Biophysical Journal Volume 71 August 1996 858–867

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

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ABSTRACT Complex formation between horse heart cytochrome *c* (cyt c) and bovine cytochrome *c* oxidase (cco) incorporated into a supported planar egg phosphatidylcholine membrane containing varying amounts of cardiolipin (CL) (0–20 mol%) has been studied under low (10 mM) and medium (160 mM) ionic strength conditions by surface plasmon resonance (SPR) spectroscopy. Both specific and nonspecific modes of cyt c binding are observed. The dissociation constant of the specific interaction between cyt c and cco increases from approximately 6.5 μM at low ionic strength to 18 μM at medium ionic strength, whereas the final saturation level of bound protein is independent of salt concentration and corresponds to approximately 53% of the total cco molecules present in the membrane. This suggests a 1:1 binding stoichiometry between the two proteins. The nonspecific binding component is governed by electrostatic interactions between cyt c and the membrane lipids and results in a partially ionic strength-reversible protein-membrane association. Thus, hydrophobic interactions between cyt c and the membrane, which are the predominant mode of binding in the absence of cco, are greatly suppressed. Both the amount of nonspecifically bound protein and the binding affinity can be varied over a broad range by changing the ionic strength and the extent of CL incorporation into the membrane. Under conditions approximating the physiological state in the mitochondrion (i.e., 20 mol% CL and medium ionic strength), 1–1.5 cyt c molecules are bound to the lipid phase per molecule of cco, with a dissociation constant of 0.1 μM. The possible physiological significance of these observations is discussed.

INTRODUCTION

Cytochrome *c* oxidase (cco), the terminal enzyme in the respiratory chain, is an oligomeric metalloprotein located in the mitochondrial inner membrane of eukaryotic cells or in the plasma membrane of bacteria (Wikström et al., 1981; Babcock and Wikström, 1992; Calhoun et al., 1994; Trumpower and Gennis, 1994; Malatesta et al., 1995). It functions to store metabolic energy by coupling the transfer of electrons from cytochrome *c* (cyt c) to dioxygen with the translocation of protons across the inner membrane (Wikström et al., 1981; Copeland and Chan, 1989; Capaldi, 1990; Malmström, 1990; Trumpower and Gennis, 1994; Malatesta et al., 1995). The cco from bovine heart, which consists of 13 polypeptide subunits (see Malatesta et al., 1995, for a review), has recently been characterized at atomic resolution by x-ray diffraction methods (Tsukihara et al., 1995). Although the data analysis is not yet complete, the basic features of the oxidase structure, including the overall molecular dimensions and the detailed atomic structure of the metal sites (two hemes A, three coppers, one magnesium, and one zinc), are now available. The structural arrangement of the metal-containing components is in excellent agreement with previous studies (see Gennis and Ferguson-Miller, 1995, and references therein), as well as with the high-resolution x-ray structure of the simpler cco from the bacterium *Paracoccus denitrificans* (Iwata et al., 1995). The two heme A groups (cytochromes *a* and a_3) and one of the copper atoms (Cu_B) are associated with subunit I; a dinuclear copper center (Cu_A) with an unusual iron-sulfur type structure is associated with subunit II (Tsukihara et al., 1995). Cu_B and cytochrome a_3 form the dioxygen-binding site; Cu_A and cytochrome *a* participate in electron transfer from cyt c (Wikström et al., 1981; Copeland and Chan, 1989; Malmström, 1990; Panda and Robinson, 1995; Einarsdóttir, 1995).

The four-electron reduction of dioxygen by cco is initiated by binding of cyt c to subunit II on the external side of the membrane. Despite considerable effort, the number of cyt c binding sites on cco and the mechanism of electron entry have not been fully established (for reviews see Malatesta et al., 1995; Einarsdóttir, 1995). Three different experimental approaches have been employed to evaluate the stoichiometry of the cyt c/cco complex. Although one of these, the gel filtration method introduced originally by Hummel and Dryer (1962), directly monitors the binding equilibrium, it has two important disadvantages: 1) it does not provide an accurate evaluation of the stoichiometry of complex formation; and 2) any binding interaction is detected, regardless of the affinity or the location of the binding site, provided that the association is stable under the experimental conditions used. Two other techniques, steady-state kinetic measurements of electron transfer using both polarographic (e.g., Ferguson-Miller et al., 1978) and

Received for publication 4 December 1995 and in final form 12 April 1996.
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0006-3495/96/08/858/10 $2.00

optical (e.g., Antalis and Palmer, 1982) methods, and spectrophotometric titration (e.g., Michel and Bosshard, 1984), have also been widely used to investigate the cyt c/cco interaction. These approaches also have limitations. In the case of steady-state kinetics, a primary difficulty is incomplete reduction of cco redox centers by cyt c, due to the rate-limiting interaction between tightly bound cyt c molecules and the nonphysiological reductant normally used to initiate the reaction (Morgan and Wikström, 1991; Nicholls, 1993; Ortega-Lopez and Robinson, 1995). Because optical methods are usually based upon changes in the absorption spectrum of cco, which is dominated by heme a, such spectroscopic monitoring can only measure binding events related to this center (Malatesta et al., 1995).

The most extensive studies of the cyt c/cco interaction have been done using steady-state kinetic methods (Ferguson-Miller et al., 1976; Wikström et al., 1981; Cooper, 1990; Malmström, 1990; Witt et al., 1995); these have revealed a complex multiphasic pattern. Three models have been proposed to explain such complicated kinetics: 1) the presence of two catalytically active cyt c-binding sites per cco monomer, with different binding affinities ranging from 10^{-8} M to 10^{-5} M (Ferguson-Miller et al., 1976; Rieder and Bosshard, 1978; Witt et al., 1995); 2) a single large binding site that can accommodate two molecules of cyt c; although in this model, the second bound cytochrome is not catalytically active, its presence regulates the binding of the first molecule (Nalecz et al., 1983; Speck et al., 1984; Bolli et al., 1985); and 3) a single binding site with two different affinities for cyt c, depending on cco conformation (Brzezinski and Malmström, 1987; Michel and Bosshard, 1989; Garber and Margoliash, 1990). It has to be emphasized, however, that none of these three models gives a completely satisfactory explanation of the experimental data. Furthermore, in a recent study, Ortega-Lopez and Robinson (1995) have clearly demonstrated that the biphasic kinetics and the requirement for additional electron transfer from excess cyt c during the low-affinity phase are a direct consequence of the limited rate of electron input into cco via a high-affinity cyt c molecule. In addition, the concept of two nonequivalent active sites on cco has been challenged repeatedly (Michel and Bosshard, 1989; Garber and Margoliash, 1990; Ortega-Lopez and Robinson, 1995).

In the present experiments we have utilized self-assembled, solid-supported planar lipid films and surface plasmon resonance (SPR) spectroscopy to directly measure the binding constants and the stoichiometry of the interaction between cyt c and a cco-containing lipid bilayer under different lipid composition and ionic strength conditions. SPR is a highly sensitive method for the optical characterization of thin films deposited on solid metal supports. It allows the evaluation of some of the structural properties of such films (specifically, thickness and density) and permits the measurement of the mass of deposited material with a high degree of accuracy. It is therefore an excellent optical method for the direct observation of interactions between large biological molecules. Originally, the methodology was applied to determining the properties of thin-film mirror coatings (Rothenhauser et al., 1988) and to the characterization of metal-electrolyte interfaces (Abeles, 1976; Rothenhauser et al., 1988). The first biological application of the method involved an analysis of arachidate monolayer assemblies on silver films (Pockrand et al., 1977). Since that time SPR has been extensively used to optically characterize lipid layers (Hickel and Knoll, 1990; Haussling et al., 1991; Plant et al., 1995), chemisorbed protein multilayers, and protein-protein interactions (Morgan et al., 1992; Schuster et al., 1993; Terrettaz et al., 1993; Swanson et al., 1993; Bondeson et al., 1993). In previous work from this laboratory, we have used SPR spectroscopy to characterize the structural properties of planar, solid-supported lipid membranes (Salamon et al., 1994b) to investigate the mechanism of binding of an apolipoprotein to a diacylglycerol-containing phosphatidylcholine (PC) bilayer (Soulages et al., 1995), to follow the interaction between photoactive yellow protein and lipid bilayers (Salamon et al., 1995), to monitor rhodopsin reconstitution into a supported lipid membrane and to detect conformational events occuring as a result of rhodopsin photoexcitation (Salamon et al., 1994a), to observe rhodopsin-G protein (transducin) interactions (Salamon et al., 1996), and in the accompanying paper, to characterize the binding of cyt c to lipid bilayers in the absence of cco (Salamon and Tollin, 1996). The results described below demonstrate that SPR can monitor the binding of cyt c to both the lipid and the cco components of a proteolipid film under a variety of conditions and can provide accurate values for the binding affinities and the stoichiometries of these interactions.

MATERIALS AND METHODS

Cyt c from horse heart was purchased from Sigma Chemical Co. (St Louis, MO) and used in the oxidized form without further purification. Bovine heart cco, kindly provided by L. P. Pan and S. I. Chan of the California Institute of Technology, was isolated by the method of Hartzell and Beinert (1974) and had been used previously in this laboratory in both flash photolysis and electrochemical studies (Pan et al., 1991; Salamon et al., 1993). The enzyme in its oxidized form was stored in 10 mM Tris buffer (pH 7.4) containing 0.1% lauryl maltoside frozen in liquid nitrogen. Egg phosphatidylcholine (PC) and cardiolipin (CL) were obtained in solid form from Avanti Polar Lipids (Alabaster, AL).

Surface plasmon resonance measurements

The techniques used for SPR spectroscopy (including details of the theoretical principles of the method, and the apparatus and data analysis procedures) have been described elsewhere (Salamon et al., 1994a,b, 1995). Those aspects of the methodology that are unique to the present application are discussed in the preceeding paper (Salamon and Tollin, 1996). The mass of the material deposited on the metal surface has been calculated using the optical parameters obtained from the SPR measurements, and applying the following Lorentz-Lorenz relation describing dilute films (Salamon et al., 1994b; Salamon and Tollin, 1996):

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

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 proteolipid 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.

Reconstitution of cytochrome *c* oxidase into planar supported lipid membranes

The functional reconstitution of integral membrane proteins into phospholipid membranes is, in general, based on detergent removal from protein solution in the presence of a lipid material. Among the different techniques applied for this purpose, the following are the most frequently used: dialysis, gel filtration, and dilution (Tiede, 1985; Levitzki, 1985). Of these various reconstitution procedures, detergent dilution is the most appropriate in our experiments, where we have a preformed solid surface-bound lipid bilayer in an SPR cell. In previous work we have applied this method to reconstitute two integral membrane proteins: rhodopsin and cco. As we have clearly demonstrated (Salamon et al., 1993, 1994a, 1996), in both cases incorporation of proteins into a solid-supported lipid bilayer by diluting concentrated solutions of the detergent-solubilized proteins into an aqueous medium generates an active protein layer. In the case of rhodopsin, photoactivity and G-protein-binding activity, as well as regeneration of photolyzed rhodopsin with 11-*cis*-retinal, have been demonstrated (Salamon et al., 1994a, 1996). For cco, its electrochemical activity and redox parameters under these conditions (Salamon et al., 1993) compared quite well to the values reported both for detergent solutions, measured by conventional titration methods, and for cco incorporated into supported lipid films by cholate dialysis and measured by cyclic voltammetry (Cullison and Hawkridge, 1994).

In the present work the detergent dilution technique of cco incorporation into a solid supported lipid bilayer has been used for SPR measurements. Lipid bilayer membranes on silver supports were prepared according to methods described previously (Salamon and Tollin, 1996). Small aliquots of frozen cco solutions were thawed, and the stock enzyme was exchanged into 10 mM Tris plus 0.5 mM EDTA (pH 7.4) containing 200 mM octyl glucoside. The protein concentration was determined spectrophotometrically (Hazzard et al., 1991). After a stable self-assembled lipid bilayer membrane was formed, small aliquots (usually 2–5 μl) of a concentrated solution of cco in the octyl glucoside-containing buffer 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). This diluted both the cco and detergent by a factor of 400-1000, to a final concentration that was considerably below the critical micelle concentration of the detergent (25 mM), causing transfer of cco from the aqueous phase to the membrane phase. The SPR spectrum was then recorded over a 20–30-min interval (i.e., until the spectrum stabilized). After saturation of the membrane with cco, small aliquots of cyt c solution were added to the SPR cell, and spectra were recorded as described in the preceding paper (Salamon and Tollin, 1996). Control experiments have shown no measurable effect of addition of comparable amounts of the detergent without cco, either on the lipid bilayer SPR spectrum or on cytochrome *c* interaction with the membrane. 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); 150 mM NaCl added (medium ionic strength conditions, I = 160 mM).

RESULTS AND DISCUSSION

Incorporation of cytochrome *c* oxidase into lipid membranes

A typical example of the SPR spectra obtained during reconstitution of cco into a lipid membrane is shown in Fig. 1. The resonance curve obtained when a bare silver film (curve 1) is modified by depositing a PC membrane (curve 2) is characteristically altered by consecutive additions of small aliquots of concentrated cco in detergent solution to the aqueous compartment of the SPR cell (curves 3 and 4). We interpret these shifts in the position of the resonance angle and small increases in the reflected intensity as being due to the incorporation of cco into the lipid phase as a consequence of the dilution of the detergent to below its critical micelle concentration. The SPR changes saturate at high concentrations of added cco (see below), indicating that no additional protein molecules can be incorporated into the lipid phase. All experiments involving cyt c additions have been carried out in the saturation range of cco concentration. Similar SPR spectral changes have previously been observed upon incorporation of the smaller integral membrane protein rhodopsin into a PC membrane by analogous methods (Salamon et al., 1994a, 1996).

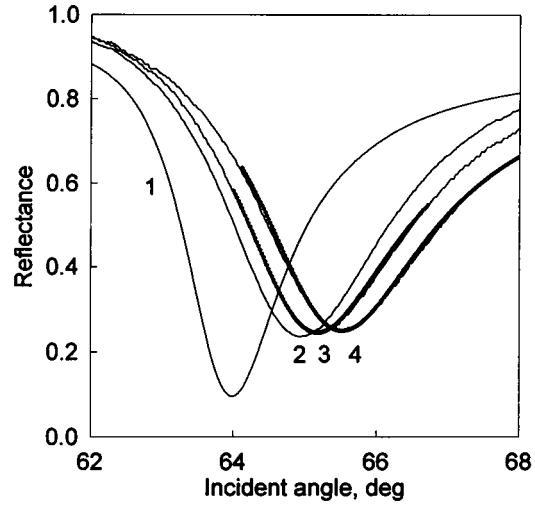

FIGURE 1 Typical SPR spectra obtained with a silver film of 55 nm average thickness and coated with dielectric layers as indicated below. The buffer solution in contact with the film contained 10 mM Tris and 0.5 mM EDTA at pH 7.4 (T = 23 ± 1°C). Reflectance values measure the fractional decrease in the total reflected light. Curve 1: Bare metal film in contact with buffer solution. Curve 2: Metal film coated with an egg PC bilayer membrane, described by the following optical parameters obtained from a theoretical fit to the SPR curve (not shown): thickness, t = 5.9 nm; refractive index, n = 1.51, and extinction coefficient, k = 0.075. Curves 3 and 4: After two incremental additions of cytochrome *c* oxidase to the aqueous compartment (final total concentrations added to the aqueous phase are 1.75 μM and 8.75 μM, respectively). Dotted curves represent theoretical fits to curves 3 and 4 (see text for details).

To evaluate the structure (i.e., average thickness and density) of the reconstituted proteolipid (PC/cco) film, we have calculated the optical parameters t (thickness), n (refractive index), and k (extinction coefficient) from theoretical fits (examples shown for curves 3 and 4 in Fig. 1) to the SPR spectrum obtained at saturation, using a two-layer model consisting of the metal layer plus the PC/cco film (see preceding paper for details). The t value obtained from this fit (t = 11.0 ± 0.1 nm; for additional details see legend to Fig. 1) is very close to the length of the cco molecule obtained from the recent x-ray structure (11.7 nm; Tsukihara et al., 1995), clearly indicating that the cco molecules

have been incorporated into the lipid membrane with their long axis perpendicular to the membrane surface. Based on this, one can construct a model of the proteolipid membrane structure, as shown schematically in Fig. 2. As can be seen from the model, two lipid/protein layers with different sets of optical parameters can be distinguished. One of these includes the lipid bilayer and the transmembrane portion of the cco molecule and is described by the optical parameters t_1 and n_1. The second layer includes the peripheral part of the cco, which extends into the aqueous buffer within the SPR cell and is characterized by t_2 and n_2. Using the value of the cco length given by the x-ray structure, we have recalculated the values of n at different levels of cco incorporation from the SPR data. There is a simple relationship between the optical parameters for the model shown in Fig. 2 and those obtained from fitting the SPR curves (i.e., t and n, which represent the average values for the entire PC/cco membrane):

$$t = t_1 + t_2, \tag{2}$$

and if we assume a linear approximation:

$$n = (n_1 + n_2)/2. \tag{3}$$

Incorporation of cco into a lipid membrane generates an excess of lipid material. This can be squeezed into the Plateau-Gibbs border, which anchors the bilayer film to a Teflon spacer and provides a large volume where lipid bilayer forming material is stored, or it can be displaced into the buffer solution (the same way lipid bilayer membrane is formed; cf. Salamon and Tollin, 1996; Ivanov, 1988). The final consequence of these processes is replacement of lipid with cco molecules, with some alterations in the surface structure of the remaining lipid bilayer membrane, which (as we will demonstrate below) affects cyt c-lipid membrane interactions.

Assuming that the thickness of the lipid portion of the PC/cco membrane is not changed by cco incorporation (i.e., t_1 = 5.9 nm; see legend to Fig. 1), one can obtain a value for

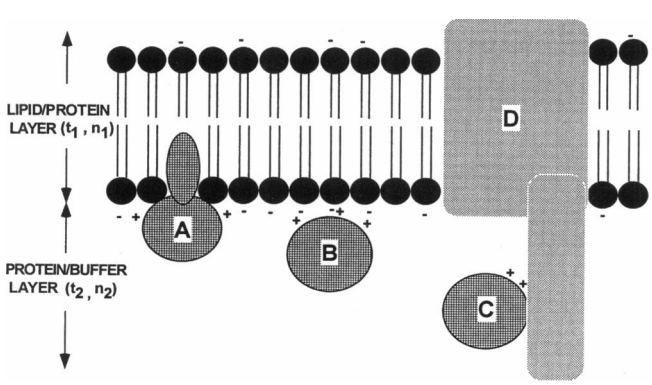

FIGURE 2 Schematic model of a negatively charged lipid membrane showing cytochrome *c* oxidase (D) interacting with a cytochrome *c* molecule (C), and cytochrome *c* molecules (A, B) interacting electrostatically and hydrophobically with the lipid membrane.

t_2 of 5.8 nm. It is worthwhile to note that, although in Fig. 2 we have depicted t_2 only on one side of the lipid membrane, in principle this value can be divided between both sides of the film, but probably with a disproportionately larger fraction extending into the buffer solution. Such a minor modification of the model presented in Fig. 2 will not have any measurable consequences on the calculated cco mass, because both sides of the membrane have aqueous buffer present with the same optical parameters. One can also safely assume that the refractive index of the lipid region of the membrane is not appreciably changed by incorporation of cco, inasmuch as lipids and proteins have quite similar n values. Therefore, the major contribution to the alterations in the SPR spectrum that are caused by insertion of a transmembrane protein such as cco into a lipid membrane must be related to the changes that occur in the protein-buffer layer (and therefore to the t_2 and n_2 parameters), rather than to the lipid-protein part of the PC/cco membrane. For these reasons we have calculated the mass of the cco molecule in the protein-buffer layer from Eq. 1 above, using the n_2 values obtained from Eq. 3 and taking n_1 = constant = 1.51 (see optical parameters for PC from legend to Fig. 1). The other two parameters in Eq. 1, i.e., *A/M* and *V*, have been assumed to be 0.27 and 0.7, respectively (Cuypers et al., 1983). Using the additional assumption that the amount of cco mass located in the protein-buffer layer is between one-third and one-half of the total cco mass (we have used the value 0.42, estimated from the x-ray structure), we can then calculate the entire cco mass that is inserted into the membrane. The results of such calculations, expressed as molar surface concentration (based on a molecular mass of 160 kDa for cco), are shown in Fig. 3 for PC membranes containing different amounts of CL in a buffer of medium salt concentration (160 mM ionic strength). As can be seen from Fig. 3, all of the reconstitution curves appear to saturate at approximately the same concentration of cco in the membrane (see below), which indicates that there is a final structure with a surface protein concentration that cannot be exceeded, regardless of the lipid composition. Furthermore, it is evident that the incorporation of cco molecules into a PC membrane is more efficient in the presence of CL, i.e., the apparent binding affinity is larger and thus saturation occurs at a lower cco concentration. Although it is not surprising that CL molecules, which interact strongly with cco both electrostatically and hydrophobically (see review by Hoch, 1992, for details), and which create discontinuities in the PC membrane surface (Hoch, 1992), cause the process of cco incorporation to become more efficient, further studies are required to understand this in more detail.

To quantitate these results we have generated least-squares hyperbolic fits to the experimental curves in Fig. 3 (shown by solid lines). These allow two parameters to be obtained, the final (extrapolated) surface concentration of cco and an apparent reconstitution constant. The final cco surface concentration is similar in all of the curves and is about 60 nmol m^{-2}, whereas the reconstitution constant

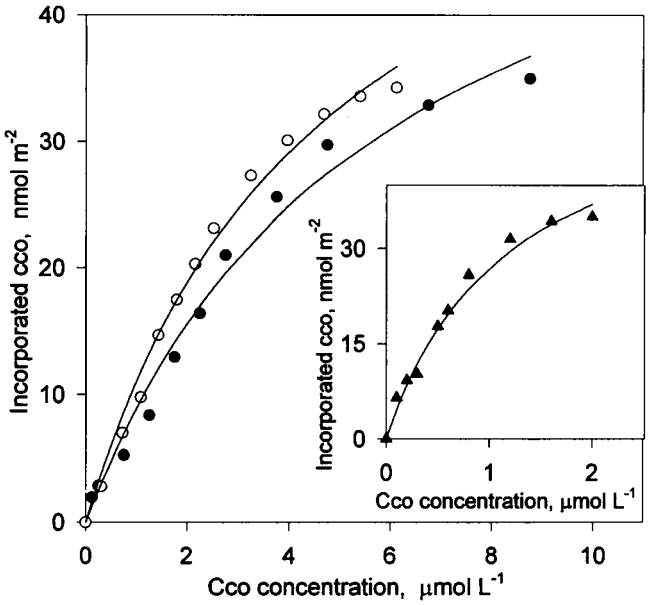

FIGURE 3 Dependence on the cytochrome *c* oxidase concentration in the aqueous phase of protein incorporation into PC bilayer membranes containing varying amounts of cardiolipin: ●, 0 mol%; ○, 5 mol%; ▲, 20 mol%. All other conditions are as in Fig. 1. Solid curves show nonlinear least-squares fits to a hyperbolic function; these yield the apparent incorporation constants and the final protein surface coverages given in the text.

becomes smaller with increasing CL (6 μM for a pure PC membrane and about 1.5 μM for PC + 20 mol% CL). The final surface concentration obtained for cco allows us to calculate an average surface area per protein molecule in the saturated membrane. The value obtained for this parameter (approximately 35 nm^2 per cco molecule of 160 kDa molecular mass) is in good agreement with both electron microscopy and the recent x-ray structural data (Tihova et al., 1993; Iwata et al., 1995). This strongly indicates that the model used to analyze the SPR data is a reasonable one.

Cytochrome *c* binding to a lipid membrane containing cytochrome *c* oxidase

Binding isotherms obtained for cyt c incorporation into CL and cco-containing membranes under low and medium ionic strength conditions are shown in Fig. 4. The amount of adsorbed cyt c was calculated based on the model presented in Fig. 2 and using Eq. 1. To understand the results in Fig. 4 it is important to recall two observations that were described in the preceding paper (Salamon and Tollin, 1996). First, it was found that cyt c interacts biphasically with pure PC and PC/CL membranes, and that the initial phase of the interaction is electrostatic. After the initial binding occurs, cyt c molecules are irreversibly bound in two separate layers by varying degrees of incorporation into the hydrophobic interior of the membrane. The formation of the first layer follows a simple Langmuir isotherm and can be represented by a hyperbolic function, whereas formation of the second layer exhibits cooperativity and can be represented by a

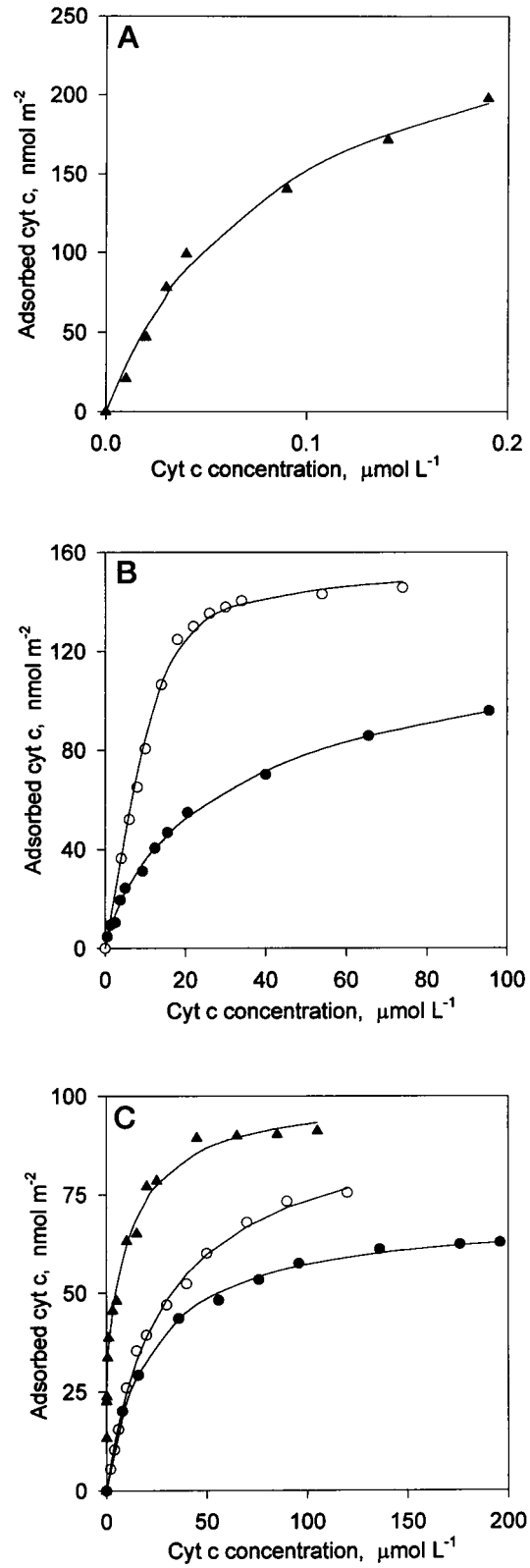

FIGURE 4 Cytochrome *c* binding isotherms measured at low ionic strength (I = 10 mM; *A* and *B*), and at medium ionic strength (I = 160 mM; *C*), with PC membranes containing varying amounts of cardiolipin: ●, 0 mol%; ○, 5 mol%; ▲, 20 mol%. All other experimental conditions are as in Fig. 1. Solid lines represent hyperbolic fits to the data.

sigmoidal function. As a consequence, the experimental binding isotherms show a plateau region between two saturation levels. Second, although the binding constants for both modes depend on the ionic strength and the amount of negative charge on the membrane surface, the final levels of adsorbed cyt c in both layers do not, and full saturation of the membrane with densely packed cyt c molecules is always achieved. The results in Fig. 4 clearly demonstrate that, in the presence of cco, there is generally a much lower sensitivity of the binding affinity to the electrostatic conditions of the experiment (both ionic strength and level of CL incorporation) than is the case when cco is absent. Furthermore, the binding isotherms no longer contain a plateau region, as observed for lipid membranes without cco. In addition, the final cyt c saturation level depends significantly on the experimental conditions and results in a surface density that is much smaller than was observed without cco.

In general, it is reasonable to conclude from these observations that the binding isotherms in the presence of cco contain at least two components: one that corresponds to a specific cco/cyt c interaction and is relatively insensitive to the electrostatic conditions of the experiment, and another that is electrostatic in nature and represents nonspecific binding of cyt c to the lipid membrane by mechanisms characterized in the preceding paper (Salamon and Tollin, 1996).

To deconvolute the binding curves shown in Fig. 4 into the individual components, we have carried out least-squares hyperbolic fits to the data. A summary of the parameters obtained from the fits shown in Fig. 4 is given in Table 1. There are several important conclusions that can be drawn from these calculated parameters. First, at low ionic strengths (10 mM) the binding isotherms can only be resolved into two hyperbolic components for the membrane without CL. Second, both binding parameters (K_D and C) are significantly altered by the addition of varying amounts of CL. Thus, increasing the concentration of CL causes a decrease in K_D (from 6.5 μM and 55 μM for a PC membrane, to 0.06 μM for a PC + 20 mol% CL membrane) and an increase in C (from 132 nmol m^{-2} for PC only, to 285 nmol m^{-2} for PC + 20 mol% CL). Third, both parameter values are changed by increasing the salt concentration in the buffer solution. Specifically, the final surface concentration of cyt c is decreased, with the largest effect obtained at 20 mol% of CL (100 nmol m^{-2} versus 285 nmol m^{-2}), and the smallest effect at 0 mol% of CL (70 nmol m^{-2} versus 132 nmol m^{-2}). Fourth, at medium ionic strength the binding isotherms can be deconvoluted into one component for the pure PC membrane and two components for the PC + CL membranes.

Under conditions with the largest electrostatic forces (i.e., 20 mol% CL at low ionic strength), the binding dissociation constant reaches a value that is very similar to that obtained for the first component under the same electrostatic conditions but without cco (K_D = 0.06 μM for membranes with cco versus K_D = 0.05 μM for membranes without cco; see preceding paper). This demonstrates that the electrostatic binding component (binding predominantly to the CL in the membrane) entirely masks any binding to cco under these conditions. In contrast, for membranes without CL at medium ionic strength, one can clearly see two components, the second of which is dominant (see Table 1). Based on the results obtained without cco in the preceding paper, we assume that the component with the highest binding affinity (K_D = 6.5 μM) at low ionic strength in pure PC membranes containing cco represents a specific binding of cyt c to cco (there was no such strongly bound component in the absence of cco). The second component seen under these conditions (K_D = 55 μM) is much closer in its affinity to the previously observed first component (K_D = 92 μM; see preceding paper, Salamon and Tollin, 1996), which we have interpreted as resulting from a purely electrostatic interaction between the lipid membrane and cyt c. Although in the present situation K_D is smaller, this can be rationalized as a consequence of alterations in the electrostatic interactions between the membrane and cyt c resulting from the incorporation of cco molecules. Such incorporation probably generates discontinuities in the polar surface of the membrane, thereby exposing additional charges that can interact with cyt c and lower the value of K_D. According to this interpretation, the final surface concentration obtained for the high-affinity component (32 nmol m^{-2}) represents the number of active cco-binding sites. Given the total concentration of cco incorporated into the lipid layer (60 nmol m^{-2}; see above), one can use the ratio of 32/60 = 0.53 as a measure of the stoichiometry of the cco/cyt c interaction. Because the stoichiometry is less than one, this strongly suggests that this component does not result from two binding sites associated with the cco molecule itself, as has

TABLE 1 Cyt c binding parameters [binding affinity constant (K_D) and final surface concentration (C)] to PC membranes containing cco and 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	6.5	55	32	100	20	ND	70	ND
5	12	ND	160	ND	20	45	48	48
20	0.06	ND	285	ND	0.07	15	65	35

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

been concluded from the majority of the steady-state kinetic measurements. The lack of a precise 1:1 stoichiometry in the present experiments can be readily rationalized on the assumption that the insertion of cco into the solid-supported lipid membrane results in about 50% of the protein being incorporated with the cyt c binding site facing the external solution. This is in agreement with previous results obtained with supported membrane systems (Cullison and Hawkridge, 1994).

At medium ionic strength in the absence of CL, the final surface concentration of adsorbed cyt c is decreased from 132 nmol m^{-2} to 70 nmol m^{-2}, implying that the electrostatic component has been diminished (from 100 nmol m^{-2} to 25 nmol m^{-2}). This decrease, together with the observation that the electrostatic component is characterized by a rather low binding affinity under such conditions (K_D = 525 μM; see preceding paper), accounts for the apparent absence of the second (i.e., electrostatic) component in the deconvolution. Although the final surface concentration of cyt c is higher than expected for the specific cco interaction (70 nmol m^{-2} versus 32 nmol m^{-2} for the sample at low ionic strength), which indicates some electrostatic binding, it seems clear that the value of K_D (20 μM) obtained under such conditions represents mainly the specific cco/cyt c binding process. Therefore, the difference between this value and K_D = 6.5 μM for the PC membrane at low ionic strength can be ascribed to the effect of ionic strength on the cco/cyt c interaction.

Upon the addition of 5 mol% CL to the lipid phase at medium ionic strength, a second component can be resolved in the deconvolution of the binding isotherm (the ratio of specific binding to electrostatic binding is 1:1; see Table 1). It is also important to note that the dissociation constant for the electrostatic component is approximately the same as it was for the same membrane without cco (45 μM versus 55 μM; see preceding paper). Further addition of CL (20 mol%) generates a much higher level of electrostatic binding at medium ionic strength (about 65 nmol m^{-2}), with a dissociation constant that again is the same as that for the membrane without cco (both 0.07 μM; see preceding paper). The dissociation constant for the cco/cyt c binding is also in good agreement with the value obtained for the membrane containing no CL (15 μM versus 20 μM). The average value for the K_D of binding cyt c to cco at medium ionic strength, independent of the presence of varying amounts of CL, is therefore ~18 μM, i.e., there is an approximately threefold increase in this value between the two ionic strength conditions (I = 10 mM and I = 160 mM).

Although steady-state kinetic studies generally agree that the rate constant for the reaction of cco and cyt c decreases with increasing ionic strength (Malatesta et al., 1995), which is consistent with the binding results presented in this paper, there are some conflicting data regarding the variation of the rate constant at ionic strengths less than 100 mM. Davis and co-workers (Bolgiano et al., 1988) have observed either a decrease or no change in the steady-state rate of cyt c oxidation by bovine or *P. denitrificans* cco for ionic strengths below 100 mM. Furthermore, results obtained by laser flash photolysis in this laboratory (Hazzard et al., 1991; Pan et al., 1991), using detergent solutions of cco, have demonstrated that the first-order electron transfer rate constant from reduced cyt c to oxidized cco shows biphasic behavior with respect to ionic strength, decreasing both below and above an ionic strength of 110 mM. In contrast, the kinetically determined protein/protein binding affinity does not change below 110 mM and decreases above this ionic strength. These results were interpreted in terms of nonproductive complex formation at low ionic strengths. Although the existence of such unreactive complexes can significantly influence kinetic measurements, they may be much more difficult to detect from thermodyamic determinations of binding interactions. There is also another significant difference between the kinetic and thermodynamic measurements of K_D, i.e., the kinetic experiments measure binding affinity for reduced cyt c, whereas the present experiments utilize the oxidized form of cyt c.

There are also experimental results with detergent solutions of cco which indicate that an increase in ionic strength causes the apearance of biphasic kinetics Antalis and Palmer, 1982). These latter observations correspond especially well to the results described in the present paper from membranes containing 20 mol% CL, i.e., at low ionic strength only one very strongly bound complex can be detected, and increasing the ionic strength causes an overall decrease in the binding affinity and a separation of the binding process into two components, one involving electrostatic association with the membrane lipids and the other a specific cco/cyt c interaction. This will be discussed further below.

To further substantiate the binding model described above, we have characterized the extent of reversibility of cyt c adsorption by increasing the ionic strength (to 250 mM by NaCl addition) of the aqueous solution in contact with a membrane containing 5 mol% CL, after reaching saturation with cyt c under low ionic strength conditions (see Fig. 4 *B, open circles*). As is shown by the results in Fig. 5, the SPR spectrum is partially shifted back to smaller resonance angles. This clearly corresponds to dissociation of some cyt c molecules from the cco-containing membrane. This result is in contrast with data obtained in the preceding paper with lipid membranes in the absence of cco (Salamon and Tollin, 1996), in which no cyt c dissociation could be induced by increasing the ionic strength. Although one cannot determine from the spectra which component of bound cyt c is reversible (lipid-associated or cco-bound), the amount of cyt c released as calculated from the SPR spectrum (about 90 nmol m^{-2}) is larger than the cco-bound component (about 32 nmol m^{-2}), indicating that at least some of the lipid-bound component, and most likely a combination of both components, has been dissociated from the cco-containing membrane.

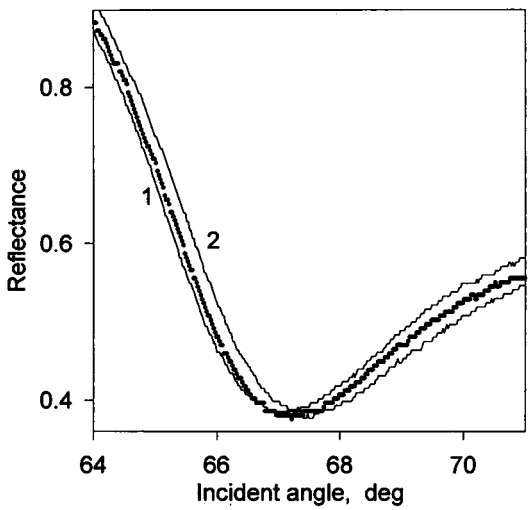

FIGURE 5 Curve 1: SPR spectrum measured at low ionic strength (I = 10 mM) with a PC membrane containing 5 mol% CL after the addition of saturating amount of cytochrome c oxidase. Curve 2: SPR spectrum obtained after the addition of saturating concentration of cytochrome c to sample, giving spectrum shown in curve 1 (final concentration of cytochrome c in aqueous phase is 74 μM). ●, SPR spectrum after increasing ionic strength of the buffer solution to 250 mM by the addition of NaCl to the aqueous compartment subsequent to measurement of curve 2. All other conditions as in Fig. 1.

CONCLUSIONS

As has been demonstrated above, the process of binding cyt c to a lipid membrane containing cco is complex. At least two components are involved, one of which is governed by electrostatic interactions involving the membrane lipids, and the other reflects a specific cco/cyt c interaction. The dissociation constant of the lipid-binding component changes dramatically with variation of the ionic strength between 10 mM and 160 mM (K_D varies from 0.06 μM to approximately 20 μM; see Table 1). This component is also very sensitive to the CL concentration, with K_D changing from 55 μM with a pure PC membrane to 0.06 μM with 20 mol% CL under low ionic strength conditions. As noted above, steady-state kinetic experiments have also been interpreted in terms of two different binding processes, one with $K_D \approx 10-60$ nM, and the other with $K_D \approx 1-5$ μM (Ferguson-Miller et al., 1976; Witt et al., 1995). It is important to stress that these kinetic parameters have been obtained with relatively low ionic strength detergent solutions, and the effect of ionic strength on the apparent binding constants is not well documented, as noted above. Despite these caveats, these K_D values agree surprisingly well with the present results for the biphasic binding obtained under low ionic strength conditions (K_D values of 60 nM and 6.5 μM for 20 mol% CL and 0 mol% CL, respectively; see Table 1). If one takes into account observations which indicate that cco extracted from mitochondrial membranes by detergent treatment requires the presence of CL to be fully functional (for review see Hoch, 1992), the correspondence between these two sets of values raises the possibility that the high-affinity binding constant obtained from the steady-state kinetic experiments reflects the CL/cyt c electrostatic interaction, whereas the lower affinity binding constant reflects the specific interaction between the two proteins. Although the idea that the electrostatic interaction between CL and cyt c can account for the second binding site has previously been suggested by Vik et al. (1981), the results presented here by direct measurement of cyt c binding to lipid membranes containing CL and cco provide additional strong support for this interpretation. Furthermore, as the results in Table 1 indicate, the nonspecific electrostatic binding is even stronger than the binding due to the specific cco/cyt c interaction under conditions similar to those in the mitochondrion, i.e., PC membranes containing 20 mol% CL at 160 mM ionic strength.

The results described above allow us to further elaborate the model presented in schematic form in Fig. 2. Under low ionic strength conditions, a lipid membrane containing 20 mol% CL is able to bind as much as 285 nmol m^{-2} of cyt c, of which approximately 32 nmol m^{-2} is bound directly to cco and the remaining 253 nmol m^{-2} is bound to the lipid phase. Given the total cco concentration in the lipid membrane (approximately 60 nmol m^{-2}), one can calculate that four or five cyt c molecules are bound to the lipid phase per cco molecule incorporated under these experimental conditions. As noted above, this nonspecific binding is clearly dependent on both the amount of negative charge on the membrane surface, as well as on the ionic strength of the buffer solution, and thus varies from between four and five molecules of cyt c per cco at low ionic strength with the membranes containing 20 mol% CL, to about 0.5 molecules of cyt c/cco at medium ionic strength with membranes without CL. Under electrostatic conditions corresponding to the physiological state of the mitochondrion, about 1–1.5 cyt c molecules are bound to the lipid phase per molecule of cco (see Table 1). These results are in contrast with those obtained without cco (see preceding paper), where the final saturation level of cyt c bound to the lipid membrane was clearly independent of the electrostatic conditions of the experiment. Two additional observations also differ from those obtained with membranes in the absence of cco (Salamon and Tollin, 1996). First, a large fraction of the nonspecifically bound cyt c can be dissociated from the membrane by increasing the ionic strength of the buffer solution. Second, the binding affinity for the nonspecific type of binding is higher with cco present in the membrane. These differences between membranes with and without cco indicate that incorporation of cco molecules into the lipid membrane induces changes in the lipid/cyt c interactions. Based on the model of this interaction derived from the results described in the preceding paper, it is possible to rationalize this as follows: binding of cyt c to lipid membranes containing cco does not lead to the cyt c unfolding process to as great an extent, which allows the protein to be anchored irreversibly to the hydrophobic interior of the membrane. Although the physical basis for this is unclear, it is probably a consequence of the fact that the cco molecule

is itself strongly associated with the membrane interior. Physiologically, the alterations in the binding process discussed here may play a critical role in providing an additional pool of reversibly bound cyt c in the mitochondrion, which can then be utilized for transport of electrons along the lipid membrane surface, as has previously been suggested based on steady-state kinetic analysis (Hackenbrock et al., 1986). Furthermore, under conditions that emulate those in the mitochondrion, cyt c is able to associate with both the oxidase and the lipid components of the membrane, which is clearly favorable in terms of its role as an electron shuttle between membrane-bound redox proteins.

This work was supported by a grant from the National Science Foundation (MCB-9404702).

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