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Studies on protein-lipid interactions in cytochrome *c* oxidase by differential scanning calorimetry

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The interaction between cytochrome *c* oxidase and phospholipids was studied by differential scanning calorimetry. The active, lipid-sufficient cytochrome *c* oxidase undergoes thermodenaturation at 336 K with a relatively broad and concentration dependent endothermic transition. The delipidated enzyme shows an endothermic denaturation temperature at 331.3 K. When the delipidated cytochrome *c* oxidase was treated with chymotrypsin, a lowered thermodenaturation temperature was observed. When the delipidated cytochrome *c* oxidase was reconstituted with asolectin to form a functionally active enzyme complex, the thermodenaturation shifted to a higher temperature, with a sharper transition thermogram. The increase in thermotransition temperature and enthalpy change of thermodenaturation of the asolectin-reconstituted enzyme is directly proportionate to the amount of asolectin used, up to 0.5 mg asolectin per mg protein. The thermotransition temperature and enthalpy changes of thermodenaturation for the phospholipid-reconstituted cytochrome *c* oxidase are affected by the phospholipid headgroup and the fatty acyl groups. Among phospholipids with the same acyl moiety but different head groups, phosphatidylethanolamine was found to be more effective than phosphatidylcholine in protecting cytochrome *c* oxidase from thermodenaturation. An exothermic transition thermogram was observed for delipidated cytochrome *c* oxidase embedded in phospholipid vesicles formed with phospholipids containing unsaturated fatty acyl groups. The increase in exothermic transition temperature and exothermic enthalpy change of thermodenaturation of the oxidase-asolectin vesicles is proportional to the amount of asolectin used, up to 1.0 mg per mg protein. Formation of a cytochrome *c*-cytochrome *c* oxidase complex destabilized cytochrome *c* but not cytochrome *c* oxidase toward thermodenaturation.

Introduction

Cytochrome *c* oxidase [1], a well characterized integral protein complex in the inner mitochondrial membrane, functions as a terminal oxidase in the mitochondrial electron transfer system. The electron transfer from cytochrome *c* to molecular oxygen catalyzed by cytochrome *c* oxidase is believed to be coupled with proton translocation [2], a key step in the energy conservation of biological oxidation. The protein subunit structure and topological arrangement of cytochrome *c* oxidase in the membrane has been characterized, and is more or less accepted by most investigators [3,4]. The active, purified cytochrome *c* oxidase contains about 20% phospholipids. Although the delipidated form of cytochrome *c* oxidase has enzymatic stability and spectral properties similar to those of the

Abbreviations: dCO, delipidated cytochrome *c* oxidase; DSC, differential scanning calorimetry; DLPC, dilinoleoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DPG, diphosphatidylglycerol; DPPC, dipalmitoylphosphatidylethanolamine; DSPC, distearoylphosphatidylcholine.

intact enzyme, it is not functionally active in the absence of phospholipids. The requirement of phospholipids for enzymatic activity [5], and the mode of interaction between protein and phospholipid have been subjects of intensive study. Recently, many physical and chemical methods have been employed for the study of the protein-lipid interaction in this particular enzyme complex. However, the question regarding the existence of a specific boundary phospholipid [6–9] surrounding the protein remains unanswered. Recent results [10,11] from a differential scanning calorimetry (DSC) study of the phospholipid perturbation seem to favor the existence of a specific boundary phospholipid in this enzyme complex. In this investigation, we extended the DSC study to examine the thermodenaturation properties of cytochrome *c* oxidase in the presence and absence of phospholipids, to gain more insight into the protein-phospholipid interaction in this enzyme complex. To avoid possible complications resulting from protein-lipid interactions of other segments of the mitochondrial electron transfer chain, a highly purified cytochrome *c* oxidase preparation was used throughout this study.

Experimental procedures

Materials

Horse cytochrome *c*, type III, dilinoleoylphosphatidylcholine (DLPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylethanolamine (DPPE), diphosphatidylglycerol (DPG), sodium cholate and chymotrypsin are products of Sigma. Asolectin was obtained from Associated Concentrates Inc. Other chemicals were obtained commercially at the highest purity available.

Methods

Phospholipid-sufficient and phospholipid-depleted cytochrome *c* oxidases were prepared by the method reported previously [5], except that submitochondrial particles were used as starting material instead of the Keilin-Hartree heart muscle preparation. To ensure complete delipidation, the delipidated cytochrome *c* oxidase (dCO), as prepared, was subjected to two more cycles of ammonium sulfate precipitation in the presence of 1.5% sodium cholate. The purity of cytochrome *c* oxidase was about 10–12 nmoles heme *a* per mg protein.

Replenishment of phospholipids to the delipidated cytochrome c oxidase. Two methods were used: Method I involved direct addition of the phospholipid micellar solution to the delipidated enzyme, which was in 50 mM phosphate buffer, pH 7.4. No detergents other than the residual sodium cholate which was precipitated with the enzyme during ammonium sulfate fractionation were present in the preparation. The resulting preparation is called the phospholipid-reconstituted cytochrome *c* oxidase in this paper. The phospholipid micellar solution was prepared by sonification of phospholipid in H_2O. Method II involved addition of the phospholipid solution in 1.5% sodium cholate to the delipidated oxidase, which was in 50 mM phosphate buffer (pH 7.4), containing 1.5% sodium cholate. The protein concentration was about 10 mg/ml. The mixture was incubated for 20 min at 0°C, and subjected to extensive dialysis. After dialysis, the enzyme-phospholipid complex was collected by centrifugation, and the precipitated complex was suspended in 50 mM phosphate buffer (pH 7.4). The resulting preparation is referred to as the cytochrome *c* oxidase/phospholipid vesicle.

Formation of the cytochrome c-cytochrome c oxidase complex. Formation of the cytochrome *c*-cytochrome *c* oxidase complex in the presence and absence of phospholipids was carried out according to the method reported previously [5].

Proteolytic enzyme digestion. Delipidated cytochrome *c* oxidase, 22 to 88 mg/ml, was treated with chymotrypsin for 1 h at room temperature before the DSC experiments were carried out. The final ratio of chymotrypsin to protein was 3% (w/w).

DSC measurements were carried out in the Perkin-Elmer DSC-2C equipped with a Haake constant temperature bath. The bath temperature was set at 0°C unless otherwise stated. The temperature at the peak of the exo- or endo-thermogram was the measured T_m, and the change of enthalpy for thermodenaturation was calculated from the area covered by the endo- or exo-thermogram. The sample and reference capsules were weighed before and after scanning to ensure the

balance and to detect the possible leakage during scanning. Each sample was repeatly scanned twice in order to detect any reversible transition. The second scan was used as the base line for the calculation of the change of enthalpy for thermodenaturation. The DSC-2C was calibrated with Indium and checked with a DPPC-H_2O suspension, assuming the enthalpy change of the DPPC phase transition to be 8.5 kcal/mol [12].

The protein concentration was determined by the biuret method, using serum albumin as standard. Absorption spectra were measured in a Cary spectrophotometer, model 219. The enzymatic activity of cytochrome *c* oxidase was determined according to the method reported in Ref. 5.

Results and Discussion

Thermotropic properties of phospholipid sufficient- and phospholipid depleted-cytochrome c oxidases

Fig. 1 shows the differential scanning calorimetry thermograms for phospholipid-sufficient, delipidated, and chymotrypsin-treated delipidated cytochrome *c* oxidases. A relatively broad transition or thermodenaturation peak at 336.0 and 331.3 K were observed for phospholipid sufficient (42 mg/ml) and delipidated (44 mg/ml) cytochrome *c* oxidases in 50 mM phosphate buffer (pH 7.4), respectively. When a higher concentration of delipidated cytochrome *c* oxidase was used, a broader transition peak at 332.3 with a shoulder at 326 K was observed, indicating that the enzyme complex may exist in more than one physical state [13,14]. The enzyme complex may exist in different degrees of aggregation because the delipidated enzyme, as prepared, contains trace amounts of sodium cholate. The shoulder at 326 K became more apparent when the protein concentration was further increased to 88 mg/ml. This suggests that the endothermic transition temperature at 326 K may due mainly to the aggregation of proteins. At a lower concentration, the endothermic denaturation occurred at a higher temperature, 336.9 and 333 K for phospholipid sufficient (28 mg/ml) and delipidated (22 mg/ml) cytochrome *c* oxidases, respectively. The enthalpy changes of endothermic denaturation of phospholipid sufficient and delipidated cytochrome *c* oxidases increased as the protein concentrations increased (see Table I).

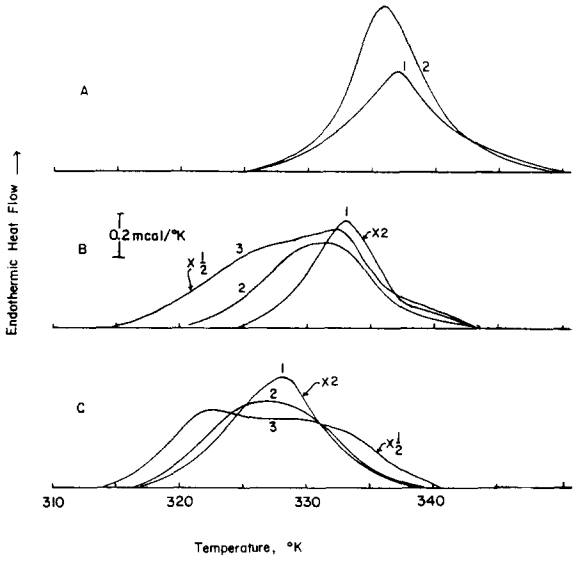

Fig. 1. DSC thermograms of phospholipid-sufficient, delipidated and chymotrypsin-treated cytochrome *c* oxidase at various protein concentrations. Aliquots of phospholipid sufficient (A), delipidated (B), and chymotrypsin treated-delipidated (C) cytochrome *c* oxidase in 50 mM Na/K phosphate buffer (pH 7.4), at various protein concentrations were used. The protein concentrations were 28 and 42 mg/ml for curves 1 and 2 in (A), and 22, 44, and 88 mg/ml for curves 1, 2, and 3 in (B) and (C), respectively. A heating rate of 2.5 K/min and a sensitivity of 0.1 mcal/s were used. A 62-μl sample in a large volume capsule was placed in the sample holder and a 62-μl 50 mM Na/K phosphate buffer (pH 7.4), was placed in the reference holder. The recorder scanning speed was 100 s per inch.

When the delipidated cytochrome *c* oxidase was treated with chymotrypsin at room temperature for one hour, the enzyme became more labile toward thermodenaturation. The DSC thermograms of the chymotrypsin-treated enzyme at various protein concentrations are given in Fig. 1C. At a protein concentration of 44 mg/ml, the DSC thermogram shows an endothermic denaturation peak at 327.1 K. When a higher concentration of cytochrome *c* oxidase (88 mg/ml) was used, the endothermic denaturation of the treated enzyme shows a peak at 322.5 K with a shoulder at 332 K. This indicates that some polypeptides in this enzyme complex are more susceptible to proteolytic enzyme digestion than others, and the cleavage of the peptide bonds does not cause the decrease in the degree of aggregation especially when the enzyme is in a highly aggregated state. Due to the

TABLE I

THERMOTROPIC PROPERTIES OF VARIOUS PREPARATIONS OF CYTOCHROME *c* OXIDASE

CO, phospholipid-sufficient cytochrome *c* oxidase. dCO, delipidated cytochrome *c* oxidase prepared by the method developed in this laboratory [5].

Preparations	T_m (K)	ΔH (kcal/mol)
In 50 mM phosphate buffer (pH 7.4)		
CO (28 mg/ml)	336.9	251
CO (42 mg/ml)	336.0	266
In 50 mM phosphate buffer (pH 7.4)		
dCO (22 mg/ml)	333.0	268
dCO (44 mg/ml)	331.3	270
dCO (88 mg/ml)	332.3, 326 (sh)	401
Treated with 3% chymotrypsin		
dCO (22 mg/ml)	328.1	278
dCO (44 mg/ml)	327.1	278
dCO (88 mg/ml)	322.5, 332 (sh)	342
At 44 mg/ml in different buffer concentrations		
dCO in 25 mM buffer	331.3	265
dCO in 50 mM buffer	331.3	270
dCO in 275 mM buffer	332.3	280
Sodium dithionite reduced form		
dCO in 50 mM buffer at 40 mg/ml	328.0	327

sensitivity of the DSC instrument used, we are unable to resolve patterns of the digestion products of the specific subunits of cytochrome *c* oxidase.

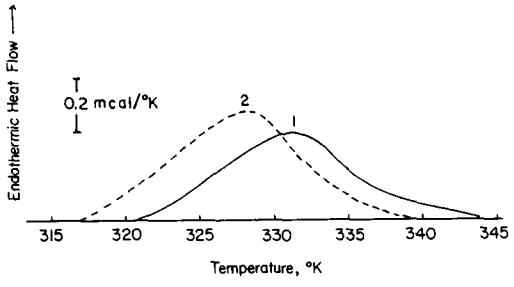

Fig. 2. DSC thermograms for oxidized and reduced forms of the delipidated cytochrome *c* oxidase. The delipidated cytochrome *c* oxidase, 40 mg/ml, in 50 mM phosphate buffer (pH 7.4), was reduced by a few grains of sodium dithionite. 62-μl aliquots of delipidated cytochrome *c* oxidase were used. Curves 1 and 2 represent the oxidized and reduced forms of delipidated cytochrome *c* oxidase. The DSC settings are as given in Fig. 1.

As indicated in Fig. 2, the thermotropic properties of cytochrome *c* oxidase are also affected by the redox state of the preparation. Reduction of the delipidated cytochrome *c* oxidase by dithionite caused a shift of the endothermic transition temperature of about 3.3 K downwards and increased the enthalpy change about 21%. In contrast to the effect of ionic strength on thermotropic properties of cytochromes *c* and c_1, changes in ionic strength had only a slight effect on the T_m and ΔH of cytochrome *c* oxidase. Table I summarizes the thermotropic data of various preparations of cytochrome *c* oxidase.

Thermotropic properties of the phospholipid-reconstituted cytochrome c oxidase preparations

It has been demonstrated [5] that addition of phospholipids, individually or in combination, restores enzymatic activity to the phospholipid-depleted cytochrome *c* oxidase. Among the phospholipids tested, asolectin was the most effective in restoration of enzymatic activity to the delipidated cytochrome *c* oxidase. Fig. 3 shows the DSC thermograms for the asolectin-reconstituted cytochrome *c* oxidase preparations at various ratios of asolectin to protein. Addition of asolectin to the delipidated cytochrome *c* oxidase protected the enzyme complex from heat denaturation, and the

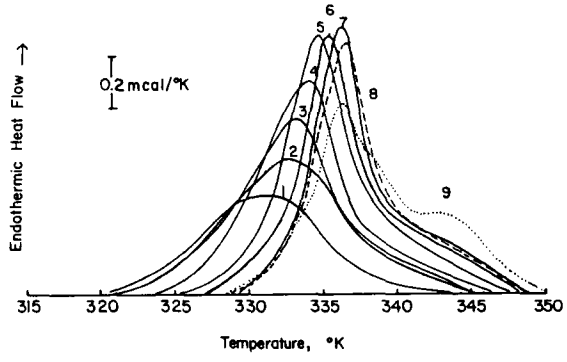

Fig. 3. Effect of asolectin concentrations on the thermodenaturation of cytochrome *c* oxidase. Asolectin micellar solution was added directly to the delipidated cytochrome *c* oxidase, which was in 50 mM Na/K phosphate buffer (pH 7.4). Curves 1 to 9 represent 0, 0.81, 1.62, 3.25, 7.5, 10, 15, 22, and 25 mg asolectin per ml of protein solution, and the final protein concentration was 44 mg/ml in 50 mM Na/K phosphate buffer (pH 7.4). 62-μl aliquots were used. The DSC instrument settings were as given in Fig. 1.

reconstituted complex showed a relatively sharp endothermic transition peak. As the amount of asolectin added to the delipidated cytochrome *c* oxidase increased, the thermodenaturation temperature for the enzyme complex increased, up to a ratio of 0.5 mg asolectin per mg protein. When the ratio of asolectin to protein in the reconstituted enzyme was higher than 0.5, no further change in the transition temperature was observed, but the thermodenaturation was broadened. A maximum increase of 5.5 K in thermodenaturation temperature was observed upon addition of asolectin to the delipidated cytochrome *c* oxidase. This result correlates well with the results obtained from activity restoration by asolectin to the delipidated cytochrome *c* oxidase, since the maximal restoration of activity to delipidated cytochrome *c* oxidase by asolectin was reached when the ratio of asolectin to protein was 0.5. About 43% increase in enthalpy change of thermodenaturation of asolectin-reconstituted cytochrome *c* oxidase was observed (385 kcal/mol) relative to the delipidated enzyme (270 kcal/mol).

Three major phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG), are present in intact cytochrome *c* oxidase. We investigated the effect of each individual phospholipid on the thermotropic properties of cytochrome *c* oxidase. Table II summarizes the thermotropic properties of the DPPC-, DPPE- and DPG- reconstituted cytochrome *c* oxidase preparations. Data of the asolectin-reconstituted cytochrome *c* oxidase are also included for comparison. The thermotropic properties of these phospholipid-reconstituted cytochrome *c* oxidase complexes differ significantly. The asolectin-reconstituted enzyme shows an endothermic transition peak at 336.8 K, with an enthalpy change for thermodenaturation of 385 kcal/mol. Although the DPPC-reconstituted cytochrome *c* oxidase underwent thermodenaturation at the same temperature as the asolectin-reconstituted cytochrome *c* oxidase complex, a slight decrease in enthalpy change for thermodenaturation was observed relative to the asolectin reconstituted enzyme.

TABLE II

THERMOTROPIC PROPERTIES OF VARIOUS PHOSPHOLIPIDS RECONSTITUTED CYTOCHROME *c* OXIDASE

Preparations	Phospholipid/protein	T_m (K)	ΔH (kcal/mol)
dCO + asolectin	0.5	336.8	385
dCO + DPPC	1.0	336.8	368
dCO + DPPE	0.2	338.0	1140
dCO + DPG	0.36	331.0	558

The DPPE-reconstituted cytochrome *c* oxidase shows an endothermic denaturation peak at 338 K with an enthalpy change for thermodenaturation of 1140 kcal/mol. Although a significantly greater enthalpy change of thermodenaturation was observed for the DPPE-reconstituted cytochrome *c* oxidase than for other phospholipid-reconstituted enzyme preparations this does not correlate with a difference in enzymatic activity, because the activity of the DPPE-reconstituted cytochrome *c* oxidase is similar to that of the other single phospholipid-reconstituted enzyme preparations. Since DPPC and DPPE contain the same fatty acyl groups, the different effects on the thermotropic properties of cytochrome *c* oxidase must be attributed to the phospholipid headgroups. Perhaps the polarity of the head group in the phospholipid molecule plays an important role in protein-phospholipid interaction.

It has been shown that DPG binds tightly to cytochrome *c* oxidase [15], and is the phospholipid most difficult to remove from the enzyme complex. The DSC thermogram for the DPG-reconstituted cytochrome *c* oxidase shows and endothermic denaturation peak at 331 K, with an enthalpy change for thermodenaturation of 558 kcal/mol.

Thermotropic properties of cytochrome c oxidase embedded in various phospholipid vesicles

Delipidated cytochrome *c* oxidase was mixed with phospholipids in the presence of 1.5% sodium cholate at a protein concentration of approx. 10 mg/ml in 50 mM phosphate buffer (pH 7.4) followed by extensive dialysis against the same buffer. Cytochrome *c* oxidase/phospholipid vesicles were formed, and collected by centrifugation and resuspended in 50 mM phosphate buffer (pH 7.4). The thermodenaturation behavior of this cytochrome *c* oxidase/asolectin vesicle was found to be completely different from that of the asolectin-recon-

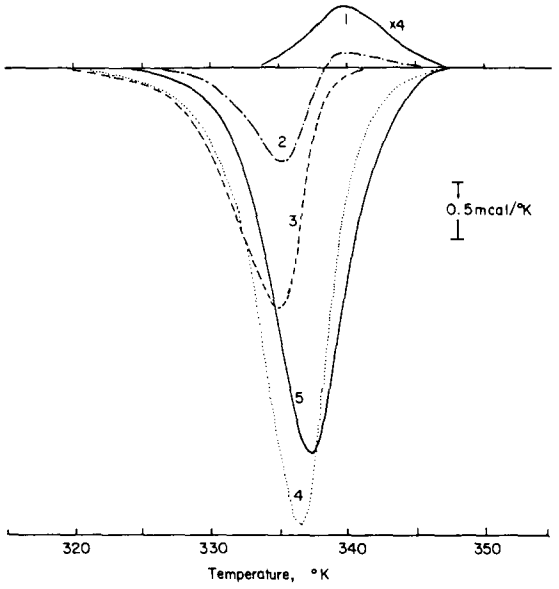

Fig. 4. Phospholipid concentration-dependent exothermic denaturation of cytochrome *c* oxidase embedded in phospholipid vesicles. Aliquots of delipidated cytochrome *c* oxidase were mixed with various amounts of asolectin in the presence of 1.5% sodium cholate. The mixture was then dialyzed extensively against 50 mM Na/K phosphate buffer (pH 7.4) overnight, with two changes of buffer. The cytochrome *c* oxidase/asolectin vesicles formed were collected by centrifugation and resuspended in the same buffer before DSC measurement. The instrument settings were: heating rate, 2.5 K/min and sensitivity, 0.2 mcal/s. Curves 1 to 5 represent cytochrome *c* oxidase/asolectin vesicles with asolectin to protein ratios of 0, 0.125, 0.25, 1.0 and 2.0, respectively. The final protein concentration in all samples was 18 mg/ml. 65-μl aliquots were used.

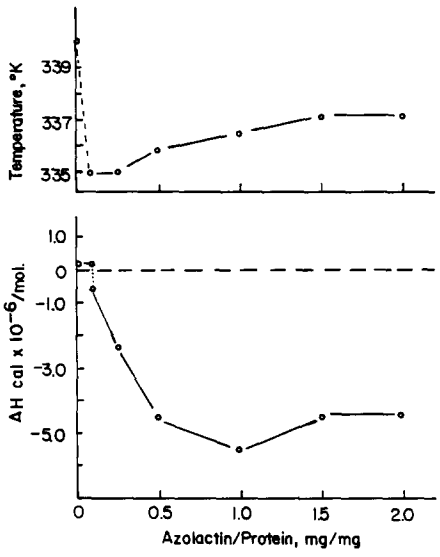

Fig. 5. Phospholipid concentration dependent T_m and enthalpy changes of cytochrome *c* oxidase/asolectin vesicles. Experimental conditions and DSC settings were the same as those given in Fig. 4.

stituted cytochrome oxidase. An exothermic transition was observed for the cytochrome oxidase/asolectin vesicle, in contrast to the endothermic transition observed for the asolectin-reconstituted cytochrome oxidase. Fig. 4 shows the DSC thermograms for cytochrome *c* oxidase/asolectin vesicles prepared with various concentrations of asolectin. The dialyzed delipidated cytochrome *c* oxidase showed an endothermic transition at 340 K. When the ratio of asolectin to protein in the cytochrome *c* oxidase/asolectin vesicles was increased, the exothermodenaturation temperature and the enthalpy change of thermodenaturation increase, up to a ratio of 1.0 mg asolectin per mg protein. Beyond that, the exothermic transition for the cytochrome *c* oxidase/asolectin vesicles increased slightly but the enthalpy change for thermodenaturation decreased. The maximal exothermodenaturation temperature for the cytochrome *c* oxidase/asolectin vesicles was 337.3 K (Fig. 4, curve 5) which is about 0.5 K higher than the maximal endothermodenaturation temperature for the asolectin-reconstituted cytochrome *c* oxidase in the dispersed form, and about 2.7 K lower than that of the dialyzed delipidated cytochrome *c* oxidase. Fig. 5 shows the asolectin concentrations dependent denaturation temperature and enthalpy changes of thermodenaturation of the cytochrome *c* oxidase vesicles.

When single phospholipids such as DPPC and DPPE, which possess saturated fatty acyl groups, were used for preparation of cytochrome *c* oxidase/phospholipid vesicles, the resulting cytochrome oxidase/phospholipid vesicles showed endothermic transition DSC thermograms. When DPG was used, the resulting cytochrome *c* oxidase/DPG vesicle showed an exothermic denaturation. Although the thermotropic properties of the cytochrome *c* oxidase/DPG vesicle are similar to those of the cytochrome *c* oxidase/asolectin vesicle (see Table III), a lower amount of DPG was needed to reach the maximal exothermic enthalpy change of thermodenaturation.

TABLE III

THERMOTROPIC PROPERTIES OF VARIOUS PHOSPHOLIPIDS VESICLES OF CYTOCHROME *c* OXIDASE

Preparations	Phospholipid/ protein	T_m (K)	ΔH (kcal/mol)
dCO + Asolectin	1.0	337.3	−5500
dCO + DSPC	1.0	308.4	116
dCO + DOPC	1.0	327.9, 341.5	171
dCO + DLPC	1.0	336.5	−808
dCO + DPG	1.0	341.3	−296

A well defined exothermic transition was observed in the cytochrome *c* oxidase/DLPC vesicle, but not in the cytochrome *c* oxidase/DOPC vesicle (see Fig. 6). The cytochrome *c* oxidase/DLPC vesicle showed an exothermodenaturation at 336.5 K, with an enthalpy change of thermodenaturation of −808 kcal/mol. The DSC thermogram of the cytochrome *c* oxidase/DOPC vesicle shows two endothermic transition peaks: one at 327.9 K, about 3.4 K lower than the endothermic denaturation temperature of the delipidated cytochrome *c* oxidase, and another peak at 341.5 K, about 4.7 K higher than the maximal endothermic denaturation temperature of the asolectin-reconstituted cytochrome *c* oxidase. It is possible that the two endothermic transition peaks observed in the cytochrome *c* oxidase/DOPC vesicle result from the overlap of a broad endothermic transition with a rather weak exothermic transition occurring at around 335 K. The thermotropic properties of various phospholipid vesicles of cytochrome *c* oxidase are summarized in Table III.

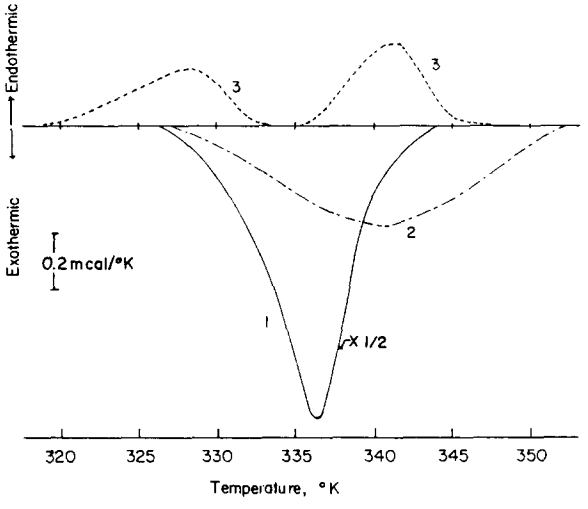

Fig. 6. DSC thermograms for various cytochrome *c* oxidase/phospholipid vesicle preparations. Delipidated cytochrome *c* oxidase (10 mg/ml) in 50 mM Na/K phosphate buffer (pH 7.4) was mixed with the indicated phospholipids in the presence of 1.5% sodium cholate at 1 mg phospholipid per mg protein. The mixtures were dialyzed extensively against 50 mM phosphate buffer (pH 7.4). The cytochrome *c* oxidase/phospholipid vesicles were collected by centrifugation and suspended in 50 mM phosphate buffer (pH 7.4) to a protein concentration of 50 mg/ml. 62-μl aliquots were used for DSC measurement. Curves 1, 2 and 3 represent the DLPC/, DPG/ and DOPC/ cytochrome *c* oxidase vesicles, respectively.

Since phospholipids such as asolectin, DPG, DLPC and DOPC all contain unsaturated fatty acyl groups, it is possible that the unsaturated fatty acyl groups in these phospholipids are responsible for the exothermic transition in the cytochrome *c* oxidase/phospholipid vesicles. This exothermic transition could not result from the chemical decomposition of the cytochrome *c* oxidase protein or the phospholipid components, as it was observed only when cytochrome *c* oxidase was embedded in the phospholipid vesicles, not when cytochrome *c* oxidase/phospholipid complex was in the dispersed form. Both preparations contain the same amount of phospholipids. One possible source of the energy for this exothermic transition is the collapse of a 'meta-stable' state upon the thermodenaturation of protein. This 'meta-stable' state could be created by a thermodynamically unfavorable interaction between a portion of the protein surface and the surrounding phospholipids during the formation of the cytochrome *c* oxidase/phospholipid vesicles. The production of such a meta-stable state during vesicle formation would depend upon both protein and the fatty acyl components of phospholipids. For instance, the cytochrome *c* oxidase/DLPC vesicle shows an endothermic transition, whereas the cytochrome oxidase/DPPC vesicle exhibits only an endothermic transition of protein thermodenaturation.

Thermotropic properties of the cytochrome c and cytochrome c oxidase complex

It has been observed [16] that formation of a complex between cytochrome *c* and c_1 at low ionic strength causes a significant destabilization of the

former and a slight stabilization of the latter toward thermodenaturation. It is, therefore, of interest to investigate the effect of complex formation between cytochrome *c* and cytochrome *c* oxidase on the thermotropic properties of these two enzymes. Since cytochrome *c* oxidase requires phospholipids for activity, the effect of phospholipids on the thermotropic properties of cytochrome *c* in the cytochrome *c*/phospholipid mixture was first examined by DSC. As indicated in Fig. 7, curve 2, the presence of phospholipids caused cytochrome *c* to become more labile toward thermodenaturation. The DSC thermogram of the cytochrome *c*-phospholipid complex shows both exo- and endothermic transitions. The endothermic transition temperature for cytochrome *c* in the presence of phospholipids was 341 K, compared to 357 K, measured in the absence of phospholipid. The exothermic transition was found at 321 K. When cytochrome *c* was complexed with cytochrome oxidase in the presence of phospholipids little effect was observed on the exothermic transition temperature, but the enthalpy change was significantly increased. The endothermic denaturation temperature was further decreased to 335 K. This is about the same as the endothermic denaturation temperature of the cytochrome *c* oxidase-phospholipid complex, suggesting that the complex formation between cytochrome *c* and cytochrome *c* oxidase resulted in no significant conformational change of the cytochrome *c* oxidase entity. Formation of the cytochrome *c* oxidase-cytochrome *c* complex in the absence of phosholipids also show little effect on the thermodenaturation of the former. Whether or not the formation of a cytochrome *c*-cytochrome *c* oxidase complex is a prerequisite for electron transfer remains an open question, although the formation of, at least, a transient complex is probably physiologically important for the electron transfer from cytochrome *c* to cytochrome *c* oxidase. Favorable evidence for the formation of a complex between cytochrome *c* and cytochrome *c* oxidase has been obtained from binding kinetic data [13] and structural studies with cross-linking reagents [17]. At present we offer no explanation for the destabilization of cytochrome *c* but not cytochrome *c* oxidase during their complex formation.

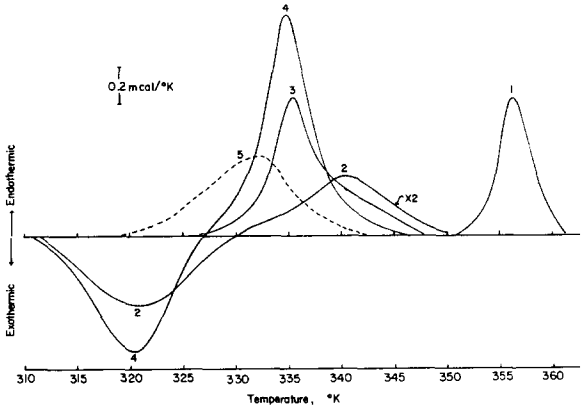

Fig. 7. DSC thermograms of cytochrome *c*, cytochrome *c*-phospholipid complex, cytochrome *c* oxidase-phospholipid complex, the cytochrome *c*-cytochrome *c* oxidase-phospholipid complex and the cytochrome *c*-cytochrome *c* oxidase complex. Curve 1 is cytochrome *c* [12]; curve 2, cytochrome *c*-asolectin complex (4.8:12); curve 3, cytochrome *c* oxidase-asolectin complex (35.2:12); curve 4, the cytochrome *c*-cytochrome *c* oxidase-asolectin complex (4.8:35.2:12) and curve 5, the cytochrome *c*-cytochrome *c* oxidase complex (2.4:35.2). The numbers given in the parentheses are the concentrations of proteins or phospholipid used. 62-μl aliquots were used. the DSC settings are the same as those given in the Fig. 1.

Acknowledgements

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