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Interaction of Horse Heart Cytochrome *c* with Lipid Bilayer Membranes: Effects on Redox Potentials

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Cyclic voltammetry has been used to study the effects of interactions between horse cytochrome *c* and solid-supported planar lipid membranes, comprised of either egg phosphatidylcholine (PC) or PC plus 20 mol.% cardiolipin (CL), on the redox potential and the electrochemical electron transfer rate between the protein and a semiconductor electrode. Experiments were performed over a wide range of cytochrome *c* concentrations (0–440 μM) at low (20 mM) and medium (160 mM) ionic strengths. Three types of electrochemical behavior were observed, which varied as a function of the experimental conditions. At very low cytochrome *c* concentration (≈0.1 μM), and under conditions where electrostatic forces dominated the protein–lipid membrane interaction (i.e., low ionic strength with membranes containing CL), a redox potential (≈265 mV) and an electrochemical electron transfer rate constant (0.09s $^{-1}$)were obtained which compare well with those measured in other laboratories using a variety of different chemical modifications of the working electrode. Two other electrochemical signals (not reported with chemically modified electrodes) were also observed to occur at higher cytochrome *c* concentrations with this membrane system, as well as with two other systems (membranes containing CL under medium ionic strength conditions, and PC only at low ionic strength). These involved positive shifts of the cytochrome *c* redox potential (by 40 and 60 mV) and large decreases in the electron transfer rate (to 0.03 and 0.003 s^{-1}). The observations can be rationalized in terms of a structural model of the cytochrome *c*–membrane interaction, in which association involves both electrostatic and hydrophobic forces and results in varying degrees of insertion of the protein into the hydrophobic interior of the membrane.

KEY WORDS: Mitochondrial membranes; supported lipid bilayers; lipid membranes; vesicles; redox potential; electrochemical electron transfer.

INTRODUCTION

Many peripheral membrane-associated proteins interact both with their physiological reaction partners and with proteolipid membranes. Thus, their mode of action can be influenced by the physicochemical properties of the membrane. Protein/membrane interactions may have both thermodynamic and structural consequences, mediated by effects on the conformation of the protein as well as on the structure of the membrane. It is therefore of considerable interest to characterize the coupling between lipid and protein structural and thermodynamic states (for reviews see Nall, 1995; Jordi and de Kruijff, 1995; Gunner and Honig, 1995; Pinheiro, 1994).

Cytochrome *c* (cyt *c*),[2] one of the most widely studied of the peripheral membrane redox proteins, has been shown to bind both to its physiological partners, cytochrome *c* oxidase and the cytochrome bc_1 complex, as well as to lipid bilayer membranes, especially

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[2] Abbreviations: cyt *c*, cytochrome *c*; PC, egg phosphatidylcholine; CL, cardiolipin; CV, cyclic voltammetry; $E_{1/2}$, midpoint redox potential; ΔE, separation of the reduction and oxidation peak currents; ET, electron transfer; k_{et} electron transfer rate; SPR, surface plasmon resonance.

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to those which are negatively charged. Cyt *c*/membrane binding studies have been carried out with a variety of lipid species and experimental conditions, using both single- and multi-component lipid bilayers, either in oriented lipid films or in dispersed multilayers (Shipley *et al.*, 1969; Gulik-Krzywicki *et al.*, 1969; Papahadjopoulos *et al.*, 1975; Van and Griffith, 1975; Brown and Wüthrich, 1977; MacNaughtan *et al.*, 1985; Morse and Deamer, 1973; Tessie, 1981; Szebeni and Tollin, 1988; Cullis and de Kruijff, 1979). These investigations have demonstrated the dual nature of the interaction between cyt *c* and the lipid membrane. Thus, experiments have shown that binding is initiated by an electrostatic interaction with the membrane surface, and that this is followed by hydrophobic interactions occurring between the protein and the lipid acyl chains in the membrane interior (Gulik-Krzywicki *et al.*, 1969; Papahadjopoulos *et al.*, 1975; Tessie, 1981; Szebeni and Tollin, 1988; Cullis and de Kruijff, 1979). The binding of cyt *c* to acidic phospholipids (including cardiolipin, which is an important component of the inner mitochondrial membrane; cf. Daum, 1985; Hoch, 1992 and references cited therein) is known to induce local protein structural changes involving the immediate environment and coordination of the heme group (Vincent and Levin, 1986; Vincent *et al.*, 1987; Hildebrandt and Stockburger, 1986; 1989a,b; Heimburg *et al.*, 1991), as well as more extensive alterations in the conformation of the protein backbone, including loosening and destabilizing of the overall protein structure (Jori *et al.*, 1974; Muga *et al.*, 1991; Spooner and Watts, 1991a; de Jongh *et al.*, 1992; Heimburg and Marsh, 1993). Correspondingly, cyt *c* binding can alter the structure of the lipid phase of such negatively charged membranes, inducing the formation of inverted hexagonal phases, and possibly an inverted micellar structure (Cullis and de Kruijff, 1979; de Kruijff and Cullis, 1980; Rietveld *et al.*, 1983; Mustonen *et al.*, 1987; Spooner and Watts, 1991b, 1992; Pinheiro and Watts, 1994a,b). The latter observation has given support to the suggestion that acidic phospholipids may be functionally involved in the import of proteins into mitochondria (Jordi and de Kruijff, 1995; Hennig and Neupert, 1981; Pfanner *et al.*, 1987; Jordi *et al.*, 1989).

Whereas electrostatic interactions between negatively charged membranes and cyt *c* are well documented and have never been challenged, the involvement of hydrophobic interactions has been a contentious issue. This concept was originally proposed based on observations that cyt *c* promotes ionic permeability in phospholipid vesicles (Kimelberg and Papahadjopoulos, 1971), and can induce significant changes in the hydrocarbon interior (Gulik-Krzywicki *et al.*, 1969) and modifications of the phase transition temperature of the lipid membrane to which it is complexed (Papahadjopoulos *et al.*, 1975). Although there are spectroscopic and X-ray diffraction studies which argue against protein penetration into the hydrophobic region of the lipid bilayer (Van and Griffith, 1975; MacNaughtan *et al.*, 1985), there are also spectroscopic results which indicate that such penetration does occur (Brown and Wüthrich, 1977; Morse and Deamer, 1973; Tessie, 1981; Szebeni and Tollin, 1988). Especially strong support for bilayer penetration has been provided by the finding that there is a subpopulation of cyt *c* molecules that remains bound to mitochondrial as well as to artificial membranes at high, physiological, ionic strength, and which can only be released from the membranes by treatments that result in a loss of membrane structure (Cortese *et al.*, 1992; Cortese and Hackenbrock, 1993; Cortese *et al.*, 1995; Rytömaa and Kinnunen, 1995). In addition, Heimburg and Marsh (1995) have recently shown that the binding capacity of cyt *c* to a negatively charged 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 finding is particularly difficult to explain by a purely electrostatic model.

Studies from this laboratory utilizing a variety of experimental techniques, optical kinetic measurements (Szebeni and Tollin, 1988), electrochemical methods (Salamon and Tollin, 1991a,b), and more recently surface plasmon resonance (SPR) spectroscopy (Salamon and Tollin, 1996a; 1996b), have also supported the idea that both electrostatic and hydrophobic interactions occur between cyt *c* and a lipid membrane. SPR spectroscopy, which provides a highly sensitive method for *direct* measurement of protein binding to (Salamon *et al.*, 1994a, 1995; Soulages *et al.*, 1995), or incorporation into (Salamon and Tollin, 1996b; Salamon *et al.*, 1994b; 1996), a lipid bilayer, and at the same time allows the detection of structural changes occurring within such a proteolipid membrane (Salamon *et al.*, 1994b), has clearly demonstrated that cyt *c* binds to phospholipid membranes in two distinct phases, yielding two adsorbed layers of protein characterized by widely different affinity constants (Salamon and Tollin, 1996a). Such results have provided strong support for a mechanism in which the initial binding

interaction involves electrostatic association between cyt *c* and the lipid membrane surface, followed by hydrophobic processes resulting in both partial and complete penetration of the protein into the membrane interior. SPR studies (Salamon and Tollin, 1996a) have also indicated that no major structural transitions in the proteolipid membrane occurred over the entire binding curve, i.e., the bilayer character of the lipid phase was conserved during these interactions.

The present work extends these investigations by using cyclic voltammetry (CV) to follow the thermodynamic properties (redox potentials) of cyt *c* during the protein/lipid complexation process. The electrochemical studies reported in the present paper involve interactions between cyt *c* and lipid films supported on a solid surface (indium oxide). The structural properties of such self-assembled lipid membranes have been previously evaluated using both electrical (Salamon and Tollin, 1991a,b; 1993) and optical (Salamon *et al.*, 1994a) measurements. Thus cyclic voltammetry of pure solid-supported lipid membranes demonstrate two important characteristics: (i) very small ionic currents, which indicate a high resistance to ion flow through the membrane, and (ii) a large-capacitance component, which is related to the thickness of the membrane. These two electrical parameters are quite comparable to those obtained with conventional freely suspended lipid bilayers, indicating that the structures of these two types of membranes are very similar. In addition, reconstitution of integral or peripheral membrane proteins into such supported lipid films does not significantly alter these two electrical parameters (cf. Salamon and Tollin, 1991b; 1993; Salamon *et al.*, 1993; this can also be seen from the results presented in this paper). Thus, any structural changes which might occur due to the interactions between the lipid membrane and the incorporated protein molecules does not appreciably affect either the ionic conductivity of the lipid film or the bilayer structure (Salamon and Tollin, 1991a,b; 1993; Salamon *et al.*, 1993). These results suggest that a self-assembled solid-supported lipid membrane can accommodate varying amounts of protein either on its surface or within its interior without losing its integrity. Such a structural model of these membranes has been also substantiated by optical measurements using SPR spectroscopy (Salamon *et al.*, 1994a). The structural parameters for both pure lipid and lipoprotein membranes (e.g., thicknesses and mass densities) obtained from these studies agree very well with the results obtained using electrical measurements (Salamon *et al.*, 1994a; Salamon and Tollin, 1996a). As will be demonstrated below, the results clearly demonstrate that the characteristics of the electrochemical electron transfer (ET) reaction between cyt *c* and a lipid membrane-modified electrode change significantly as a consequence of protein–lipid membrane association. Specifically, at high cyt *c* concentrations two distinct CV waves can be observed, corresponding to two species of adsorbed protein, both of which have their redox potentials shifted to higher values compared to that of cyt *c* in aqueous solution by varying degrees of inclusion into the hydrophobic interior of the lipid membrane.

MATERIALS AND METHODS

Oxidized horse heart cytochrome *c* (type VI) was obtained from Sigma Chemical Co. (St. Louis, Missouri) and was used without further purification. As has been demonstrated by Heimburg and Marsh (1993), the small amount of deamidated cytochrome *c* present in commercially available material has no appreciable effect on interactions with negatively charged phospholipids. Small aliquots of concentrated cyt *c* solution were added to the aqueous compartment of the electrochemical cell using a microliter syringe. The cell contained 2 ml of 10 mM Tris buffer (pH 7.4), with 0.5 mM EDTA and either 10 mM NaCl (low ionic strength) or 150 mM NaCl (medium ionic strength). Egg phosphatidylcholine (PC) and cardiolipin (CL) were obtained in solid form from Avanti Polar Lipids, Inc. (Alabaster, Alabama).

Electrochemical measurements were performed using a previously described SPR cell (Salamon and Tollin, 1996a), in which the silver-coated glass prism was replaced by a tin-doped indium oxide film on a glass microscope slide, which functioned as the working electrode (Salamon and Tollin, 1993). The cell also accommodated reference (silver/silver chloride) and auxiliary (platinum wire) electrodes. Lipid membranes were formed on the surface of the indium oxide working electrode as previously described (Salamon and Tollin, 1996a). This involved spreading a small amount of lipid bilayer-forming solution across an orifice in a Teflon barrier which separated the working electrode from the aqueous phase. Two lipid bilayer-forming solutions were used, containing either 10 mg/ml of PC, or 10 mg/ml of PC plus 20mol.% CL, dissolved in squalene (Fluka)/butanol (0.2:10, v/v). The indium oxide electrode was cleaned immediately before use

by successive 30-min sonications in Alconox solution in 95% ethanol, and in purified water.

All experiments were performed at room temperature (22±1°C) using a home-made function generator with a current interface connected to a DAS 1601 Keithley Metrabyte I/O computer board. Apparent electron transfer rate constants, k_{et}, were determined from cyclic voltammetry peak separations measured with a slow scan rate, (5.5 mV/s), using the simplest model of Laviron (1979).

RESULTS AND DISCUSSION

Figure 1 shows typical cyclic voltammograms for various concentrations of cyt *c* in 10 mM Tris buffer, pH 7.4 and 20 mM ionic strength, obtained at an indium oxide working electrode modified with a lipid membrane formed from a mixture of egg PC and 20 mol.% CL. As can be seen, the most notable feature of these spectra is that, at cyt *c* concentrations greater than about 1 μM, the voltammogram consisted of two distinct redox waves which strongly overlap each other. In contrast, at very low protein concentrations (≈0.1 μM), where the electrochemical response first became measurable, only a single wave was observed (curve 2). This had a halfwave potential value of approximately 265 mV (marked by an arrow), with a peak-to-peak separation of about 80 mV. These values are comparable to those obtained for cyt *c* in solution (Koller and Hawkridge, 1985, 1988).

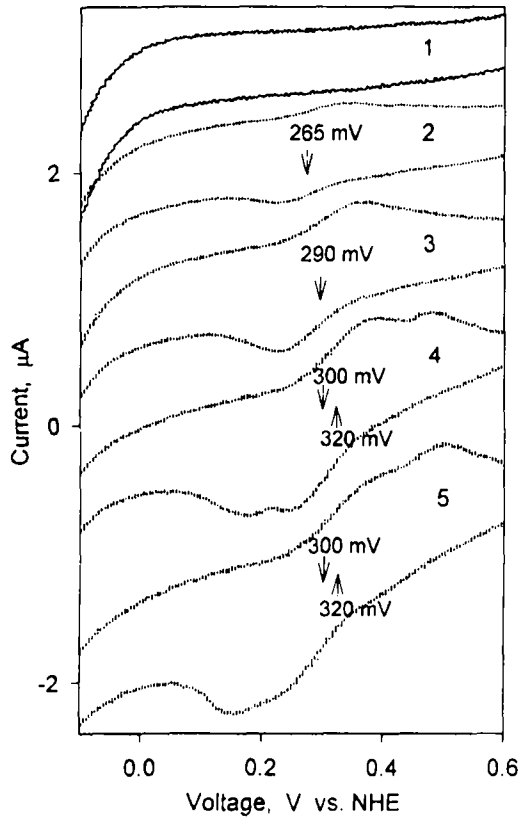

Fig. 1. Cyclic voltammograms obtained at 5.5 mV/s for cyt *c* in 10 mM Tris, 0.5 mM EDTA, and 10 mM NaCl (pH 7.4) at indium oxide working electrode coated with a lipid bilayer membrane made of 10 mg/ml PC plus 20 mol.% CL as a function of protein concentration: (1) buffer solution without cyt *c;* (2) 0.1 μM cyt *c;* (3) 0.6 μM cyt *c;* (4) 1.2 μM cyt *c;* (5) 6.4 μM cyt *c*. Curves are offset for clarity. Arrows indicate halfwave (redox) potentials measured vs. normal hydrogen electrode (NHE).

The first phase of the CV response shifted toward more positive potential values upon increasing the cyt *c* concentration to above 0.1 μM, along with a change in the separation distance between the peaks, and reached a halfwave potential of 290–300 mV and a peak-to-peak separation of about 145 mV at approximately 0.6 μM (curve 3, Fig. 1). We will refer to this below as the second CV phase. Further increases in the cyt *c* concentration produced a third CV phase, characterized by a visibly altered voltammogram and the appearance of a second redox wave (occurring at about 1 μM cyt *c;* curve 4, Fig. 1). This had a halfwave potential positively shifted to about 320 mV, and a much larger peak separation (about 350 mV). As is evident, the two waves overlap each other. Additional increases of protein concentration resulted in a decrease of the magnitude of the lower potential wave relative to the higher potential one, finally yielding a broadened waveform such as shown in curve 5, Fig. 1. At still higher cyt *c* concentrations, voltammograms similar to curve 5 were obtained. Significantly, no measurable changes in the electrochemical responses shown in curve 5 were obtained either by dilution of the bulk cyt *c* in the aqueous phase by addition of buffer, or by increasing the ionic strength of the buffer solution (data not shown). The pattern of electrochemical behavior illustrated in Fig. 1 reveals the complexity in the cyt *c*–lipid membrane interaction, and suggests the existence of (at least) three protein populations having different redox potentials (approximately 265, 300, and 320 mV), and having very different ET rates as indicated by the variation in peak-to-peak separations (see below for additional discussion).

Although a similar pattern of CV changes with protein concentration was also observed in two other systems, i.e., PC + 20 mol.% CL at 160 mM ionic

strength, and PC alone at 20 mM ionic strength, there are important distinctions between these systems which need to be emphasized. First, the protein concentration range within which electrochemical responses could be detected changed appreciably with variation of both the ionic strength of the buffer and the amount of cardiolipin present in the lipid membrane. Specifically, an increase of the buffer ionic strength shifted this range to higher cyt *c* concentrations, as did a decrease of the amount of CL in the lipid bilayer. Second, phase one of the electrochemical response (with halfwave potential of approximately 265 mV, as described above) was not observable in the other two systems. These results reflect the strong influence of electrostatic interactions between cyt *c* and the lipid membrane, which appear to be essential in order to observe the first phase of electrochemical ET, as well as to facilitate the subsequent phases of the protein–lipid membrane interaction (see below).

It is also important to note that, although the relative positions and shapes of the oxidation and reduction peaks changed from one lipid membrane to another, the redox potentials remained the same. For less well-resolved cyclic voltammograms, the potentials can be determined by subtracting the spectra measured at two different cyt *c* concentrations. An example of such a procedure is presented in Fig. 2 for the cyt *c*/PC system in low ionic strength buffer. The voltammograms obtained in this way were similar to those determined under the conditions of Fig. 1, and corresponded to phases two (curve 1) and three (curve 2) as defined above. Thus, at low cyt *c* concentration (in this case < 150 μM), a single wave was obtained (curve 1, Fig. 2) with a redox potential of 300 mV. A second wave, with a more positive halfwave potential (315–320 mV), appeared at higher cyt *c* concentrations (> 280 μM; curve 2, Fig.2).

Such an analysis also enables us to evaluate other characteristic parameters (i.e., peak currents and potentials and peak separations) for the ET reaction between the cyt *c*–lipid membrane components and the working electrode. Figure 3 shows the dependence of the oxidation peak current as a function of the cyt *c* concentration for the second (closed circles) and third electrochemical phases (closed triangles), determined under the conditions of Fig. 1. These data can be correlated with the binding isotherms obtained with these same systems using SPR spectroscopy (Salamon and Tollin, 1996a). Thus, both the electrochemical and the SPR responses showed two levels of saturation, which occurred in approximately the same protein concentration ranges. This suggests that the two experimental methods reflect the same aspects of the cyt *c*/lipid membrane binding process. It is also significant that although the SPR measurement showed that the amounts of cyt *c* bound during these two phases were

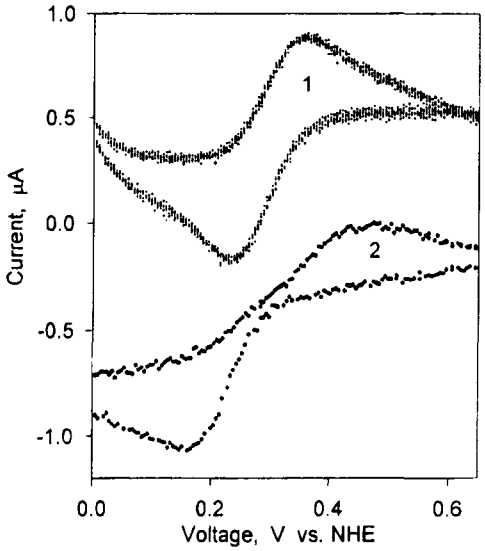

Fig. 2. Cyclic voltammograms obtained for cyt *c* at working electrode coated with 10 mg/ml PC bilayer membrane. Curve 1: CV obtained with 120 μM cyt *c* after background (no cyt *c*) subtraction. Curve 2: difference between cyclic voltammograms obtained with 280 μM and 120 μM cyt *c*. Other experimental conditions as in Fig. 1.

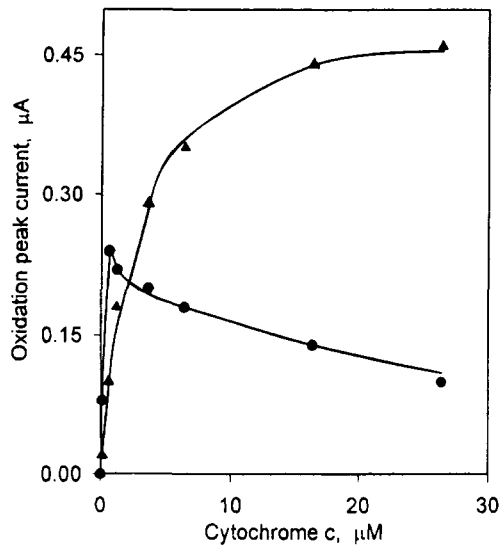

Fig. 3. Oxidation peak currents, obtained under the experimental conditions given in Fig. 1. as a function of cyt *c* concentration for the second (circles) and third (triangles) phases of the protein–lipid membrane interaction (see text and Fig. 4).

quite similar, the electrochemical responses indicated that the first component was strongly "quenched" by the second one, i.e., before the first CV response was fully saturated, its amplitude began to be diminished by the second CV wave (see Fig. 3). This will be discussed further below.

A summary of the parameters characterizing the electrochemical waves obtained at various cyt *c* concentrations under the three experimental conditions used here is given in Table I. Note that the electrochemical responses for the three systems were separated by several orders of magnitude in cyt *c* concentration, and strongly depended upon the electrostatic conditions of the experiment. Furthermore, as noted above for the cyt *c*/(PC + 20 mol.% CL) system at low ionic strength, the concentration dependences for all of the systems are in good agreement with the binding isotherms measured by SPR spectroscopy (Salamon and Tollin, 1996a).

The large positive shifts in redox potential observed here from that of cyt *c* in solution (about 60 mV for phase three) have not been obtained with other types of working electrode modifications. In contrast, it has commonly been observed that the adsorption of cyt *c* onto an electrode surface (especially an unmodified metallic surface) induces a *negative* redox potential shift, whose magnitude depends on the strength of the adsorption forces and varies over a wide range (Dutton *et al.*, 1970; Kimelberg and Lee, 1970; Vanderkooi and Erecinska, 1974; Peterson and Cox, 1980; Willit and Bowden, 1990; Song *et al.*, 1993). The negative shift is a consequence of the oxidized form of cyt *c* being more stabilized by the polar environment and binding more strongly to anionic surfaces than the reduced form (Dutton *et al.*, 1970; Kimelberg and Lee, 1970; Vanderkooi and Erecinska, 1974; Peterson and Cox, 1980; Willit and Bowden, 1990; Song *et al.*, 1993). It is also important to note that the value of the apparent electron transfer rate constant decreased from 0.09 s^{-1} for the phase one cyt *c* species (having a redox potential value of 265 mV), to 0.03 s^{-1} for the phase two species (with a redox potential of about 300 mV), to 0.003 s^{-1} for the cyt *c* species giving rise to phase three (with a redox potential value between 315–330 mV). It is also worth noting that the larger k_{et} value compares rather well with those obtained for cyt *c* bound to other types of chemically modified working electrode surfaces (Song *et al.*, 1993).

The above CV results can be interpreted in terms of the schematic model of the cyt *c*–lipid membrane interaction presented in Fig.4, which is similar to that proposed previously based on SPR measurements of cyt *c* binding to supported planar lipid membranes (Salamon and Tollin, 1996a). According to this model, three stages of binding can be recognized. In the first stage, an electrostatic interaction occurs resulting in transient binding to the lipid membrane surface (molecule 1). This is a relatively weak, easily reversed bind-

Table I. Electrochemical Parameters Obtained for Various cyt *c*–Lipid Membrane Systems. Peak to Peak Separations (ΔE); Redox Potentials vs. Normal Hydrogen Electrode ($E_{1/2}$); Apparent Electron Transfer Rate Constants (k_{et})

Sample	Cyt *c* (μM)	ΔE (mV)			$E_{1/2}$ (mV)			k_{et} (S^{-1})		
PC + 20 Mol. %		1	2	3	1	2	3	1	2	3
CL; 10 mM	0.1	80	—	—	265	—	—	0.09	—	—
NaCl	0.6	—	145	—	—	290	—	—	0.030	—
	1.2	—	150	315	—	305	330	—	0.030	0.003
	6.4	—	150	340	—	305	325	—	0.030	0.003
	16.4	—	160	360	—	305	325	—	0.030	0.003
PC + 20 Mol. %	1	—	130	—	—	285	—	—	0.035	—
CL; 150 mM	2	—	135	—	—	300	—	—	0.035	—
NaCl	4	—	135	—	—	300	—	—	0.035	—
	10	—	150	340	—	305	320	—	0.030	0.003
	20	—	150	350	—	305	320	—	0.030	0.003
	30	—	160	360	—	305	320	—	0.030	0.003
PC; 10 mM NaCl	40	—	130	—	—	290	—	—	0.035	—
	200	—	135	—	—	300	—	—	0.035	—
	280	—	140	340	—	300	315	—	0.035	0.003
	360	—	150	350	—	300	325	—	0.030	0.003
	440	—	160	350	—	305	325	—	0.030	0.003

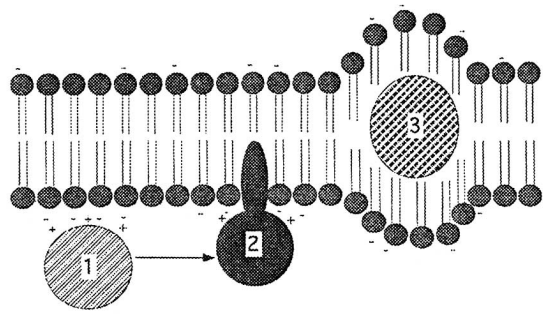

Fig. 4. Schematic model indicating the three phases of the cyt *c*–lipid membrane interaction. In phase 1, cyt *c* is electrostatically associated with the hydrophilic region of the membrane. In phase 2, cyt *c* is partially inserted into the hydrophobic interior of the membrane as a consequence of structural changes which expose hydrophobic surfaces of the protein. In phase 3, cyt *c* is completely incorporated into the membrane following extensive structural alterations. Note that this scheme should not be interpreted as an attempt to provide a detailed structural model of the lipid–protein interactions (see text for additional comments).

ing, occurring without any significant changes in protein structure. Subsequent to this initial interaction, structural changes in the protein molecule occur which allow some penetration into the hydrophobic interior of the membrane. This is induced by the initial membrane–protein interaction and is accompanied by at least a partial unfolding of the protein molecule, thereby generating an irreversibly bound layer of cyt *c* (molecule 2). We associate this process with the observed shift in cyt *c* redox potential to higher positive values during phase two. In this context, it is important to note that general agreement exists that adsorption of cyt *c* onto conventional electrode surfaces leads to structural changes which open the heme crevice, resulting in an increase in the solvent (i.e., water) accessibility of the heme. This increases the local dielectric constant, thereby stabilizing the oxidized form of the protein and causing a negative shift in the redox potential (Song *et al.*, 1993; Hildebrandt, 1995 and references cited therein). In contrast, as Kassner (1972, 1973) has demonstrated, placing the heme in a low dielectric environment would destabilize the more positively charged oxidized heme and increase the redox potential relative to heme in an aqueous medium. In the present case, penetration of cyt *c* into the interior of the lipid membrane exposes the heme to a hydrophobic environment of low dielectric constant, which should shift the heme redox potential to higher positive values, as is observed.

The processes of unfolding and penetration continue until the molecules of cyt *c* are totally buried in the hydrophobic interior of the lipid membrane (molecule 3). This results in additional changes in the cyt *c* molecular structure and in its thermodynamic properties, the latter reflected in a shift to still higher redox potentials. It should be emphasized that, although the three binding phases are in general both time and concentration dependent, the electrochemical measurements and the earlier SPR experiments have been done under "steady-state" conditions, i.e., measurements were performed 10–15 min after addition of an aliquot of cyt *c* to the cell compartment. Thus, the time dependence has not yet been characterized.

This schematic model of the cyt *c*–lipid membrane interaction is based on two important assumptions. First, the interaction of cyt *c* with the lipid membrane is assumed to induce changes in the folding state of the protein molecule. Second, the model presumes that hydrophobic interactions between cyt *c* and the hydrophobic interior of the lipid membrane occur, which result in either partial or total insertion of the protein into the lipid membrane. The extent of insertion would depend, in general, on the degree of molecular unfolding and on the physicochemical properties of the lipid membrane.

The occurrence of both hydrophobic and electrostatic interactions between cyt *c* and a lipid bilayer can be rationalized based on the molecular structure of the protein, as presented in Fig. 5. Although cyt *c* has a largely hydrophilic surface, there are quite extensive hydrophobic patches on the front of the molecule (i.e., near the exposed heme edge; see Fig. 5), indicating that hydrophobic interactions can take place when the protein binds electrostatically to the membrane surface. However, the proposed insertion of cyt *c* into the interior of the membrane requires additional assumptions. Either protein unfolding occurs to expose additional hydrophobic surfaces, or, as has been proposed by de Kruijff and Cullis (1980), hexagonal or inverted micelle structures are formed in the lipid phase in the presence of CL which can accommodate cyt *c* in the membrane interior. We have previously excluded the latter possibility based on SPR spectra (Salamon and Tollin, 1996a), which have not indicated any changes in the lipid membrane caused by cyt *c* binding which could be related to the formation of non-bilayer structures. The presumed unfolding of cyt *c* which occurs upon binding is not only consistent with the electrochemical results presented in this work (especially the observed positive shift of the redox potential of cyt *c*), but is also in agreement with results obtained from studies of folding pathways and translocation of

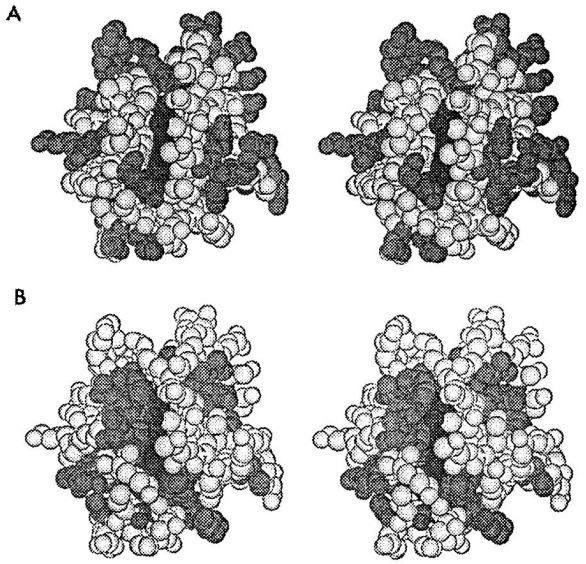

Fig. 5. Stereo views of front surface of horse heart cyt *c*. The darkly shaded residues in panel A correspond to the polar (hydrophilic) amino acids (Lys, Arg, His, Glu, Asp). In panel B, the darkly shaded residues are the nonpolar (hydrophobic) amino acids (Gly, Ala, Val, Ile, Leu, Phe, Trp, Tyr, Pro, Met). In both panels, the heme is shown with the darkest shading.

soluble proteins across lipid membranes. Thus, there is evidence that an aqueous–lipid interface induces a common intermediate folding state in soluble proteins upon interaction with lipid membrane surfaces (Eilers *et al.*, 1988; Buchkova *et al.*, 1988; van der Goot *et al.*, 1991, 1992; de Jongh *et al.*, 1992; Elöve *et al.*, 1992). Although these studies, in which cyt *c* has often been used as a model molecule, have not yet completely elucidated the mechanisms of protein unfolding and translocation, they have nevertheless shown that non-native states of protein molecules are involved in these processes (van der Goot *et al.*, 1991; Bychkova *et al.*, 1996), and that the membrane surface can lead to a partial denaturation of proteins transforming them into a much more flexible state, a process which can be strongly influenced by negative charges on the membrane (Endo and Schatz, 1988). Based on these considerations, we presume that both hydrophilic and electrostatic interactions between cyt *c* and the lipid membrane result in a partial unfolding of the protein, leading to penetration of cyt *c* into the hydrophobic interior of the membrane. These unfolding and penetration processes continue until cytochrome molecules become totally incorporated into the hydrophobic interior of the lipid membrane.

We can also associate the decrease in the electrochemical ET rate constant (cf. Table I) with these changes in the heme environment. In fact, the ET rate for the high potential species decreased to such a low level that it became the rate-limiting factor at higher scan rates in the CV experiment. Figure 6 clearly demonstrates this effect, showing the variation of the oxidation peak currents as a function of the potential scan rate for two CV waves (the phase three redox species having a potential of about 320 mV—closed triangles; and the phase two species with redox potential about 300 mV—closed circles), measured with PC +20 mol.% CL in low ionic strength buffer. Typically, when the ET rate is high enough, the current limitation arises either from diffusion (resulting in a square root dependence of the peak current on the scan rate), or adsorption (producing a linear relationship between the peak current and the scan rate) (Nicholson and Shain, 1964). Although, as the data presented in Fig. 6 indicate, both components showed a nonlinear dependence, the character of the deviation clearly correlates with the rate of ET, i.e., the CV component with the slower ET rate (closed triangles) had a more marked deviation which occurred at lower scan rates than that of the higher ET rate component (closed circles). Furthermore, although the amounts of cyt *c* adsorbed in these two layers were similar, as indicated by the SPR measurements (Salamon and Tollin, 1996a), and the ET rate was higher for the partially buried protein layer, the amplitude of the CV response for phase two decreased as the magnitude of the response from the

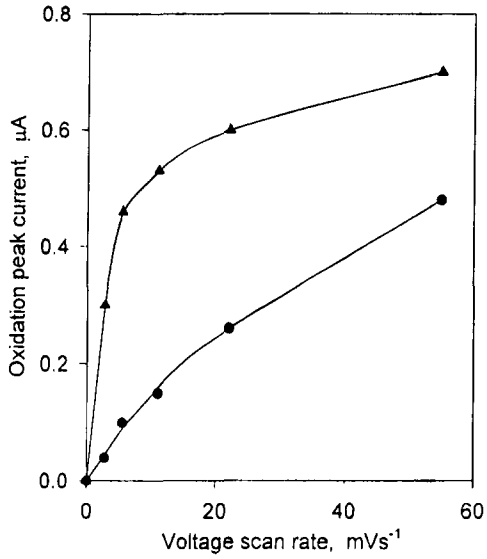

Fig. 6. Dependence of the oxidation peak current on the voltage scan rate for a 26.5 μM solution of cyt *c* (see Fig. 3) for the second (circles) and third (triangles) phases of the adsorption process (see Fig. 4). Experimental conditions as described in Fig. 1.

phase three component increased (cf. Fig. 3). This again supports a two-layer model, in which the component which is closer to the surface of the working electrode (i.e., phase three) introduced a barrier to the communication of the outer layer with the working electrode, thereby diminishing its electrochemical response.

The model of cyt *c*–lipid membrane interaction described in this and the previous work (Salamon and Tollin, 1996a) agrees qualitatively with recent measurements of the binding reversibility of cyt *c* to both liposomal and mitochondrial membranes (Cortese *et al.*, 1995; Rytömaa and Kinnunen, 1995). Thus, Rytomaa and Kinnunen (1995) have demonstrated that cyt *c* bound to CL-containing phospholipid liposomes does not readily equilibrate with subsequently added liposomes. Furthermore, they have shown that this irreversible binding involves hydrophobic interactions, which they associate with extended lipid anchorage, in which one acyl chain of the acidic diacyl phospholipid is accommodated within the hydrophobic cavity of cyt *c* while the other chain remains intercalated within the bilayer (Rytömaa and Kinnunen, 1995; Kinnunen *et al.*, 1994). Additionally, Cortese *et al.* (1995) have demonstrated that when inner mitochondrial membranes were obtained through controlled digitonin treatment at low ionic strength, most of the cyt *c* was electrostatically bound and could be readily dissociated by washing at physiological ionic strength. There was, however, a small but significant percentage of cyt *c* (11–17% of the total amount) which remained bound to the membranes after such treatment. They have noted that cyt *c* was able to form such a membrane-bound species when it interacted with regions of the membrane in which the hydrophobic core was transiently exposed, thereby allowing the protein to penetrate into membrane structural defects. Significantly, such binding of cyt *c* also occurred to large unilamellar phospholipid vesicles having a neutral surface charge, indicating that a negative surface was not a prerequisite for this type of binding. Spectroscopic analysis of the membrane-bound subpopulation of cyt *c* suggested that it consisted of a monomeric form of the protein with similar spectroscopic characteristics to those of soluble cyt *c* (Cortese *et al.*, 1995). It is also important to emphasize that such membrane-bound cyt *c* had a much decreased ET activity, compared to that of either electrostatically bound or unbound protein, which is consistent with the observations described in the present study. This result, taken together with the observation that there is only a small portion ($\approx$ 10%) of the total cyt *c* which is hydrophobically bound to the mitochondrial membranes under physiological conditions (Cortese *et al.*, 1995), rationalizes the fact that ET *in vivo* is dominated by electrostatic forces between cyt *c* and the mitochondrial membrane, and that the oxidation–reduction potential of cyt *c* measured in mitochondria is negatively shifted by about 30–40 mV in comparison with its solution value (Dutton *et al.*, 1970). The physiological role of this subpopulation of membrane-bound cyt *c* is not entirely clear at this time.

We conclude from these results that, despite important differences (compositional, structural, and physicochemical) between bilayer vesicles, inner mitochondrial membranes, and the self-assembled solid-supported bilayers studied in the present experiments, there is a basic consistency between the results obtained with these various membrane systems. Thus, all of the experiments indicate a much more complex pattern of cyt *c*–lipid membrane binding than can be accounted for by purely electrostatic, and therefore fully reversible, interactions.

CONCLUSIONS

The binding of cyt *c* to various types of membranes has been studied extensively, using biophysical techniques such as optical spectroscopy (Van and Griffith, 1975; Brown and Wüthrich, 1977; Szebeni and Tollin, 1988), nuclear magnetic or electron spin resonance spectroscopy (Van and Griffith, 1975; Brown and Wüthrich, 1977; Spooner and Watts, 1991b, 1992; Pinheiro and Watts, 1994a,b), and X-ray diffraction (Shipley *et al.*, 1969; Gulik-Krzywicki *et al.*, 1969; MacNaughtan *et al.*, 1985). These investigations have yielded several models, including those in which the protein forms a transient electrostatic complex with the membrane and does not penetrate into the bilayer (Gulik-Krzywicki *et al.*, 1969; Brown and Wüthrich, 1977), models in which the protein penetrates partially into the bilayer (Tessie, 1981; de Jongh *et al.*, 1992; Kimelberg and Papahadjopoulos, 1971), and those in which it penetrates deeply into the hydrophobic core of the membrane (Szebeni and Tollin, 1988; de Kruijff and Cullis, 1980; Rietveld *et al.*, 1983). The recent results obtained in our laboratory with SPR spectroscopy have demonstrated that all three of these modes of interaction can in fact be observed (Salamon and Tollin, 1996a). The present CV experiments also indicate the occurrence of three distinct modes of electron transfer, which correlate quite well with these three

types of protein–lipid membrane interactions. Furthermore, we have shown that the three subpopulations of cyt *c* have redox potentials ranging from 265 to 320 mV, depending upon the extent of the hydrophobic interactions. It is not clear at the present time whether these different thermodynamic forms of cyt *c* have any physiological significance. Functional cyt *c* molecules appear to be peripherally associated with the mitochondrial membrane, and to interact with extra-membrane domains of the physiological cyt *c* ET partners. The hydrophobic sequestering of cyt *c* observed in these and other studies therefore may represent a mechanism for maintaining an adequate population of cytochrome molecules at the membrane–cytosol interface. They may also provide an alternative slower pathway for electron transfer, as suggested by Cortese *et al.* (1995). Further studies will be required to clarify this.

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