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Calorimetric studies of the interactions of cytochrome *c* with dioleoylphosphatidylglycerol extruded vesicles: ionic strength effects

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Abstract

Cytochrome *c* has been studied as an example of a peripheral membrane protein which interacts with the lipids as well as the proteins of the inner mitochondrial membrane. In order to elucidate the thermodynamic properties of these interactions, isothermal titration calorimetry and differential scanning calorimetry (DSC) were used to study the binding of cytochrome *c* to negatively charged dioleoylphosphatidylglycerol (DOPG) extruded vesicles as a function of ionic strength. The binding constant and enthalpy of association decrease with increasing ionic strength, with no binding detected above 0.5 M NaCl. The enthalpy of the binding of cytochrome *c* to DOPG-extruded vesicles was 15 kcal/mol, and the binding constant was $6 \cdot 10^6$ M^{-1} at the lowest ionic strengths. The minimum size of the lipid cluster to which the protein bound was found to be approx. 9 lipid molecules in the titration calorimetry measurements and as low as 5 lipid molecules in the DSC measurements. The stability of the bound cytochrome *c* was found to be reduced; the thermal denaturation temperature was lowered from 83 to 50 degrees when bound to DOPG. The results of this study support previous suggestions that cytochrome *c* may undergo a conformational change when it binds to charged lipids such as DOPG. The results also support the suggestion that the protein penetrates partially into the lipid bilayer.

Key words: Cytochrome *c*; Titration calorimetry; DSC; Lipid interaction

1. Introduction

Protein lipid interactions in biological membranes may have important roles in the regulation of the function of both the lipids and the proteins. There have been many studies of the influence of lipids on the structure and function of intrinsic membrane proteins; however, the possibility of functionally important mutual effects occurring as a result of the interactions of the peripheral or extrinsic membrane proteins with lipids has only recently become of interest. Cytochrome *c* is an example of a soluble protein whose biological role involves interactions with membranes as it carries out its role of accepting electrons from cytochrome *c* reductase, and passing them to cytochrome oxidase. Consequently, cytochrome *c* has been widely studied as a representative peripheral membrane protein. Cytochrome *c* contains a net positive charge at neutral pH, and it binds to negatively charged phospholipids such as phosphatidylglycerol (PG) [1], cardiolipin (CL) [2,3], and phosphatidylserine (PS) [4]; it has been shown that these interactions involve electrostatic interactions with surface lysines on the protein.

Several studies have been reported in the literature concerning the interactions of cytochrome *c* with lipids [5–17,33]. Many of these studies have been reviewed by McElhaney [18]. Most of the studies have been directed at the effect of the binding of cytochrome *c* on the phase behavior of the lipids. It was shown that cytochrome *c* lowered the get to liquid-crystal phase transition of DPPG by several degrees, as well as broadening the transition and reducing the enthalpy of

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Abbreviations: DOPG, dioleoyl-*sn*-glycero-3-phosphoglycerol; CL, cardiolipin; PG, phosphatidylglycerol; PS, phosphatidylserine; DSC, differential scanning calorimetry.

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this transition [14]. Reitveld et al. [13] showed that it reduced the temperature of the phase transition of DMPG, as well as broadened it. It was also found that apo cytochrome *c* has a much greater effect on the properties of the PG phase transitions than the holo cytochrome *c* [13,14]. These results suggested that cytochrome *c* penetrates into the bilayer to some extent so that the interaction is more than just a superficial charge interaction, but also includes interactions between the protein and the acyl chain region of the lipid. Recently more studies have been focused on the influence of membrane lipid on the structure and function of cytochrome *c*. DSC studies showed that the interaction of cytochrome *c* with DOPG reduces the denaturation temperature of the protein by thirty degrees [1]. Nuclear magnetic resonance (NMR) [2,3,19], Resonance raman spectroscopy [20], circular dichroism (CD) [21] and differential scanning calorimetry (DSC) [1] studies suggest that binding of cytochrome *c* to acidic phospholipids induces changes in the conformation of cytochrome *c*.

In the present investigation, the thermodynamics and stoichiometry of the binding of cytochrome *c* to extruded vesicles of dioleoylphosphatidylglycerol (DOPG) have been studied using isothermal titration calorimetry. The effects of the binding to DOPG extruded vesicles on the stability of cytochrome *c* have been studied by differential scanning calorimetry. Because of the involvement of charges in these interactions, it is expected that ionic strength will play a role in these interactions. Most of the previous studies were performed at a single ionic strength, which often is not specified. In the present investigation, the effect of ionic strength on each of the parameters measured has also been investigated.

2. Material and methods

2.1. Samples

Dioleoylphosphatidylglycerol was obtained from Avanti, Birmingham, AL. Bovine heart cytochrome *c* was obtained from Sigma St Louis, MO. The protein was studied in the oxidized state, which was documented by spectrophotometry. Gel filtration chromatography was performed to ensure that there were no significant dimers present in the cytochrome *c* preparation. Cytochrome *c* concentrations were determined by absorption at 280 nm using $E^{1\ \mathrm{cm},\ 1\%} = 19$ [22]. The pH of cytochrome *c* for the DSC and titration calorimetric studies was adjusted to 7.8. The ionic strength of the solutions was controlled by added NaCl; the contributions of the lipids and proteins to the ionic strength were neglected.

Extruded vesicles were prepared from multilamellar vesicles by the standard method [23,24]. Chloroform stock solutions of DOPG were dried under a steady stream of nitrogen gas, and then kept overnight on a vacuum pump to remove all residual chloroform. The stock suspensions were hydrated with distilled water at room temperature for at least 2 h with occasional vortexing. The lipid concentrations used for these studies were about 25 mM. Extruded vesicles were prepared by extrusion of multilamellar vesicles through two stacked polycarbonate filters of 400 nm pore size at room temperature, using the thermostatted Extruder from Lipex (Vancouver, B.C.) [24]. The extrusion was repeated ten times. The sizes of the extruded vesicles were determined by NICOMP Model 370 Submicron Particle Sizer (Pacific Scientific, Silver Spring, MD) to be 230 ± 95 nm at room temperature. Lipid concentrations were determined by phosphorus assay by the method of Bartlett [25].

2.2. Differential scanning calorimetry

DSC was performed using the MC2 scanning Calorimeter from Microcal, Amherst, MA. The calorimeter is interfaced with a computer, and the software used is that provided by Microcal. A Haake FC3 refrigerated bath controlled by the computer is connected to the calorimeter for temperature control. The final cytochrome *c* concentration for DSC studies was approximately 0.36 mM. The scan rate for the cytochrome *c* denaturation studies was 90°C/h. The data were analyzed using ORIGIN software from Microcal, Amherst, MA.

2.3. Titration calorimetry

Titration experiments were carried out using the OMEGA titration calorimeter from Microcal, (Northampton, MA). The calorimeter is interfaced with an IBM PS/2 Model 30 286 computer, and the software used is ORIGIN provided by Microcal. The Omega Titration Calorimeter is described in detail in the literature [26]. The data for the interactions of cytochrome *c* with lipid were fit to a model in which each protein molecule P binds to a lipid cluster C containing n lipid molecules L with enthalpy ΔH and binding constant K described as follows:

$$\mathrm{P} + \mathrm{C} \Leftrightarrow \mathrm{PC}, \text{ and } K = [\mathrm{PC}]/[\mathrm{C}][\mathrm{P}], \text{ where } \mathrm{C} = n\mathrm{L}.$$

The interactions between DOPG extruded vesicles and cytochrome *c* were studied by placing the lipid suspension in the syringe and the cytochrome *c* solutions in the sample cell. A sequence of injections of DOPG extruded vesicles was made at 3-min intervals into the reaction cell which contained 1.4172 ml cytochrome *c* solution. The ionic strength was controlled

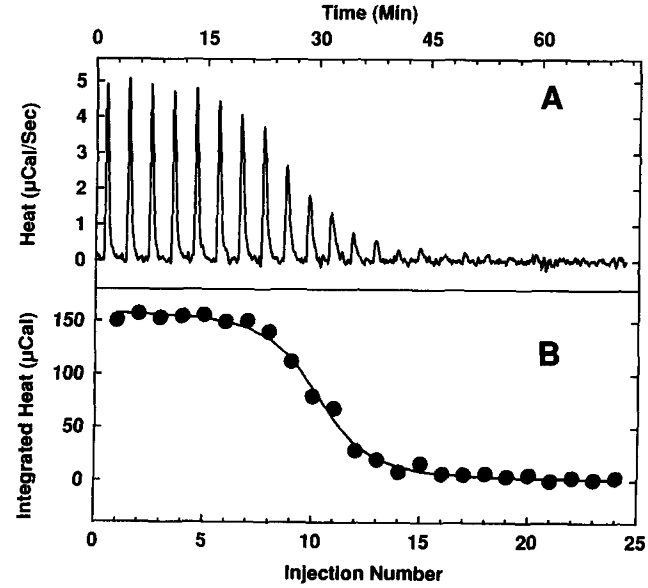

Fig. 1. A typical isothermal titration calorimetry experiment. (A) A sequence of injections, 4.55 μl each, at 3-min intervals, of 24.0 mM DOPG-extruded vesicles into 1.4172 ml 0.084 mM cytochrome *c* in the cell. NaCl concentration is 1 mM, and temperature is 25 C. (B) Integrated heat for each injection in panel A.

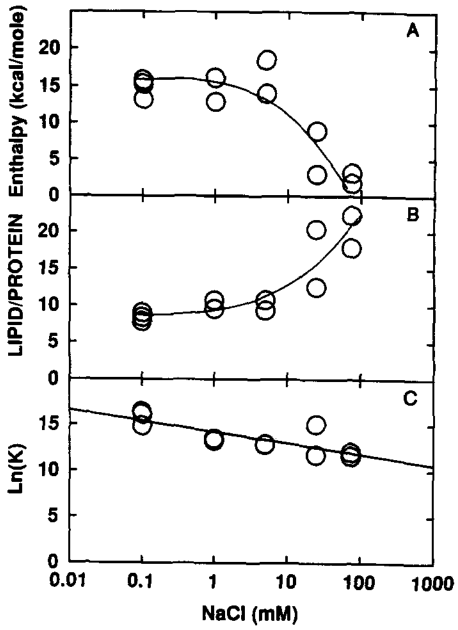

Fig. 2. (A) The enthalpy of binding per mol protein, (B) Number of lipids per mol of protein, (C) Ln (*K*), as a function of NaCl concentration.

by adding NaCl to both the cytochrome *c* and DOPG solutions. The titration calorimetry experiments were carried out at 25°C.

3. Results

3.1. Titration calorimetry

The binding of cytochrome *c* to DOPG extruded vesicles was studied by titration calorimetry. Fig. 1 shows a typical experiment in which the vesicle suspension was injected into the protein solution in the sample cell. Fig. 1A shows the raw data and Fig. 1B shows the integrated heat from each injection plotted against injection number. These data show that the binding of cytochrome *c* to DOPG is an endothermic process, and the shape of the curve in Fig. 1B is a typical titration curve for a system of individual non-interacting saturable binding sites. These data were fit to the model as described above and the solid line in Fig. 1B represents the best fit of these data. In this example, the binding constant $K = 1 \cdot 10^6$ M^{-1}, enthalpy of binding $\Delta H = 13$ kcal/mol, and the number of lipids in the lipid cluster for each bound protein molecule $n = 8.9$.

Similar experiments were carried out as a function of ionic strength. The results are summarized in Figs. 2A–C. The enthalpy of binding remains fairly constant up to 10 mM NaCl and then drops to zero above 100 mM NaCl, where the heat evolved upon mixing of cytochrome *c* and lipid becomes undetectable. As the ionic strength is increased there is an increase in the number of lipid molecules in the binding cluster to a maximum of 18 as the binding heat becomes undetectable.

The binding constant is plotted as a log function of NaCl concentration in Fig. 2C, showing a linear decrease in binding constant with increasing ionic strength. These effects of ionic strength are expected for interactions which are primarily due to charge-charge interactions [27]. The thermodynamic parameters determined in this study are summarized in Table 1. These results show that the enthalpy for the binding of cytochrome *c* to DOPG is positive, and that the driving force for the interaction is entropic.

3.2. Differential scanning calorimetry (DSC)

The effect of the binding of cytochrome *c* to DOPG vesicles on the stability of cytochrome *c* was measured

Table 1
Thermodynamic parameters for the binding of cytochrome *c* to DOPG at 25°C

NaCl (mM)	*n*	*K* (10^5 M^{-1})	ΔH (kcal/mol)	ΔG (kcal/mol)	ΔS (cal/mol per K)
0.1	7.8±0.3	60±30	15±0.5	−9.2±0.4	81± 5
1	9.4±0.5	8± 3	14±1	−8.0±0.1	73± 5
5	9.6±0.1	4± 1	16±3	−7.6±0.1	78±10
25	16 ±1	3± 1	7±2	−7.4±0.6	48± 9
75	18 ±1	1± 1	2±2	−7.0±1	31± 7

Error ranges were determined from the range of observed measurements in multiple determinations.

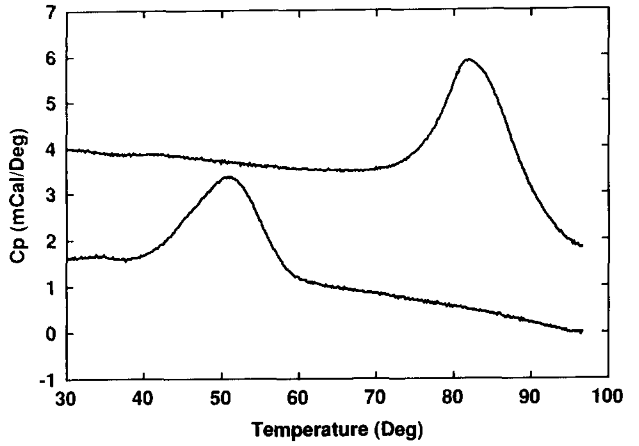

Fig. 3. DSC scans of cytochrome *c* in the absence (upper trace) and presence (lower trace) of DOPG extruded vesicles at a DOPG/cytochrome *c* ratio of 20:1. Scan rate was 90°C/h.

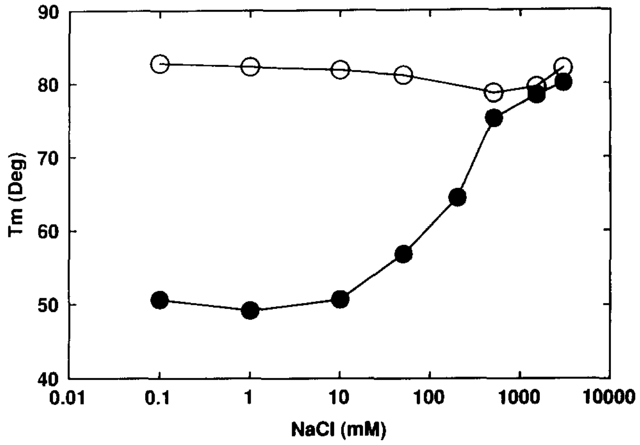

Fig. 4. Denaturation temperature of cytochrome *c* in the absence (open circles) and presence (filled circles) of DOPG extruded vesicles as a function of NaCl concentration. The ratio of DOPG/cytochrome *c* was 20:1.

by DSC. Fig. 3 shows DSC scans in the presence and absence of DOPG extruded vesicles. The DOPG is in the liquid crystalline L_α phase throughout the temperature range, so there are no lipid phase transitions. The lipid to protein mole ratio is 20:1, so that all of the protein is bound according to the titration calorimetry results. Fig. 3 shows that the denaturation temperature of cytochrome *c* is decreased from 82.8 to 50.2, showing substantial destabilization of the protein. The enthalpy for the transition is 62.8 kcal/mol in the absence of lipid and 42 kcal/mol in the presence of lipid. The van't Hoff enthalpies, calculated from the calorimetric scans, were 94.1 kcal/mol in the absence of lipid and 80.9 kcal/mol in the presence of lipid. For both scans the ratio of the van't Hoff to the calorimetric enthalpy was greater than 1, suggesting that there is some aggregation of the protein associated with denaturation under these conditions. Rescanning of these samples gave 80% of the original enthalpy in the absence of lipid and only 41% of the first scan enthalpy for the lipid-bound protein. This irreversibility suggests that the unfolded protein may remain associated with lipid such that refolding is inhibited. The van't Hoff enthalpy remains the same for the repeat scans.

Fig. 4 shows the denaturation temperature of cytochrome *c* in the presence and absence of DOPG extruded vesicles as a function of NaCl concentration. The DOPG decreases the denaturation temperature of cytochrome *c* by about 30 degrees at lower NaCl concentration. The decrease in denaturation temperature of cytochrome *c* becomes smaller as NaCl concentration increases from 10 mM to 500 mM. At these NaCl concentrations, only one denaturation transition was detected for each NaCl concentration. This result suggests that there is a fast equilibrium between the cytochrome *c* which is bound to DOPG extruded vesicles and free cytochrome *c* under these experimental conditions. There is no significant difference in denaturation temperature for cytochrome *c* in the presence and absence of DOPG at NaCl concentrations higher than 500 mM. In both the presence and absence of DOPG, there is a slight increase in denaturation temperatures at NaCl concentrations above 1 M, suggesting some stabilization of cytochrome *c* at high NaCl concentration.

Fig. 5 shows the enthalpy of cytochrome *c* denaturation as a function of NaCl concentration in the presence and absence of DOPG. In the NaCl concentration range from 0.1 to 50 mM, the enthalpy of cytochrome *c* denaturation is 61.5 ± 2.7 kcal/mol without DOPG extruded vesicles and 46.3 ± 4.1 kcal/mol in the presence of DOPG extruded vesicles. Above 500 mM NaCl the enthalpies for both are increased to 70–80 kcal/mol. This again suggests that high NaCl concentration increases the stability of cytochrome *c*.

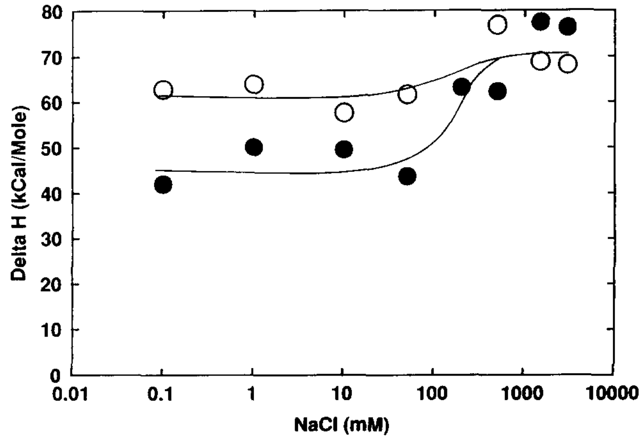

Fig. 5. The enthalpy of cytochrome *c* denaturation in the absence (open circles) and presence (filled circles) of DOPG extruded vesicles as a function of NaCl concentration.

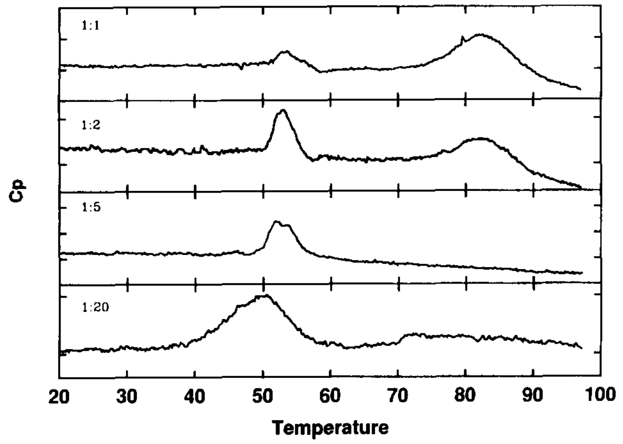

Fig. 6. Dsc scans of cytochrome *c* in the presence of increasing DOPG/cytochrome *c* ratios, from 1:1 to 20:1, at 1 mM NaCl concentration.

Fig. 6 shows the progressive change in the stability of cytochrome *c* as it binds to DOPG extruded vesicles at increasing mol ratios, at 1 mM NaCl. DSC scans of cytochrome *c* were carried out at DOPG to cytochrome *c* mol ratios of 1:1, 2:1, 5:1, and 20:1 respectively. At the lower DOPG/cytochrome *c* ratios, two denaturation transitions of cytochrome *c* occurred, one at about 52°C, the other about 82°C. The low temperature denaturation transition is due to the cytochrome *c* associated with DOPG extruded vesicles, and the higher temperature one is due to free cytochrome *c*. As the DOPG/cytochrome *c* ratio increases, the lower temperature transition becomes larger, and the higher temperature transition becomes smaller and finally disappears. This sequence of scans shows that the binding of cytochrome *c* to lipid is not rapidly equilibrating at this low ionic strength. It also shows that a mol ratio of only five lipid molecules per protein is sufficient to bind all of the protein. This can be compared to the value of 9:1 obtained in the titration calorimetry results.

4. Discussion

The calorimetric results reported here indicate that the association of cytochrome *c* with DOPG is a complex one which probably involves changes in the protein conformation and some penetration into the acyl chain region of the bilayer, in addition to the well documented charge-charge interactions. The association constants shown in Table 1 can be compared with those determined previously by conventional methods involving centrifugation and physical separation of reactants. For example, at 100 mM NaCl, Reitveld et al. [13] reported an inverse association constant for cytochrome *c* with DMPG which converts to an association constant of $3.3 \cdot 10^4 M^{-1}$; which is within the error of the value we measured at 75 mM NaCl. Heimburg et al. [33] reported a value of -9.3 kcal/mol for ΔG for the interaction of cytochrome *c* with DPPG at low ionic strength, also in good agreement with our value at low ionic strength.

The large positive enthalpy of 15 kcal/mol indicates that the association involves more than the simple charge-charge interactions, which would be expected to be exothermic. The positive enthalpy may be due to a conformational change in the protein upon binding to the lipid. Our DSC results showed that the lipid-bound protein has a denaturation temperature reduced by nearly 30 degrees compared to the unbound protein. Muga and co-workers also observed a 30 degree reduction in the denaturation temperature of cytochrome *c* bound to DMPG [1]. This observation supports the suggestion that a conformational change or 'loosening' of the cytochrome *c* structure occurs upon binding to DOPC. There is evidence from other laboratories that the interaction of cytochrome *c* with lipids results in conformational differences. For example, Spooner and Watts [2,3] showed by deuterium NMR that cytochrome *c* is loosened or 'unfolded' upon interaction with cardiolipin bilayers in the liquid crystal phase. They suggested that the protein 'reversibly denatures' when bound to cardiolipin [2]. Muga et al. [1] also provided evidence from FTIR spectroscopy that there is a loosening of the structure of cytochrome *c* when bound to DMPG, DOPG and mixtures of these with phosphatidylcholines, based on a greater accessibility of the protein backbone hydrogens to exchange. However, their interpretation was that there was only slight perturbation of the protein secondary structure. In addition to the conformational change upon binding to lipids, it has also been shown that there are two conformational states of cytochrome *c* bound to negatively charged surfaces [31,32], including charged lipid suspensions [20]. Heimburg et al. [33] showed that the conformational transition between two functionally different states occurs in cytochrome *c* when bound to various charged lipids and that the equilibrium between these states is sensitive to the lipid composition and phase state of the lipid.

Titration calorimetry measurements of interactions of some other proteins with lipids have been measured, which gives some basis for comparison of the results determined in the present study. For example, for the association of myelin basic protein with sonicated unilamellar vesicles of brain phosphatidylserine in the liquid-crystal phase, a large negative enthalpy was observed, with an average value of -72 kcal/mol [29]. In this system at temperatures below the gel to liquid crystal transition, a positive enthalpy was observed, which was interpreted to be due to changes in the lipid including a reversible aggregation of the vesicles. This did not occur in the fluid phase vesicles. These authors concluded that protein–lipid interactions themselves are exothermic, with the positive enthalpy arising due to lipid energetic changes. Similarly, in a study of the interaction of ornithine transcarbamylase leader peptide and gel phase phospholipids a relatively large negative enthalpy of −59.9 kcal/mol peptide was observed [37]. A small positive enthalpy of 1.5 kcal/mol was obtained in a titration calorimetry study of the interactions of apolipoprotein A-I with dimyristoylphosphatdylglycerol (DMPG) at 35°C which is above the phase transition temperature [30]. This heat was attributed to the rearrangement of the lipid as it forms the lipoprotein particles [30]. In another example the interaction of glucagon with DMPC was studied, and found to give positive enthalpy below the phase transition of the lipid and negative enthalpy above the lipid phase transition; this result was interpreted in terms of the effect of the glucagon binding on the phase transition of the lipid, such that the heat measured came from the lipid phase changes [39]. In the present study, the lipid was in the liquid crystal phase at all temperatures, so the finding of a large positive enthalpy is not likely to come from further melting of the lipid. These considerations support the suggestion that the positive enthalpy is due to a conformational change in the protein, such as a partial unfolding or loosening of the cytochrome *c* upon binding to DOPG.

The stoichiometry of the binding ratios of cytochrome *c* and lipid is reliably determined in the titration calorimetry experiment. This study gave a lipid to protein ratio of 9:1 for the maximum binding of protein to lipid. The DSC results also gave a small lipid to protein ratio of only 5:1, based on the minimum ratio needed to shift all of the protein from the free DSC peak to the bound DSC peak (Fig. 6). Previous studies of cytochrome *c* lipid interactions have reported various values, some of them much larger than this, but many of these were prepared as MLVs where the cytochrome *c* was added after formation of the liposomes, so that much of the lipid was not accessible to the protein (e.g. Ref. 13). Heimburg et al. [33] also observed ratios as low as 8.4 to 9.6 for vesicles of DMPG and DOPG, respectively.

The interactions of cytochrome *c* with negatively charged lipids is often considered as a typical peripheral protein-membrane interaction in which the protein is interacting superficially with the lipid surface. Cytochrome *c* has generally been considered to be interacting with lipids in such a superficial surface model. However, the low ratio of lipid to protein we have measured is not really consistent with a single layer of protein molecules bound to the surface of the DOPG vesicles without affecting the lipid packing; the diameter of a cytochrome *c* molecule indicates that it would block access to more than ten lipid molecules on a single surface of the bilayer. Moreover, in a closed vesicle, the lipid molecules on the opposing side would also be counted in its binding cluster. This consideration supports the possibility that the cytochrome *c* penetrates partially into the bilayer, separating the lipids and in effect increasing the area of the membrane. This is consistent with the data discussed above, as well as some previously reported data indicating that cytochrome *c* penetrates into the membrane surface, interacting with the acyl chain region [5], and that the cytochrome *c* bound to certain vesicles can be labelled by a photoreactive dye inside the vesicle [6]. The possible configurations of the lipid in a protein-perturbed vesicle include a protein-induced interdigitation of the lipids, similar to that which occurs in phosphatidyl glycerols when bound to myelin basic protein or polymyxin B [34–36].

The effects of ionic strength on the interactions of cytochrome *c* with DOPG were consistent with an ionic interaction. The enthalpy of the interaction and the size of the lipid cluster per protein molecule remained constant up to 10 mM NaCl. Above this concentration the enthalpy decreases and the number of lipids per bound protein molecule decreases as the ionic strength increases, due to the shielding effect of the ionic species in the solvent. The titration calorimetry results indicate that there is no detectible interaction above 100 mM NaCl, whereas the DSC results show an effect of DOPG on the cytochrome *c* denaturation temperature up to 500 mM NaCl. This difference may be due to differences in the sensitivities of the two methods. An alternative possibility is that there are two types of interactions of cytochrome *c* with DOPG, one of which has little or no measurable enthalpy and which persists to a greater ionic strength than the one which was directly observed in the titration calorimetry experiments. Muga and co-workers have shown that the binding of cytochrome *c* to anionic lipids is dependent on the charge density by using lipid mixtures [1]. It is likely that the particular ionic strength cut-offs for detectable binding would be influenced by the charge density of the lipid. This sensitivity of the binding to both ionic strength and charge density on the lipid could be an important factor in the biological activity of cytochrome *c*.

The binding of cytochrome *c* to the lipid is not rapidly equilibrating at low ionic strength, as shown in Fig. 6; at non-saturating amounts of lipid, there are two separate DSC denaturation events for the bound and free protein at the two characteristic temperatures for these two species. In contrast, in excess lipid conditions, as ionic strength is increased there is a single DSC peak which progressively moves to higher temperature as the ionic strength is increased (Fig. 4). This shows that the binding is rapidly equilibrating at the intermediate ionic strengths, which are biologically important, since the ionic strength is 150 mM inside the mitochondrial intra membrane space [38].

In summary, the results of this study show that cytochrome *c* is destablilized by binding to DOPG, and they suggest that cytochrome *c* undergoes a conformational change when it binds to the lipid. The results also provide evidence that cytochrome *c* penetrates into the lipid bilayer. These results are biologically significant because they provide some further insight into possible means of the regulation of the activity of cytochrome *c*. At biological ionic strengths we have shown the protein binds weakly to the lipid and is rapidly equilibrating; this ionic strength effect, along with the effects of lipid charge density, may be a key factor in the activity of the protein in interacting with various membrane components. Our results supporting a conformational change on binding provides a possible mechanism of regulation of the activity of cytochrome *c*. Other authors have found that the equilibrium between two different conformations is affected by various aspects of the lipid as well. Whether such conformational effects of the binding of peripheral proteins to lipids are common or are unique to cytochrome *c* will become clear as more protein–lipid interactions are studied.

Acknowledgements

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