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Studies of protein-phospholipid interaction in isolated mitochondrial ubiquinone-cytochrome *c* reductase

Sung-Hee Gwak, Linda Yu and Chang-An Yu *

Department of Biochemistry, Oklahoma State University, Stillwater, OK 74078 (U.S.A.)

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The interaction between phospholipids, ubiquinone and highly purified ubiquinol-cytochrome *c* reductase was studied using differential scanning calorimetry. The enzyme complex and its delipidated forms undergo thermodenaturation at 337.3 and 322.7 K, respectively. The reduced reductase is more stable toward thermodenaturation than is the oxidized enzyme. While phospholipids restored enzymatic activity to the delipidated enzyme complex and stabilized the enzyme toward thermodenaturation, ubiquinone showed little effect on the thermostability of ubiquinol-cytochrome *c* reductase. The effect of phospholipids on the thermotropic properties of ubiquinol-cytochrome *c* reductase is dependent upon the molecular properties of the phospholipid. When ubiquinol-cytochrome *c* reductase was embedded in closed asolectin vesicles, an exothermic transition peak was observed upon thermodenaturation. When the asolectin concentration in the reconstituted preparation was less than 0.3 mg/mg protein, an amorphous structure was observed in the electron micrograph and the preparation showed an endothermic transition upon thermodenaturation. The thermotropic properties of the enzyme-phospholipid vesicles were affected by the phospholipid head groups as well as the fatty-acyl chains, with those phospholipids having the most highly unsaturated fatty-acyl chains having the greatest effect. The energy for the exothermic transition may be derived from the collapse, upon thermodenaturation, of a strained interaction between the unsaturated fatty-acyl groups of phospholipids and protein molecules resulting from vesicle formation. The exothermic transition of the enzyme-phospholipid vesicle was abolished when cholesterol was included in the vesicles and when reductase was treated with a proteolytic enzyme prior to incorporation into the phospholipid vesicles.

Introduction

Ubiquinol-cytochrome *c* reductase is a segment of the mitochondrial respiratory chain which catalyzes electron transfer from ubiquinol to cytochrome *c* coupled with proton translocation. This complex is the most complicated and the least understood among the four resolved mitochondrial electron-transfer complexes. The highly purified enzyme (the cytochrome b-c_1 III complex) [1] con-

* To whom correspondence should be addressed.

Abbreviations: DSC, differential scanning calorimetry; DPG, diphosphatidylglycerol or cardiolipin; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; PLPC, 1-palmitoyl-2-linoleoylphosphatidylcholine; DLPC, dilinoleoylphosphatidylcholine; DSPG, distearoylphosphatidylglycerol; DSPA, distearoylphosphatidic acid; DOPG, dioleoylphosphatidylglycerol; DOPC, dioleoylphosphatidylcholine; DOPA, dioleoylphosphatidic acid.

tains 7–8 protein subunits, ubiquinone and 20% phospholipids, by weight. The major phospholipids present in this enzyme complex are phosphatidylcholine, phosphatidylethanolamine and diphosphatidylglycerol in a molar distribution of 40, 30 and 20%, respectively [2]. When ubiquinol-cytochrome *c* reductase was embedded in phospholipid vesicles, proton translocation activity was observed [3]. The absolute requirement for phospholipids for enzymatic activity and for maintenance of the structural integrity of this enzyme complex was demonstrated by delipidation and replenishment of phospholipids [4,5] and by circular-dichroism spectroscopy [6] studies. The phospholipids in ubiquinol-cytochrome *c* reductase can be reversibly removed by several methods: repeated ammonium-sulfate precipitation in the presence of cholate [6], Phenyl-Sepharose [7], and Sephadex LH-20 or hydroxyapatite column chromatography in the presence of Triton X-100 [8]. The resulting delipidated preparation contains about 2–7.5% phospholipids, depending on the method used, and the residual phospholipids are tightly bound cardiolipins [4,9]. The delipidated reductase is enzymatically inactive. Addition of phospholipids such as asolectin, mitochondrial phospholipids, or of phospholipids prepared from ubiquinol-cytochrome *c* reductase, completely restored enzymatic activity to the delipidated preparation. However, the reconstitutive ability of the delipidated reductase, unlike that of delipidated cytochrome *c* oxidase, is unstable, with a half-life of about 2 h at 0°C. Due to the unstable nature of the delipidated enzyme and the complexity of the purification procedure, studies of the phospholipid-protein interaction and the phospholipid-Q-protein interaction in this segment of the electron-transfer chain are rather insufficient. Recently, we have employed the differential scanning calorimetry, a useful technique for probing the conformational transitions of biomembranes and lipoprotein complexes, to study the lipid-protein interaction in ubiquinol-cytochrome *c* reductase. In this paper, we report the thermotropic properties of intact and delipidated ubiquinol-cytochrome *c* reductases, and the reductase embedded in different phospholipid vesicles.

Experimental procedures

Materials. Dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), dimyristoylphosphatidylcholine (DMPC), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), 1-palmitoyl-2-linoleoylphosphatidylcholine (PLPC), dilinoleoylphosphatidylcholine (DLPC), distearoylphosphatidylglycerol (DSPG), distearoylphosphatidic acid (DSPA), dioleoylphosphatidylglycerol (DOPG), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidic acid (DOPA), diphosphatidylglycerol or cardiolipin (DPG), sodium cholate, cholesterol and protease form *Streptomyces griseus* are products of Sigma. Asolection was obtained from Associated Concentrates Inc.

Preparations. A highly purified bovine heart mitochondrial ubiquinol-cytochrome *c* reductase and its phospholipid-depleted form were prepared according to the reported methods [1,6]. The ubiquinol-cytochrome *c* reductase activity was assayed in a Cary spectrophotometer, model 219, at room temperature using Q_2H_2 as substrate. Q_2 and Q_2H_2 were synthesized in this laboratory according to the reported method [10]. The enzyme preparations were dissolved in 50 mM Na/K phosphate buffer (pH 7.4), containing 10% glycerol and stored at −80°C.

Reconstitution methods. Phospholipids were incorporated into the delipidated ubiquinol-cytochrome *c* reductase by several methods: sonication [11], direct incorporation [12], freeze–thaw and sonication [13], and cholate dialysis [14]. The cholate-dialysis method was performed as follows: 5.3 mg of the delipidated ubiquinol-cytochrome *c* reductase in 50 mM Na/K phosphate buffer (pH 7.4), containing 10% glycerol was mixed with the indicated concentraiton of the phospholipid micellar solution (20 mg/ml in 50 mM phosphate buffer, pH 7.4) and sodium cholate. The mixture was adjusted to a total volume of 1.8 ml and a concentration of 1% sodium cholate, then dialyzed against 50 mM potassium phosphate buffer (pH 7.4), overnight, with four changes of buffer. After dialysis, the phospholipid reductase complex was collected by centrifugation at $80\,000 \times g$ for 1 h and suspended in 0.2 ml 50 mM phosphate buffer (pH 7.4).

The cholesterol-containing asolectin-reductase vesicle was prepared by mixing the delipidated ubiquinone-cytochrome *c* reductase with a sonicated mixture of asolectin and cholesterol (1 mol cholesterol per 2 mol asolectin) in the presence of 1% cholate, followed by extensive dialysis against 50 mM phosphate buffer (pH 7.4).

Proteolytic enzyme digestion of the delipidated ubiquinol-cytochrome c reductase. Delipidated reductase (15 mg/ml), in 50 mM phosphate buffer (pH 7.4), containing 10% glycerol, was treated with protease from *Streptomyces* for 30 min at room temperature. The ratio of protease to protein used was 0.02 (w/w).

Differential scanning calorimetry. All calorimetric measurements were performed with a Perkin Elmer DSC-2 equipped with a Haake constant temperature bath. The bath temperature was set at 5°C unless otherwise stated. A 65 μl sample in a large volume capsule was placed in the sample holder and the same amount of 50 mM sodium potassium phosphate buffer (pH 7.4), was placed in the reference holder. The recorder scanning speed was 100 s per inch. All differential calorimetry scans in this paper were performed at a rate of 2.5 K/min and a sensitivity of 0.4 mJ/s. After the first scan, the samples were cooled to the original temperature, and rescanned again. Since after the first can the protein is completely and irreversibly denatured, therefore, no thermotransiton peaks was observed in the second scan, and it can thus be used as a baseline. In the protein/lipid reconstitution studies, the sample concentrations were based on protein. A concentration of 26.7 mg/ml was routinely used. The temperature at the peak of the exo- or endothermogram was recorded as T_m, without correction. The enthalpy change of thermodenaturation was calculated from the area covered by the peaks in the endo- or exothermogram. The DSC-2C was calibrated with indium, and chekced with a DPPC-H_2O suspension, assuming the enthalpy change of the DPPC phase transition to be 35.6 kJ/mol [15].

Electron microscopy. Phospholipid-reconstituted ubiquinol-cytochrome *c* reductase preparations containing 25% glycerol were used for freeze-fracture [16] and quenched from 25°C. They were fractured and visualized using a Balzero freeze-fracture apparatus and a Philips 200 electron microscope.

Additional analytical methods. The protein concentration was determined by the biuret method, using bovine serum albumin as the standard [17]. Absorption spectra were measured in a Cary spectrophotometer, model 219. Phospholipids in the enzyme complex were extracted and quantitated by total phosphorous [18]. Activity of the phospholipid-reconstituted reductase was assayed according to the reported method [6].

Results

Thermotropic properties of intact, delipidated, and proteolytic enzyme digested ubiquinol-cytochrome c reductase preparations

The highly purified ubiquinol-cytochrome *c* reductase (the cytochrome *b*-c_1 III complex) is in the dispersed form as the preparation contains a residual amount of the detergent used during isolation. It contains about 20% phospholipids by weight and catalyzes antimycin-A-sensitive electron transfer from ubiquinol to cytochrome *c* with a specific activity of 160 μmol cytochrome *c* reduced per min per mg protein at 23°C [1]. The electron-transfer activity of ubiquinol-cytochrome *c* reductase was abolished upon removal of phospholipids from the preparation, and could be completely restored by replenishing phospholipids (asolectin or mitochondrial phospholipids) to the delipidated preparation [6].

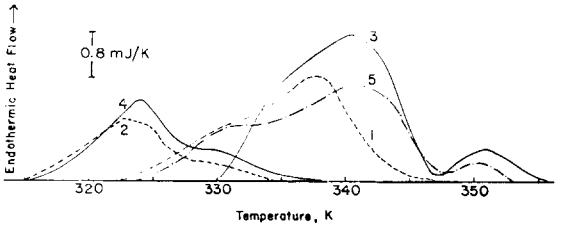

Fig. 1. DSC thermograms of intact and delipidated ubiquinol-cytochrome *c* reductases. 65 μl aliquots of intact (curve 1), 11.2 mg/ml, and delipidated (curve 2), 18.8 mg/ml, preparations in 50 mM sodium potassium phosphate buffer (pH 7.4) were placed in a large volume capsule and 65 μl of sodium sodium phosphate buffer (pH 7.4) was used as reference. Curves 3 and 4 represent the dithionite-reduced form of intact and delipidated reductases, respectively. Curve 5 represents the ascorbate-reduced form of the intact preparation. The DSC scannings were taken from 305 K to 370 K, with a heating rate of 2.5 K per min and sensitivity of 0.4 mJ/s.

Fig. 1 shows the differential scanning calorimetric (DSC) thermograms of intact and delipidated ubiquinol-cytochrome *c* reductases. The intact reductase showed a relatively broad DSC thermogram with an endothermic transition at 337.3 K and a shoulder at 330 K (curve 1 of Fig. 1). The enthalpy change for the endothermodenaturation of the intact preparation was calculated to be 1775 kJ per mol cytochrome c_1 in the complex. When the phospholipids in the reductase were removed, the delipidated preparation became less stable toward thermodenaturation, showing an endothermic transition at 322.7 K with a shoulder at 324.5 K (curve 2 of Fig. 1). The enthalpy change for the endothermodenaturation of the delipidated preparation was calculated to be 921 kJ per mol of the complex, based on cytochrome c_1. These results indicate that phospholipids protect ubiquinol-cytochrome *c* reductase from thermodenaturation. These results are consistent with the previous findings [6] that phospholipids play an important role in maintaining the structural integrity of ubiquinol-cytochrome *c* reductase.

The reduced form of ubiquinol-cytochrome *c* reductase, either in the phospholipid-containing or phospholipid-depleted preparation, was more stable toward thermodenaturation than the comparable oxidized enzyme. When intact reductase was reduced either by dithionite or by ascorbate, the DSC thermogram of the reduced enzyme showed two endothermic transition peaks: one broad peak at 340 K, about 2.7 K higher than the endothermic denaturation temperature of the oxidized enzyme, and another small peak at 350.8 K (see curves 3 and 5 of Fig. 1). Although similar endothermic transition temperatures were observed for the ascorbate reduced- and the dithionite reduced-reductases, the enthalpy change for the thermodenaturation of the latter (2469 kJ/mol) was larger than that of the former (2013 kJ/mol), indicating that reduction of cytochrome *b* in ubiquinol-cytochrome *c* reductase significantly increases the thermostability of the enzyme complex. When the delipidated reductase was reduced by dithionite (curve 4 of Fig. 1), the endothermic denaturation temperature was shifted 1.5 K upward and the enthalpy change for the thermodenaturation was increased about 84 kJ/mol, as compared to the oxidized enzyme. These results complement the observation that reduced ubiquinol-cytochrome *c* reductase was more stable toward detergent treatment than was the oxidized enzyme [19]. Since no double thermodenaturation peaks were observed in the DSC thermograms of the reduced or oxidized delipidated preparations or the oxidized intact enzyme, the second thermodenaturation peak observed in the reduced intact preparation probably results from the collapse of a special lipid-protein interaction which occurred only in the reduced enzyme. Nevertheless, the nature of the second thermodenaturation peak of the reduced intact sample remains to be clarified.

It should be mentioned that all the endothermic transitions disappeared after the samples were subjected to DSC scanning. This indicates that all the transitions are irreversible and due to the thermodenaturation of the proteins in the reductase.

When the delipidated ubiquinol-cytochrome *c* reductase was treated with protease (2% by weight) at room temperature for 30 min before DSC scanning, the digested enzyme complex showed no thermotransition peaks between 300 and 360 K. This suggests that the secondary and tertiary structure of the enzyme complex was completely collapsed by the protease treatment. This observation differs from the results observed with cytochrome *c* oxidase, which showed only a slight change in the endothermic transition temperature after the enzyme complex was treated with protease [20].

Table I summarizes the thermotropic properties of ubiquinol-cytochrome *c* reductases under various conditions.

Effect of the phospholipid incorporation methods on the thermotropic properties of the asolectin-reconstituted ubiquinol

Since asolectin has been shown to be as effective as phospholipids prepared from bovine heart mitochondria or from intact ubiquinol-cytochrome *c* reductase in restoring enzymatic activity to the delipidated reductase [6], it was chosen to use for the study of the incorporation methods. Fig. 2 shows the DSC thermograms of the asolectin-reconstituted ubiquinol-cytochrome *c* reductases prepared by the sonication [11], direct incorporation [12], freeze–thaw and sonication [13], and cholate-dialysis methods [14], at an asolectin-to-protein ratio of 2, by weight. Regardless of the

TABLE I

EFFECT OF OXIDATION, REDUCTION AND Q ON THERMAL STABILITY OF UBIQUINOL-CYTOCHROME *c* REDUCTASE

The reconstituted enzyme was prepared by the cholate-dialysis method. Oxi, oxidation; red, reduction; QCR, quinone-cytochrome *c* reductase.

QCR	mg phospholipids/mg protein		T_m (K)	ΔH (kJ/mol c_1)
Intact	0.19	oxi	337.3	1774
		red [a]	340.7	2469
Delipidated	0.02	oxi	322.7	921
		red	324.2	1003
Reconstituted (QCR + Azo)	0.25	oxi	341.8	810
		red	344.3	1350
	1.50	oxi	332.5	−4861
		red	331.2	−2238
Reconstituted (QCR + Azo + Q)	0.25	No Q	341.8	818
		Q [b]	341.9	857
	1.50	No Q	332.5	−4861
		Q [b]	330.0	−5710

[a] Enzyme was reduced by $Na_2S_2O_4$.

[b] 5 mole Q per mole c_1 (6.7 nmol c_1/mg protein) was used for reconstitution.

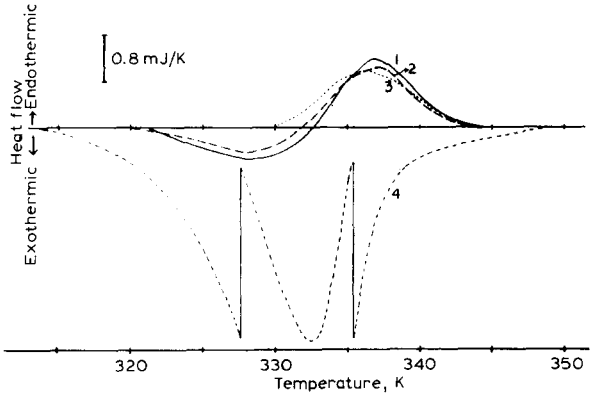

Fig. 2. DSC thermograms of the asolectin-reconstituted ubiquinol-cytochrome *c* reductase prepared by different phospholipid incorporation methods. The concentrations of asolectin and the delipidated reductase in the reconstituting system were 53.4 mg/ml and 26.7 mg/ml, respectively, in 50 mM sodium potassium phosphate buffer (pH 7.4). Curves 1, 2, 3 and 4 represent the asolectin-reductase complex prepared by the sonication, direct incorporation, freeze–thaw and sonication, and cholate-dialysis methods, respectively. The DSC settings are as given in Fig. 1.

methods used to incorporate asolectin into the delipidated ubiquinol-cytochrome *c* reductase, all the asolectin-reconstituted preparations were more stable toward thermodenaturation than was the delipidated preparation. However, the DSC thermograms of these asolectin-reconstituted preparations varied significantly. When asolectin was incorporated into the delipidated reductase by the sonication method, the DSC thermogram of the resulting asolectin-reductase complex showed two thermodenaturation peaks: one relatively broad exothermic transition peak at 329 K, about 6.3 K higher than the endothermic transition temperature of the delipidated enzyme, and an endothermic transition peak at 337.5 K. When asolectin was incorporated into the delipidated reductase by direct incorporation, the resulting preparation showed a DSC thermogram almost identical to that of the sample prepared by the sonication method, except that the former has a slightly higher endothermic transition temperature (0.5 K) than the latter. However, when the phospholipids were incorporated into the delipidated reductase by the freeze–thaw and sonication method, the resulting

complex showed only an endothermic transition with the endothermodenaturation temperature at 335.5 K, which is slightly lower than that of the asolectin-reductase complex prepared either by sonication method or by the direct incorporation method. Surprisingly, when asolectin was incorporated into the delipidated reductase by the cholate-dialysis method, the resulting preparation exhibited a huge exothermic denaturation peak at 332.5 K. This is about 9.8 K higher than the endothermic denaturation temperature of the delipidated reductase, and about 3.5 K higher than the exothermic denaturation temperature observed in the asolectin-reductase complexes prepared by the sonication or direct incorporation methods.

It is possible that the different thermotropic properties of the asolectin-reductase complexes prepared by different phospholipid incorporation methods result from differences in the physical contact between phospholipid and ubiquinol-cytochrome *c* reductase proteins. This deduction was substantiated by electron microscopy. When these asolectin-reconstituted preparations, with a phospholipids-to-protein ratio larger than 0.5, were examined by electron microscopy, a tight vesicle structure was found only in the asolectin-reductase complex prepared by the cholate-dialysis method (see Fig. 3B). When low azolectin to protein ratio (0.3) was used in the reconstitution an amorphous structure (Fig. 3A) was observed and the preparation showed only endothermic transition upon thermodenaturation. The exothermic DSC thermogram, which is unusual for the thermodenaturation

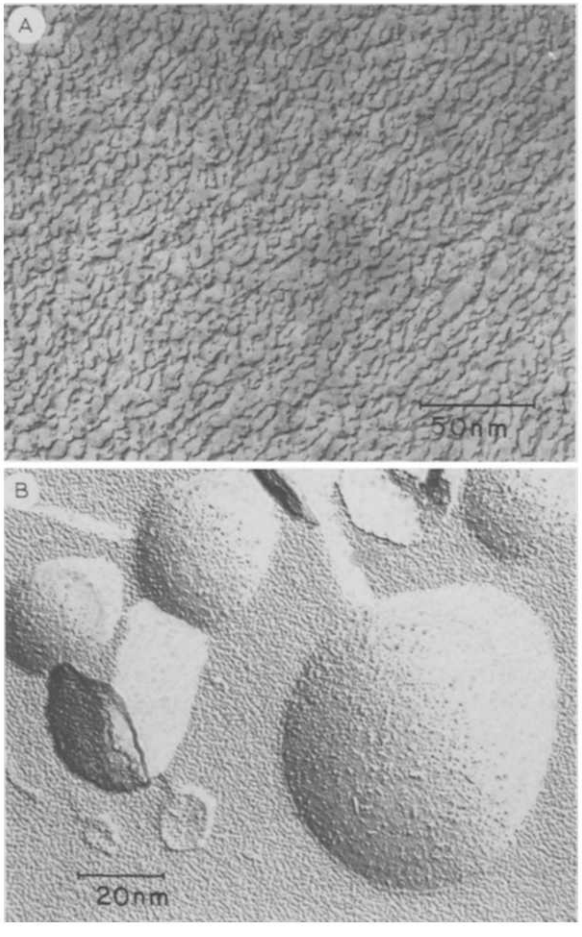

Fig. 3. Freeze-fracture electron microscopy of the asolectin-reductase complex containing different asolectin concentrations. The asolectin-reductase complexes were prepared by thecholate-dialysis method. The asolectin concentrations in the reconstituting systems were 0.3 (A) and 0.8 mg/mg protein (B).

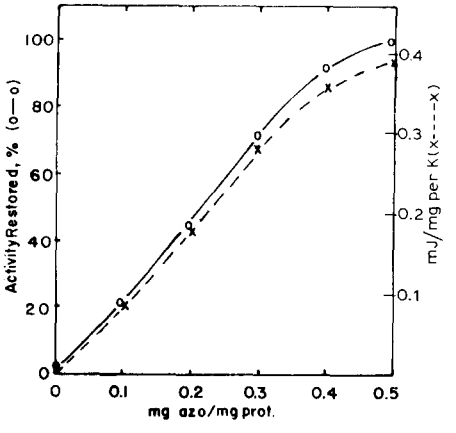

Fig. 4. Effect of the phospholipid concentrations on the enzymatic activity and enthalpy changes at 333 K upon thermodenaturation of the asolectin-reductase complexes. 2-ml aliquots of the delipidated reductase, 2.0 mg/ml in 50 mM sodium potassium phosphate buffer (pH 7.4) were incubated with the indicated concentrations of asolectin in the presence of 1% sodium cholate for 10 min and then diluted with 8 ml of phosphate buffer. The samples were then subjected to 4 h centrifugation at 100000 × *g*. The reductase-azolectin complexes were collected as precipitates and redissolved in 0.4 ml of 50 mM phosphate buffer containing 0.5% sodium cholate. The resulting asolectin-reductase complexes were determined for ubiquinol-cytochrome *c* reductase activity (O——O) using Q_2H_2 as substrate. The enthalpy change of thermodenaturation at 333 K (×——×) was determined by DSC using the same conditions as given in Fig. 1.

of a protein complex, was also observed for cytochrome *c* oxidase embedded in asolectin vesicles [20].

Effect of asolectin concentrations on the enzymatic activity and thermotropic properties of the reconstituted ubiquinol-cytochrome c reductase

Fig. 4 shows the correlation between the enzymatic activity and the endothermic transition enthalpy changes at 333 K of the azolectin-reductase complexes formed at low azolectin to protein ratios. The phospholipid concentrations in the reconstituted preparations varied from 0 to 0.5 mg phospholipid per mg protein. The asolectin-reductase complexes were prepared by the direct incorporation method. As the phospholipid in the reconstituted complex progressingly increased, the endothermic enthalpy change at 333 K increased and the enthalpy change at 323 K decreased. The increased in enthalpy change at 333 K is proportional to the activity restoration. The maximal activity restoration was reached when 0.5 mg azolectin per mg protein was used.

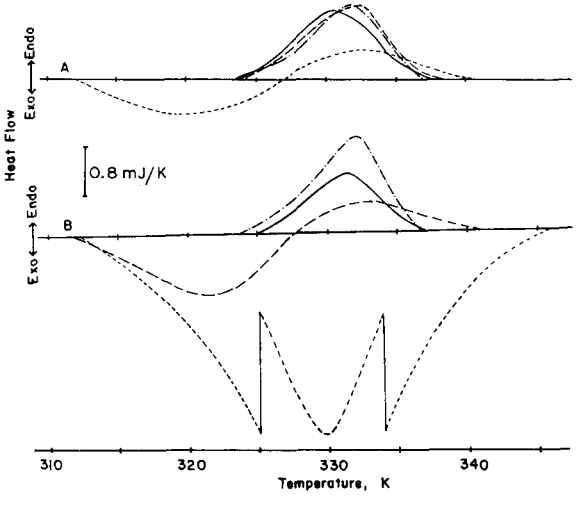

Fig. 5. The DSC thermograms of the delipidated ubiquinol-cytochrome *c* reductase reconstituted with phosphatidylcholine containing different unsaturated fatty acyl groups. The delipidated reductase in 50 mM sodium potassium phosphate buffer (pH 7.4) was reconstituted with DPPC (———), POPC (·–·–·), PLPC (— — —) and DLPC (- - - - -) by the cholate-dialysis method. The phospholipid concentrations were 0.2 (A) and 0.8 mg/mg protein (B), respectively. The protein concentration in samples was 26.7 mg/ml. 65-μl aliquots of samples were used and the DSC settings were as in Fig. 1.

The effect of the unsaturation and chain length of the fatty-acyl groups in the phospholipid molecule on the thermotropic properties of the phospholipid-reconstituted ubiquinol-cytochrome c reductase

When phosphatidylcholines, such as DPPC, POPC, PLPC and DLPC, which possess different degrees of unsaturation in the fatty-acyl groups, were reconstituted with the delipidated reductase, the cholate-dialysis method, at phospholipid-to-protein ratios of 0.2 and 0.8 (w/w), the resulting phospholipid-reductase complexes showed significant differences in the DSC thermograms (see Fig. 5A and B). The DPPC-reductase or the POPC-reductase complexes, regardless of the phospholipid concentration in the system, showed endothermic DSC thermograms. The endothermic transition temperature of the DPPC-reductase or the POPC-reductase complexes increased as the phospholipid concentration in the system increased. When a low concentration of PLPC was used (0.2 mg per mg protein), the resulting PLPC-reductase complex showed an endothermic transition peak at 332.7 K. When the PLPC concentration was increased to 0.8 mg per mg protein, the resulting preparation showed two broad thermodenaturation peaks: one exothermic peak at 321.7 K, and an endothermic

TABLE II

EFFECT OF FATTY-ACID COMPOSITION AND POLAR HEAD GROUP OF PHOSPHOLIPID ON T_m AND ΔH OF RECONSTITUTED UBIQUINOL-CYTOCHROME *c* REDUCTASE

The enzyme concentration for DSC measurement was 26.7 mg/ml in 50 mM potassium phosphate buffer (pH 7.4). PL, phospholipid. Pd, thermodenaturation peak of the enzyme disappeared.

PL used for reconstitution	PL/protein (mg/mg)	T_m (K)	ΔH (kJ/mol c_1)
DMPC	0.6	331.4	648
DPPC	0.6	331.7	791
DSPC	0.6	331.3 [a]	543 [a]
DSPG	0.8	Pd	Pd
DSPC	0.8	332.5 [a]	347 [a]
DSPA	0.8	332.5	636
DOPG	0.8	337.7	1043
DOPC	0.8	331.9	1050
DOPA	0.8	346.9	1512

[a] Some part of thermodenaturation curve of the enzyme was overlapping with the peak from DSPC.

peak at 332.7 K. When low concentrations of DLPC (0.2 mg/mg protein) were used, the reconstituted preparation showed a DSC thermogram nearly identical to that obtained for the PLPC-reductase vesicle containing 0.8 mg PLPC per mg protein. However, when the DLPC concentration in the reconstituted system was increased to 0.8 mg per mg protein, the preparation showed a large exothermic transition at 329.4 K.

When phosphatidylcholines, such as DMPC, DPPC and DSPC, which possess different fatty-acyl chain lengths, were reconstituted with delipidated reductase, the resulting phospholipid-reductase vesicles showed almost identical endothermic transition temperatures (see Table II). The enthalpy changes for thermodenaturation of the DMPC-, DPPC- and DSPC-reductase vesicles were 649, 791 and 545 kJ/mol reductase, respectively. This result suggests that the chain length of the fatty-acyl groups has little effect on the thermotropic properties of the phospholipid-reductase vesicles.

The effect of the phospholipid head group on the thermotropic properties of the phospholipid reductase vesicles

When different kinds of phospholipids, such as DSPC and DSPA, which possess the same saturated fatty-acyl groups, were used for preparation of the phospholipid-reductase vesicle, the resulting preparations showed identical endothermic transition temperatures of 332.5 K (see Table II). The enthalpy change for thermodenaturation of the DSPA-reductase vesicle, however, was about double that obtained for the thermodenaturation of the DSPC-reductase vesicle. When DSPG was used for reconstitution, the resulting DSPG-reductase vesicle showed no thermotransition peaks between 300 and 360 K. It is possible that the lack of thermotransition peaks observed for the DSPG reductase vesicle results from the cancellation of an exothermic transition peak with an identical endothermic transition peak.

When different kinds of phospholipids, such as DOPG, DOPC and DOPA, which possess the same unsaturated fatty-acyl groups, were incorporated into the delipidated reductase, the resulting phospholipid-reductase vesicles varied in their thermotropic properties (see Table II). The endothermic denaturation temperatures of these phospholipid-reductase vesicles descreased as the negative charge on the head group of the phospholipid decreased, in the following order: DOPA, DOPG, DOPC. This tendency was not observed in the phospholipid-reductase vesicles formed with different phospholipids containing the same saturated fatty acyl groups, suggesting that the effect of the phospholipid head group on the thermotropic properties of the phospholipid-reductase vesicles depended on the unsaturated fatty-acyl groups.

Effect of phospholipid mixtures on the thermotropic properties of the phospholipid-reductase vesicle

Table III compares the thermotropic properties of delipidated reductase reconstituted with single and mixed phospholipids. When DPPC and DLPC, at a phospholipid concentration of 0.8 mg per mg protein, were separately reconstituted with the delipidated reductase, the resulting DPPC-reductase vesicle showed an endothermic transition peak at 331.9 K, with an enthalpy change for the thermodenaturation of 930.0 kJ/mol. The DLPC-reductase vesicle showed an exothermic transition temperature at 329.4 K, with an enthalpy change for thermodenaturation of −1691 kJ/mol. When a mixture of DPPC and DLPC was used in the reconstituting system, the resulting DPPC-DLPC-reductase vesicles, regardless of the DPPC-to-DLPC ratios in the vesicle, showed an exothermic transition, with an enthalpy change of thermodeaturation greater than that of the DLPC-reductase vesicle. This result suggests that DPPC may not be homogeneously distributed in

TABLE III

T_m AND ΔH CHANGE OF UBIQUINOL-CYTOCHROME *c* REDUCTASE-PHOSPHOLIPID VESICLES

PL, phospholipids.

PL_1/PL_2/ mg protein	DPPC/DLPC		DPG/DLPC	
	T_m (K)	ΔH (MJ/mol)	T_m (K)	ΔH (MJ/mol)
0.8/0.0	331.9	0.920	340.0	1.177
0.0/0.8	329.3	−1.689	329.4	−1.689
0.8/0.8	330.4	10.764	330.2	−29.166
1.6/0.8	331.8	11.119	330.4	−19.280

the reconstituting system, and that DLPC interacts preferentially with the delipidated reductase at these areas which produced an exothermic transition upon thermodenaturation. The DPPC, however, binds equally or even preferentially to DLPC with the delipidated reductase at the areas having higher phospholipid affinity.

Cardiolipin is one of the major phospholipids in ubiquinol-cytochrome *c* reductase, and is the last one to be removed from the reductase during the delipidation process. The residual phospholipids (less than 2%) in the delipidated reductase are mainly cardiolipin, indicating the preferential binding of cardiolipin to the reductase. When cardiolipin from bovine heart mitochondria, 0.8 mg per mg protein, was used in the reconstituting system, the resulting cardiolipin-reductase vesicle showed an endothermic transition temperature of 340 K with an enthalpy change of thermodenaturation of 117 kJ/mol. When a mixture of equal concentrations of cardiolipin and DLPC was used in the reconstituting system, the resulting cardiolipin-DLPC-reductase vesicle showed an exothermic transition at 330.2 K with an enthalpy change for thermodenaturation of -29.166 MJ/mol, which is about 17 times the exothermic enthalpy change for the thermodenaturation of the DLPC-reductase vesicle. When the cariolipin-to-DLPC ratio in the cardiolipin-DLPC-reductase vesicle was increased to 2, a decrease in the exothermic enthalpy change for thermodenaturation was found. This differs from the thermotransitions obtained using a mixture of DPPC and DLPC for reconstitution, in which the exothermic enthalpy change for thermodenaturation of the DPPC-DLPC-reductase vesicle increased as the DPPC-to-DLPC ratio increased. The lowered exothermic enthalpy change observed for the thermodenaturation of the cardiolipin-DLPC-reductase vesicle formed with a higher cardiolipin-to-DLPC ratio, as compared to the enthalpy change for thermodenaturation of those vesicle formed with lower cardiolipin-to-DLPC ratios, indicates that cardiolipin interacts with the delipidated reductase preferentially at the native phospholipid binding sites. DLPC is more likely to be located at those protein surface areas where protein-lipid interaction is less favorable and occurs only when a closed vesicle is formed. When the concentration of cardiolipin is higher than that of DLPC, part of the cardiolipin may interact at sites on the protein surface, where interaction with DLPC is favored when cardiolipin concentration is low, thus decreasing the binding of DLPC to those particular protein surface areas.

Effect of ubiquinone and cholesterol on the thermotropic properties of the phospholipid-reductase vesicles

When ubiquinone-10 alone, or in combination with asolectin, was incorporated into the delipidated reductase by the cholate-dialysis method, the resulting Q-containing preparation had thermotropic properties nearly identical to those of the complex prepared in the absence of Q (see Table I). This result suggests that Q exerts little effect on the interaction between phospholipids and ubiquinol-cytochrome *c* reductase protein, and exerts no protection of ubiquinol-cytochrome *c* reductase toward the thermodenaturation. This result is in line with the fact that Q has little effect on the stabilization of the ubiquinol-cytochrome *c* reductase activity [6].

Cholesterol has been reported to act as a plasticizer in the lipid bilayer. It reduces the freedom of motion of the lipid hydrocarbon chains in the liquid crystal state above the transition temperature, and thus abolishes phospholipid phase transition [21–23]. It is of interest to see whether or not cholesterol affects the interaction between re-

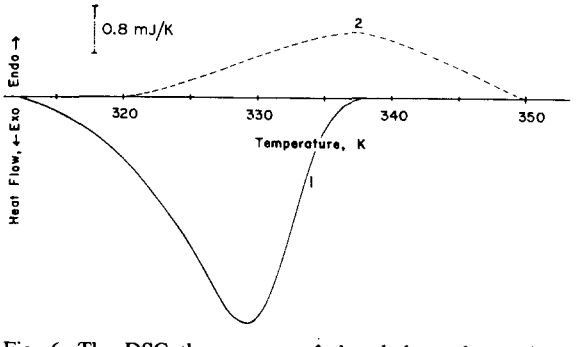

Fig. 6. The DSC thermogram of the cholesterol containing asolectin-reductase vesicle. The delipidated reductase in 50 mM sodium potassium phosphate (pH 7.4) was embedded in asolectin vesicles by the cholate-dialysis method in the presence (curve 2) and absence (curve 1) of cholesterol. 1 mg asolectin per mg protein, and 1 mol of cholesterol per 2 mol asolectin was used. 65-μl samples, 26.7 mg/ml, were used for the DSC scanning. The DSC settings were as in Fig. 1.

ductase and phospholipids. Fig. 6 shows the DSC thermograms for the asolectin-reductase vesicles prepared in the presence and absence of cholesterol. The cholesterol-containing asolectin-reductase vesicle, 1 mol cholesterol per 2 mol of asolectin, showed a broad endothermic DSC thermogram while the asolectin-quinone-cytochrome *c* reductase vesicle, in the absence of cholesterol, showed an exothermic DSC thermogram, indicating that cholesterol affects the interaction between reductase and phospholipids. The endothermic denaturation temperature of the cholesterol-containing asolectin-reductase vesicle is 337.5 K, which is about 8.5 K higher than the exothermic denaturation temperature of the asolectin-reductase vesicle. It is possible that the cholesterol molecules in the reconstituting system are homogeneously dispersed among the phospholipid molecules [28], and occupy the space between the unsaturated fatty-acyl groups, thus abolishing the interaction between phospholipids and protein which would express an exothermic transition upon thermodenaturation. It is also possible that cholesterol molecules may interact with the delipidated reductase at the protein surface areas where only those phospholipids containing highly unsaturated fatty-acyl groups would react, to generate the exothermic transition.

Discussion

Phospholipid has been shown to play dural roles in ubiquinol-cytochrome *c* reductase [6]. It is not only required for catalytic activity, but also required for maintaining structural stability. The results present in this study confirm the structural role of phospholipids in ubiquinol cytochrome *c* reductase complex. The higher transition temperature and enthalpy change observed in the reduced form complex of the intact reductase upon thermodenaturation indicate that it processes a more stable structure than that of the oxidized form protein. This is consistent with the observed facts that reduced form reductase is more difficult to be cleaved into subfraction by the detergent and salt treatment [19]. The effect of the redox state on the thermostability of the phospholipid depleted reductase is still existent, but to a much less extent. Direct correlation between activity restoration to the delipidated reductase upon addition of phospholipids and the increase in the enthalpy change at 333 K or higher of the phospholipids-replenished reductase indicates that a change of the enzyme complex from more labile structure (denatured at 225 K) to a stable form must have occurred, in order to regain the catalytic activity. Such a structure change has been documentated in the circular dichroism study of the delipidated and intact ubiquinol-cytochrome *c* reductase [6].

One of the striking phenomena observed in this study is the exothermodenaturation of ubiquinol-cytochrome *c* reductase reconstituted in the phospholipids vesicles. When the reductase, either in the delipidated or intact form, was reconstituted with phospholipid at high phospholipid-to-protein ratio (> 0.5) in the presence of detergent followed by an extensive dialysis, a vesicle preparation is formed. DSC thermogram of such vesicle preparation showed exothermic transition. The transition temperature and enthalpy change of the exothermodenaturation increased as the phospholipid concentration increased. The exothermodenaturation is depending on the nature of the phospholipid used. In the case of azolectin, the exothermic enthalpy change of thermodenaturation increased as the phospholipid concentrations increased, up to 2 mg asolectin per mg protein; beyond that ratio, the exothermic enthalpy change for thermodenaturation decreased, then leveled off at 5 mg asolectin per mg protein.

Since the exothermodenaturation was not observed in the DSC thermogram of the reductase phospholipid complex in the dispersed form, the energy released during thermodenaturation can be simply resulted from collapse of protein structure. Furthermore, the exothermic transition is observed only when the reductase is embedded in the phospholipid vesicles formed with phospholipid containing unsaturated fatty-acyl groups. One possible explanation is that in formation of a closed vesicle, asolectin probably interacts with reductase not only at areas with high protein-phospholipid affinity, but also at areas of the protein which are not surrounded by phospholipids in the physiological state. Since, under these circumstances, phospholipids are in contact with protein at areas where protein-phospholipid interaction is not favorable, a strain on the phospholipid molecule

located in these areas is generated. Such a strained interaction could be accommodated by the thermodynamically favorable vesicle formation. The energy for the exothermic transition observed in the asolectin-reductase vesicle may be derived from the collapse of such a strained interaction between asolectin and protein molecules, upon thermodenaturation. If this deduction is correct, the exothermic transition should be dependent on the intactness of the protein structure and the properties of the fatty-acyl groups of the phospholipids; and such interaction should have little to do with the enzymatic activity as the maximal restoration of the activity is reached before the phospolipid-to-protein ratio is high enough to form reductase-phospholipid vesicle. Furthermore, incorporationof cholesterol into the reductase-phospholipid vesicles abolished the exothermotransition, but showed no effect on the enzymatic activity.

The necessity for the tertiary protein structure of the delipidated reductase for the exothermic transition of the reconstituted preparation was confirmed by the lack of exothermotransition in the vesicles formed with asolectin and protease-treated reductase.

As described in Results, the protein–phospholipid interaction which resulted in exothermic transition is depending on the molecular properties of the phospholipid, including the nature of the head group and the degre of unsaturation of the fatty-acyl groups. Exothermic transitions were observed only when reductase was embedded in phospholipid vesicles formed with phospholipids containing highly unsaturated fatty-acyl groups, such as asolectin and DLPC. For the reductase-phospholipid vesicles preparation to show an exothermic transition, a minimum of three double bonds in the fatty-acyl groups in phospholipids molecules is needed.

Since the exothermotransition of reductase-azolectin vesicles is not reversible, it disappears after protein become denaturaturation. The exothermotransition cannot be resulting from the phase transition of azolectin. The phase transition of azolectin is observed at temperature below 300 K.

The suggestion that the unusual exothermic transition observed is due to the collapse of an unfavorable interaction between the unsaturated fatty-acyl groups of the phospholipid with areas of the protein surface exposed by the removal of the interacting or neighboring protein complexes during the isolation of reductase is based mainly on the fact that the DSC thermogram of the inner mitochondrial membrane shows no exothermic transition. If this deduction is correct, then we would expect the DSC thermogram of isolated ubiquinol-cytochrome *c* reductase embedded in the phospholipid vesicle to differ from that of succinate-cytochrome *c* reductase embedded in the phospholipid vesicle. The DSC thermogram of phospholipid-succinate-cytochrome *c* reductase vesicle would be expected to differ from the sum of the DSC thermograms of succinate-Q reductase and ubiquinol cytochrome *c* reductase individually embedded in phospholipid vesicles. Our preliminary results on the DSC studies of the interaction between electron-transfer complexes confirm the above deduction [24]. The exothermic transition of the asolectin-succinate-cytochrome *c* reductase vesicles is much less than that of the sum of the exothermic transitions of the asolectin-ubiquinol-cytochrome *c* and solectin-succinate-Q reductases vesicles. The exothermic transition of the asolectin-reductase vesicle was not affected by the presence of cytochrome *c* oxidase, suggesting that the interaction between the two enzyme complexes is not as strong or nonexisting. The interaction between succinate-ubiquinone reductase and ubiquinol-cytochrome *c* reductase has been confirmed by the saturation transfer EPR study of spin-labeled ubiquinol cytochrome *c* reductase (Gwak, S.H., Yu, L. and Yu, C.A., unpublished results).

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