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Protein Surface-Distribution and Protein-Protein Interactions in the Binding of Peripheral Proteins to Charged Lipid Membranes

Thomas Heimburg and Derek Marsh
Max-Planck-Institut für biophysikalische Chemie, Abt. Spektroskopie, D-37077 Göttingen, Germany

ABSTRACT The binding of native cytochrome *c* to negatively charged lipid dispersions of dioleoyl phosphatidylglycerol has been studied over a wide range of ionic strengths. Not only is the strength of protein binding found to decrease rapidly with increasing ionic strength, but also the binding curves reach an apparent saturation level that decreases rapidly with increasing ionic strength. Analysis of the binding isotherms with a general statistical thermodynamic model that takes into account not only the free energy of the electrostatic double layer, but also the free energy of the surface distribution of the protein, demonstrates that the apparent saturation effects could arise from a competition between the out-of-plane binding reaction and the lateral in-plane interactions between proteins at the surface. It is found that association with nonlocalized sites results in binding isotherms that display the apparent saturation effect to a much more pronounced extent than does the Langmuir adsorption isotherm for binding to localized sites. With the model for nonlocalized sites, the binding isotherms of native cytochrome *c* can be described adequately by taking into account only the entropy of the surface distribution of the protein, without appreciable enthalpic interactions between the bound proteins. The binding of cytochrome *c* to dioleoyl phosphatidylglycerol dispersions at a temperature at which the bound protein is denatured on the lipid surface, but is nondenatured when free in solution, has also been studied. The binding curves for the surface-denatured protein differ from those for the native protein in that the apparent saturation at high ionic strength is less pronounced. This indicates the tendency of the denatured protein to aggregate on the lipid surface, and can be described by the binding isotherms for nonlocalized sites only if attractive interactions between the surface-bound proteins are included in addition to the distributional entropic terms. Additionally, it is found that the binding capacity for the native protein is increased at low ionic strength to a value that is greater than that for complete surface coverage, and that corresponds more closely to neutralization of the effective charge (determined from the ionic strength dependence), rather than of the total net charge, on the protein. Electron spin resonance experiments with spin-labeled lipids indicate that this different mode of binding arises from a penetration or disturbance of the bilayer surface by the protein that may alleviate the effects of in-plane interactions under conditions of strong binding.

INTRODUCTION

The binding of peripheral proteins to membranes is, in the main, electrostatic in nature (for a review, see Sankaram and Marsh, 1993). The strength of protein binding decreases with increasing ionic strength (see, e.g., Schwarz and Beschiaschvili, 1989; Stankowski, 1991) in a manner that can be described at least semi-quantitatively by electrostatic double-layer theory (McLaughlin, 1989; Kuchinka and Seelig, 1989). Allowance for discreteness-of-charge effects (Nelson and McQuarrie, 1975; Winiski et al., 1986) also leads to results similar to those of double-layer theory when the distance between ligands is greater than the Debye length (Mathias et al., 1988; Vorotyntsev and Ivanov, 1988). In general, it is found that the effective charge governing the electrostatic interaction is considerably less than that of the formal net charge on the protein.

A further significant feature of the electrostatic membrane association of peripheral proteins is that the protein becomes progressively displaced from the membrane with increasing ionic strength, even in the presence of a large excess of added protein (Görrissen et al., 1986; Sankaram et al., 1989). This implies that not only the strength of binding, but also the apparent saturation binding, decreases with increasing ionic strength. The mechanism for this may lie in a competition between the binding forces and the lateral interactions between the bound proteins at the membrane surface. In principle, therefore, a study of the ionic strength dependence of the binding of peripheral proteins should provide a means for investigating protein-protein interactions at the membrane surface.

Frequently, the Langmuir adsorption isotherm (or even a partitioning equilibrium) is used to describe the binding of peripheral proteins to membranes. The Langmuir isotherm corresponds to binding to fixed, localized sites, such as is appropriate for small ions or for low extents of binding. However, for the binding of large ligands at high degrees of occupancy, the Langmuir isotherm overestimates the degree of binding because it does not describe correctly the entropy of the ligand distribution on the surface, and a model for binding to nonlocalized sites is more appropriate (cf. Hill, 1946). In this paper, a statistical thermodynamic model for binding to nonlocalized sites is introduced that is generally applicable to the association with continuous surfaces, and it includes

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Address reprint requests to Dr. Thomas Heimburg, ABT.010, Max-Planck-Inst./Biophys. Chem., Am Fassberg, D-37077 Gottingen-Nikolausberg, Germany.

Abbreviations used: DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; Cyt. *c*, horse heart cytochrome *c*; 14-PGSL, 1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)]stearoyl-*sn*-glycero-3-phosphoglycerol; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulphonic acid; ESR, electron spin resonance.

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explicitly both electrostatic interactions and lateral interactions between the bound ligands. For concreteness, the electrostatic interactions are obtained from double-layer theory and the lateral interactions are obtained from the two-dimensional Van der Waals gas equation.

Binding of the basic mitochondrial membrane protein cytochrome *c* to negatively charged lipid surfaces has been investigated extensively as a model for peripheral protein-membrane interactions. For example, the protein conformation when bound to bilayers or micelles (Spooner and Watts, 1991; De Jongh et al., 1992; Heimburg and Marsh, 1993), the effects of the protein on lipid structure and mobility (De Kruijff and Cullis, 1980; Görrissen et al., 1986), and the thermodynamic effects of binding (Heimburg and Biltonen, 1994), as well as possible functional changes (Heimburg et al., 1991), have been studied. Here, the binding isotherms for native cytochrome *c* with phosphatidylglycerol bilayers have been determined over a wide range of ionic strength, to obtain details of the in-plane interactions between the surface-bound proteins. The complete dependence on protein concentration and ionic strength has been fitted to the binding model with nonlocalized sites. For comparison, cytochrome *c* that has been heat-denatured on the membrane surface (but not in solution), and is expected to display enhanced protein-protein interactions because it is thought to be aggregated in this state (Muga et al., 1991), has also been studied in parallel. The results indicate the importance of lateral surface interactions in the binding of peripheral proteins to membranes.

MATERIALS AND METHODS

Cytochrome *c* (type VI, oxidized form, Sigma Chemical Co., St. Louis, MO) was used without further purification. Dioleoyl phosphatidylglycerol (DOPG) (Avanti Polar Lipids, Birmingham, AL) was shown to be pure on thin layer chromatography and was used without further purification.

Lipid dispersions (10 mg/ml) and protein solutions (20 mg/ml) were prepared in distilled water. Various amounts of protein solution were added to 1 mg of lipid under conditions of minimal ionic strength (i.e., of maximum binding strength). The lipid-protein mixtures were then diluted to a total volume of 6.1 ml (corresponding to 238 μM lipid), with 2 mM HEPES, 1 mM EDTA buffer at pH 7.5, and various concentrations of NaCl in the range from 0 to 250 mM. The NaCl concentration was adjusted after mixing cytochrome *c* and DOPG in the absence of salt, to avoid any changes in the accessibility of the protein to the lipid (e.g., multilayer formation) that might occur at higher salt concentrations. It should be noted that the lipid as originally dispersed in buffer of minimal ionic strength consists of an extended network that allows maximum accessibility to the protein (Heimburg and Biltonen, 1994). Formation of multilayers does not occur before addition of protein (and/or salt). The lipid-protein mixtures were then equilibrated at room temperature for 48 h to allow for redistribution of protein from the membrane surface into the buffer. The ionic strength was calculated including the counterions of the HEPES and the EDTA in the buffer (this contribution corresponds to 4 mM Na^+). All preparations were under either argon or nitrogen to avoid oxidation of the unsaturated lipid chains.

The lipid-protein complexes were separated from the protein free in solution by centrifugation (Beckman L7–55, Ti-50 rotor, 50,000 rpm) for 1 h for samples of high ionic strength, and for 2 h for samples of low ionic strength. No phosphate was detectable in the supernatant after ultracentrifugation, demonstrating complete resolution of the lipid-protein complex. The concentration of free protein was determined from the spectrophotometric extinction of cytochrome *c* at 546 and 410 nm, in the supernatant. All protein other than that in the supernatant was assumed to be bound to the lipid membranes.

ESR spectra were recorded on a 9-GHz spectrometer (E-line, Varian Associates, Instrument Division, Palo Alto, CA) equipped with nitrogen gas-flow temperature regulation. The pelleted lipid/protein complexes were contained in sealed 1 mm ID 100-μl glass capillaries. Spin-labeled phosphatidylglycerol, 14-PGSL (1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)]stearoyl-*sn*-glycero-3-phosphoglycerol), was prepared from the corresponding spin-labeled phosphatidylcholine (see Marsh and Watts, 1982) by the action of phospholipase D (Comfurius and Zwaal, 1977). The concentration of spin label was 1 mol% relative to the overall lipid.

THEORETICAL BACKGROUND

The case of ligand binding to a continuous surface with nonlocalized sites is considered. Each ligand that binds to the surface occupies an area ΔA. The total area of the surface is given by $n\Delta A$, corresponding to complete saturation of the surface with n ligand molecules. The effective binding constant, defined in terms of the concentration of the state with i ligands bound, is given by $K_i = K_0\exp(-\Delta F(i)/ikT)$, where K_0 is the intrinsic binding constant. The argument i indicates that the cumulative change in free energy, ΔF, of the system depends on the number, i, of ligands bound. This surface free energy contains not only the electrostatic contribution, ΔF_{el}, but additionally a contribution, ΔF_D, from the distribution and interactions of the ligands on the surface. The latter can be expressed in terms of the work, $\Delta W(i)$, done to compress a two-dimensional gas of i ligand molecules to a surface area $n\Delta A$

$$\Delta F_D = \Delta W(i) = -\int \Pi(i)\, dA, \qquad (1)$$

where $\Pi(i)$ is the lateral pressure exerted at the surface by the bound ligands.

The mean number, ν, of ligands bound to the surface of area $n\Delta A$ can be written as (cf., e.g., Cantor and Schimmel, 1980)

$$\nu \equiv \langle i \rangle = \frac{\sum_{i=1}^{n} i \frac{1}{i!}[L]^i K_o^i \exp\left(-\frac{\Delta F(i)}{kT}\right)}{\sum_{i=0}^{n} \frac{1}{i!}[L]^i K_o^i \exp\left(-\frac{\Delta F(i)}{kT}\right)} = \frac{\sum_{i=1}^{n} iP_i}{Q}, \qquad (2)$$

where $[L]$ is the free ligand concentration and the factor $i!$ represents the number of indistinguishable arrangements of i ligands on a continuous surface (cf. Tanford, 1961). The partition coefficients P_i/Q describe the distribution of surfaces that have i ligands bound, with Q being the partition function. For the aggregation equilibria of ligands on a surface, this distribution can have more than one maximum (see Fig. 1). However, in most cases the distribution will have one distinct maximum at a particular value of i. In this case, Eq. 2 can be simplified to (see Appendix I)

$$\nu = [L]K_o \exp\left(-\frac{d}{di}\left[\frac{\Delta F(i)}{kT}\right]\right). \qquad (3)$$

This general result is valid for all binding isotherms. To obtain practical isotherms requires specific models for the

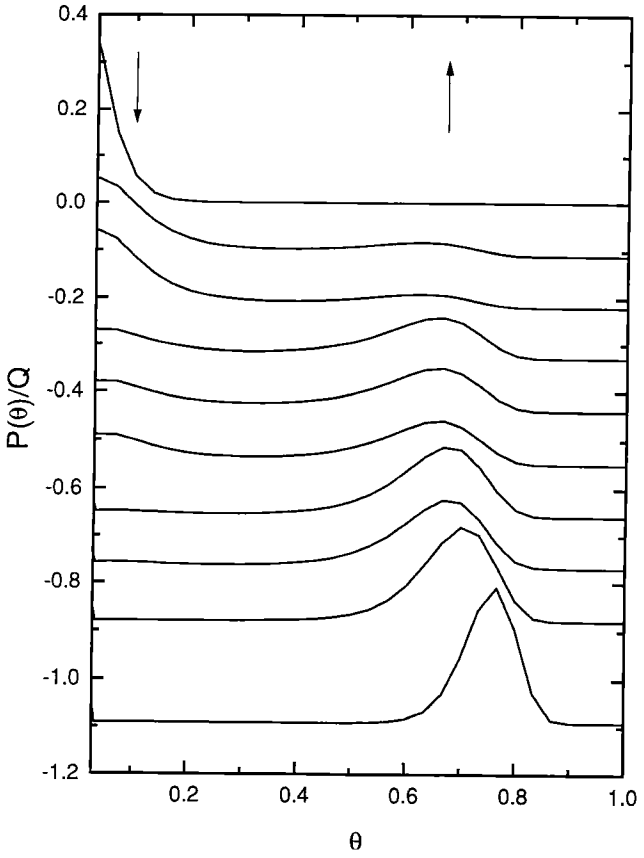

FIGURE 1 Probability distribution, P_i/Q, of bound states as a function of the degree of surface occupancy, θ, for increasing total ligand concentrations (from *top* to *bottom*), obtained from Eqs. 2, 5, and 14, 16 with $K(0) = 250$ l/mol of lipid, $a = 6$, and $Z = 4.232$. Although at low and at high total ligand concentrations (*top* and *bottom* curves) there is only one maximum in the distribution, at intermediate ligand concentrations a coexistence of two maxima that correspond to monomeric and to aggregated states of the ligands on the surface are found. The corresponding binding isotherms are given later in Fig. 3.

electrostatic interactions, defining $\Delta F_{el}(i)$, and for the intermolecular interactions, defining the work $\Delta W(i)$.

Free energy of the ligand distribution

The most general approach to define a surface pressure is a virial expansion, as has been used, e.g., by Stankowski (1991) to describe electrostatic ligand-ligand interactions on membrane surfaces. A similar approach, but restricted to relatively low surface densities, has been used by Tsien (1978) for discrete charges buried in a membrane. As a first-order approximation, the surface pressure is given here by the van der Waals gas equation in two dimensions (cf. Hill, 1946)

$$\Pi(i) = \frac{ikT}{(n-i)\Delta A} - a\left(\frac{kT}{\Delta A}\right)\left(\frac{i}{n}\right)^2, \qquad (4)$$

where a is a measure of the strength of interactions between ligand molecules (positive for attractive interactions and negative for repulsive interactions) and ΔA is the excluded area per ligand. The work $\Delta W(i)$ can be obtained from Eqs. 1 and 4 using $dA = \Delta A dn$

$$\Delta W(i) = -ikT\ln(n-i) - akT\left(\frac{i^2}{n}\right), \qquad (5)$$

where the integration constant from Eq. 1 refers to the standard state and therefore is contained already in the binding constant, K_0.

In the case of solely nonelectrostatic interactions between surface and ligand (i.e., $\Delta F_{el}(i) = 0$), it follows from Eqs. 3 and 5 that

$$\nu = [L]K_0(n-i)\exp\left(-\frac{i}{n-i} + 2a\frac{i}{n}\right), \qquad (6)$$

or in terms of the degree of surface coverage, $\theta = i/n$:

$$[L] = \frac{1}{K_0}\frac{\theta}{1-\theta}\exp\left(\frac{\theta}{1-\theta} - 2a\theta\right), \qquad (7)$$

which is equivalent to the binding isotherm derived by Hill (1946), and reduces to the familiar Langmuir adsorption isotherm for binding to localized sites, only in the limit of low saturation. Also, the exact solution of McGhee and von Hippel (1974) for binding to a one-dimensional lattice reduces to the noninteracting case of Eq. 7, in the limit of a one-dimensional continuous surface (see Appendix II). The additional two exponential terms in Eq. 7, which do not appear in the Langmuir adsorption isotherm, represent the modification of the distributional entropy, for binding to nonlocalized sites, and the nonideal surface interactions (i.e., the a-term) between the proteins, respectively.

Binding of charged ligands to charged surfaces

The total gain in electrostatic free energy upon binding i ligand molecules to the surface is the difference between that in the bound and unbound states and contains three terms:

$$\Delta F_{el}(i) = F^S_{el}(i) - F^S_{el}(0) - i \cdot F^L_{el}, \qquad (8)$$

where $F^S_{el}(i)$ is the electrostatic free energy of a surface of area $n\Delta A$ with i ligands bound, $F^S_{el}(0)$ is the electrostatic free energy of the surface without any ligands bound, and F^L_{el} is the electrostatic free energy of a ligand in solution.

The surface is assumed to have a uniform charge density, which with i ligands bound is given by

$$\sigma = -\frac{(n\alpha - iZ)}{n\Delta A}e = -\left(1 - \frac{iZ}{n\alpha}\right)\frac{e}{f_0}, \qquad (9)$$

where f_0 is the surface area per lipid molecule, $+Ze$ is the charge on the ligand, and α is the number of negative (lipid) surface charges per bound ligand at saturation. The electrostatic free energy of the surface given by double-layer theory, in the high potential limit, is (Jähnig, 1976)

$$F^S_{el}(i) = 2(n\alpha - iZ)kT\ln\left(-\frac{\Lambda_0\sigma}{\sqrt{c}}\right), \qquad (10)$$

where $\Lambda_0 = \sqrt{(1000\pi/2\epsilon N_A kT)}$. The condition for the high potential limit is that the argument of the logarithm

is $(-\Lambda_0\sigma/\sqrt{c}) > 2$. This is found to be the case for all systems considered here (cf. Results).

The ligand is approximated as a uniform spherical charge distribution that is impenetrant to solvent. The electrostatic self energy F_{el}^L of a free ligand in solution is then (Tanford, 1955; Rice and Nagasawa, 1961)

$$F_{el}^L = \frac{Z^2e^2}{2\varepsilon r_0(1 + \kappa r_0)}, \tag{11}$$

where r_0 is the radius of the ligand with net charge $+Ze$ and κ is the reciprocal Debye length:

$$\kappa = \sqrt{\frac{8\pi e^2 N_A}{\varepsilon k 10^3}}\sqrt{\frac{c}{T}}, \tag{12}$$

with e being the elementary charge, N_A Avogadro's number, ϵ the dielectric constant of the solution, and c the monovalent ion concentration in mol/l. Between 1 and 250 mM monovalent ion concentration (i.e., $2.5 \geq \kappa r_0 \geq 0.16$, with the radius of cytochrome c, $r_0 = 15$ Å), Eq. 11 is well approximated by a logarithmic expression:

$$\begin{aligned} F_{el}^L &= \frac{Z^2e^2}{4\varepsilon r_0}(1 - \tfrac{1}{2}\ln(\kappa r_0)) \\ &\equiv kTZ^2\Lambda_2(1 - \tfrac{1}{2}\ln(\kappa r_0)), \end{aligned} \tag{13}$$

where $\Lambda_2 = e^2/4\epsilon r_0 kT = 0.126$ at $T = 277$ K. This approximation is required for conformity with the ionic strength dependence of the surface contributions to the electrostatic free energy. (This self energy term is included for consistency, although it refers to a standard state that depends on ionic strength. For cytochrome c binding to negatively charged membranes, it is a minor contribution to the binding energy, but for ligands with higher effective charge it would be larger.)

The total change in electrostatic free energy on binding i ligands is then given from Eqs. 8, 9, 10, and 13 by

$$\Delta F_{el}(i) = kT\left[2(n\alpha - iZ)\ln\left(1 - \frac{iZ}{n\alpha}\right) - 2iZ\ln\left(\frac{\Lambda_1}{\sqrt{c}}\right) - i\Lambda_2Z^2 - \tfrac{1}{2}i\Lambda_2Z^2\ln\left(\frac{\Lambda_3}{\sqrt{c}}\right)\right], \tag{14}$$

where $\Lambda_1 = \Lambda_0 \cdot e/f_0 = 1.71\ \mathrm{M}^{1/2}$ with $f_0 = 82$ Å^2 at $T = 277$ K, and $\Lambda_3 = \sqrt{c}/(\kappa r_0) = 0.197\ \mathrm{M}^{1/2}$, with $r_0 = 15$ Å and $T = 277$ K. Correspondingly, the incremental change in electrostatic free energy on ligand binding is

$$\frac{d\Delta F_{el}(i)}{di} = -kT\left[2Z\ln\left(1 - \frac{iZ}{n\alpha}\right) + 2Z\ln\left(\frac{\Lambda_1}{\sqrt{c}}\right) + 2Z + \Lambda_2Z^2 + \tfrac{1}{2}\Lambda_2Z^2\ln\left(\frac{\Lambda_3}{\sqrt{c}}\right)\right]. \tag{15}$$

The latter expression, with $\theta = i/n \to 0$, is also equal to the total electrostatic free energy, $\Delta F_{el}(\theta \to 0)$, of initial ligand binding, for which there is no appreciable neutralization of the electrostatic surface charge. This leads to an "initial" binding constant, $K(0)$, for the first ligand that includes the surface electrostatics and is given by

$$\begin{aligned} K(0) &= K_o\exp\left(-\frac{\Delta F_{el}(\theta \to 0)}{kT}\right) \\ &= K_o e^{2Z + \Lambda_2Z^2}\left(\frac{\Lambda_1}{\sqrt{c}}\right)^{2Z}\left(\frac{\Lambda_3}{\sqrt{c}}\right)^{\Lambda_2Z^2/2}. \end{aligned} \tag{16}$$

From Eqs. 3, 5, 15, and 16, the binding isotherm in the presence of electrostatics is then given by

$$[L] = \frac{1}{K(0)}\left(1 - \theta\frac{Z}{\alpha}\right)^{-2Z}\frac{\theta}{1 - \theta}\exp\left(\frac{\theta}{1 - \theta} - 2a\theta\right). \tag{17}$$

This equation contains four parameters, K_0, Z, α, and a. The first two, K_0 and Z, can be obtained experimentally from the ionic strength dependence of the initial binding by using Eq. 16. The maximum lipid/protein binding stoichiometry, α, and the parameter, a, governing the strength of the interactions between the proteins on the surface, can then be obtained by fitting the entire binding isotherms at different ionic strengths.

Model calculations

Fig. 2 shows the behavior of the binding isotherms defined by Eq. 17 in the absence of surface interaction between ligands (i.e., $a = 0$), with different values of the intrinsic binding constant K_0, and compares these with isotherms obtained from a fixed-site model (i.e., the Langmuir isotherm) in the presence of electrostatics. The latter is described by Eq. 17 with both exponential terms absent. It is clear that the binding isotherm for fixed sites approaches complete occupancy of the surface (indicated by the dotted line in Fig. 2) with a very different concentration dependence from that of the isotherm for a continuous surface that is defined by Eq. 17. In the latter case, further binding is strongly inhibited at high degrees of surface occupancy, leading to an apparent saturation at levels of ligand binding that are far below complete coverage of the surface. This shows that the modification for nonlocalized binding of the entropic part of the free energy of the ligand distribution (i.e., of the logarithmic term in Eq. 5), which gives rise to the first of the two exponential terms in the binding isotherm of Eq. 17, cannot be neglected when treating binding to continuous surfaces.

Addition of a repulsive (enthalpic) interaction between ligands on the surface (i.e., $a < 0$), such as could arise from, e.g., contact of the hydration shells of the ligands, increases further the depression of the apparent saturation level (cf. Eq. 17). Attractive interactions between the bound ligands (i.e., $a > 0$), which can lead to dimerization or aggregation, result in a degenerate behavior of the binding isotherm, as is illustrated in Fig. 3. The dotted line in this figure is obtained from Eq. 17, whereas the solid line is calculated from Eq. 2 together with Eqs. 5 and 14, 16. The total area, $n\Delta A$, is chosen for the latter calculations to be equal to $n = 30$ ligand cross sections. Higher values of n result in sharper distributions

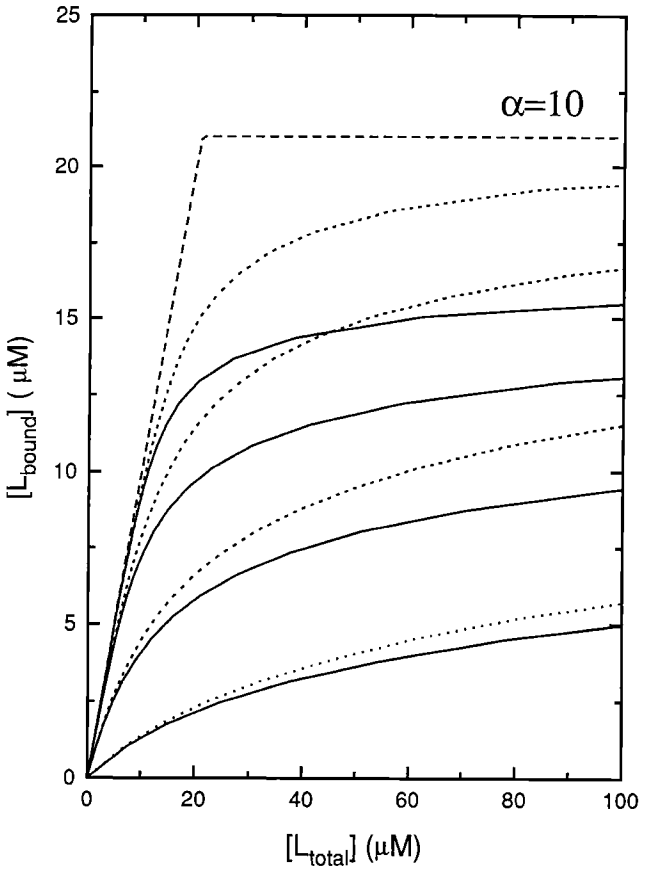

FIGURE 2 Binding isotherms calculated from Eq. 17 for ligand binding to charged lipid surfaces with binding constants (from *bottom* to *top*) of $K(0) = 10^3$, 10^4, 10^5, and 10^6 l/mol of lipid. The total lipid concentration is 210 μM. There are $\alpha = 10$ lipids per ligand at saturation surface coverage, and the effective charges of the ligand and of the lipids are $+4e$ and $-1e$, respectively. Dotted lines represent binding isotherms for localized ligand sites, and solid lines describe the binding to a continuous surface with $a = 0$, i.e., including the exponential term that corresponds to the nonlocal nature of the entropic contributions from the ligand distribution.

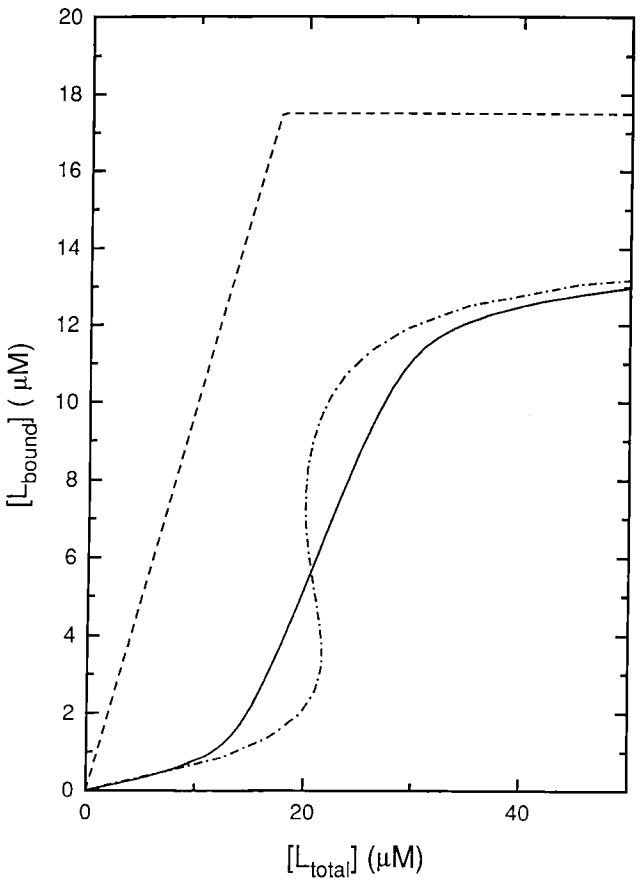

FIGURE 3 Binding isotherms obtained from Eq. 17 with an attractive interaction between the ligand molecules, i.e., $a > 0$, (– · – · –). Equation 17 cannot describe adequately the coexistence of monomeric and aggregated states. This is possible, however, with the isotherm obtained from Eqs. 2, 5, and 14, 16 (——) because this allows the coexistence of states. Parameters used are $K(0) = 250$ l/mol of lipid, $a = 6$ and $Z = 4.232$, with a lipid concentration of 210 μM. The nonmonotonic behavior caused by aggregation of ligands on the surface is evident.

than those shown in Fig. 1. Both approaches lead to similar results at low and at high total ligand concentrations, where only a single state (either all monomeric or all aggregated, see Fig. 1) of the bound ligand is present. At intermediate ligand concentrations, where both states coexist (cf. Fig. 1), Eq. 17 does not produce a unique solution and the approach using Eq. 2 is the more adequate way to represent the binding isotherms.

RESULTS AND DISCUSSION

Binding studies

The binding of cytochrome *c* to dioleoyl phosphatidylglycerol membranes has been studied over a range of ionic strengths, to vary the strength of binding relative to that of the surface interactions between the bound proteins. Further, the binding isotherms for the native protein have been compared with the results obtained for cytochrome *c* that had been heat-denatured after binding to the lipid. A significant feature of the binding of cytochrome *c* to negatively charged lipid membranes is that the denaturation temperature of the protein is reduced from 83°C in solution to approximately 50–55°C in the lipid-protein complex (Muga et al., 1991; Heimburg and Marsh, 1993). Upon surface denaturation, additional components appear in the amide I region of the infrared spectrum of the protein that have been attributed to intermolecular hydrogen bond (extended chain) formation upon aggregation of the denatured protein on the surface (Muga et al., 1991). In the present work, surface-denatured samples of cytochrome *c* have been obtained by incubating lipid-protein mixtures at 65°C, leaving the unbound (soluble) protein in its native form and, hence, allowing an effective binding isotherm to be determined for the denatured protein. The latter therefore provides a system in which the intermolecular interactions on the surface may be enhanced considerably relative to those for the native bound protein.

Initial binding

The "initial" binding constants $K(0)$ can be derived from the initial slopes of the experimental binding isotherms (see below) because, at low saturation, Eq. 17 reduces to $K(0) =$

$\theta/[L]$ ($\theta \ll 1$). The binding constants, $K(0)/\alpha$, obtained in this way and defined as $[\text{cyt.}c_{\text{bound}}]/[\text{cyt.}c_{\text{free}}] \cdot [\text{lipid}]$, are given as a function of the ionic strength in Fig. 4. The linear dependence found in a double logarithmic plot is that predicted by Eq. 16, where the slope, $-(Z + 1/4\Lambda Z^2)$, gives the effective charge, Z, on the protein. This yields values of $Z = 3.78$ and 3.23 for the native and surface-denatured proteins, respectively. The effective charge for the denatured cytochrome c is found to be reduced slightly relative to that of the native protein. Nevertheless, the overall binding constants are significantly higher in the case of the denatured protein, as might be expected because the latter effectively includes contributions from the thermodynamics of the denaturation process. From the intercepts of the ionic strength dependence in Fig. 4, values of $K_0 = 1.9 \cdot 10^{-5}$ and $1.6 \cdot 10^{-3}$ l/mol lipid are obtained for the native and denatured proteins by taking values of $\alpha = 11.9$ and 7.5, and $Z = 3.78$ and 3.25, respectively (cf. Eq. 16 and see below). From this it can be concluded that the additional contribution to the binding free energy for the lipid complex with denatured cytochrome c is energetically more favorable by -2.5 kcal/mol than for the lipid complex with the native protein. It was not possible to extend the results of Fig. 4 to much lower ionic strengths because of the extremely tight binding under those conditions.

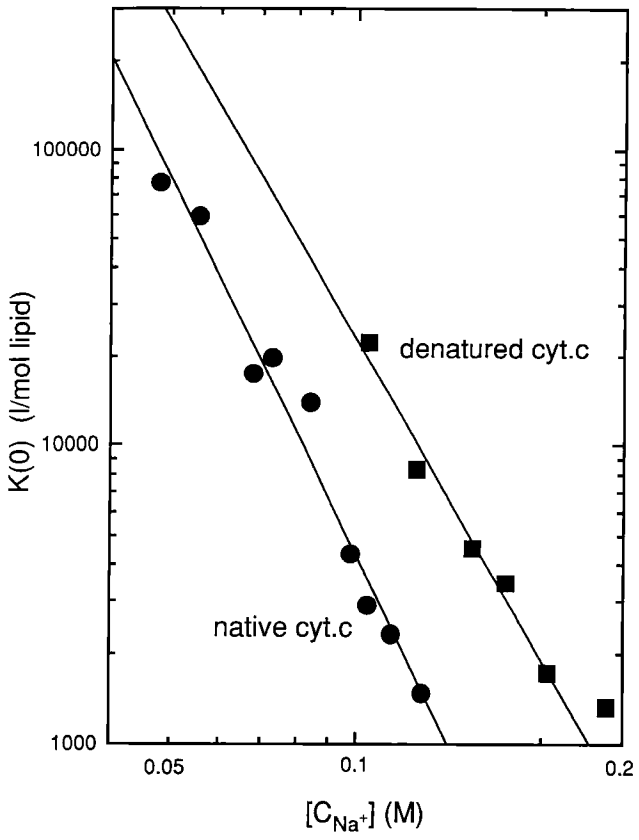

FIGURE 4 Ionic strength dependence of the binding constants $K(0)/\alpha$ [l/mol of lipid] obtained from the initial slopes of the binding isotherms at salt concentrations above 40 mM NaCl. Circles represent the binding of native cytochrome c, and squares represent the binding of surface-denatured cytochrome c, to 210 μM DOPG dispersions. Full lines are linear regressions of the double-logarithmic plots according to Eq. 16.

The primary sequence of horse heart cytochrome c (Sober, 1968) contains 19 lysine, 2 arginine, and 12 glutamic and aspartic acid residues. The net charge on the protein (+9, excluding the charge of the heme group) is therefore much larger than the effective charge derived above from the binding to negatively charged lipid bilayers. Similar reductions also have been found for the binding of small basic peptides (Kuchinka and Seelig, 1989; Schwarz and Beschiaschvili, 1989). Possible reasons that may be advanced for this reduction are electrostatic ligand-ligand repulsions (Stankowski, 1991), ion condensation at the charged interface (Manning, 1978), and the finite size of the protein relative to the Debye length (Carnie and McLaughlin, 1983; Alvarez et al., 1983).

Binding isotherms

The binding curves for the association of native cytochrome c with DOPG membranes at different ionic strengths are shown in Fig. 5. The isotherms display a systematically de-

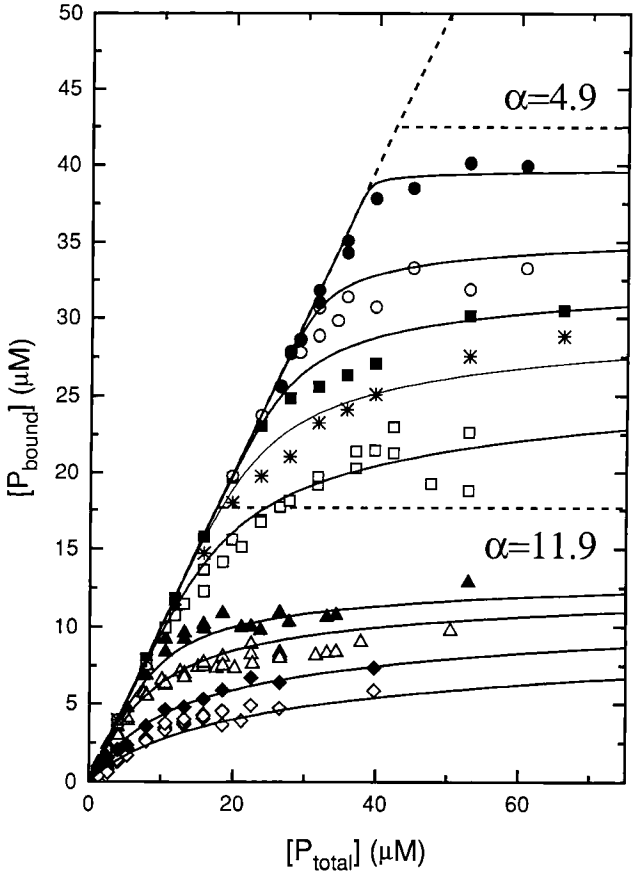

FIGURE 5 Binding isotherms for association of native cytochrome c with 210 μM DOPG dispersions at low and high ionic strengths. *Low ionic strength:* 0.21 mM (●), 4.35 mM (○), 10 mM (■), 16.9 mM (*) and 29.4 mM NaCl (□); *high ionic strength:* 42 mM (▲), 54.4 mM (△), 79.4 mM (◆) and 104.4 mM NaCl (◇). Global nonlinear least-squares fits of the isotherms to Eq. 17, with α and a as fitting parameters and using the values of Z and K_0 obtained from Fig. 4, are given by the full lines. Two different stoichiometries, $\alpha = 11.9$ and 4.9, are required for global fits in the high and low ionic strength regimes, respectively, but with a single value of $a = 0.03$.

creasing strength of binding with increasing ionic strength. This is seen from the decreasing initial slope for the high ionic strength regime from 40 mM NaCl concentration upwards. (This was the range of ionic strength, for which $K(0)$ was determined to give the data of Fig. 4.) In addition, an apparent saturation is reached at lower levels of protein binding, the higher is the ionic strength of the suspending medium. Although the ionic strength dependence of the binding constant can be accounted for adequately by electrostatic double-layer theory (cf. Fig. 4), the apparent saturation behavior most probably is determined by a competition between binding and the surface pressure of the bound molecules, which is included in the model for nonlocalized binding that is given by Eq. 7.

Fits to the experimental binding curves (Fig. 5, *solid lines*) have been obtained by using Eq. 17 with the values of Z and $K(0)$ from Fig. 4. For the isotherms at high ionic strength, a global fit to the four isotherms is obtained with a saturating lipid/protein stoichiometry of $\alpha = 11.9$ and a very low value of the interaction parameter of $a = 0.03$. This stoichiometry obtained for complete surface occupancy is compatible with the size of cytochrome *c* (Dickerson et al., 1971) and the area of a dioleoyl phospholipid molecule in the fluid phase (Gruner et al., 1988; Lis et al., 1982). The model given by Eq. 17, which includes the effects of the protein distribution on the surface is therefore capable of describing the ionic strength dependence of the binding isotherms in the regime above 40 mM NaCl in a manner that is consistent with established dimensional data. It is further found that interactions between the bound native proteins, if any, are rather small.

At low ionic strength, the effective limiting extents of binding are much higher than those obtained in the regime above 40 mM NaCl and exceed the value that corresponds to complete surface coverage based on the dimensional data. Possible reasons for this high degree of binding at low ionic strength include formation of a second layer of bound protein and/or partial penetration of the protein into the lipid membrane. A global fit of the five binding curves at low ionic strength (<40 mM NaCl) to Eq. 17 is shown in Fig. 5. This yields a reduced lipid/protein stoichiometry at saturation of $\alpha = 4.9$, with the effective charge and interaction parameter maintained at the same values as at high ionic strength. It will be noted that this value corresponds more closely to neutralization of the effective net charge on the protein that was deduced from the ionic strength dependence of the binding constant (i.e., $Z = 3.8$ from Fig. 4) than to that of the total net charge on the protein (i.e., $Z = 9$). As for the fits to the data at high ionic strength, the value of Z and the extrapolated values of $K(0)$ were taken from Fig. 4. Somewhat better fits to the individual isotherms can be obtained by slight adjustments in the values of the binding constants within the range allowed by the scatter in Fig. 4, which was found also to be the case for the high ionic strength regime.

It will be noted that a contribution to the increase in apparent saturation binding with decreasing ionic strength resulting from increases in the effective protein charge cannot be excluded entirely. Such an effect would correlate with the increasing Debye length in the low ionic strength regime (cf. above). However, the effective protein charge cannot increase above +5 to ensure effective electrostatic binding at the lowest ionic strength (see Fig. 5). Also, increases in Z would require nonzero (and varying) values of the a-parameter to obtain good fits to the binding isotherms. Thus, a consistent fit throughout the entire range of ionic strength would no longer be possible.

A description of the data over the full range of ionic strength, with an essentially vanishing value of the interaction parameter ($a \approx 0$), therefore requires that the saturation stoichiometry changes from a value close to that corresponding to complete surface coverage at ionic strengths above 40 mM to one corresponding to an increased protein binding capacity at low ionic strength. With a fixed stoichiometry, nonvanishing values of the ligand-ligand interaction parameter are needed to fit the data over the full range of ionic strengths. The values of the a-parameter that are required for the fits with different constant values of the stoichiometry ($\alpha = 3$, 4, and 5) have been calculated. The dependence of the interaction parameter on ionic strength is nonmonotonic under these assumptions, and even oscillates in sign. Such a behavior is unlikely and lends further support to the suggestion of a change in the saturation binding stoichiometry such as is depicted by the fits in Fig. 5. Additionally, it is found that a discontinuity occurs in the effective a-parameter required for fits with a constant stoichiometry, at exactly the same ionic strength as that proposed for the change in binding stoichiometry in Fig. 5.

The dependence of the binding isotherms for native cytochrome *c* on ionic strength therefore establishes possible effects of the lateral pressure arising from the surface distribution of the bound proteins, but provides no unambiguous evidence for either attractive or repulsive interactions (i.e., a nonzero a-parameter) between them. The binding isotherms for denatured cytochrome *c* in association with DOPG bilayers are given in Fig. 6. The binding curves at the higher ionic strengths achieve an apparent saturation much less readily than do those of the native protein (cf. Fig. 5), a feature that is indicative of aggregation of the denatured protein on the membrane surface. In contrast to the situation with the native protein, these binding characteristics of the denatured protein can be fit consistently only by taking a nonvanishing and positive value of the ligand-ligand interaction parameter ($a = 2.3$) in Eq. 17. Best global fits to the data at high ionic strength were obtained with a lipid/protein stoichiometry of $\alpha = 7.5$, which is consistent with complete surface coverage of the membrane, although it is somewhat smaller than that found for the native protein. It was possible to obtain reasonably consistent fits of the binding curves for the denatured protein over the complete range of ionic strengths with a constant lipid/protein stoichiometry of $\alpha = 5.7$, equal to that obtained by fitting the data at low ionic strength alone. However, better fits, with consistent values of Z and $K(0)$ and a common value of the interaction parameter ($a = 2.3$) can be obtained, if the saturation stoichiometries in the high and low ionic strength

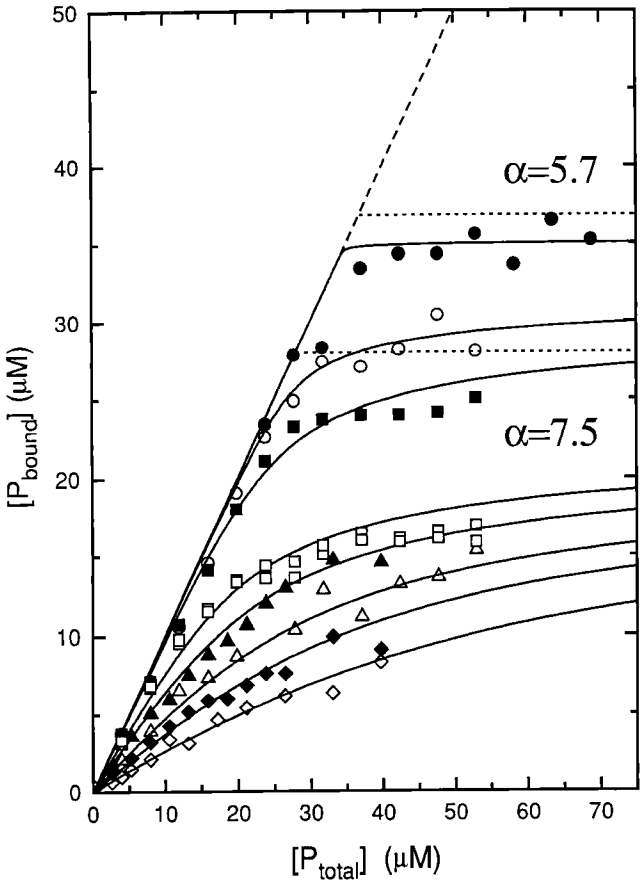

FIGURE 6 Binding isotherms for association of surface-denatured cytochrome *c* with 210 μM DOPG dispersions at low and high ionic strengths. *Low ionic strength:* 0.21 mM (●), 29.4 mM (○), and 54.4 mM NaCl (■); *high ionic strength:* 104.4 mM (□), 125 mM (▲), 154.4 mM (△), 175 mM (◆), and 204.4 mM NaCl (◇). Two different stoichiometries, α = 7.5 and 5.7, in the high and low ionic strength regimes, respectively, and a single value of a = 2.3, are obtained from global fits of the isotherms to Eq. 17, using the values of Z and K_0 obtained from Fig. 4.

regimes are allowed to differ, as was done for the native protein (see Fig. 6).

The data obtained for the surface-denatured protein must be viewed with some caution because the analysis assumes that the protein denatured on the surface is in equilibrium with nondenatured protein free in solution at 65°C, and further that the denatured protein is trapped in this state on cooling to 4°C, at which temperature the separation of bound and free protein was performed. Neither of these assumptions is established with complete certainty. However, to within these limitations, the large differences in the data obtained with the native and denatured proteins do suggest that there are nonideal interactions between molecules of the bound protein in the denatured state that are not present for the protein in the native state. These nonidealities could be fit only with a positive (i.e., attractive) value for the ligand-ligand interaction parameter, which is consistent with the tendency of the denatured protein to aggregate on the surface of the membrane.

Electron spin resonance studies

The binding isotherms obtained with the native protein suggest that the saturation stoichiometry is different at low ionic strength from that at high ionic strength. Based on available dimensional information, the binding capacity at low ionic strength is found to be greater than that expected for complete surface coverage of the lipid membrane by the protein. To obtain further information on a possible difference in the mode of protein binding at low ionic strength, experiments were performed with ESR spectroscopy of spin-labeled lipids incorporated in the membrane bilayer. Such studies are expected to be sensitive to any disturbance or penetration of the lipid bilayer by the bound protein (see, e.g., Görrissen et al., 1986).

The ESR spectra of the 14-PGSL spin probe with the label group at the ^{14}C-atom of the phosphatidylglycerol *sn*-2 chain in DOPG bilayers, at low and high ionic strength and with differing amounts of cytochrome *c* added, are given in Fig. 7. Upon addition of protein to DOPG dispersions in 4.4 mM NaCl, a second spectral component of larger hyperfine anisotropy appears in the wings of the sharper three-line spectrum that is obtained with the lipid alone (Fig. 7, *left*). This component, which is not seen in the spectra from DOPG dispersions in 54.4 mM NaCl (Fig. 7, *right*), corresponds to a considerable increase in the motional restriction of the lipid chains, relative to that in the lipid alone, and most probably arises from their coming in close contact with part of the protein (cf. Görrissen et al., 1986). It will be noted that such effects are accentuated by the relatively low temperature at which the ESR spectra are recorded, and for cytochrome *c* may be a particular feature of lipids with unsaturated chains, but nonetheless point to a very clear difference between the modes of binding of cytochrome *c* at low and high ionic strength.

The ratio of the center-field lineheight of the ESR spectra to the low-field lineheight has been used as an empirical indicator of the relative populations of the two spectral components. The dependence of this lineheight ratio on the total concentration of cytochrome *c* added to the lipid dispersion is given in Fig. 8 (*top*). The corresponding protein binding curves for these samples are given also in Fig. 8 (*bottom*). For the samples at high ionic strength, the ESR lineheight ratio was measured at the position at which the second component appears in the spectra of the samples at low ionic strength. The slight increase that is observed in the lineheight ratio for these samples at high ionic strength corresponds to a progressive linebroadening of the single fluid component in the spectra with increasing protein binding (cf. Fig. 8, *bottom*). At comparable degrees of binding, the lineheight ratios at low ionic strength are considerably larger than those at high ionic strength. For the samples of low ionic strength, a steep increase in the lineheight ratio is observed up to protein/lipid ratios of approximately 1:14 mol/mol. (The apparent sigmoidal shape of the increase may reflect a nonlinear relation with the degree of binding.) This increase in the lineheight ratio corresponds to a progressive increase in

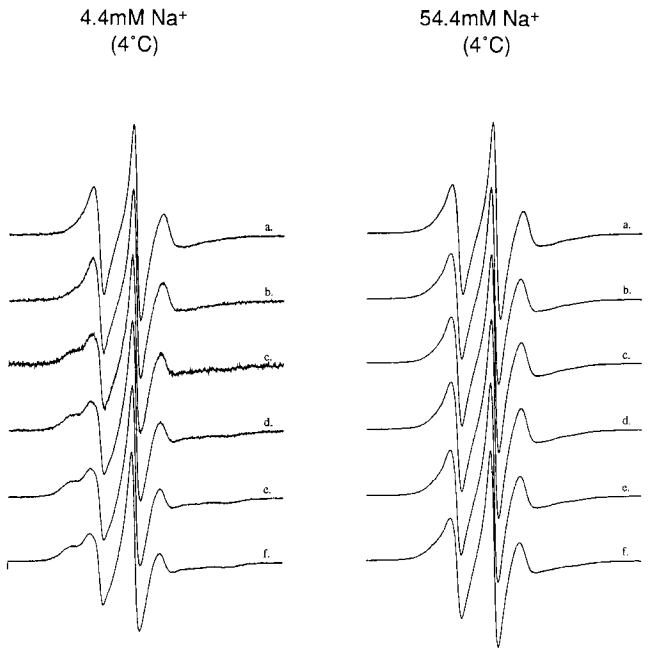

FIGURE 7 ESR spectra of the 14-PGSL phosphatidylglycerol spin label in lipid-protein complexes pelleted from 210 μM DOPG dispersions with increasing concentrations of cytochrome *c* added. (*left*) Lipid-protein complexes in 4.4 mM NaCl with lipid/protein ratios of: (*a*) 106:1, (*b*) 35:1, (*c*) 17.6:1, (*d*) 10.6:1, (*e*) 7.6:1, and (*f*) 5.3:1 mol/mol. (*right*) Lipid-protein complexes in 54.4 mM NaCl with lipid/protein ratios of: (*a*) 106:1, (*b*) 35:1, (*c*) 21.2:1, (*d*) 13.2:1, (*e*) 8.8:1, and (*f*) 5.3:1 mol/mol. Total scan width = 100 gauss, T = 4°C.

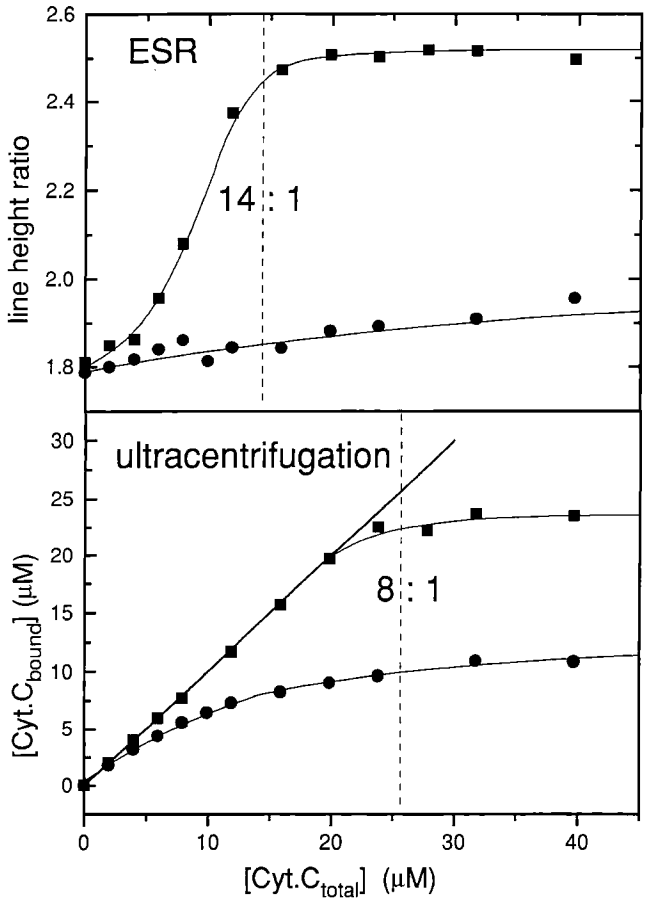

FIGURE 8 (*top*) Dependence of the ratio of the center-field lineheight to the low-field lineheight, in the ESR spectra of Fig. 7, on the concentration of cytochrome *c* added to the DOPG dispersions. (*bottom*) Dependence of the concentration of bound protein on the concentration of cytochrome *c* added obtained from the centrifugation assay for the same samples as in the upper panel. Squares represent dispersions in 4.4 mM NaCl, and circles represent dispersions in 54.4 mM NaCl.

the proportion of the motionally restricted, protein-associated lipid component with increasing amounts of protein bound at low ionic strength. Beyond a protein/lipid ratio of ~1:14 mol/mol, however, the ESR lineheight ratio remains essentially constant when further protein is added. This indicates that, although the subsequent protein added continues to bind up to protein/lipid ratios of approximately 1:8 mol/mol (see Fig. 8, *bottom*), the bound protein no longer has the pronounced effect on the lipid mobility that is observed on binding at lower protein/lipid ratios. In principle, this could correspond to the binding of a second layer of protein, which would be possible because the Debye length at low ionic strength exceeds the protein diameter.

In general, the significant difference between the apparent stoichiometry of binding and that for the influence on the ESR spectra, for the samples at low ionic strength, appears to correlate with the high protein-binding capacity found under these conditions. The first stages of binding involve a large perturbation of the lipid mobility, possibly resulting from partial penetration of the protein or from some other disturbance of the lipid surface that results effectively in an increase in the membrane area. This mode of binding, which is not present for samples at high ionic strength, could also account for the additional binding capacity that is obtained for samples at low ionic strength, and most probably is driven by the strong binding acting to reduce the in-plane interactions between the bound proteins.

CONCLUSIONS

The lateral interactions between molecules of cytochrome *c* bound to bilayers of dioleoyl phosphatidylglcerol have been studied by fitting consistently the binding isotherms obtained over a wide range of ionic strengths. The results indicate not only the likely importance of such interactions in controlling the binding of peripheral proteins to membranes, but also the necessity to take these interactions into account explicitly when analyzing membrane binding isotherms in general. A model with nonlocalized binding sites is that most appropriate for the binding of large ligands to continuous surfaces and, in the case of cytochrome *c*, indicates that the distributional entropy of the protein at the surface plays a dominant role. For cytochrome *c* that has been denatured on the lipid surface, attractive interactions between the bound proteins are additionally of importance and lead to a much greater extent of binding than for the native protein at high ionic strength.

At low ionic strength, the binding of native cytochrome *c* to DOPG membranes is very strong and, as evidenced

by the spin-label ESR results, leads to a penetration of the protein, or to some other similar distortion of the membrane surface, that is not observed under conditions of weaker binding at high ionic strength. The result of this almost certainly is to reduce energetically unfavorable in-plane (entropic) interactions between the bound proteins, which gives rise to a much larger capacity of the lipid surface for binding further protein at low ionic strength. The differences in binding characteristics between low and high ionic strength are not so pronounced for the surface-denatured protein as they are for the native protein, presumably because the attractive in-plane interactions already mitigate the effects of the entropic in-plane interactions (cf. Eq. 17).

It should now be possible to extend these methods to investigate the interactions between other peripheral membrane proteins, and to membranes of more complex composition, approaching more closely those of natural membranes.

APPENDIX I

Derivation of Eq. (3)

Eq. 2 can be rewritten as

$$\nu = [L]K_0 \frac{\sum_{i=1}^{n} \frac{1}{(1-i)!}[L]^{i-1}K_0^{i-1}\exp\left(-\frac{\Delta F(i-1)}{kT}\right)\exp\left(-\frac{\Delta F(i)-\Delta F(i-1)}{kT}\right)}{\sum_{i=0}^{n}\frac{1}{i!}[L]^iK_0^i\exp\left(-\frac{\Delta F(i)}{kT}\right)}$$

$$=[L]K_0 \frac{\sum_{i=0}^{n-1}\frac{1}{i!}[L]^iK_0^i\exp\left(-\frac{\Delta F(i)}{kT}\right)\exp\left(-\frac{\mathrm{d}}{\mathrm{d}i}\left(\frac{\Delta F(i)}{kT}\right)\right)}{\sum_{i=0}^{n}\frac{1}{i!}[L]^iK_0^i\exp\left(-\frac{\Delta F(i)}{kT}\right)}.$$

If the distribution has only one distinct maximum and $n\to\infty$ (i.e., the maximum becomes infinitely sharp), then the summations reduce to the largest overall term, i.e.,

$$\nu = [L]K_0\exp\left(-\frac{\mathrm{d}}{\mathrm{d}i}\left(\frac{\Delta F(i)}{kT}\right)\right).$$

Under certain special conditions the distribution can have more than one maximum (cf. Fig. 1), and this simplification then cannot be made.

APPENDIX II

Binding to one-dimensional lattices

McGhee and von Hippel (1974) have given an analytical expression for the binding of multivalent ligands to one-dimensional lattices. For a lattice of n sites, and a ligand that occupies m adjacent sites and has a binding constant K per site, the binding isotherm in the absence of ligand overlap but otherwise no interaction between ligands (i.e., $a = 0$) is

$$[L] = \frac{1}{mK}\left(\frac{\theta}{1-\theta}\right)\left(\frac{1-\theta}{1-\theta+\theta/m}\right)^{1-m}.$$

The limit of a one-dimensional continuum is obtained by letting $m \to \infty$. Using the identity, $\lim(1 + 1/n)^n = e$ as $n \to \infty$, the limiting value of the final term in the above expression becomes $\exp(\theta/1-\theta)$. Thus, the binding isotherm for a one-dimensional continuum is given by

$$[L] = \frac{1}{K_0}\left(\frac{\theta}{1-\theta}\right)\exp\left(\frac{\theta}{1-\theta}\right),$$

where K_0 is the finite limiting value of the product mK.

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