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Surface Plasmon Resonance Studies of Complex Formation Between Cytochrome *c* and Bovine Cytochrome *c* Oxidase Incorporated into a Supported Planar Lipid Bilayer. I. Binding of Cytochrome *c* to Cardiolipin/Phosphatidylcholine Membranes in the Absence of Oxidase

Zdzislaw Salamon and Gordon Tollin
Department of Biochemistry, University of Arizona, Tucson, Arizona 85721 USA

ABSTRACT The mechanism of interaction between cytochrome *c* and a solid-supported planar phosphatidylcholine membrane containing varying amounts of cardiolipin (0–20 mol%) has been studied over a wide range of protein concentrations (0–450 μM) and ionic strength conditions (10–150 mM), by direct measurement of protein binding using surface plasmon resonance (SPR) spectroscopy. The results demonstrate that cytochrome *c* binds to such phospholipid membranes in two distinct phases characterized by very different (approximately one order of magnitude) affinity constants. The second phase is dependent upon the prior occurrence of the first binding process. Although the binding affinities for both modes of binding are highly sensitive to both the cardiolipin concentration and the ionic strength of the buffer solution, indicating that electrostatic forces are involved in these processes, binding cannot be reversed by salt addition or by dilution. Furthermore, the final saturation levels of adsorbed protein are independent of ionic strength and cardiolipin concentration. These observations suggest that binding involves more than a simple electrostatic interaction. Invariance in the shapes of the SPR spectra indicates that no major structural transitions occur in the proteolipid membrane due to cytochrome *c* binding, i.e., the bilayer character of the lipid phase appears to be preserved during these interactions. Based on these results, a model of the lipid membrane-cytochrome *c* interaction is proposed that involves varying degrees of protein unfolding and subsequent binding to the membrane interior via hydrophobic forces.

INTRODUCTION

Cytochrome *c* (cyt c) is an essential component of the mitochondrial respiratory electron transport chain. It functions to shuttle electrons between two of the inner membrane-bound electron transfer complexes, cytochrome bc_1 and the terminal enzyme, cytochrome *c* oxidase (cco). It is well established that complex formation between cyt c and cco, as well as between cyt c and the lipid membrane, plays an important role in both recognition (i.e., specificity) and in electron transport (Nicholls, 1974; Chan and Li, 1990; Malmström, 1990; Capaldi, 1990; Babcock and Wikström, 1992; Calhoun et al., 1994; Trumpower and Gennis, 1994; Pinheiro, 1994; Malatesta et al., 1995). Cyt c-bilayer interactions have also been considered as a paradigm for the electrostatic binding of peripheral proteins to biological membranes (Nicholls, 1974; Brown and Wüthrich, 1977; Rytömaa et al., 1992; Sankaram and Marsh, 1993; Heimburg and Marsh, 1995). As a consequence, the mechanisms of these associations have been intensively studied in vitro, using both lipid monolayers and lipid vesicles as model systems (De Kruijff and Cullis, 1980; Hackenbrock et al., 1986; Szebeni and Tollin, 1988; Gupte and Hackenbrock, 1988; Senthilathipan and Tollin, 1989; Spooner and Watts, 1991; Cheddar and Tollin, 1991; Heimburg et al., 1991; Pinheiro, 1994; Rytömaa and Kinnunen, 1995).

Cyt c has been shown to bind strongly via electrostatic interactions to negatively charged membrane surfaces, and it has been established that such binding may have both a structural and a functional role, mediated by effects on the conformation of the protein as well as on the structure of the membrane surface. Spectroscopic studies have shown that the binding of cyt c to acidic phospholipids (including cardiolipin, which is an important component of the inner mitochondrial membrane; cf. Hoch, 1992) induces both local protein structural changes involving the immediate environment and coordination of the heme group (Vincent and Levin, 1988; Hildebrandt and Stockburger, 1989; Spooner and Watts, 1991; Heimburg et al., 1991; Choi and Swanson, 1995), as well as more extensive alterations in the conformation of the protein backbone, including loosening and destabilizing of the overall protein structure (Muga et al., 1991). It has also been demonstrated that cyt c binding alters the structure of the lipid phase of negatively charged membranes, inducing the formation of inverted hexagonal phases, and possibly an inverted micellar structure (De Kruijff and Cullis, 1980). The latter observation has given rise to the suggestion that acidic phospholipids may be functionally involved in the import of proteins into mitochondria (Demel et al., 1989). Although the electrostatic interactions between negatively charged membranes and cyt c are well documented and have been extensively discussed within the context of specific mechanisms of cyt c binding, there are also results that indicate the existence of hydro-

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Address reprint requests to Dr. Gordon Tollin, Department of Biochemistry, University of Arizona, Tucson, AZ 85721. Tel.: 520-621-3447; Fax: 520-621-9288; E-mail: gtollin@ccit.arizona.edu.

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phobic interactions of the protein with a lipid bilayer (Brown and Wüthrich, 1977; Szebeni and Tollin, 1988). Especially strong experimental support for this has been provided by the finding that bound cyt c cannot be dissociated from such membranes, either by increasing the ionic strength of the buffer solution, or by dilution of the bulk protein. This is despite the fact that the initial binding itself is very sensitive to both the ionic strength of the solution and the amount of charge incorporated into the lipid membrane, consistent with the electrostatic nature of the interaction (Rytömaa and Kinnunen, 1995). In addition to these observations, Heimburg and Marsh (1995) have recently shown that the binding capacity of cyt c to the negatively charged surface of a lipid dispersion is increased at low ionic strength to a value that is greater than that for complete surface coverage, suggesting that there is more than one layer of protein adsorbed onto, or incorporated into, the membrane. This is difficult to rationalize by a purely electrostatic model. In summary, there appears to be a general consensus on two points: 1) the initial binding step of cyt c to the surface of a lipid membrane is governed by electrostatics, and 2) subsequent to the initial stage of binding, changes in the structure of the protein and lipid components of the membrane occur, leading to a new and more complex situation involving penetration of cyt c into the lipid phase (cf. Pinheiro, 1994; Szebeni and Tollin, 1988).

In the present experiments, we have utilized surface plasmon resonance (SPR) spectroscopy to further investigate the mechanism of interaction between cyt c and a lipid membrane. As has recently been demonstrated and discussed in detail below, SPR is a highly sensitive method for the direct measurement of protein binding to (Salamon et al., 1994b, 1995; Soulages et al., 1995) or incorporation into (Salamon et al., 1994a, 1996) a solid-supported lipid bilayer, and is capable of detecting structural changes involving mass redistribution occurring within a proteolipid membrane (Salamon et al., 1994a). In previous work from this laboratory, kinetic methods have been used to study electron transfer between cyt c and other proteins in a lipid vesicle system (Fang and Tollin, 1988; Zhao and Tollin, 1991), and electrochemical techniques have been applied to measuring direct electron transfer from cyt c to a gold electrode, the surface of which was modified with a planar lipid membrane (Salamon and Tollin, 1991, 1993). These studies have demonstrated the importance of electrostatics in the electron transfer processes and have provided evidence which indicates that insertion of cyt c into the lipid film must occur to allow electron transfer through the membrane from the aqueous phase to the electrode surface. The present work extends these investigations by using steady-state SPR spectroscopy to obtain protein/membrane binding isotherms over a wide range of protein concentration, bilayer composition, and buffer ionic strength, and to directly monitor structural changes in the proteolipid membrane that occur during the binding process. The results clearly demonstrate that cyt c binds to phospholipid membranes in two distinct phases characterized by widely different affinity constants. In agreement with the previous studies, an analysis of the SPR spectral changes indicates that the initial binding interaction involves a purely electrostatic association between cyt c molecules and the lipid membrane surface, and is followed by hydrophobic association processes accompanying both partial and complete penetration of the protein into the membrane interior. The shapes of the SPR curves indicate the absence of any major structural transitions in the proteolipid membrane over the entire binding curve, i.e., the bilayer character of the lipid phase appears to be conserved during these interactions.

MATERIALS AND METHODS

Horse heart cytochrome *c* (type VI) (cyt c) was obtained from Sigma Chemical Co. (St. Louis, MO) and was used in the oxidized form without additional purification. Using a microliter syringe, small aliquots of a concentrated cyt c solution were added to the aqueous compartment of the SPR cell, which contained 2 ml of 10 mM Tris buffer and 0.5 mM EDTA (pH 7.4). The buffer ionic strength was changed by the addition of varying amounts of sodium chloride, as follows: no added salt (low ionic strength conditions, I = 10 mM), and 150 mM NaCl (medium ionic strength conditions, I = 160 mM). Egg phosphatidylcholine (PC) and cardiolipin (CL) were obtained in solid form from Avanti Polar Lipids (Alabaster, AL) and were used in membrane-forming solutions as described below.

Preparation of lipid membranes on silver supports

There are two principal techniques that can be employed to form a variety of solid-supported lipid membranes: 1) transfer onto a solid surface of either monolayers generated on a liquid-gas interface by the Langmuir-Blodgett method (cf. Peterson, 1990) or lipid bilayer vesicles suspended in an aqueous solution (cf. Brian and McConnell, 1984); 2) assembly techniques that directly form either a monolayer or a bilayer on a solid surface. Two very different experimental protocols for direct assembly can be utilized. The first of these involves chemical modification of the solid surface (usually a metal) to allow the covalent binding of a substrate. This generates a fully packed, solid-like monolayer on the surface. The most frequently used materials for this purpose are gold or silver metallic films interacting with organosulfur compounds (Nuzzo and Allara, 1983). The second method employs the principles that govern the spontaneous formation of a freely suspended lipid bilayer membrane within an orifice in a hydrophobic barrier separating two aqueous solutions (Mueller et al., 1962). This type of assembly allows the formation of more fluid lipid bilayer membranes.

In the present study, self-assembled solid-supported lipid membranes have been used. The method of preparation involves spreading a small amount of lipid bilayer-forming solution (about 2–4 μl) across an orifice (about 4 mm in diameter) in a Teflon sheet that separates the silver film from the aqueous phase (Salamon et al., 1994a,b). The hydrophilic surface of the metal attracts the polar groups of the lipid molecules, thus forming an adsorbed lipid monolayer with the hydrocarbon chains oriented toward the bulk lipid phase. Subsequent to this first step of lipid membrane formation, the main body of the SPR cell is filled with the appropriate aqueous solution. This initiates the second step, which involves a thinning process, i.e., formation of both the second monolayer and a Plateau-Gibbs border that anchors the bilayer film to the Teflon spacer, allowing the excess of lipid and solvent to move out of the Teflon orifice (see Fig. 1). In previous work from this laboratory involving electrochemical measurements (Salamon and Tollin, 1991, 1993; Salamon et al., 1993) with several redox proteins (including cyt c and cco), we have demonstrated that this technique generates a membrane that provides a biocompatible medium for binding and immobilizing both peripheral and integral membrane proteins,

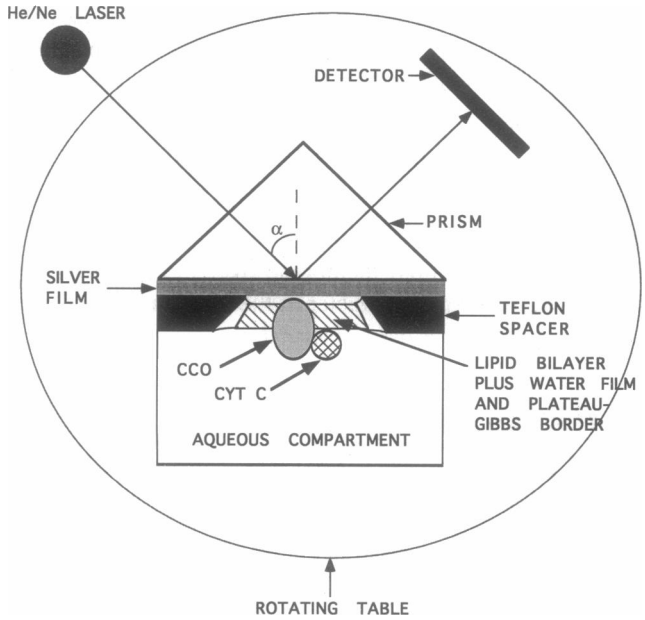

FIGURE 1 Top view of the experimental geometry used for surface plasmon resonance spectroscopic studies of self-assembled solid-supported lipid bilayer membranes containing integral membrane proteins interacting with their soluble biochemical partners. The lipid bilayer and its Plateau-Gibbs border are schematically shown in contact with an adsorbed layer of water on the surface of the silver film. Also shown are a molecule of cytochrome *c* oxidase incorporated into the lipid membrane and a molecule of cytochrome *c* adsorbed on the surface of the proteolipid layer. α (measured with an accuracy of $\pm 0.1°$) is the incident angle of the exciting light at the prism-silver film interface. See text for further details.

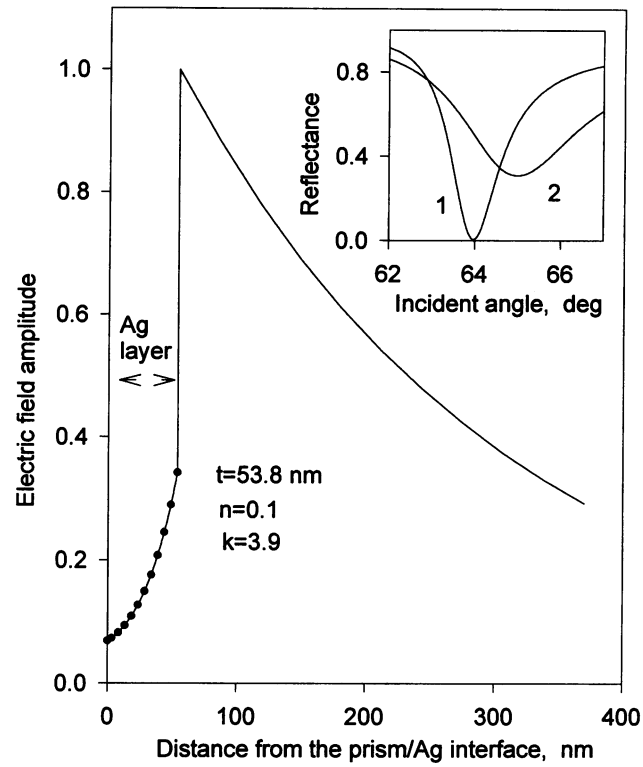

FIGURE 2 Calculated decay of the amplitude of the evanescent electric field (normalized at its largest value) generated within a silver film (●), as a function of the distance from the prism/silver film interface. Optical parameters are as indicated in the figure. The field was obtained using a helium-neon laser as an excitation source (λ = 632.8 nm) under total internal reflection conditions (α = 64°, i.e., at the minimum of the resonance spectrum under the conditions used; see curve 1 in the inset). (*Inset*) Theoretical SPR spectra obtained under these conditions for a bare silver film (curve 1) and for a silver film coated with a dielectric layer with optical parameters within the range typical of lipid bilayer membranes, i.e., t = 6.0 nm, n = 1.5, and k = 0.20 (curve 2).

and which has an arbitrarily large area, the composition and properties of which can be systematically varied. Such membranes are accessible to optical measurement techniques as well, especially SPR spectroscopy (Salamon et al., 1994b). In the present experiments, the lipid films were formed on a hydrated metallic silver surface from a solution containing 10 mg/ml egg PC in squalene (Fluka)/butanol (0.2:10, v/v), using silver films prepared at the Optical Sciences Center of the University of Arizona by vacuum deposition onto a glass prism (refractive index 1.575). Inasmuch as each film has slightly different thicknesses and optical parameters, the angle at which resonance occurs in an SPR experiment varies over a range of $\pm 1°$; the spectral shape is also variable. Therefore, each new SPR experiment begins with control measurements that characterize the optical parameters and quality of the silver film. Varying amounts (0–20 mol%) of CL were incorporated into the egg PC bilayers to produce membranes containing different numbers of negative charges.

Surface plasmon resonance measurements

SPR spectroscopy uses photons from a CW laser (He-Ne; λ = 632.8 nm with a beam diameter of 0.63 mm), passing through a glass prism under total internal reflection conditions, to resonantly excite collective electronic oscillations (plasmons) in a thin metal (or semiconductor) film deposited on the external surface of the prism. This can be in contact either with air or, as in the present experiments, with an aqueous solution (Fig. 1). The evanescent electromagnetic field thereby generated in the metallic layer propagates into the aqueous space (Fig. 2), whereas the incident laser light is totally reflected back into the prism. The field can couple to electronic motions in dielectric materials (proteolipid films in the present case) deposited on the front surface of the metal (Fig. 1). This coupling, which diminishes the intensity of reflected light, can be treated by classical electromagnetic theory (cf. Macleod, 1986), resulting in a relationship between reflectance (R), defined as the ratio of the reflected and incident light intensities, and the optical properties of the deposited dielectric material. This relationship allows an evaluation of the optical parameters of the metal and dielectric films, from which one can deduce the structure and the mass of the deposited material (Salamon et al., 1994a,b). Inasmuch as the method uses an evanescent electromagnetic field (which rapidly decays with increasing distance from the metal surface; see Fig. 2) to probe the optical properties of materials, it is highly sensitive to changes in the properties of the metal/semiconductor surface layer. This is manifested over distances that correspond to a fraction of the wavelength of the light used to monitor the SPR effect, without interference from the bulk volume within the sample compartment. As a consequence, SPR spectroscopy is ideally suited to studying solid-supported lipid membranes, lipid membrane-protein interactions, and interactions between integral membrane and peripheral, water-soluble proteins (including receptor-ligand binding).

The details of the experimental procedures for SPR measurement and data analysis have been described elsewhere (Salamon et al., 1994a,b; Soulages et al., 1995; Salamon et al., 1995, 1996). Here we will provide a brief summary of the technique, together with some additional aspects unique to the present application. The theoretical analysis of the coupling between the evanescent electromagnetic field and the dielectric properties of a deposited surface layer utilizes a relationship between reflectance and the optical admittance (Y) of the multilayer system (Salamon et al., 1994b, 1996). This leads to the generation of a resonance spectrum (i.e., a curve

of reflectance versus incident angle; cf. inset in Fig. 2), which allows curve-fitting procedures to be applied. Y is defined by the ratio of the amplitudes of the magnetic (B) and electric (C) fields of the electromagnetic wave:

$$Y = C/B. \quad (1)$$

Using Maxwell's equations to describe the propagation of the electromagnetic field, one can replace this ratio with the following matrix equation (Macleod, 1986), which includes the refractive index, n, the deposited film thickness, t, the nonresonant absorption coefficient, k, and the incident light angle, α:

$$\begin{bmatrix} B \\ C \end{bmatrix} = \prod_{r=1}^{p} \begin{bmatrix} \cos \delta_r & i(\sin \delta_r)/y_r \\ y_r/\sin \delta_r & \cos \delta_r \end{bmatrix} \begin{bmatrix} 1 \\ y_{r+1} \end{bmatrix}. \quad (2)$$

In Eq. 2, $\delta_r = 2(n - ik)_r t_r(\cos \alpha_r)/\lambda$, $y_r = (n - ik)_r/\cos \alpha_r$, and p is the number of dielectric layers deposited on the incident medium (in the present case, the medium for which $r = 0$ is a glass prism, whereas (r + 1) is an aqueous solution; see Fig. 1). The reflectance of such a multilayer system is given by the following relationship involving the admittance:

$$R = (y_0 - Y)^2/(y_0 + Y)^2, \quad (3)$$

where y_0 is the admittance of the incident medium (i.e., the glass prism). Equation 3 describes the SPR curve. Examples are shown in the inset in Fig. 2 for a silver metal layer deposited on a glass prism in contact with water (curve 1) and coated with a dielectric film, characterized by typical parameters (t, n, and k) obtained with either lipid or proteolipid membranes (curve 2). The high sensitivity of such SPR spectra to the values of the optical parameters is demonstrated by the plots in Fig. 3, which illustrate the calculated alterations of both the position of the resonance minimum (curves 1) and the shape of the SPR curve described by the ratio of the half-width to the depth of the resonance (curves 2), as a function of either thickness (Fig. 3 *A*), refractive index (Fig. 3 *B*), or nonresonant extinction coefficient (Fig. 3 *C*). Curves 3 and 4 in Fig. 3 *A* have been calculated for the same conditions as curves 1 and 2, except that the dielectric layer has been shifted an additional 20 nm from the surface of the metal film, to demonstrate the decrease in sensitivity that occurs with increasing distance from the metal where the evanescent field is generated (see also Fig. 2). Furthermore, the curves shown in Fig. 3 also clearly illustrate that the resonance is controlled in a different mannner by each of the three optical parameters, indicating that they are readily separable by curve-fitting procedures. Thus, the parameters that describe a dielectric layer deposited on a metallic surface can be evaluated from the experimental resonance curves uniquely, and with a high degree of sensitivity and accuracy.

In the present study, the Kretschmann procedure is followed (Kretschmann, 1971; Haltom et al., 1979), using computer-based optimization methods that allow a systematic search for a global minimum in the fitting error (Salamon et al., 1996). A theoretical fit is initially obtained for the SPR spectrum of the bare metal film in contact with aqueous buffer, and results in evaluation of the optical parameters of the silver film. Next, the SPR spectrum obtained after a lipid membrane is formed on the metal surface is similarly fit with a theoretical curve, assuming a two-layered system consisting of the metallic film and the deposited membrane, each having characteristic t, n, and k values (cf. Eqs. 1–3). Binding of cyt c to the bilayer generates a new SPR spectrum, which is analyzed by application of a three-layer model. The parameters obtained for the uncoated metal and the metal coated with a lipid membrane are used in the latter fitting procedure to evaluate t, n, and k for the protein layer.

The values of the optical parameters describe the thickness and density (the latter by the refractive index) of the deposited dielectric (lipid or proteolipid) film, thereby allowing the construction of a two-dimensional model of the film, and the calculation of the mass of the deposited material using the Lorentz-Lorenz relationship (Salamon et al., 1994a,b). As noted previously (Salamon et al., 1994b), the Lorentz-Lorenz relation can be applied to two different situations: 1) dilute films that include a contribution from the solvent, and 2) films of pure, solvent-free lipid and/or protein.

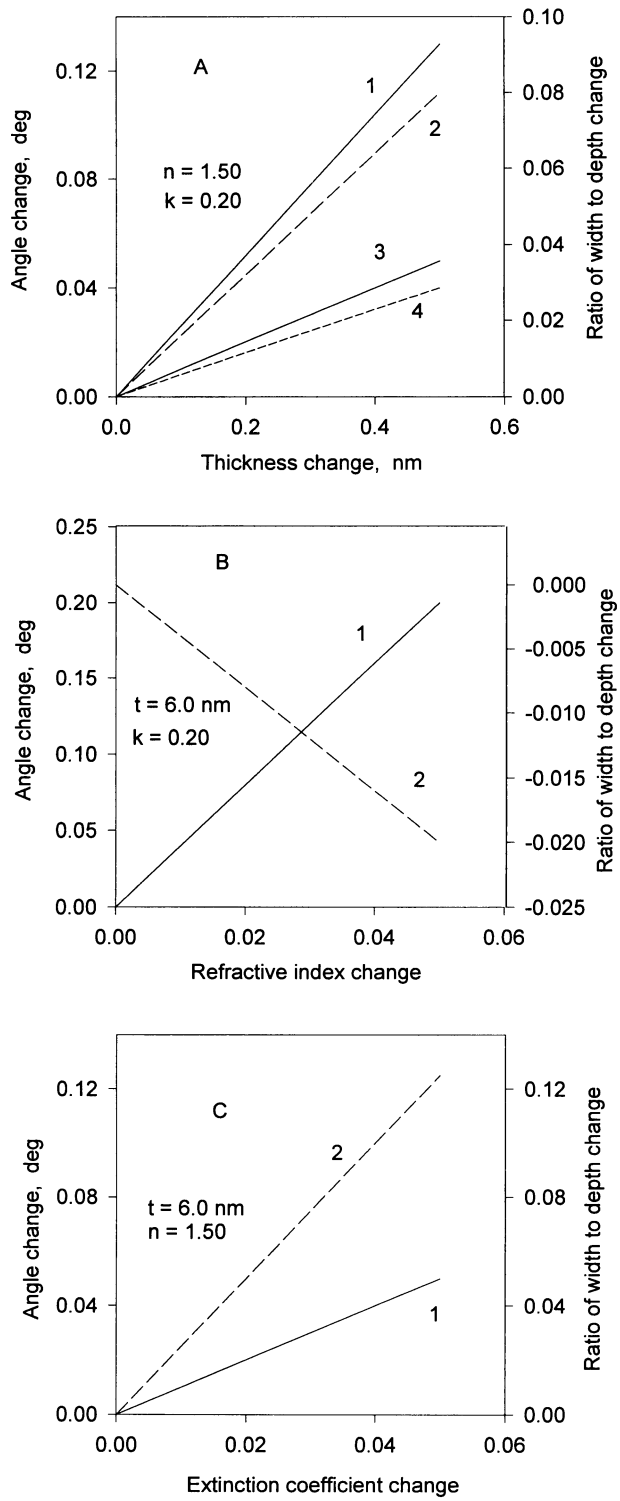

FIGURE 3 Calculated sensitivity of the SPR spectrum to changes in either the position of the resonance (curves 1), or the ratio of the half-width to the depth of the resonance spectrum (curves 2), as a function of the optical parameters of the dielectric layer, i.e., thickness (*A*), refractive index (*B*), and nonresonant extinction coefficient (*C*). Values were calculated using a two-layer model (silver film deposited on a glass prism and coated with a dielectric layer), as in Fig. 2. *A* also shows the decreased sensitivity (resonance angle change, curve 3, and the ratio of half-width to depth, curve 4) obtained by a 20-nm shift in the position of the dielectric layer away from the silver/water interface (see Fig. 2).

Previous evaluations of these two approaches for mass calculations involving phospholipid layers and various proteins have shown that the adsorbed biological materials, as characterized by refractive index values between those of an aqueous buffer (n = 1.33) and values in the range of 1.5–1.6, should be treated as dilute films (Cuypers et al., 1983). Therefore, in the present work the following equation describing such films has been applied:

$$m = 0.3tf(n)(n_p - n_b)/[D/M - V(n_b^2 - 1)/(n_b^2 + 2)], \quad (4)$$

where $f(n) = (n_p + n_b)/[(n_p^2 + 2)(n_b^2 + 2)]$; n_p and n_b are the refractive indices of the dielectric layer (protein or lipid layer in our case) and of solvent (buffer), respectively; m is the mass; D is the molar refractivity; M is the molar mass; V is the partial specific volume; and t is the thickness, of the layer of dielectric material.

The errors associated with these procedures have been discussed extensively in our previous papers (Salamon et al., 1996). These result in overall uncertainties of ±0.1 nm, ±0.02, and ±0.02 for thickness, refractive index, and extinction coefficient, respectively. Assuming the largest experimental errors in the optical parameters, we estimate that the resulting mass errors are approximately ±10%.

Determination of binding parameters (i.e., dissociation constant, K_D, and final surface concentration, C)

As will be shown below, the binding isotherms (i.e., mass of adsorbed protein per unit surface area as a function of the aqueous buffer protein concentration) for cyt c on a deposited lipid bilayer are biphasic, consisting of two well-defined components. To deconvolute these binding curves, the following analysis has been applied. The initial portion of the binding isotherm was fit to a single hyperbolic function using a nonlinear least-squares iterative procedure. The mass values obtained from this fit were then subtracted from the experimental data, and the resulting S-shaped curve was modeled using a nonlinear least-squares fit to a sigmoidal function (see below for discussion). In all cases, the sum of the two theoretical curves fit the experimental results quite well. The parameters obtained from this analysis allow a determination of the dissociation constant, K_D, and the final concentration of adsorbed protein, C, for each of the two binding processes. We estimate that such a computer analysis results in uncertainties of approximately ±20% in both of these parameters.

RESULTS AND DISCUSSION

Cyt c binding isotherms were obtained from SPR curves measured under steady-state conditions, i.e., after each addition of an aliquot of protein solution to the aqueous compartment of the SPR cell, the spectrum was monitored until no further changes occurred (this usually required approximately 10–15 min). A typical set of such SPR spectra, obtained upon incremental additions of small aliquots of a concentrated cyt c stock solution to an aqueous buffer solution in contact with a silver-supported egg PC membrane at low ionic strength, is presented in Fig. 4. Curve 1 was obtained with a lipid membrane without cyt c, whereas curve 6 was determined at saturating concentrations of added cyt c. Both of these curves are shown superimposed upon theoretical spectra (dotted curves) obtained by fitting the experimental data using the procedures described above.

It is important to emphasize that no major changes occur in the shape of the resonance curves over the entire cyt c concentration range; this indicates that large structural alterations in the proteolipid membrane do not occur (see below for further discussion). The shift in resonance position to larger incident angles and the decrease in reflected light intensity that occur during the binding process are readily interpreted in terms of changes in the refractive index (n) and the thickness (t) of the proteolipid film, without an appreciable change in the k parameter (cf. Fig. 3). Similar results have been obtained under all of the conditions used in the present study.

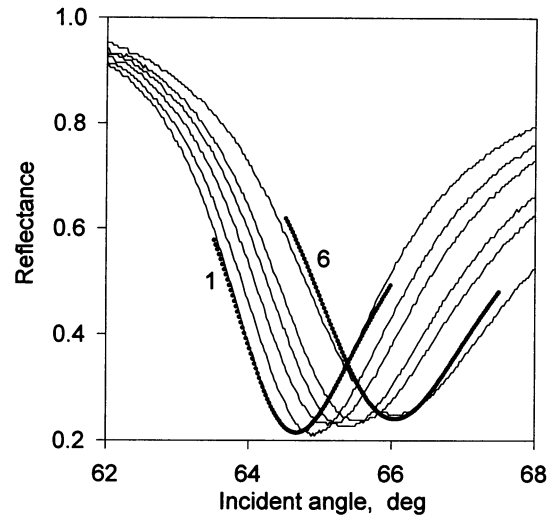

FIGURE 4 Experimental SPR spectra (T = 23 ± 1°C) obtained with a silver film of 52.5 nm average thickness and a lipid layer made from a solution containing 10 mg/ml PC in squalene/butanol (0.2:10, v/v; curve 1), and after five incremental additions of cyt c to the aqueous compartment of the SPR cell (see Fig. 1), reaching a final protein concentration of 400 μM (curve 6). Dotted curves represent theoretical fits to curves 1 and 6. The buffer solution in the aqueous compartment of the SPR cell (Fig. 1) contained 10 mM Tris and 0.5 mM EDTA at pH 7.4 with no added salt, i.e., under low ionic strength conditions as defined in the text.

Fig. 5 shows the concentration dependence of the calculated optical parameters obtained by fitting the SPR curves resulting from cyt c binding. These are expressed as the change in the n value of the lipid membrane/aqueous buffer interface in Fig 5 A, and the change in film thickness, t, in Fig 5 B, assuming a three-layer model (see above). As can be seen, the changes of both optical parameters occur in a characteristic biphasic manner with increasing cyt c concentration. These are associated with two distinct levels of thickness change (approximately 3.0 nm and 6.5 nm), as well as two levels of refractive index change (approximately 0.1 and 0.25). Such alterations in optical parameters can be related to the structure of the deposited layer. Thus, the thickness changes obtained at both saturating levels are in good agreement with the structure of the cyt c molecule obtained from x-ray crystallography (about 3.4 nm for the longest dimension; Takano et al., 1973) and therefore strongly indicate that cyt c binds so as to generate two separate protein layers. The values of the refractive indices for the protein layers (n_p) at the first and second saturation

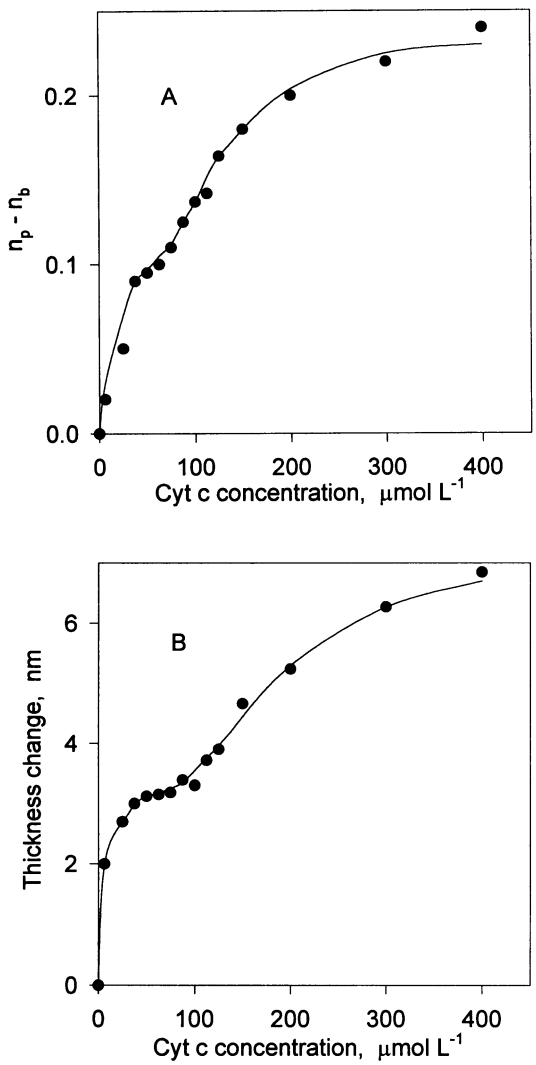

FIGURE 5 Dependence of the optical parameters (refractive index change, $n_p - n_b$, where n_p is the refractive index of the cyt c layer and n_b is the refractive index of the buffer solution (*A*) and change in average thickness of the cyt c layer (*B*) on the added cyt c concentration. Spectra were obtained for a PC membrane under low ionic strength conditions (cf. Fig. 4). Parameter values were obtained by fitting the SPR curves to a theoretical model as discussed in the text; examples are shown in Fig. 4. Solid lines are smooth curves drawn through the data points.

levels are approximately 1.43 and 1.48, respectively; in comparison, the value of the refractive index for a pure protein is estimated to be approximately 1.5 (Cuypers et al., 1983). Thus, this parameter is also in agreement with the formation of two separate protein layers.

Using the values of t and n, one can calculate the mass of adsorbed cyt c, by applying Eq. 4 and assuming $V = 0.7$ and $D/M = 0.27$ (Cuypers et al., 1983). The results of such calculations are presented in Fig. 6 for experiments performed with a variety of lipid compositions under low ionic strength conditions, and in Fig. 7 for results obtained in medium ionic strength buffer. As can clearly be seen, the curves shown in these figures correspond to a family of biphasic cyt c binding isotherms, with a characteristic pla-

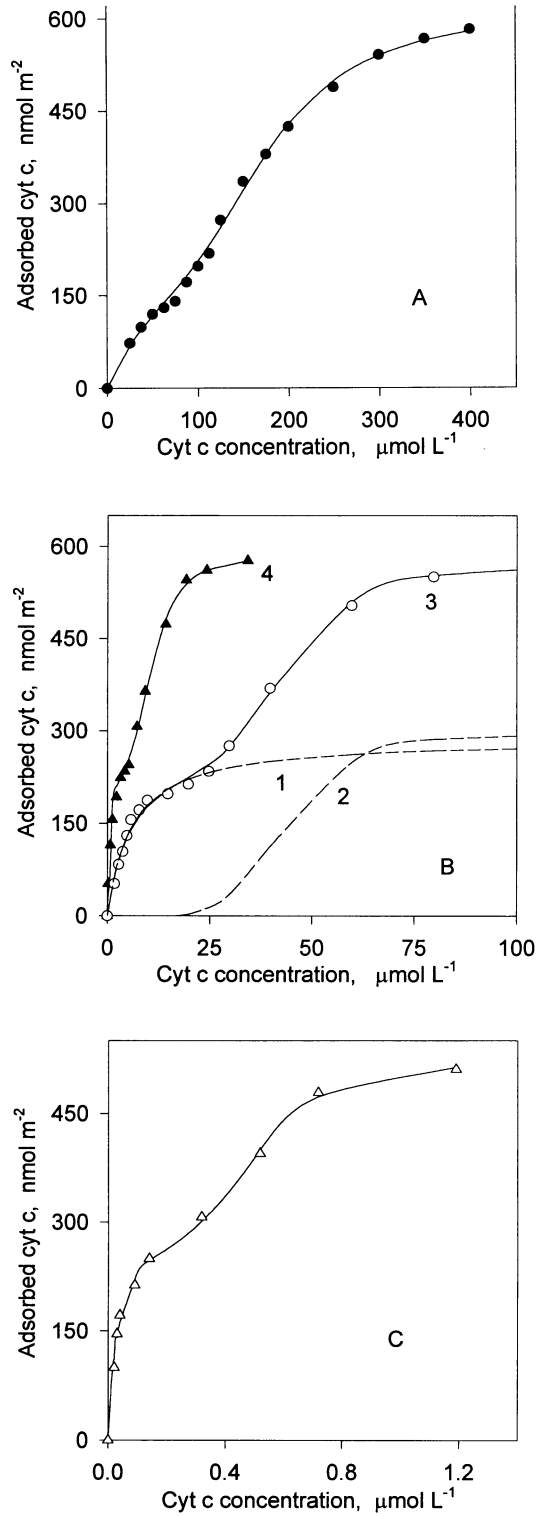

FIGURE 6 Binding isotherms for cyt c obtained under low ionic strength conditions (see Fig. 5). Symbols represent experimental data, whereas solid lines show theoretical fits (see Materials and Methods). (*A*) PC alone. Solid curve is a theoretical fit to the sum of hyperbolic and sigmoidal functions. (*B*) PC + 5 mol% CL (○); PC + 15 mol% CL (▲). Dashed curves labeled 1 and 2 represent deconvoluted hyperbolic and S-shaped components, respectively, for PC + 5 mol% CL data; solid curves labeled 3 and 4 represent the sum of the two deconvoluted components superimposed on the experimental data. (*C*) PC + 20 mol% CL. Solid curve is a theoretical fit to the sum of hyperbolic and sigmoidal functions.

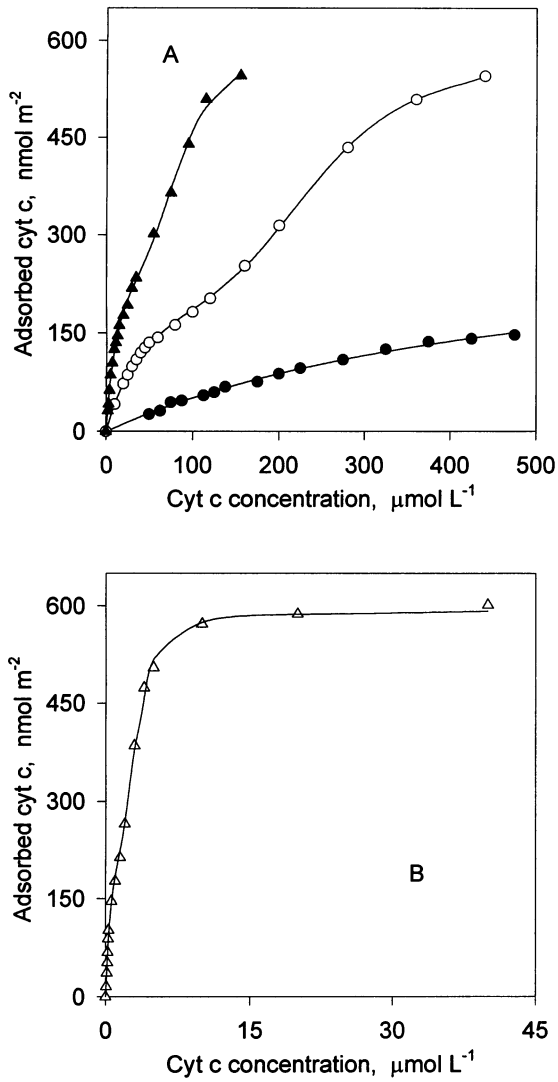

FIGURE 7 Binding isotherms for cyt c obtained under medium ionic strength conditions (see text). Symbols represent experimental data, and solid lines show theoretical fits to the sum of hyperbolic and sigmoidal functions. (*A*) PC alone (●); PC + 5 mol% CL (○); PC + 15 mol% CL (▲). (*B*) PC + 20 mol% CL.

teau between the two binding phases. This plateau region not only distinctively separates the two adsorption processes, but also indicates that they are not independent of one another, i.e., that the first process must occur before the second can begin. This type of cooperative binding mechanism can be modeled by assuming that the first phase of adsorption occurs according to a simple Langmuir isotherm, and can therefore be described by a hyperbolic function, whereas the second phase can be represented by a sigmoidal function. Such a theoretical deconvolution (an example of which is shown in Fig. 6 *B*) of the experimental binding isotherms allows an evaluation of the binding parameters for these two phases (cf. Materials and Methods). Similar theoretical deconvolutions have been done for all of the binding isotherms, and the resulting data points and fitted curves for adsorbed cyt c as a function of the concentration of added cyt c are shown in Figs. 6 and 7; the calculated values for K_D and the final adsorbed cyt c concentrations (C) are given in Table 1.

There are three important conclusions that can be drawn from these data. First, all of the binding isotherms (except for cyt c binding to a PC membrane without CL under medium ionic strength conditions, which can be fit with just a single hyperbolic function) show two levels of saturation with relative binding affinities that differ by about one order of magnitude, indicating two separate (although dependent) binding processes. The amounts of cyt c bound by these two processes are quite similar, resulting in average values of 300 nmol m^{-2} and 310 nmol m^{-2} for low and medium ionic strength conditions, respectively. Second, the dissociation constants for both binding processes are very sensitive to both the CL concentration and the ionic strength of the buffer solution, clearly indicating that electrostatic forces are involved in the binding processes. An exponential relationship between K_D values and CL concentration (see Fig. 8) agrees with predictions of the double layer theory (Heimburg and Marsh, 1995) and thus confirms this conclusion. Third, the magnitudes of the final saturation levels (Table 1) are clearly independent of both the CL concentration and ionic strength. This last result suggests that the binding processes involve more than a simple electrostatic interaction between negative charges exposed on the membrane surface and the positively charged cyt c molecules.

To further explore the nature of the binding between the membrane and cyt c, the extent of reversibility of the SPR changes once the two saturation levels were reached was determined, either by increasing the salt concentration in the buffer solution, or by decreasing the cyt c concentration in

TABLE 1 Cyt c binding parameters [dissociation constant (K_D) and final surface concentration (C)] to PC membranes containing different amounts of cardiolipin

	Low ionic strength				Medium ionic strength			
	K_D (μM)		C (nmol m^{-2})		K_D (μM)		C (nmol m^{-2})	
mol% CL	I	II	I	II	I	II	I	II
0	92	175	333	320	525	ND	320	ND
5	6	43	290	300	55	250	280	320
15	0.5	11	250	336	14	86	310	300
20	0.04	0.5	311	220	0.7	3.2	304	295

I and II refer to deconvoluted binding components (see text). ND, Not detected.

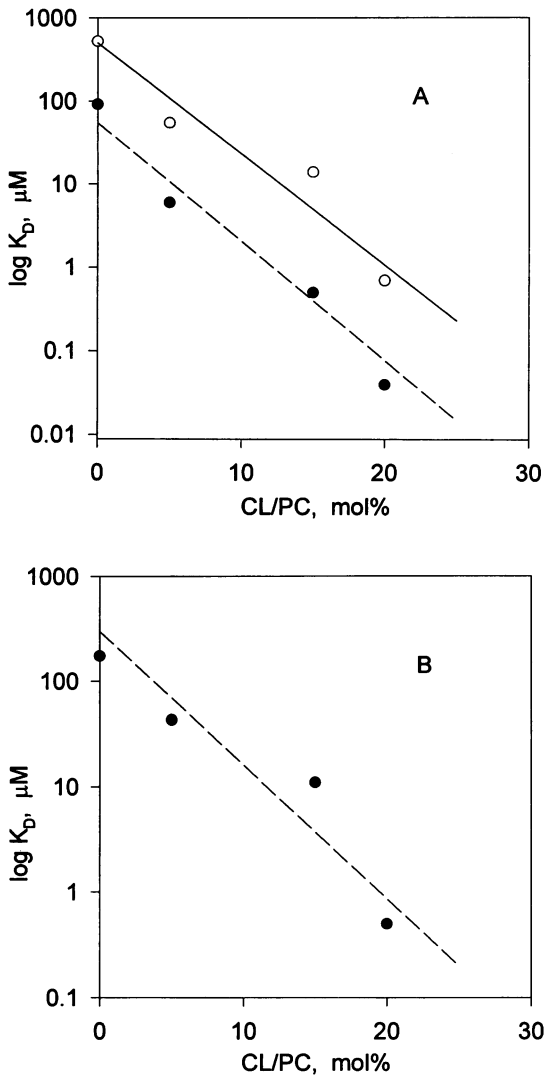

FIGURE 8 Dependence of the binding dissociation constants (K_D) obtained for cyt c on the extent of incorporation of CL. (*A*) K_D values for the first phase of cyt c binding. ●, Low ionic strength conditions; ○, medium ionic strength conditions. (*B*) K_D values for the second phase of cyt c binding under low ionic strength conditions.

the aqueous compartment of the SPR cell by addition of buffer solution. The results demonstrate a complete lack of reversibility at both levels of saturation, i.e. no detectable changes in the SPR spectra resulted from either treatment (data not shown). This finding is in agreement with results obtained in similar experiments involving binding of cyt c to liposomes (Rytömaa and Kinnunen, 1995). The lack of reversibility implies that at least partial penetration of the initially electrostatically bound protein molecules into the lipid phase occurs. This penetration would allow cyt c molecules to interact hydrophobically with the interior of the lipid membrane, resulting in a binding mode that is no longer reversible by increases in the ionic strength of the buffer or by decreases in the cyt c concentration in the aqueous phase.

A mechanistic question about the nature of this penetration into the hydrophobic interior of the membrane can be asked: is it related to the formation of hexagonal or inverted micelle structures in the lipid phase in the presence of CL (cf. De Kruijff and Cullis, 1980), or is it a consequence of destabilization of the protein structure by electrostatic interactions that expose hydrophobic surfaces and thereby allow cyt c molecules to associate with the interior of the membrane (cf. Muga et al., 1991)? The following observations provide some insight into this question. The fact that similar biphasic binding is obtained with membranes containing only PC (at low ionic strength) suggests that CL is not introducing any additional specific features into the binding process. Furthermore, similar binding isotherms have been obtained by using the nonphysiological, negatively charged lipid dihexadecylphosphate incorporated into the PC membrane instead of CL (data not shown). Furthermore, as noted above, there is no evidence from the SPR spectra for the formation upon protein binding of any structures within the proteolipid film larger than cyt c itself. The generation of large nonbilayer structures should produce a separate resonance signal shifted toward greater incident angles. Lipid aggregate formation should also produce holes in the membrane phase due to lipid depletion, resulting in contact between a portion of the silver film and the aqueous phase, and the appearance of a new resonance minimum occurring at incident angles considerably smaller than that for a metal/lipid phase. Such a three-resonance SPR spectrum, observed under conditions in which a large nonbilayer structure was induced by the interaction between a PC membrane containing 6 mol% of diacylglycerol and apolipophorin III, has recently been reported (Soulages et al., 1995).

The overall picture that emerges from the analysis of these SPR results is as follows. Cyt c initially interacts electrostatically with the lipid membrane, as indicated by the molecule labeled A in the model shown in Fig. 9. This results in a loosely packed protein surface layer. In the next phase of the binding process, hydrophobic interactions with the interior of the lipid membrane arise because of unfolding of cyt c molecules. This allows penetration of the protein into the lipid membrane, which can be either partial, as indicated in Fig. 9 by molecule B, or complete, as indicated by molecule C, depending upon the extent of the unfolding process. We presume that the initial binding event triggers these secondary events by changing the lipid surface structure, perhaps by producing discontinuities that facilitate both electrostatic and hydrophobic association with the membrane. The final amount of adsorbed cyt c will be determined by the capacity of both the interior membrane volume and the membrane surface area to accommodate protein molecules. Although it is not entirely clear which of these two binding modes corresponds to phase I and which to phase II in the binding isotherms, it seems simpler to envision that complete incorporation occurs first, followed by cyt c association with the membrane surface via partial unfolding. It has to be emphasized that, although there is an

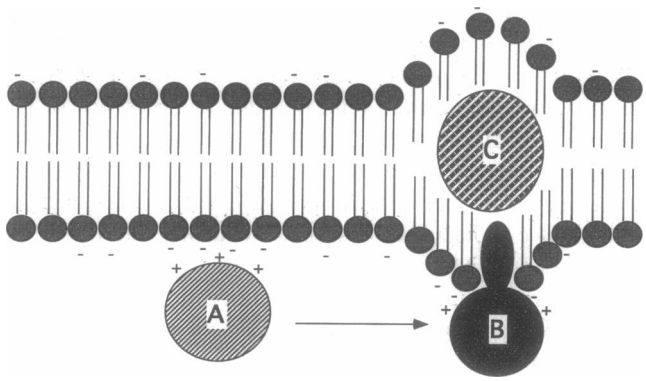

FIGURE 9 Schematic model illustrating three modes of cyt c binding to a PC membrane containing negatively charged lipid molecules. (*A*) Cyt c is electrostatically associated with the membrane surface. (*B*) Cyt c is partially inserted into the membrane interior as a consequence of structural changes that expose hydrophobic surfaces in the protein. (*C*) Cyt c is shown completely incorporated into the membrane interior after extensive structural rearrangement.

apparent dependence of the binding constant of the second phase on the electrostatic conditions of the experiment (see Table 1), this is actually a consequence of the fact that the initial binding process occurs via electrostatic forces, which then induces the second binding phase. Whereas in the present study we have only used equilibrium titrations to experimentally resolve these two binding modes, it may also be possible to separate them by using time-resolved SPR spectroscopy. Such studies would clearly be desirable.

At low ionic strength and high levels of CL incorporation, the range of cyt c concentrations over which the initial binding phase is observable is quite narrow (cf. Fig. 6, *B* and *C*). This is probably the reason why at low ionic strengths Heimburg and Marsh (1995) observed two layers of cyt c bound to a negatively charged lipid dispersion of dioleoylphosphatidylglycerol, whereas at higher ionic strengths they obtained only a single bound layer over the range of cyt c concentrations used.

The present SPR results correlate very well with our previous electrochemical measurements and provide support for the model that was proposed to explain those observations (Salamon and Tollin, 1991). In the electrochemical studies, it was demonstrated that electron transfer between cyt c and a PC bilayer-coated gold electrode occurs only at rather high protein concentrations (>100 μM) and relatively low PC concentrations in the film. This was ascribed to limitations associated with the number of available anionic functional groups that could electrostatically attract the positively charged cyt c. Furthermore, the electrode current was found to be very sensitive to both the proportion of negative charges (using dihexadecylphosphate) incorporated into the PC membrane, as well as to the ionic strength of the buffer solution. Based on the present results, it seems reasonable to infer that these observations are a consequence of a requirement for cyt c incorporation into the membrane interior to allow communication with the electrode surface to occur.

CONCLUSIONS

The results described above clearly demonstrate the utility of SPR spectroscopy as a tool for investigating peripheral protein-lipid interactions. The application of this methodology has allowed additional quantitative and structural insights into the binding process to be obtained, and has yielded a binding model that is consistent with earlier work using other methodologies (cf. Pinheiro, 1994). Of particular physiological interest in terms of protein transport mechanisms are the results which indicate that the initial electrostatic interaction between cyt c and negatively charged sites on the lipid membrane surface induce structural alterations in the protein molecule, which lead to varying degrees of penetration into the hydrophobic interior and to associations with the membrane that are not readily reversed by changes in the solvent environment, without appreciably altering the lipid bilayer structure. As will be demonstrated in the following paper, prior insertion into the lipid bilayer of a transmembrane protein such as cytochrome *c* oxidase makes this mode of binding less likely, thereby ensuring that appropriate interactions between integral and peripheral proteins are maintained, while still allowing the peripheral protein to remain sequestered at the membrane surface, so as to maximize its functional properties.

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