L^-], concentration of free negative lipid (not associated with protein) (M); [L_f], concentration of free lipid (not associated with protein) (M); [L_b], concentration of lipid bound to protein (M); $[L]_m$, total lipid concentration at point of maximum curvature in the titration function (M); λ, effective quenching coverage per lipid molecule by a lipophilic quencher; n, lipid/protein binding stoichiometry (mol/mol); n_{lo}, limit for the determination of n (mol/mol); $[Na^+]_o$, concentration for free sodium at the membrane surface (M); [NaL], concentration for sodium−lipid ionic complex (M); [P], total protein concentration (M); [P_f], free protein concentration (M); [P_b], concentration of protein bound to lipid (M); ψ_o, potential at the membrane surface (V); R_o, characteristic Förster quenching distance (Å); σ, membrane surface charge density (Coulomb/Å^2); V_{aq}, total aqueous volume (L); V_L, total lipid volume (L); V_{Pb}, volume associated with bound protein (L); V_{Pf}, volume associated with free protein (L); $\bar{v}_L$, molar lipid volume (M^{-1}); $\bar{v}_q$, molar quenching volume (M^{-1}); x_-, fraction of negative lipid in the membrane; x_a, fraction of lipid accessible from external aqueous phase; z_{eff}, effective binding charge.

The relevant component of the fluorescence signal arises from the fraction of protein not bound to the vesicles:

$$f_f \equiv [P_f]/[P] \quad (2)$$

where $[P_f]$ and $[P]$ are the free and total protein concentrations, respectively. This model assumes the simultaneous interaction of multiple groups of the protein with the membrane, with minimal cooperativity on the time scale of the binding measurement. This is in agreement with previous studies showing the independent binding of polyvalent cationic peptides to negatively charged lipid vesicles (Kim *et al.*, 1991).

The dissociation constant, K_d, for the interaction of the polypeptide with one binding unit of n lipid molecules on the vesicle surface is

$$K_d = [L_f][P_f]/n[P_b] \quad (3)$$

where n is the lipid/protein stoichiometry, $[P_f]$ and $[P_b]$ are the free and bound protein concentrations, $[L_f]$ is the "free" lipid concentration (i.e., not bound to polypeptide), and the concentration of lipid bound to protein is $[L_b] = n[P_b]$. The total protein, [P], and total lipid, [L], concentrations are

$$[P] = [P_f] + [P_b] \quad (4)$$

$$[L] = [L_f] + [L_b] = [L_f] + n[P_b] \quad (5)$$

Solving eqs 3−5 for the fraction of free protein, f_f, at equilibrium,

$$f_f = \frac{1}{2}\left[1 - \frac{[L]}{n[P]} - \frac{K_d}{[P]} + \sqrt{\left(1 - \frac{[L]}{n[P]} - \frac{K_d}{[P]}\right)^2 + 4\frac{K_d}{[P]}}\right] \quad (6)$$

This relationship indicates that for titrations (i.e., protein titrated with lipid, [L]) carried out at different protein concentrations, [P], there is a reciprocity between the values of n and [P], and parallel changes of K_d with [P], because the terms $n[P]$ and $K_d/[P]$ remain constant between experiments differing in protein concentration.

One would like to describe the energy involved in the transfer of one protein molecule from one infinite phase (aqueous solution) to another infinite phase (the membrane) in a concentration-independent formalism. This can be described by a partition coefficient, K_b, in terms of the reactant volumes:

$$K_b = \frac{V_{Pb}/(V_L + V_{Pb})}{V_{Pf}/(V_{aq} + V_{Pf})} \approx \frac{V_{Pb}/V_L}{V_{Pf}/V_{aq}} \quad (7)$$

where V_{Pb}, V_{Pf}, V_L, and V_{aq} are the partial volumes associated with bound and free polypeptide, total lipid, and total aqueous volume, respectively. The right-hand expression applies for $V_{Pf} \ll V_{aq}$ and concentrations of protein such that $V_{Pb} \ll V_L$. In this case, K_b can be expressed in terms of K_d and n, (a) under the condition of excess lipid, $[L_f] \approx [L]$, (b) the assumption that the ratio of protein volumes are proportional to the ratio of their concentrations, i.e., $V_{Pb}/V_{Pf} \approx [P_b]/[P_f] = [L_f]/nK_d$ (the equality from eq 3), (c) the use of the relation $V_L = \bar{v}_L[L]\ V_{aq}$ ($\bar{v}_L$ is the specific lipid molar volume, ~0.8 L/mol), and (d) substitution into eq 7:

$$K_b = \frac{1}{\bar{v}_L n K_d} \quad (8)$$

The bimolecular interaction and partitioning approaches to protein−membrane interactions therefore converges to the same solution for very dilute protein. The total binding energy is then

$$\Delta G = -RT \ln K_b = RT \ln(\bar{v}_L n K_d) \quad (9)$$

Membrane−Protein Interaction Assay Based on Trp Fluorescence Quenching. The binding of protein to membrane vesicles (Figure 1A) was assayed by the quenching of the intrinsic tryptophan (Trp) fluorescence of the protein with LUV containing as the quenchers, either (trinitrophenyl)phosphatidylethanolamine (TNP-PE; Figure 1B) or 1,2-distearoyl(9,10-dibromo)phosphatidylcholine (Br_4-DSPC, Figure 1C). The TNP headgroup of TNP-PE has an absorbance band centered at 339 nm (Figure 1D, spectrum a), which overlaps the emission spectrum of Trp substantially in the wavelength range 320−340 nm (Figure 1D, spectrum b) and quenches Trp fluorescence by nonradiative energy transfer (Figure 1D, spectra c,d).

The Förster distance, R_o, for FRET from colicin tryptophans to TNP-PE is 27 ± 1 Å (A. Szabo and J. Brennan, unpublished data, personal communication), comparable to the radii of monomeric globular proteins (15−30 Å). The Trp residues of a protein bound to the membrane should therefore be quenched to an extent mostly dependent on the distribution of quencher in the plane of the membrane and less dependent on the exact positions of the three Trp residues at the membrane surface. In the binding assays reported here, TNP-PE constituted 10% (w/w ≡ 8.4 mol %) of the lipid in the LUV, with the remainder consisting of DOPG and DOPC. This amount of TNP-PE in the membrane causes >90% quenching of the Trp fluorescence of colicin E1 and its channel polypeptides at high lipid/protein ratios (Figure 1D, spectrum d). LUV with 5% TNP-PE quenched P190 80−90% (data not shown), which was considered insufficient for these studies.

At pH 4, rapid quenching occurs within 1−5 s after addition of TNP-PE LUV (anionic lipid content, $x_- = 0.6$; pH 4, $I = 0.3$ M) to the protein, followed by a slow decay (Figure 2A, trace a). Equilibrium is reached within ~2 min for tight binding, while under conditions allowing only weak binding the time required for equilibration was typically 5−10 min in a stirred cuvette. At equilibrium, the remaining signal could be attributed to (i) light scattering and (ii) residual Trp fluorescence that is not quenched (Figures 2A, trace a, 3c,d). At pH 7.5−8.0, where electrostatic interactions of proteins with neutral or negatively charged membranes are minimized, there is a small component of the fluorescence quenching and binding that occurs rapidly during mixing upon the addition of vesicles (Figure 2A, trace b).

The ability of the TNP headgroup to quench the fluorescence of proteins bound to the membrane surface contrasts with the inability of the buried Br quencher of Br_4-DSPC to do so. This is illustrated for the water-soluble basic protein, lysozyme C, whose Trp fluorescence is not quenched by the brominated lipid (Figure 2B, trace a), whereas that of P178 is largely quenched at low pH (Figure 2B, trace b).

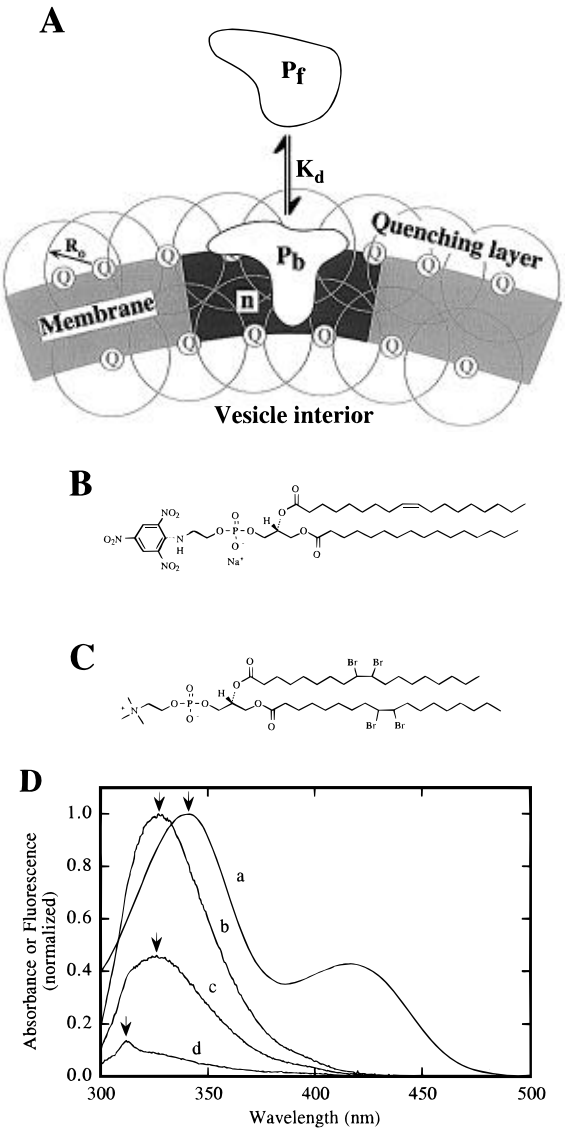

FIGURE 1: (A) Binding equilibrium between free (P_f) and bound (P_b) protein to a membrane, with a lipid/protein stoichiometry of "*n*". Binding is assayed as the quenching of the protein's intrinsic tryptophan fluoresence by lipids with quenching headgroups (Q ≡ TNP-PE) or acyl chains (Br_4-DSPC). K_d is the dissociation constant for binding of polypeptide to a unit of *n* lipid molecules on the membrane surface; R_o, Förster distance for energy transfer from TNP quencher to Trp residue. (B) Structure of TNP-PE [*N*-(trinitrophenyl)phosphatidylethanolamine]. (C) Structure of Br_4-DSPC [1,2-distearoyl-(9,10-dibromo)phosphatidylcholine]. (D) Spectrofluorimetric properties of the colicin E1 channel polypeptide, P190, and the absorbance of TNP-PE: (a) Absorbance spectrum of TNP-PE. (b) Fluorescence emission spectrum of P190 (8 μg/mL ≡ 0.39 μM). (c) Emission spectrum of P190 after partial quenching by TNP-PE LUV (20 μM lipid). (d) Complete quenching by excess LUV (100 μM lipid). The emission maxima of curves a−d are at 339, 326, 325, and 312 nm (indicated by arrows). Excitation, 280 nm; LUV composition: 30% DOPG/60% DOPC/10% TNP-PE. Buffer: 20 mM DMG, 0.13 M NaCl, pH 4.0.

Dibrominated phospholipid quenches Trp fluorescence through a dipole−dipole interaction, with an $R_o \approx 9$ Å (Bolen & Holloway, 1990). With 60% Br_4-DSPC LUV, ~75% quenching of the intrinsic Trp residues of the channel polypeptides was obtained, leaving a significant fraction of residual fluorescence. This may indicate that not all three Trp residues in the channel polypeptides penetrate deeply enough into the membrane to be fully quenched by the Br.

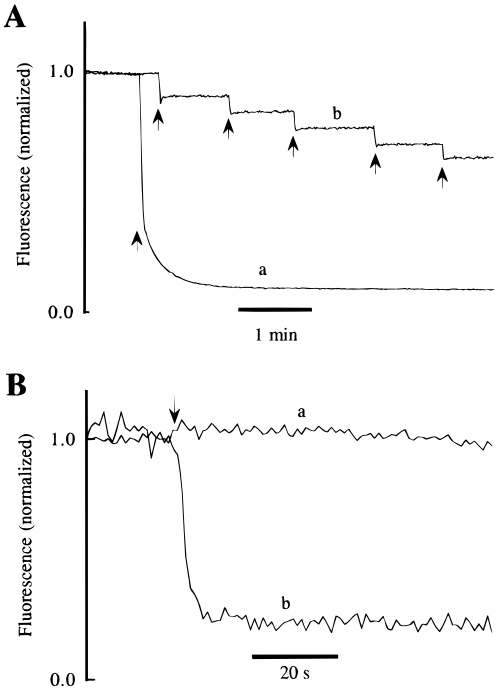

FIGURE 2: (A) Time course for the quenching of P178 (4 μg/mL ≡ 0.19 μM) fluorescence with TNP-PE LUV (solid arrows indicate addition of vesicles, 50 μM lipid, 50% DOPG/10% TNP-PE/40% DOPC) showing (a) binding at pH 4.0, and (b) rapid nonelectrostatic binding at pH 8.0 (I = 0.3 M). (B) Time course for the quenching of lysozyme C (8 μg/mL ≡ 0.56 μM) fluorescence (a) and P178 (4 μg/mL ≡ 0.19 μM) fluorescence (b) with Br_4-DSPC LUV. 40 μM lipid; 60% Br_4-DSPC/40% DOPG; pH 4.0; I = 0.12 M; excitation, 293 nm; emission, 340 nm.

Relationship between Protein Bound to Vesicles and Quenching of Its Fluorescence by a Lipophilic Quencher. Given the features of the binding assay mentioned above, the experimentally determined fluorescence signal as a function of lipid concentration, [L], can be described by

$$\frac{F}{F_o} \equiv f_x = \frac{f_f}{1 + K_q[L]} + \frac{K_s}{F_o}[L] + (1 - f_f)e^{-\lambda} \quad (10)$$

where F and F_o are the absolute magnitudes of the fluorescence intensity in the presence and absence of quenching vesicles, and components contributing to the intensity are as follows: (a) f_f, the fraction of free protein (eq 6); (b) $1 + K_q[L]$, a correction for the nonspecific nonelectrostatic binding; (c) $(K_s/F_o)[L]$, vesicle light scattering including inner filter effects; and (d) $(1 - f_f)e^{-\lambda}$, Trp fluorescence arising from incomplete quenching which is independent of lipid concentration and is a function of quencher distribution in the membrane. A calculated fluorescence titration curve from typical system parameters is shown in Figure 3a, as well as its nonspecific (Figure 3b) and nonbinding (Figure 3c,d) components. The theoretical basis for the individual terms in eq 10 is discussed further in the following.

Residual Fluorescence and Light Scattering. At the end of a titration experiment when all protein should be bound to the vesicles ($f_f = 0$), a significant signal is observed arising from vesicle light scattering and incomplete quenching of bound protein arising from the random distribution of quencher molecules in the membrane surface. Titration of buffer with 0.1 μm LUV of various lipid compositions in the absence of protein yielded a light scattering constant,

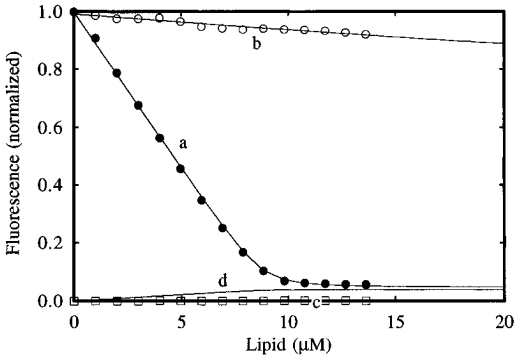

FIGURE 3: (a, ●) Experimental titration of P178 (0.20 μM) with TNP-PE LUV, showing a fit (f_x in eq 10, solid line, $r = 0.98$) with $K_d = 1$ nM and $n = 44$, also including (b, ○) quenching due to nonelectrostatic binding, determined from titration of the protein with LUV at pH 8.0 ($K_q = 0.002\ \mu M^{-1}$, $r = 0.97$), (c, □) light scattering determined from titration of buffer with LUV ($K_s/F_0 = 0.0002\ \mu M^{-1}$, $r = 0.87$), and (d) light scattering plus nonquenched fluorescence estimated from the last three points on the titration curve (solid line, $e^{-\lambda} = 0.04$).

K_s, of $36 \pm 5\ mM^{-1}$, typically representing 0.5−2% of the unquenched protein fluorescence intensity (F_o) during binding assays.

The complement of the residual fluorescence signal is a function of the fraction of bound protein, $(1 - f_f)$, and the fractional volume, $e^{-\lambda}$, within the quenching layer excluded from the spheres of influence of the quenchers randomly arranged within the membrane plane (Lakowicz, 1983). The exponent describes the effective quenching coverage per lipid molecule and can be calculated as $\lambda = (2/3)\pi R_o^2(x_q/A_L)$, where x_q is the fraction of quenching lipids in the membrane, and $A_L = 65$ Å^2 (Lantzsch *et al.*, 1994) is the lipid surface area. This fractional excluded volume was calculated as 0.07 for 10% TNP-PE and 0.21 for 60% Br_4-DSPC, in good agreement with the measured residual fluorescence values of 5−10% and 20−30%, respectively. This agreement suggests that significant lateral phase separation of the different lipids in the LUV does not contribute to the residual signal. Additionally, the binding studies were conducted far above the gel−liquid crystalline phase transition temperature for DOPG, DOPC, and TNP-PE, the most likely point at which domain formation would be expected (Welti & Glaser, 1994). The only exception is the saturated Br_4-DSPC, although the measured residual fluorescence indicates this to be a minor consideration.

Nonspecific Binding. Significant quenching of the intrinsic Trp fluorescence of various proteins at high pH (i.e., conditions which preclude significant electrostatic binding) by TNP-PE-containing vesicles was observed (data shown only for P178, Figure 2A, trace b). Quenching due to the nonspecific, nonelectrostatic binding was derived from the equilibrium equation, $K_q = [P_b]/[P_f][L_f]$. For weak binding, the free lipid concentration, $[L_f] \approx [L]$, yielding a Stern−Volmer type equation:

$$\frac{F}{F_o} = \frac{[P_f]}{[P]} = \frac{1}{1 + K_q[L]} \tag{11}$$

The energy associated with this interaction can be calculated from the relation between K_q and the partitioning of the protein between the aqueous and quenching layer above the membrane surface. This relationship is described by the specific quenching volume above the membrane surface,

Table 1: Determination of the Nonspecific, Nonelectrostatic Binding Energy, ΔG_{ns}, of P178 and Lysozyme C to TNP, and TNP-PE LUV with Low Surface Charge Density

			ΔG_{ns} (kcal/mol)			
			pH 4.0		pH 7.7	
protein	concentration (μM)	quencher	$I =$ 0.1 M	$I =$ 1.0 M	$I =$ 0.1 M	$I =$ 1.0 M
P178	0.21	TNP[a]	−4.3	−4.3	−4.1	−4.2
		LUV[b]	−5.1	−5.1	−5.1	−5.1
lysozyme C	0.56	TNP[a]	−4.5	−4.5	−4.4	−4.4
		LUV[b]	nd[c]	nd	−5.0	−5.0

[a] Quenching by TNP alone in aqueous solution. TNP, 2,4,6-trinitrophenol. [b] LUV: 10% TNP-PE/90% DOPC; $x_- = 0.084$. [c] nd, not determined.

$\bar{v}_q \approx x_a N_A R_o A_L$ ($\approx 0.5\ M^{-1}$), where x_a is the accessible lipid (LUV consistently showed fractional lipid accessibilities of, $x_a = 0.52 \pm 0.03$), $N_A = 6.02 \times 10^{23}$, and $A_L = 65$ Å^2:

$$\Delta G_{ns} = -RT \ln \frac{K_q}{\bar{v}_q} \tag{12}$$

The nonspecific nature of this binding was inferred from the similar ΔG_{ns} values for a variety of hydrophilic proteins (data not shown), including lysozyme, as compared to that for P178 (Table 1). Interaction of lysozyme and P178 with trinitrophenol (TNP) free in solution compared well with the nonspecific binding energy determined with TNP-PE LUV with low surface charge due to the 10% TNP-PE reporter lipid (Table 1). This indicates a nonspecific interaction of the protein with the TNP-PE headgroups. A lack of significant variation of ΔG_{ns} with respect to pH and ionic strength (Table 1) emphasizes its nonelectrostatic nature. TNP quenching has previously been used to qualitatively study peptide binding to membranes, but this significant nonspecific component to the binding had not been recognized (Suga *et al.*, 1991).

Determination of Thermodynamic Binding Parameters from Equilibrium Titration Curves. From the titration curve of protein with TNP-PE LUV shown in Figure 3, along with the components contributed by nonelectrostatic binding and residual fluorescence, it can be seen that the nonquenched fluorescence and light scattering contributions have an approximately linear relationship with respect to lipid or vesicle concentration. The second derivative of the titration function (eq 10, f_f substituted from eq 6) mathematically eliminates such linear terms and excludes or reduces the contribution of the irrelevant light scattering and incomplete quenching terms, to the calculation of the binding parameters. Since the second derivative emphasizes local curvature, it was found that consistent parameters could be calculated from the high-curvature region associated with binding, while avoiding problems associated with the estimation of residual fluorescence and any other constant or linear dependence on lipid concentration not included in the model.

The major term in the second derivative of eq 10 with respect to lipid concentration is

$$f_x'' \approx \frac{2K_d}{(1 + K_q[L])[P]^3 n^2 \left[\left(1 - \frac{[L]}{n[P]} - \frac{K_d}{[P]}\right)^2 + 4\frac{K_d}{[P]}\right]^{3/2}} \tag{13}$$

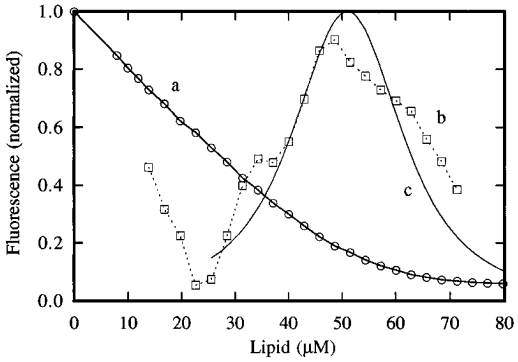

FIGURE 4: Titration of P178 (0.40 μM) with TNP-PE LUV (a, ○), showing its second derivative, calculated numerically (b, □), and the second derivative (c, eq 13) calculated from the fitted parameters, K_d (12 nM) and n (130 mol/mol). $x_- = 0.4$; pH 4.0; $I = 0.3$ M.

An example of a titration curve is shown in Figure 4a, where the protein was titrated with small aliquots (0.8−4 μM lipid) of the TNP-PE-containing LUV. The second derivative of this curve calculated numerically, shown in Figure 4b, exhibits a typical decrease in slope at low lipid concentrations, followed by an approximate bell shape. The analytical fit obtained using eq 13 to the numerical second derivative function (Figure 4b) is shown in Figure 4c. The shape and size of the bell curve of the second derivative determine K_d, while the position of its maximum determines n. Thus, at the maximum, f_m'', of f_x'' relative to [L], the denominator of eq 13 has a minimum value, i.e., $[(1 - [L]/n[P] - K_d/[P])] = 0$. At this lipid concentration, $[L]_m$, n, and K_d can be calculated from

$$n = \frac{[L]_m}{[P] - K_d} \tag{14}$$

$$K_d = \frac{1}{16n^4[P]^3}\left[\frac{1}{f_m''(1 + K_q[L]_m)}\right]^2 \tag{15}$$

The experimental titration data was analyzed by taking the slope of a moving 3 or 5 point linear fit as an approximation of the first derivative (effectively also smoothing the data). Since smoothing decreases the curvature (a measure of K_d), derivatives for tight binding cases were approximated by linear slopes between adjacent points. The second derivative was obtained in the same way from the first derivative.

The parameters n, K_d, and K_q were iteratively determined from the initial estimates (eqs 14 and 15 and $K_q = 2200$ M^{-1}) using the Downhill Simplex method of Nelder and Mead (Press *et al.*, 1992) to fit the second derivative data to eq 13, written as an Excel 4.0 macro (Microsoft Corp., WA).

For the colicin E1 channel polypeptides, P178 and P190, typical thermodynamic binding parameters are $n = 50-60$, $K_d = 2-3$ nM, and $\Delta G = -9.9$ to -9.3 kcal/mol (Tables 2 and 3). Under the same conditions the larger 293 residue channel−receptor domain, P293 (Zhang, 1993), and the colicin E1 molecule of 522 residues show an increase in n values to 95 and 211, respectively. This qualitative scaling according to molecular size supports the notion of n as an indication of the size of a putative binding site. It should be noted that $n = 220$ for the water-soluble protein, lysozyme C (MW = 14 300) is appreciably larger than that of the colicin channel polypeptides (MW ≈ 20 000) [Table 2]. This contradicts the simple interpretation of n as a binding site size.

Table 2: Thermodynamic Binding Parameters (n, K_d, and Total Binding Energy, ΔG) of Colicin E1 and Its Component Polypeptides, P178 and P190 (Channel), P293 (Channel and Receptor), and Lysozyme C, with TNP-PE LUV at pH 4.0[a]

protein	concentration (μM)	K_d (nM)	n (mol/mol)	ΔG (kcal/mol)
P178	0.21	1.9 ± 0.4	50 ± 4	−9.9
P190	0.19	3.1 ± 0.4	57 ± 3	−9.3
P293	0.20	0.4 ± 0.2	95 ± 21	−10.2
colicin E1	0.20	1.3 ± 0.2	211 ± 10	−9.1
lysozyme C	0.56	61 ± 28	220 ± 42	−6.8

[a] TNP-PE LUV $x_- = 0.4$; $I = 0.12$ M; $\psi_o = -29.8$ mV.

Table 3: Energy Components of the Binding of the Colicin E1 Channel Polypeptide P178 to TNP-PE LUV and Br_4-DSPC LUV at pH 4.0[a]

LUV	ΔG[b] (kcal/mol)	ΔG_{nel}[c] (kcal/mol)	ΔG_{ns}[d] (kcal/mol)	ΔG_{el}[e] (kcal/mol)	z_{eff}[f]
10% TNP-PE	−9.9	−5.0	−4.9	−4.9	7.1
60% Br4-DSPC	−9.8	−7.0	0.0	−2.8	4.1

[a] LUV $x_- = 0.4$; $I = 0.12$ M; $\psi_o = -29.8$ mV. [b] ΔG is the total binding energy determined at pH 4.0. [c] ΔG_{nel} is the nonelectrostatic energy calculated at $\psi_o = 0$ in Figure 5. [d] ΔG_{ns} is the nonspecific interaction energy determined at pH 7.7. [e] $\Delta G_{el} = \Delta G - \Delta G_{nel}$ is the electrostatic energy. [f] z_{eff} is the effective binding charge calculated from eq 1.

Lower Limits for the Determination of the Thermodynamic Parameters. The lipid/protein stoichiometry, n, can be determined with great accuracy, since it is a function of the position of the high-curvature region of the titration curve. The lower limit for the resolution of differences in n is simply related to the ratio of the titration step size, $\Delta[L]$, and the total protein concentration, [P], multiplied by a factor of two (from general considerations of sampling theory):

$$n_{lo} = 2(\Delta[L]/[P]) \tag{16}$$

For [P] = 200 nM and $\Delta[L] = 1$ μM, $n \geq n_{lo} = 10$ can be determined. Monomeric globular proteins have radii ∼15−30 Å, which means they should cover about 700−2800 Å^2 of membrane surface per molecule. This corresponds to ∼10−40 lipid molecules of area 65 Å^2 (Lantzsch *et al.*, 1994), giving $n \sim 20-80$ (for an accessible fraction, $x_a = 0.5$), well above n_{lo}.

On the other hand, K_d is a function of the actual curvature and is determined with less certainty. At the point of highest curvature, defined by $[L]_m$ in eq 14, K_d relative to the free peptide concentration at this point, $[P_f]_m$, is obtained by substituting eq 14 into eq 6:

$$K_d = \frac{[P_f]_m^2}{[P]} = f_m^2[P] \tag{17}$$

This indicates that, for the titration step at $[L]_m$, the relative fluorescence change must be less than f_m to be able to determine $K_d[\sim(f_m/2)]$. In this paper, with [P] ∼ 200 nM, and the relative fluorescence change for tight binding typically 0.02 per titration step, values of $K_d \geq K_{dlo} = (0.04)^2 \times 200$ nM = 0.3 nM can be determined. From n_{lo} and K_{dlo},

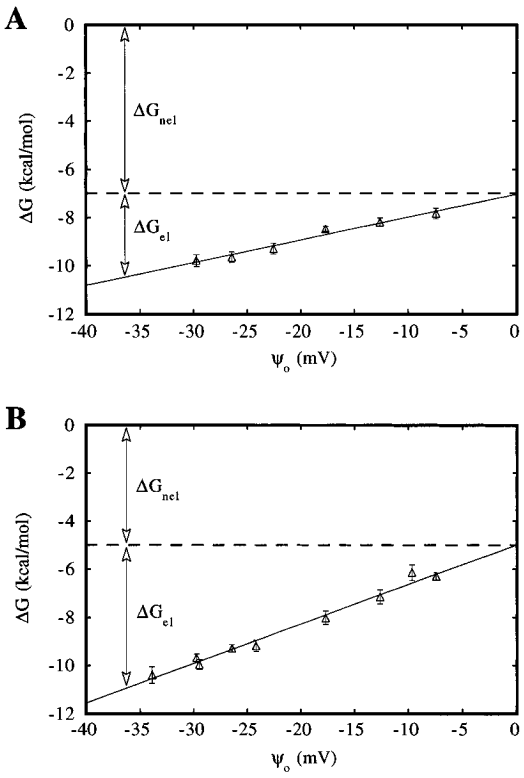

FIGURE 5: Dependence of the electrostatic membrane-binding free energy, ΔG_{el}, for the colicin E1 channel polypeptide on membrane surface potential, defining the nonelectrostatic, ΔG_{nel}, component as the remainder. (A) Binding of P178 to 60% Br_4-DSPC/40% DOPG LUV with the total energy (eq 1): $\Delta G = 0.094\psi_o - 7.0$ ($r = 0.98$), and giving an effective charge $z_{eff} = 4.1$. (B) Binding of P178 to 10% TNP-PE LUV with the total energy (eq 1): $\Delta G = 0.164\psi_o - 5.0$ ($r = 0.99$), and giving an effective charge $z_{eff} = 7.1$. [P] = 0.21 μM P178; 20 mM DMG; pH 4.0; ψ_o was varied by changing the ionic strength, $I = 0.1-1.0$ M, as well as the vesicle negative lipid content, $x_- = 0.2-1.0$.

the lower limit of the binding energy that can be determined is calculated (eq 9) as $\Delta G_{lo} \approx -12$ kcal/mol.

Free Energy of Protein−Membrane Binding; Electrostatic and Nonelectrostatic Components. The free energy, ΔG, as defined in eq 1 and determined from the measured n and K_d as in eq 9, is composed of electrostatic and nonelectrostatic components (Figure 5). The electrostatic component is a function of the vesicle surface potential, ψ_o, and the effective interacting charge, z_{eff}. The surface potential and the surface charge density are interrelated parameters dependent on the ionic strength and the fraction of charged lipid in the membrane. The surface charge density, σ, can be derived from the interaction of the major cation in the system, Na^+, with negative lipid on the membrane surface:

$$K_{NaL} = \frac{[NaL]}{[Na^+]_o[L^-]} \tag{18}$$

where [NaL], $[Na^+]_o$, and $[L^-]$ are the concentrations for the sodium−lipid ionic complex, free sodium at the membrane surface, and noncomplexed negative lipid, and $K_{NaL} = 0.6\ M^{-1}$ (Seelig *et al.*, 1993) is the sodium−negative lipid association constant. Given the fraction of negative lipid in the membrane, x_-, and the concentration of sodium at the membrane surface, $[Na^+]_o = [Na^+]e^{-F\psi_o/RT}$, where $[Na^+]$ is the bulk sodium concentration, the relation between surface charge density and surface potential is

$$\sigma = \frac{e_- x_-}{A_L[1 + K_{NaL}[Na^+]e^{-F\psi_o/RT}]} \tag{19}$$

where e_- is the elementary charge (-1.6×10^{-19} C), and $A_L = 65$ Å^2 (Lantzsch *et al.*, 1994). The surface charge density is also related to the ionic strength (Seelig *et al.*, 1993):

$$\sigma = -\sqrt{2\epsilon\epsilon_o RT[[Na^+](e^{-F\psi_o/RT} - 1) + [Cl^-](e^{F\psi_o/RT} - 1)]} \tag{20}$$

To simplify the analysis, only NaCl is considered as electrolyte, with the 20 mM DMG buffer adjusted for pH with NaOH, treated as behaving like NaCl. Equations 19 and 20 were numerically solved for σ and ψ_o, using Mathcad 3.1 (MathSoft Inc., Cambridge, MA), arriving at values closely corresponding to those in McLaughlin (1989). The validity of this approach to determine membrane surface potential has been confirmed many times, and most recently by Kraayenhof *et al.* (1993), using membrane surface fluorophores.

Using ionic strength-dependent values of ψ_o and ΔG, eq 1 can be used to derive (i) the effective charge of interaction, z_{eff}, of the protein from the slope of the ΔG-ψ_o function, and (ii) the value of the nonelectrostatic interaction, ΔG_{nel}, from the $\psi_o = 0$ intercept of the linear function (Figure 5). From the value of ΔG_{nel}, the complementary value of ΔG_{el} can be obtained immediately from eq 1.

The membrane-binding energy of the colicin E1 channel polypeptide, P178, was characterized with both TNP-PE and Br_4-DSPC LUV (Table 3 and Figure 5). The $\Delta G_{nel} = -5.0$ kcal/mol for binding to TNP-PE LUV at pH 4 (determined from extrapolation to $\psi_o = 0$; Figure 5B) is similar to the ΔG_{ns} for binding to the TNP headgroups measured at high pH and also observed for other hydrophilic proteins such as lysozyme (Table 1). With Br_4-DSPC LUV, no such nonspecific binding of P178 was observed at high pH ($\Delta G_{ns} = 0$, Table 3) and $\Delta G_{nel} = -7.0$ kcal/mol at pH 4.0 (Figure 5A), while lysozyme was not quenched at all (Figure 2B).

The higher specific nonelectrostatic binding of P178 to the Br_4-DSPC LUV containing more saturated lipid compared to TNP-PE LUV is accompanied by a decreased electrostatic interaction (Table 3 and Figure 5). Whereas the total binding energy under these conditions (which are typically used in *in vitro* channel-forming activity assays) are about the same, $\Delta G \approx -9.9$ kcal/mol, the shift to more pronounced nonelectrostatic interaction appears to be associated with increased amounts of saturated lipid.

DISCUSSION

Analysis of Protein−Membrane Interactions. The measurement of protein−membrane interactions employing a membrane-bound quencher system can be used to study the binding to and penetration of proteins into lipid bilayers. Most of the quenchers that have been used previously are localized within the membrane bilayer, e.g., pyrene (Suga *et al.*, 1991), brominated lipid (Abrams & London, 1992; Berkhout *et al.*, 1987; Bolen & Holloway, 1990; de Kroon *et al.*, 1990; González-Mañas *et al.*, 1992, 1993), or spin-labeled lipid (Abrams & London, 1992; Palmer & Merrill, 1994). These quenchers may be more suitable to assess membrane penetration by proteins rather than or, in addition to, binding at the membrane surface (Figure 2B).

Phospholipids with quenching headgroups cover the membrane surface more efficiently than submerged quenchers and, in principle, offer a better means for quantitation of binding parameters. Examples include dansyl-PE (Bardelle *et al.*, 1993) and TNP-PE (Suga *et al.*, 1991), although submerged quenchers with long effective quenching distances can also be used, e.g., anthroyl stearic acid (Shaklai *et al.*, 1977).

Analysis of Trp Quenching Titration Curves. The design of the quenching experiments will invariably involve background contributions to the fluorescence signal, negative and positive, that do not arise from the act of protein binding to the membrane surface. It is important to correct for such background processes as otherwise they can influence the accuracy of the binding parameters derived from the Trp fluorescence quenching function. In the present study, this has been approached by (i) deconvoluting the quenching function into the conceptually well-defined physical processes that could be identified, (a) nonelectrostatic quenching, (b) light-scattering, and (c) unquenched residual fluorescence (eq 10). (ii) Because processes b and c are approximately linear functions of lipid concentration, the use of the second derivative of the quenching function (eq 13), which mathematically eliminates terms that are linear in lipid concentration, increased the accuracy of determination of the dissociation constant, K_d. The value of the second derivative approach lies in the emphasis of the curvature associated with K_d as the titration function approaches its asymptotic limit at high lipid concentrations (Figures 3a and 4a). The analysis presented here has utilized both TNP-PE and Br_4-DSPC quenching as assays for protein binding and has been applied in the present primarily to colicin E1 and its component polypeptides as examples of membrane-interactive proteins, and lysozyme C as an example of a soluble protein. However, the approach is general and can be applied to Trp-containing membrane proteins that bind with sufficient affinity.

Nonspecific Binding by TNP: ΔG_{ns} and ΔG_{nel}. Analysis of TNP-PE LUV quenching of a wide variety of soluble proteins (not shown) and of the membrane-interactive colicin E1 polypeptides at alkaline pH where previous data showed it does not bind to membranes (Davidson *et al.*, 1985) revealed that part of the observed binding and quenching of fluorescence by TNP-PE LUV arose from nonspecific interaction with TNP, ΔG_{ns}. This had not been taken into account in previous studies using this technique (Suga *et al.*, 1991).

It is not known what fraction of ΔG_{ns} measured with TNP-PE LUV at high pH contributes to the nonelectrostatic binding component, ΔG_{nel}, at low pH where the mechanism of binding includes a large electrostatic component, ΔG_{el}. The similarity of the ΔG_{ns} and ΔG_{nel} values suggests that a significant fraction of the ΔG_{nel} could be ΔG_{ns}. Evidence for a large nonelectrostatic specific (probably hydrophobic) component is provided by the absence of nonspecific binding to Br_4-DSPC LUV.

Quenching by Br_4-DSPC requires that the Trp residues in the protein penetrate at least through the lipid headgroup region, and the measured $\Delta G_{ns} = 0$ may reflect either an absence of nonspecific binding or an inability to determine such binding. The similarity of the ΔG_{nel} values measured with the two quenchers implies that while the magnitude of the nonelectrostatic interaction is comparable, the $\Delta G_{ns} \approx -5$ kcal/mol measured with TNP-PE arises from a different stage of interaction (i.e., with the lipid headgroups) than that for the ΔG_{nel} detected with bromine quencher (i.e., insertion). The latter distinguishes colicin from a nonmembrane protein such as lysozyme that does not penetrate the bilayer and is believed to largely reflect interaction of the protein with the interfacial region of the bilayer subsequent to the initial electrostatic binding.

Binding Model: Complexity of n and K_d. A central concept used in this study is the binding model of Hille *et al.* (1981), which treats the system as an interaction between two multivalent entities. The binding energy is a function of both the dissociation constant, K_d, and the lipid/protein stoichiometry, *n*. It is important to note that the concept of "*n*" as a parameter describing the size of the binding site or the number of lipid molecules binding to a protein molecule (Berkhout *et al.*, 1987; de Kroon *et al.*, 1990; Hille *et al.*, 1981; Rietveld *et al.*, 1986) must take into account its dependence on protein concentration, as indicated in eq 6, and in Beschiaschvili and Seelig (1990). In addition, the difference between K_d values obtained for apocytochrome *c* binding to SUV, $K_d = 0.04$ μM for 100% PS (Berkhout *et al.*, 1987), and LUV, $K_d = 15$ μM for 100% PS (Rietveld *et al.*, 1986) may be a manifestation of the dependence on protein concentration because our studies have not shown a large difference in K_d values measured in SUV or LUV (data not shown). On the other hand, for measurements at constant or similar protein concentration, the utility of the model and the meaning of "*n*" is demonstrated by the proportionality between *n* values and molecular size for the binding of the same concentrations of proteins of similar nature, colicin E1 and its component polypeptides (Table 2). Furthermore, the reciprocal relationship between *n* and K_d with respect to protein concentration (eq 6) shows that concentration effects will not affect the calculation of the binding energy (eq 9), because the [P] terms cancel out.

Loss of Translational and Rotational Degrees of Freedom on Binding: Hydrophobic Transfer Energy. When a rigid, charged molecule such as a protein is concentrated at the membrane surface because of Boltzmann accumulation, it loses at least one translational degree of freedom. Binding to the membrane surface and insertion leads to the loss of an additional two rotational degrees of freedom. When the colicin E1 channel domain binds to a membrane, the ΔG of binding is increased by approximately 15 kcal/mol because of a decrease in this entropic component (Janin & Chothia, 1978). The true ΔG of binding to the membrane must therefore be larger than the measured ΔG by the same amount. Using the measurement of $\Delta G_{nel} \approx -7$ to -5 kcal/mol shown in Figure 5, this implies that, for the colicin E1 channel domain, the total $\Delta G_{nel} \sim -20$ kcal/mol.

This leads to two interesting conclusions: (i) the ΔG_{nel} substantially exceeds the largest values of the electrostatic binding component, $\Delta G_{el} \approx -3$ to -6 kcal/mol, determined in Figure 5; (ii) $\Delta G_{nel} \approx -20$ kcal/mol is much less than the -60 kcal/mol hydrophobic binding energy calculated for the insertion of a 40 residue hydrophobic α-helical hairpin (Engelman & Steitz, 1981). This implies either that the colicin helical hairpin is not inserted during the binding process (Lakey *et al.*, 1993) or that, in calculating the hydrophobic transfer energy, one must take into account the hydrophobic environment of the hairpin in the initial state, the soluble colicin channel domain. Because it is known

that the helical hairpin forms the hydrophobic core of the soluble channel polypeptide (Elkins *et al.*, 1995; Parker *et al.*, 1992), this can readily explain a hydrophobic transfer energy smaller than calculated by Engelman and Steitz (1981).

Information on ΔG, ΔG_{el}, *and* ΔG_{nel}. In the present study, a quantitative description is presented of the application of the protein binding assay, with TNP-PE or Br_4-DSPC as the lipophilic quencher, to extract significant details about the nature of this interaction, e.g., free energy of binding, values of electrostatic and nonelectrostatic binding components, and the effective charge of the protein participating in the binding interaction. This approach has been used to determine the conditions of electrostatic binding that are correlated with the ability of the colicin E1 ion channel to insert into the membrane and express channel activity (Zakharov *et al.*, submitted for publication).

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