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CALORIMETRIC STUDIES OF CYTOCHROME OXIDASE-PHOSPHOLIPID INTERACTIONS

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Thermotropic phase transitions in phospholipid vesicles reconstituted with mitochondrial cytochrome oxidase (EC 1.9.3.1) were studied using differential scanning calorimetry. Both dimyristoylphosphatidylcholine (DMPC) and mixtures of DMPC and cardiolipin were used at different lipid-to-protein ratios. The incorporated protein reduces the energy absorbed during phase transitions of DMPC vesicles, and causes a small decrease in the transition temperature (t_m). ΔH depends on the amount of protein in the vesicles. This dependence indicates that about 72 DMPC molecules are influenced per cytochrome aa_3 monomer. The transition parameters remain unaffected by changes in ionic strength or by reduction of the enzyme. Incorporation of cytochrome oxidase depleted of subunit III into DMPC liposomes resulted in a larger decrease of t_m, but the amount of perturbed phospholipids remains similar to that in the case of the intact enzyme. Incorporation of cytochrome oxidase into DMPC/cardiolipin vesicles counteracts the effect of cardiolipin in decreasing the enthalpy of the DMPC transition. Thus cytochrome oxidase segregates the phospholipids by attracting cardiolipin from the bulk lipid. Cytochrome *c* does not significantly affect this apparent cardiolipin 'shell' around membranous cytochrome oxidase.

Introduction

Cytochrome *c* oxidase (ferrocytochrome *c*:O_2 oxidoreductase, EC 1.9.3.1) is one of the best characterized components of the electron-transfer chain. It catalyses electron transfer from cytochrome *c* to O_2 as well as proton translocation [1]. Cytochrome oxidase is an integral membrane protein which is 'plugged through' the membrane. Therefore its interaction with phospholipids has been extensively studied.

Incorporation of cytochrome oxidase into phosphatidylcholine bilayers leads to the appearance of a fraction of the total lipid the motion of which is constrained by the protein, at least within the time domain of ESR spin-label measurements. The immobilized phospholipids were suggested to form a boundary layer around the membranous protein [2,3]. However, ^{2}H-NMR measurements with deuterated phosphatidylcholine failed to reveal such an immobilized layer [4]. This difference may be reconciled by taking into account the different time domains of the two techniques, viz. 10^{-8} s for ESR and 10^{-5} s for ^{2}H-NMR [4–6]. Recently, evidence was obtained for protein-associated lipids in rhodopsin-DMPC mixtures using ^{2}H-NMR [7].

In cytochrome oxidase, subunits I, II and III in particular may be expected to perturb the lipid layer because these are most certainly in contact with the lipid phase [8–10]. The interaction of

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Abbreviations: DMPC, L-α-dimyristoylphosphatidylcholine; DSC, differential scanning calorimetry; t_m, phase transition temperature.

phospholipids with the hydrophobic part of cytochrome oxidase could be of importance in the function of the enzyme. This is suggested by the phospholipid requirement for activity [11,12]. Bovine cytochrome oxidase contains 2–3 molecules of tightly bound cardiolipin per cytochrome aa_3 [13,14]. This phospholipid seems to be essential for the enzyme [15]. Its removal results in loss of activity which may be fully recovered by exogenous cardiolipin, but not by other phospholipids such as phosphatidylcholine or -ethanolamine [14]. It has been suggested that it is the overall structure of cardiolipin, and not its negative charges or the composition of the hydrocarbon tails, that controls this functional specificity [15]. Cardiolipin has also been implicated in binding of cytochrome *c* to the enzyme [16]. This proposal is weakened by the fact that active cytochrome oxidase isolated from dogfish contains no tightly bound cardiolipin [17].

Differential scanning calorimetry (DSC) has been used to study protein-lipid mixtures such as bacteriorhodopsin [18,19], (Ca^{2+},Mg^{2+})-ATPase [20], myelin proteolipid apoprotein [24], cytochrome *P*-450 [22] and glycophorin [23]. This method gives information about the nature of the interaction between proteins and lipids [24]. It may yield a quantitation of the perturbed lipid and possibly demonstrate a lipid phase separation induced by the protein [20,25–27].

Here we use DSC to study cytochrome oxidase-phospholipid interactions. We have determined the number of phospholipid molecules influenced by the enzyme in DMPC vesicles, and the effect of cytochrome oxidase on cardiolipin-containing liposomes. It is concluded that the membranous enzyme surrounds itself by a 'shell' of cardiolipin molecules.

Materials and Methods

Preparation of cytochrome oxidase

Mitochondria were isolated from fresh bovine hearts [28]. Cytochrome oxidase was purified by repeated ammonium sulphate precipitations, as described by Kuboyama et al. [29] and slightly modified by Saari et al. [30]. The enzyme was stored in small aliquots under liquid nitrogen. Protein was determined by the biuret method [31]. The concentration of haem *A* was measured spectrophotometrically using a molar absorptivity $\Delta\epsilon_{605-630}(\text{red-ox}) = 13.5\ \text{mM}^{-1} \cdot \text{cm}^{-1}$. The enzymic activity was assayed polarographically in 50 mM Tris-phosphate (pH 7.0)/1% (w/v) Tween-80 at 25°C. The electron-donating system consisted of 3 mM ascorbate and 40 μM horse heart cytochrome *c*. Cytochrome oxidase lacking subunit III was prepared as in Ref. 32 or by repeated precipitation of the enzyme with ammonium sulphate in the presence of sodium cholate. The polypeptide compositions were determined by polyacrylamide gel electrophoresis, as described by Kadenbach et al. [33].

Reconstitution of cytochrome oxidase into liposomes

DMPC (Sigma) or DMPC and cardiolipin (Sigma) were dissolved in 8 mM potassium phosphate buffer (pH 7.5), containing 1.5% (w/v) sodium cholate, and mixed with the protein solution to yield the desired protein/lipid ratio. This solution was dialysed at 4°C against the above buffer, but without the cholate, for 24–40 h with three or four changes of buffer. The last dialysis buffer further contained 50 g/l of Amberlite XAD-2 resin (BDH Chemicals Ltd, Poole, U.K.).

The reconstituted membranes were mainly small unilamellar liposomes, containing particles attributable to reconstituted protein, as shown by electron microscopy of freeze-fractured samples.

Cytochrome oxidase activity was preserved after reconstitution: 298 and 370 nmol cytochrome *c* oxidized $\cdot\ \text{min}^{-1}$ per nmol cytochrome aa_3 for DMPC and DMPC/cardiolipin vesicles, respectively, at 25°C. This is somewhat lower than the reported activity of the enzyme reconstituted with phosphatidylcholine containing unsaturated fatty acids [34].

Differential scanning calorimetry

The calorimetric measurements were carried out using the differential scanning calorimeter DASM-1M [35]. The scanning rate was 1 K/min. The phospholipid content of the samples was 0.78 mg/ml. The transition temperature, t_m, is defined as the temperature of the maximum in the endothermic transition.

Results

Effect of cytochrome oxidase on the thermotropic properties of DMPC

Incorporation of cytochrome oxidase into DMPC liposomes leads to a decrease in the enthalpy (ΔH) of the DMPC phase transition, a small decrease in t_m, and a broadened heat absorption (Fig. 1). Such an effect is typical of hydrophobic proteins [18–23]. Cytochrome oxidase had a greater effect on both t_m and ΔH in the second heating scan than in the first (Fig. 2A). This suggests that additional association between protein and lipid occurs in the liquid-crystalline phase of DMPC, as shown previously [36]. For this reason the proteoliposome samples were routinely incubated for 10 min at 27°C before the calorimetric measurements.

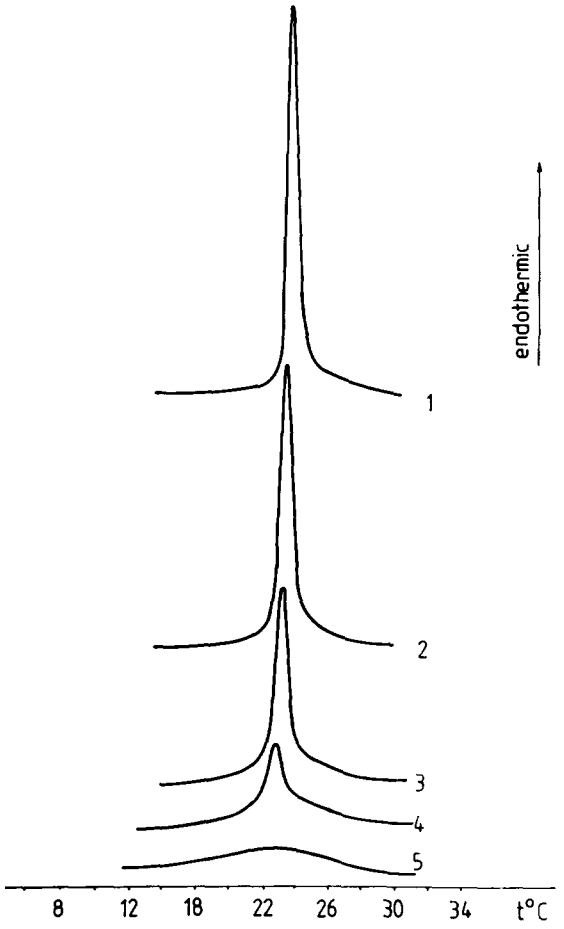

Fig. 1. Microcalorimetry of cytochrome oxidase/DMPC proteoliposomes. 1, DMPC liposomes; 2, DMPC/cytochrome oxidase ratio 1:0.27 (w/w); 3, DMPC/oxidase ratio 1:0.68 (w/w); 4, DMPC/oxidase ratio 1 : 1.35 (w/w); 5, DMPC/oxidase ratio 1:2.7 (w/w).

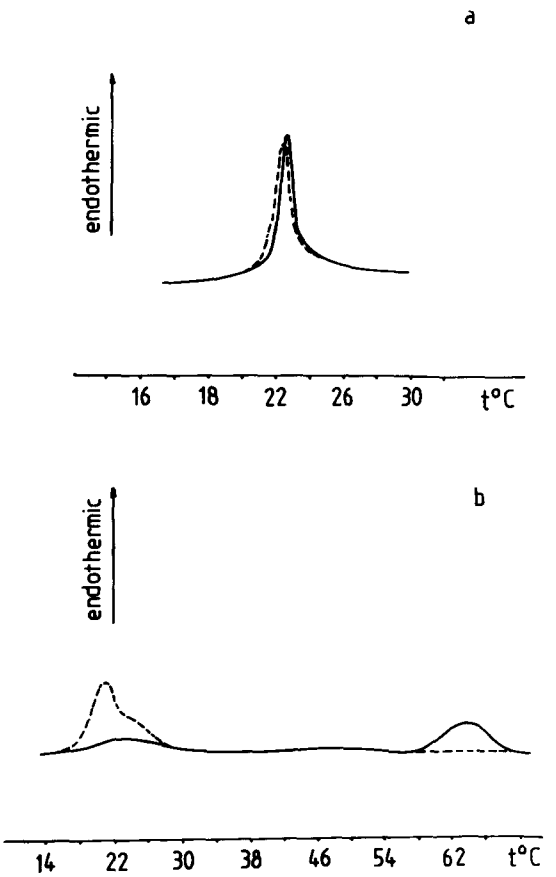

Fig. 2. Phase transitions of cytochrome oxidase/DMPC proteoliposomes. (a) Phase transition during the first (up to 30°C) and second scan. DMPC/oxidase ratio 1:0.68 (w/w), 8 mM potassium phosphate buffer, pH 7.5; ———, first scan; - - - - -, second scan. (b) Phase transitions during first (up to 70°C) and second scan. DMPC/oxidase ratio 1:2.7 (w/w), 8 mM potassium phosphate buffer, pH 7.5; ———, first scan; - - - - -, second scan.

The heating scans of the proteoliposomes showed another endothermic transition at 64°C (Fig. 2B). The position of this peak depends on the presence of cytochrome oxidase, and is no longer detected in repeated scans. As shown by repetition of the scan, preheating up to 70°C brings about an increase in the enthalpy of the lipid transition and

a decrease in t_m (Fig. 2B). Clearly, the changes in ΔH and t_m caused by heating up to 70°C are due to protein denaturation (cf. Refs. 22 and 37). Thus the endothermic transition at 64°C is probably characteristic of heat denaturation of membranous cytochrome oxidase. Denaturation of cytochrome oxidase is different in proteoliposomes and in detergent solutions, such as Triton X-100 or sodium cholate. In the latter, denaturation is accompanied by release of heat, where the maximum of the exothermic peak and its form strongly depend on the concentration of protein (Fig. 3). The exothermic transition coincides with the appearance of turbidity in the solution, and is apparently a result of protein aggregation.

Fig. 4 shows a plot of ΔH of the DMPC phase transition versus the ratio of cytochrome oxidase

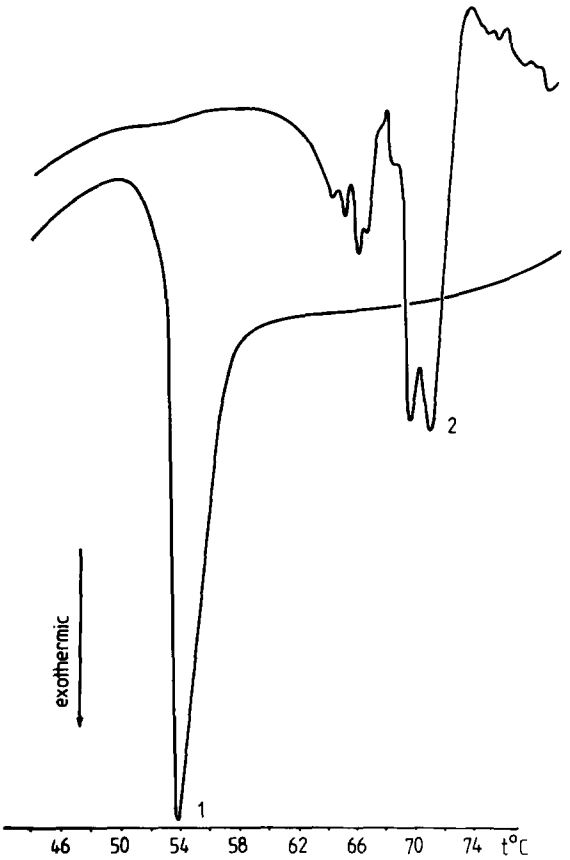

Fig. 3. Calorimetric scans of detergent solutions of cytochrome oxidase. 100 mM sodium phosphate buffer (pH 7.5)/1.5% (w/v) sodium cholate. The concentration of cytochrome oxidase was 1.6 mg/ml in trace 1 and 0.16 mg/ml in trace 2.

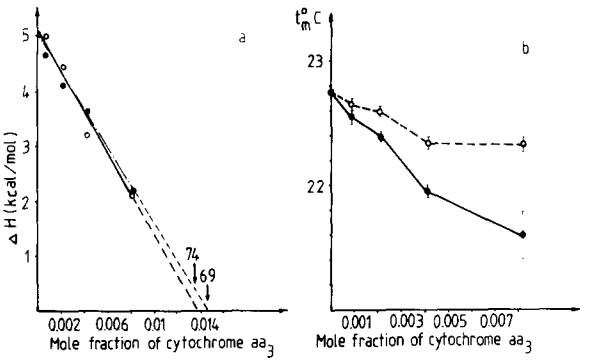

Fig. 4. Enthalpy change and temperature of phase transition of DMPC in DMPC/cytochrome oxidase proteoliposomes as a function of the molar ratio of enzyme to phospholipid. Open symbols, normal enzyme; solid symbols, subunit III-deficient enzyme.

to lipid, which yields a linear relationship. Beyond a certain protein/lipid ratio ΔH becomes too small to be measurable, but extrapolation of the straight lines to $\Delta H = 0$ allows an estimation of the number of DMPC molecules that are removed from the cooperative transition per molecule of cytochrome oxidase (cf. Refs. 18, 22, 25 and 37). From the intersection with the abscissa the number of 'withdrawn' DMPC molecules is found to be about 72 per molecule of cytochrome aa_3. This figure may be compared with that obtained by Marsh and co-workers [38,39] of 55 molecules of DMPC per 200 kDa for the yeast enzyme, using an ESR method. Since the molecular weight of the cytochrome aa_3 monomer is considerably lower than 200 000 (see Ref. 1), the two independent measurements are in good agreement.

The amount of apparently perturbed lipid is independent of ionic strength (potassium phosphate between 8 and 250 mM), and does not change upon reduction of the enzyme with dithionite, or after addition of cytochrome *c* (data not shown).

Subunits I and III are most heavily labelled after treatment of the enzyme with photoactivatable arylazido phospholipids [40]. It may therefore be of interest to test whether removal of subunit III [32] affects the enthalpy and temperature of the DMPC transition. As shown in Fig. 4B, cytochrome oxidase caused a larger decrease in t_m when subunit III was removed than in its presence.

However, the change in enthalpy and the number of affected lipid molecules were unchanged (Fig. 4A).

Interaction of cytochrome oxidase with a binary mixture of phospholipids

The apparently specific requirement of cytochrome oxidase for cardiolipin, as well as the abundance of this phospholipid in inner mitochondrial membranes (see Introduction), makes it interesting to study the influence of cytochrome oxidase on lipid membranes containing cardiolipin.

Incorporation of cardiolipin into DMPC membranes leads to a decrease in enthalpy and temperature of the DMPC transition (Fig. 5A). Incorporation of cytochrome oxidase into the DMPC membranes has qualitatively a similar effect (Figs. 1, 4 and 5B). However, when the content of cardiolipin was increased progressively in DMPC vesicles containing a constant amount of cytochrome oxidase (Fig. 5B), the enthalpy and t_m did not decrease until a limit was reached (see Fig. 6). Since cardiolipin decreases these parameters immediately in vesicles devoid of oxidase (Figs. 5A and 6), the increase of enthalpy due to the presence of protein (Fig. 6A) may be rationalized by assuming that cytochrome oxidase preferentially attracts cardiolipin, thus removing it from the bulk lipid. This indicates that in a mixture of phosphatidylcholine and cardiolipin cytochrome oxidase induces lipid class segregation, forming a cardiolipin shell around the membranous enzyme. The apparent titre of 0.025 mol cardiolipin/mol DMPC (Fig. 6), at which cardiolipin starts to influence the phase transition of DMPC, corresponds to about 10 molecules of cardiolipin per cytochrome aa_3 (see Discussion).

It is known that phase separation in mixtures of DMPC-cardiolipin can also be induced by cytochrome *c* [41,42]. We therefore studied the effect of cytochrome *c* on the phase separation induced by cytochrome oxidase.

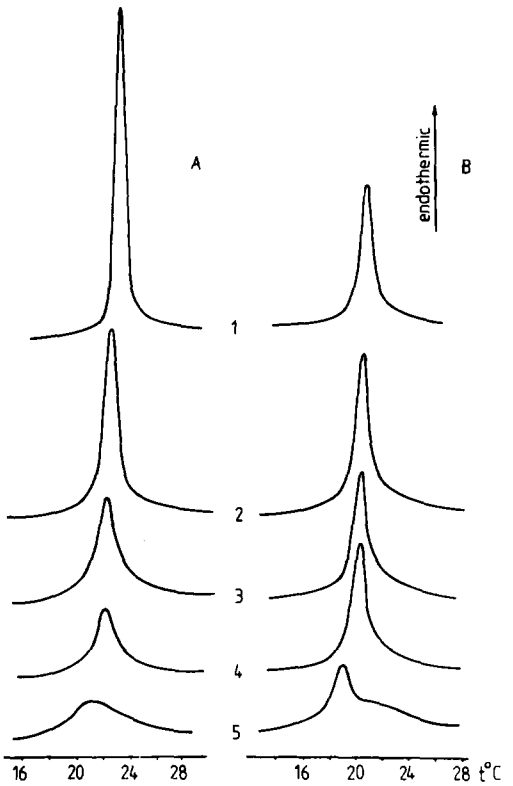

Fig. 5. Effect of cardiolipin on phase transition of DMPC liposomes, and the influence of incorporated cytochrome oxidase. 8 mM potassium phosphate buffer, pH 7.5. A: Effect of cardiolipin on DMPC vesicles. 1, DMPC vesicles; DMPC/cardiolipin ratio 40:1 (w/w), 30:1, 20:1 and 10:1 in 2, 3, 4 and 5, respectively. B: Effect of cytochrome oxidase on DMPC/cardiolipin vesicles. The concentrations of DMPC and cytochrome oxidase were held constant. DMPC/cytochrome oxidase ratio 1:0.67 (w/w). The DMPC/cardiolipin ratio was varried as in A.

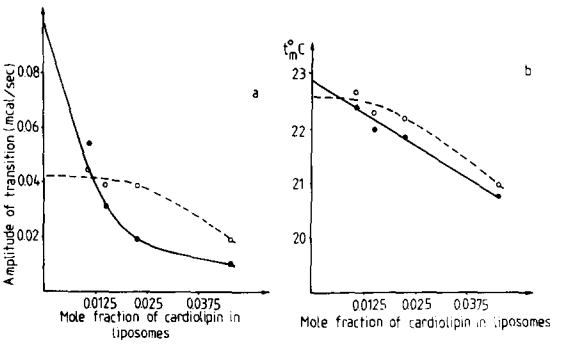

Fig. 6. The effect of cardiolipin on amplitude and temperature of phase transition in DMPC liposomes and DMPC/cytochrome oxidase proteoliposomes. 8 mM potassium phosphate buffer, pH 7.5. The concentration of cytochrome oxidase was held constant (DMPC/cytochrome oxidase ratio 1:0.67 (w/w)). The cardiolipin/DMPC molar ratio is shown on the abscissa. Solid symbols, liposomes without enzyme; open symbols, proteoliposomes with cytochrome oxidase.

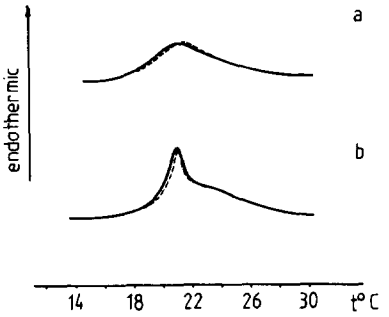

Fig. 7. Effect of cytochrome *c* on the phase transition of DMPC/cardiolipin vesicles in the presence and absence of cytochrome oxidase. 8 mM potassium phosphate buffer, pH 7.5, 0.67 mg/ml cytochrome *c*, cardiolipin/DMPC ratio 1 : 10 (w/w). (a) Without cytochrome oxidase; (b) with cytochrome oxidase. ———, without cytochrome *c*; - - - - - -, with cytochrome *c*.

Cytochrome *c* did not change either t_m or ΔH of DMPC in media of low or high ionic strength. If cardiolipin was present in the vesicles, cytochrome *c* only induced a small increase in t_m, but caused no discernible effect on ΔH. The former effect was observed only at low ionic strength (Fig. 7A). If the liposomes contained cardiolipin and cytochrome oxidase at a concentration where there remained cardiolipin molecules unattracted by the oxidase, then cytochrome *c* influenced the phase transition in the same way as when cytochrome oxidase was absent (Fig. 7B). However, when all cardiolipin is attracted by cytochrome oxidase there is no longer an influence of cytochrome *c* on the parameters of the phase transition. These results indicate that cytochrome *c* does not significantly perturb the cardiolipin shell around membranous cytochrome oxidase.

Discussion

The decrease in ΔH caused by incorporation of a hydrophobic protein in the DMPC membrane may be interpreted in terms of a model in which a fraction of the lipid is removed from the bulk lipid by the protein in such a way that it no longer participates in the phase transition of the bulk. Two different mechanisms have been proposed. According to the first, a single boundary layer of relatively immobilized lipid is formed around the protein molecule [2,43]. Alteratively, lipid could be immobilized by becoming trapped between associated protein molecules [19,37]. The rather good coincidence between the number of phospholipid molecules theoretically expected in a boundary layer and the experimental data [18,38,44] and the lack of effect of glutaraldehyde on either attached or unattached hydrocarbon spin probes despite a large immobilizing effect on the protein [4], as well as the results of studies on the correlation between the rotational dynamics of protein and lipid using spin labels [45,46], all favour the immobilized boundary layer model.

Protein aggregation may also affect lipid immobilization [18,19]. However, the presence of such phenomena may depend on the type of protein and its tendency to aggregate. With bacteriorhodopsin the formation of protein-lipid patches is accompanied by the appearance of a second component in the calorimetric scans [19]. For DMPC vesicles with cytochrome oxidase such a component was absent, and ΔH decreased gradually with increasing protein/lipid ratio. This suggests that the decrease in ΔH is simply due to formation of a boundary layer of lipid around the enzyme. In support of this proposal, the aggregation of cytochrome oxidase during heating to the temperature of denaturation led to a change in the form of the phase transition. The new form was reminiscent of the endothermic peak of bacteriorhodopsin-DMPC patches [19].

The amounts of phospholipid immobilized by cytochrome oxidase with or without subunit III are about the same (Fig. 4). Subunit III is a large polypeptide with a high degree of hydrophobicity, and is known to be in contact with membrane phospholipids [16]. Our data suggest that the total protein surface in contact with membrane lipid nevertheless remains unchanged upon removal of subunit III. On the other hand, cytochrome oxidase devoid of subunit III has a stronger effect on t_m than the intact enzyme (Fig. 4B). This may indicate a higher degree of electrostatic interactions of subunit III-depleted enzyme with lipids than is the case in the presence of this subunit [24].

The influence of cytochrome oxidase on DMPC-cardiolipin membranes is interesting. The enzyme apparently causes a phase separation of lipids, attracting cardiolipin in a 'shell' around the

protein. This boundary cardiolipin is 'weakly' bound, since the preparations of enzyme used in this work contain 'tightly' bound cardiolipin, the removal of which requires special treatment [14]. Our result is in accordance with the data of Cable and Powell [49] and Knowles et al. [50], who, using spin-labelled cardiolipin, showed its preferential association to cytochrome oxidase.

During titration of cytochrome oxidase/DMPC vesicles with cardiolipin the decrease of t_m and amplitude of the transition occurred after a 'titre' of about 10 cardiolipin molecules per cytochrome aa_3 (Fig. 6). Above this, 'free' cardiolipin molecules appear in the membrane. This might underestimate the number of binding sites for cardiolipin, since bound cardiolipin may also influence the melting of DMPC molecules nearby, decreasing the enthalpy of the transition. The apparent selectivity of first-shell lipids for cardiolipin may be due either to the negative charge or to a larger binding constant for this phospholipid. It has been shown that the negative charge of phospholipids is the cause of the phase separation in phospholipid mixtures caused by proteins such as cytochrome *c* [41,42] and lipophilin [26]. However, negative charge does not seem to be important in tight binding of cardiolipin to cytochrome oxidase [51], or in the weaker interaction of the enzyme with cardiolipin in enzyme-phospholipid mixtures [50]. Recently, a model was presented for the binding of phospholipids to membrane proteins, based on a fixed number of binding sites on the hydrophobic surfaces of the protein for fatty acyl chains [52]. This model predicts that in mixtures of phospholipids the binding of the four chains of cardiolipin will be favoured over binding of the two chains of phosphatidylcholine.

Since the inner mitochondrial membrane contains about 20% cardiolipin, cytochrome oxidase could induce a phase separation in its physiological milieu and therefore function in specific phospholipid surroundings. A shell of several negatively charged cardiolipin molecules around the membranous oxidase may help to orient properly the dipolar cytochrome *c* [53] before binding to the oxidase. This may have the effect of lowering the energy of activation of the reaction between cytochrome *c* and cytochrome oxidase.

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