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Spin-Label Electron Paramagnetic Resonance and Differential Scanning Calorimetry Studies of the Interaction between Mitochondrial Succinate–Ubiquinone and Ubiquinol–Cytochrome *c* Reductases†

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ABSTRACT: The interaction between succinate–ubiquinone and ubiquinol–cytochrome *c* reductases in the purified, dispersed state and in embedded phospholipid vesicles was studied by differential scanning calorimetry and by electron paramagnetic resonance (EPR). When the purified, detergent-dispersed succinate–ubiquinone reductase, ubiquinol–cytochrome *c* reductase, and cytochrome *c* oxidase undergo thermodenaturation, they show an endothermic transition. However, when these isolated electron-transfer complexes are embedded in phospholipid vesicles, they undergo exothermodenaturation. The energy released could result from the collapse of the strained interaction between unsaturated fatty acyl groups of phospholipids and an exposed area of the complex formed by removal of interacting proteins. The exothermic enthalpy change of thermodenaturation of a protein–phospholipid vesicle containing both succinate–ubiquinone and ubiquinol–cytochrome *c* reductases was smaller than that of a mixture of protein–phospholipid vesicles formed from the individual electron-transfer complexes. This suggests specific interaction between succinate–ubiquinone reductase and ubiquinol–cytochrome *c* reductase in the membrane. This idea is supported by saturation transfer EPR studies showing that the rotational correlation time of spin-labeled ubiquinol–cytochrome *c* reductase is increased when mixed with succinate–ubiquinone reductase prior to embedding in phospholipid vesicles. These results indicate that succinate–ubiquinone reductase and ubiquinol–cytochrome *c* reductase are indeed present in the membrane as a supermacromolecular complex. No such supermacromolecular complex is detected between NADH–ubiquinone and ubiquinol–cytochrome *c* reductases or between succinate–ubiquinone and NADH–uniquinone reductases.

The electron-transfer system of the mitochondrial inner membrane, which catalyzes the oxidation of NADH and succinate and generates the membrane potential and proton gradient for ATP synthesis (Mitchell, 1968), has been fractionated into four functionally well-defined multisubunit lipoprotein complexes—NADH–ubiquinone, succinate–ubiquinone, and uniquinol–cytochrome *c* reductases and cytochrome *c* oxidase—by treatment with detergents and salts (Green, 1966). These electron-transfer complexes are commonly known as complexes I, II, III, and IV, respectively. Fragments containing two adjacent complexes, such as NADH–cytochrome *c* reductase (complexes I and III) (Hatefi et al., 1962a) or succinate–cytochrome *c* reductase (Complexes II and III) (Takemori & King, 1964), have also been obtained. Much information about the subunit structures (Smith et al., 1978; Earley & Ragan, 1980; Millett et al., 1982) and topological arrangements (Mendel-Hartvig & Nelson, 1978; Ludwig et al., 1979; Gutweniger et al., 1981) of these complexes in the mitochondrial inner membrane has been obtained by using isolated complexes (Trumpower & Katki, 1979; Wikstrom et al., 1981; Wainio, 1983).

The native functional arrangement of these electron-transfer complexes in the mitochondrial inner membrane is not yet established. Whether these complexes are present individually (Schneider et al., 1980; Kawato et al., 1981; Gupte et al., 1984) or as macromolecular assemblies (Hochman et al., 1982, 1983, 1985) in the mitochondrial inner membrane is a matter of controversy. Early electron microscopic observation of the mitochondrial inner membrane suggested an order array arrangement (Klingenberg, 1964; Sjostrand & Cassel, 1978) of these electron-transfer complexes. The lack of a specific stoichiometric relationship between these complexes, however, raises questions concerning a supermacromolecular assembly. The facts that cytochrome *c* is diffusible on the surface of the membrane and ubiquinone is diffusible in the membrane also argue against the existence of a supermacromolecular assembly. The recent estimation of the lateral diffusion coefficients (Gupte et al., 1984) of each individual electron-transfer complex, such as cytochrome b–c_1 complex and cytochrome *c* oxidase, and of electron-transfer components such as cytochrome *c* and ubiquinone by the fluorescence recovery after photobleaching (FRAP)[1] technique has further supported the

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early proposal of Hackenbrock (1981), in which mitochondrial electron transfer occurs by a diffusion-mediated mechanism. The diffusion coefficients obtained were greater than those needed for the theoretical diffusion-controlled collision frequencies of the redox complexes or components at the maximum observed electron-transfer rate (Gupte et al., 1984). No actual measurements of the lateral diffusion coefficients of succinate–ubiquinone and NADH–ubiquinone reductases were made by the FRAP technique.

Interestingly, similar values of the diffusion coefficients obtained by Hochman et al. (1985) with the same FRAP technique were used to support a dynamic aggregate mechanism using slightly different assumptions in calculation. The essence of the dynamic aggregate model is that electron transfer can occur by random diffusion but a more rapid rate is achieved by the formation of a transitory functional aggregate between the electron-transfer components.

The idea that a free diffusion and random collision mechanism is functioning in the electron transfer between NADH–ubiquinone and ubiquinol–cytochrome *c* reductases or between succinate–ubiquinone and ubiquinol–cytochrome *c* reductases is mainly derived from the phospholipid dilution experiment of Schneider et al. (1982). The electron-transfer activity of NADH–cytochrome *c* reductase was found to be directly proportional to the molar ratio of redox components to phospholipid, and the decrease in the electron-transfer activity by the phospholipid dilution can be compensated for by the addition of exogenous ubiquinone (Schneider et al., 1982). In a similar experiment, the electron transfer from succinate to cytochrome *c* was also affected by the phospholipid dilution, but restoration of the activity by the addition of ubiquinone was not as apparent as that of NADH–cytochrome *c* reductase, suggesting that a different electron-transfer mechansim is functioning between NADH–ubiquinone and ubiquinol–cytochrome *c* reductases and between succinate–ubiquinone and ubiquinol–cytochrome *c* reductases.

Among the four electron-transfer complexes, interaction between succinate–ubiquinone (Ziegler & Doeg, 1962) and ubiquinol–cytochrome *c* (Rieske et al., 1962) reductases is probably the strongest because a stable succinate–cytochrome *c* reductase, with fixed stoichiometry between succinate–ubiquinone and ubiquinol–cytochrome *c* reductases, can be isolated from submitochondrial particles. It is highly likely that succinate–cytochrome *c* reductase exists as an independent entity in the mitochondrial membrane since further resolution into succinate–ubiquinone and ubiquinol–cytochrome *c* reductases can be achieved only under rather strong dissociating conditions (Yu & Yu, 1982).

To study the interaction between succinate–ubiquinone and ubiquinol–cytochrome *c* reductases in the mitochondrial inner membrane, dual approaches, differential scanning calorimetry (DSC) and saturation transfer EPR, were used. We have extended our recent DSC studies (Gwak et al., 1984, 1985; Yu et al., 1985) of the protein–phospholipid interaction of individual electron-transfer complexes to the interaction between electron-transfer complexes. The study is based on the assumption that if each electron-transfer complex exists separately in the phospholipid vesicle, no difference in thermotropic properties will be observed between protein–phospholipid vesicles formed from a mixture of two complexes and a mixture of protein–phospholipid vesicles formed individually from each complex. A significant difference in the thermodenaturation temperature and enthalpy change of thermodenaturation was observed between a protein–phospholipid vesicle containing both succinate–ubiquinone and ubiquinol–cytochrome *c* reductases and a mixture of protein–phospholipid vesicles containing individual succinate–ubiquinone reductase and ubiquinol–cytochrome *c* reductase. This suggests a specific interaction between these two complexes. Further evidence for interaction between succinate–ubiquinone and ubiquinol–cytochrome *c* reductases is the observation of an increase of the rotational correlation time of spin-labeled ubiquinol–cytochrome *c* reductase upon mixing with succinate–ubiquinone reductase before being embedded in phospholipid vesicles. Herein, we report experimental details and results of DSC and saturation transfer EPR studies with various electron-transfer complexes embedded in phospholipid vesicles.

Materials and Methods

Materials. Dipalmitoylphosphatidylcholine (DPPC), cytochrome *c*, and sodium cholate are products of Sigma. Asolectin was obtained from Associated Concentrates Inc. Other chemicals were of the highest purity commercially available. Centricon-30 was obtained from Amicon. Biobeads SM-2 were obtained from Bio-Rad.

Enzyme Preparations and Assays. Highly purified beef heart mitochondrial succinate–ubiquinone, succinate–cytochrome *c* (Yu & Yu, 1982) and ubiquinol–cytochrome *c* (Yu & Yu, 1980) reductases were prepared and assayed according to methods reported from this laboratory. NADH–ubiquinone reductase was prepared according to Hatefi et al. (1962b).

Preparation of Maleimide-Spin-Labeled (MSL) Ubiquinol–Cytochrome c Reductases. Ubiquinol–cytochrome *c* reductase, 38 mg/mL, in 10 mM Tris-HCl, pH 7.4, containing 0.14 M sucrose and 0.5% sodium cholate was incubated with a 5 molar excess of 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (MSL) for 1 h at room temperature. The stock solution of MSL (8.5 mM) was made in 10 mM Tris-HCl/sucrose buffer, pH 7.4, containing 20% methanol. After incubation, the unreacted MSL was removed by passage through a Sephadex G-25 column, equilibrated with 10 mM Tris-HCl/sucrose buffer, pH 7.4, containing 0.5% sodium cholate. Fractions containing MSL ubiquinol–cytochrome *c* reductase were pooled and concentrated by Centricon-30 to a protein concentration of approximately 20 mg/mL.

Preparation of the Electron-Transfer Complex Phospholipid Vesicles. The protein–phospholipid vesicles were prepared by the cholate dialysis method described by Racker (1972). An electron-transfer complex, with or without MSL labeling, singly or in combination, at a protein concentration of approximately 20 mg/mL, was mixed with an asolectin micellar solution (20 mg/mL in 50 mM phosphate buffer, pH 7.4) and a sodium cholate solution (20% w/v in water). The final solution contained, per milliliter, 6.3 mg of protein, 10 mg of sodium cholate, and 13.6 mg of asolectin. After incubation at 0 °C for 30 min, the solution was dialyzed against 100 volumes of 50 mM phosphate buffer, pH 7.4, overnight, with four changes of buffer. The protein–phospholipid vesicles formed were collected as precipitates by centrifugation at 80000*g* for 1 h. These precipitates were resuspended in 50 mM phosphate buffer, pH 7.5, to a protein concentration of 28–30 mg/mL and used for the DSC or saturation transfer EPR experiments. The UQ_2-containing electron-transfer complex phospholipid vesicles were prepared under the same

[1] Abbreviations: CcO, cytochrome *c* oxidase; DPPC, dipalmitoylphosphatidylcholine; MSL, maleimide spin-label (3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy); NQR, NADH–Q reductase; Q, ubiquinone; QCR, ubiquinol–cytochrome *c* reductase; SCR, succinate–cytochrome *c* reductase; SQR, succinate–Q reductase; Tris, tris(hydroxymethyl)aminomethane; EPR, electron paramagnetic resonance; FRAP, fluorescence recovery after photobleaching; DSC, differential scanning calorimetry; ST, saturation transfer; PL, phospholipid.

conditions except UQ_2 (10 mM in 95% alcoholic solution) was added to the protein–phospholipid mixture before incubation and dialysis. a 10-fold molar excess of UQ_2 to cytochrome c_1 was used.

Differential Scanning Calorimetry. All calorimetric measurements were performed with a Perkin-Elmer DSC-2C 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/in. All DSC scans reported in this paper were run at a rate of 2.5 K/min, with a sensitivity of 0.1 mcal/s. After the first scan, the samples were cooled to the original temperature and rescanned. Since the protein is completely and irreversibly denatured after the first scan, no thermotransition peaks were observed in the second scan, which can be used, therefore, as a base line. 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 under the peaks. The instrument 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 (Wilkinson & Nagle, 1981).

EPR Measurements. All EPR measurements were made with a Bruker ER-200D spectrometer, using an aqueous quartz cell. The temperature of the microwave cavity was controlled by circulation of cooled nitrogen gas from a modified variable-temperature housing assembly equipped with an electric temperature sensor.

Conventional EPR spectra were recorded with instrument settings as follows: field modulation frequency, 100 kHz; modulation amplitude, 5 G; microwave frequency, 9.5 GHz; microwave power, 10 mW; time constant, 0.2 s; scan rate, 500 G/min. Saturation transfer EPR spectra were recorded by using the same instrument settings as those described by Thomas et al. (1976) and Poore et al. (1982). A field modulation of 5 G and a microwave frequency of 50 kHz were employed with phase-sensitive detection at 100 kHz (second harmonic), 90° out of phase. Incident microwave power was 40 mW on the dial (Poore et al., 1982). The phase was adjusted to minimize the second harmonic signal at 0.07 mW. Approximate rotational correlation times (τ_2) were obtained from the ratio of the two low-field lines (L″/L). The calibration curve of Thomas et al. (1976), derived from isotropic tumbling of MSL-labeled hemoglobin, was used in the calculation.

Thermoinactivation of Electron-Transfer Complexes. The purified, detergent-free succinate–ubiquinone, ubiquinol–cytochrome *c*, and succinate–cytochrome *c* reductases (or reconstituted succinate–cytochrome *c* reductase), at a protein concentration of approximately 5 mg/mL, in 50 mM Tris–acetate buffer, pH 7.4, were incubated in a water bath. The temperature of the bath was increased at a rate of approximately 1 °C/min. At 5 °C intervals, 0.1 mL of the incubated enzyme preparation was withdrawn and immediately diluted with 0.4 mL of 50 mM Tris–acetate buffer, pH 7.8, containing 0.1% deoxycholate and kept at 0 °C. The enzymatic activities were assayed after all the samples were withdrawn. The succinate–ubiquinone reductase activity in the succinate–cytochrome *c* reductase preparation was assayed in the presence of antimycin A. A similar procedure was used for the thermodenaturation assay of the electron-transfer complex phospholipid vesicles. The protein-embedded vesicles were prepared by the cholate dialysis method. The ratio of phospholipid to protein used was 2.

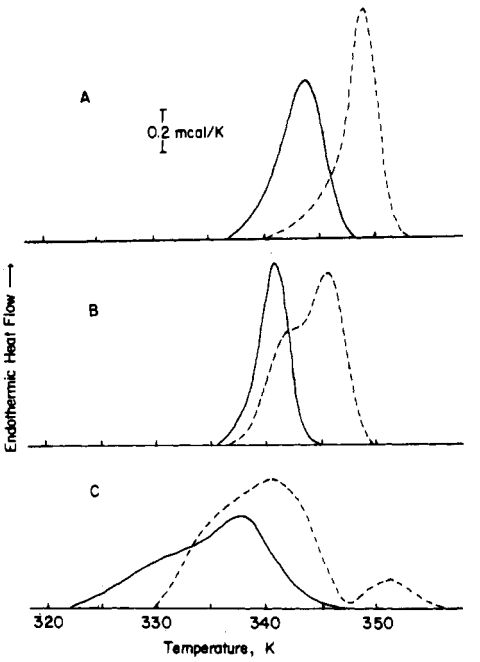

FIGURE 1: DSC thermograms of purified electron-transfer complexes. (A) Succinate–ubiquinone reductase; (B) succinate–cytochrome *c* reductase; (C) ubiquinol–cytochrome *c* reductase. The protein concentrations used were 30, 33, and 33 mg/mL, respectively. All three reductases were in 50 mM sodium/potassium phosphate buffer, pH 7.4; 65 μL of samples was used. Oxidized form (—); reduced form (---). The instrument settings are given under Materials and Methods.

Table I: Thermotropic Properties of Isolated Electron-Transfer Complexes

preparations	redox state	T_m (K)	ΔH (kcal/mol)
SQR	ox	343.9	411
	red	349.2	471
QCR	ox	337.3	424
	red	340.7	590
SCR (SQR + 2QCR)	ox	340.8	1236
	red	345.6	2103

Other Analytical Methods. Protein concentration (Lowry et al., 1951) and essential redox components (Yu & Yu, 1982) of electron-transfer complexes were determined by the methods described previously. Absorption spectra were measured in a Cary Model 219 spectrophotometer.

Results and Discussion

Thermotropic Properties of Isolated Electron-Transfer Complexes. The isolated, functionally active electron-transfer complexes succinate–cytochrome *c*, succinate–ubiquinone, and ubiquinol–cytochrome *c* reductases are in the detergent-dispersed form because of the presence of a residual amount of detergent which was used during the isolation of the complexes. These isolated electron-transfer complexes contain approximately 0.2 mg of phospholipids/mg of protein. These complexes undergo irreversible thermodenaturation when they are incubated at an elevated temperature. The thermodenaturation characteristics, such as the thermodenaturation temperature (T_m) and the enthalpy change of thermodenaturation (ΔH), however, differ significantly among these isolated complexes. Figure 1 compares the differential scanning calorimetric (DSC) thermograms of succinate–cytochrome *c* reductases in the reduced and oxidized states. The T_m and ΔH of the thermodenaturation of these three reductases are

summarized in Table I. Of the three reductases, succinate–ubiquinone reductase is the most stable toward thermodenaturation. In all cases, reduced complexes are more stable toward thermodenaturation than oxidized ones, as indicated by the higher T_m and ΔH.

Since isolated succinate–cytochrome *c* reductase can be resolved into succinate–ubiquinone and ibiquinol–cytochrome *c* reductases (Yu & Yu, 1982), it is possible that succinate–cytochrome *c* reductase merely results from copurification of succinate–ubiquinone and ubiquinol–cytochrome *c* reductases with no specific interaction between succinate–ubiquinone and ubiquinol–cytochrome *c* reductases existing in the membrane. If this were the case, the DSC parameters of isolated succinate–cytochrome *c* reductase (or reconstituted succinate–cytochrome *c* reductase) would be the same as that of the summation of succinate–ubiquinone and ubiquinol–cytochrome *c* reductases. Two thermotransition temperatures, identical with those of succinate–ubiquinone and ubiquinol–cytochrome *c* reductases, would be observed in the DSC thermogram of isolated succinate–cytochrome *c* reductase. The enthalpy change of thermodenaturation of succinate–cytochrome *c* reductase would equal the sum of twice the ΔH of ubiquinol–cytochrome *c* reductase plus the ΔH of succinate–ubiquinone reductase in isolated succinate–cytochrome *c* reductase or in the mitochondrial inner membrane is approximately 2. Results of the DSC studies of these isolated reductases, shown in Figure 1 and Table I, clearly indicate that this is not the case. The DSC thermogram of succinate–cytochrome *c* reductase has only one T_m for the oxidized form. Although there are two distinguishable T_m values in the DSC for the reduced complex, both differ significantly from the T_m of reduced succinate–ubiquinone reductase or ubiquinol–cytochrome *c* reductase. Thus, the two T_m values observed with reduced succinate–cytochrome *c* reductase most likely represent two stages of thermodenaturation of an interacting complex rather than denaturation of two different complexes. It should be noted that more than one T_m also appears in the DSC thermograms of both reduced and oxidized ubiquinol–cytochrome *c* reductase. These can be explained either by multiple stage thermodenaturation or by different degree of aggregation of the enzyme complexes, such as those observed in cytochrome *c* oxidase (Yu et al., 1985).

Although, in the oxidized form, the difference in the enthalpy change between succinate–cytochrome *c* reductase and the sum of succinate–ubiquinone and ubiquinol–cytochrome *c* reductases is small, this should be considered coincident rather than an indication of no interaction between them because, in the reduced form, succinate–cytochrome *c* reductase has 21% more heat capacity than does the sum of succinate–ubiquinone and ubiquinol–cytochrome *c* reductases, indicating that the latter are more stable in the complexed form than in their individually isolated forms.

Since the thermodenaturation of an enzyme complex observed by DSC is the thermotransition temperature that is responsible for the collapse of the tertiary structure, it is possible that the structure change related to activity may occur at a temperature lower than that required for the total collapse of the tertiary structure. If there is any interaction between succinate–ubiquinone and ubiquinol–cytochrome *c* reductases, it should be revealed by following the heat inactivation behavior of the succinate–ubiquinone reductase and ubiquinol–cytochrome *c* reductase activities in their respective isolated preparations, and in succinate–cytochrome *c* reductase. Figure 2 shows the heat inactivation pattern of activities of succinate–ubiquinone and ubiquinol–cytochrome *c* reductases in

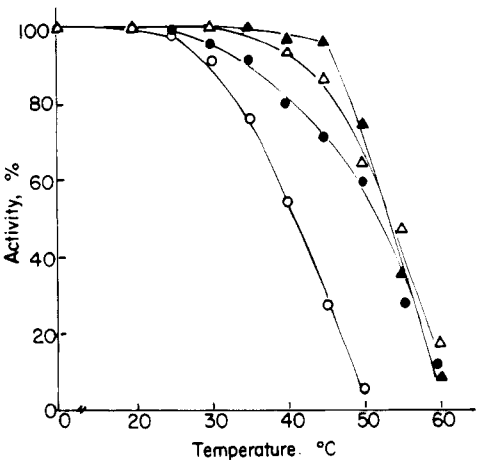

FIGURE 2: Thermoinactivation of succinate–ubiquinone reductase and ubiquinol–cytochrome *c* reductase activities in isolated succinate–ubiquinone, ubiquinol–cytochrome *c*, and succinate–cytochrome *c* reductases. The isolated, detergent-free reductases, 5 mg/mL, in 50 mM Tris–acetate buffer, pH 7.4, were used. The detailed experimental conditions were as described under Materials and Methods. Curves with the open (○) and closed (●) circles represent the ubiquinol–cytochrome *c* reductase activity in ubiquinol–cytochrome *c* and succinate–cytochrome *c* reductases, respectively. Curves with the open (△) and closed (▲) triangles represent the succinate–ubiquinone reductase activity in succinate–ubiquinone and succinate–cytochrome *c* reductases, respectively.

isolated succinate–ubiquinone, ubiquinol–cytochrome *c*, and succinate–cytochrome *c* reductases. To avoid complications that may arise from the presence of residual detergent, the detergent-free reductases were used. The residual cholate or deoxycholate in the succinate–cytochrome *c* reductase and ubiquinol–cytochrome *c* reductase was removed by extensive dialysis, and the Triton X-100 present in the succinate–ubiquinone reductase was removed by Biobeads SM-2. Ubiquinol–cytochrome *c* reductase denatures (50% loss of activity) at 41 °C and succinate–ubiquinone reductase denatures at 55 °C, when they were incubated individually. However, when these two enzyme complexes were incubated together, as in the isolated succinate–cytochrome *c* reductase or reconstituted succinate–cytochrome *c* reductase, a significant increase in the heat stability of ubiquinol–cytochrome *c* reductase activity was observed. A 50% inactivation of ubiquinol–cytochrome *c* reductase in the succinate–cytochrome *c* reductase preparation was observed at 52 °C. However, the increase in the heat stability of succinate–ubiquinone reductase activity by the presence of ubiquinol–cytochrome *c* reductase is rather insignificant. This is understandable because before the succinate–ubiquinone reductase moiety of succinate–cytochrome *c* reductase became susceptible to the heat inactivation, the ubiquinol–cytochrome *c* reductase moiety of succinate–cytochrome *c* reductase is already denatured; thus, any protection that would generate from the presence of ubiquinol–cytochrome *c* reductase is already diminished. The easier heat inactivation of ubiquinol–cytochrome *c* reductase is also evident in the DSC thermogram; ubiquinol–cytochrome *c* reductase begins its thermotransition at around 49 °C, which is 15 °C below that of succinate–ubiquinone reductase (see Figure 1). The T_m values for succinate–ubiquinone and ubiquinol–cytochrome *c* reductases are 70.9 and 64.3 °C, respectively. They are higher than the heat inactivation temperatures of these two enzyme complexes. It should be noted that incorporation of succinate–ubiquinone reductase or ubiquinol–cytochrome *c* reductase into phospholipid vesicles (asolectin) by the cholate dialysis method did not significantly protect the enzyme from heat inactivation.

Table II: Exothermic Enthalpy Changes of Thermodenaturation of Various Electron-Transfer Complexes Embedded in Phospholipid Vesicles[a]

preparations	ΔH (mcal/mg of protein)
(NQR × PL) + (SQR × PL)	2.37
(NQR + SQR) × PL	2.36
(NQR × PL) + (QCR × PL)	2.18
(NQR + QCR) × PL	2.19
(SQR × PL) + (QCR × PL)	3.63
(SQR + QCR) × PL	2.50
SCR × PL	2.45
(SQR × PL) + (CcO × PL)	3.56
(SQR + CcO) × PL	3.02
(QCR × PL) + (CcO × PL)	5.17
(QCR + CcO) × PL	4.69

[a] The protein ratios used between the electron-transfer complexes were 8:3.2:8:16 for NADH–ubiquinone reductase (NQR)/succinate–ubiquinone reductase (SQR)/ubiquinol–cytochrome *c* reductase (QCR)/cytochrome *c* oxidase (CcO). These values were calculated on the basis of molar ratios (1:2:4:8) of these complexes present in the mitochondrial inner membrane using molecular weights of 800 000, 160 000, 200 000, and 200 000 for the corresponding complexes. The ratio of phospholipids to protein is 2.0 by weight in all cases.

Thermotropic Properties of Electron-Transfer Complexes Embedded in Phospholipid Vesicles. Although the comparative DSC studies indicate a specific interaction between isolated succinate–ubiquinone and ubiquinol–cytochrome *c* reductases, it is possible that this observation stemmed from the artificial environment created during isolation of enzyme complexes or resulted from the alteration of the phospholipid–protein interaction in the presence of residual detergents. To confirm the existence of a specific interaction between succinate–ubiquinone and ubiquinol–cytochrome *c* reductases in the membrane, studies were carried out with electron-transfer complexes embedded in phospholipid vesicles. The isolated complexes, singly or in combination, were embedded in phospholipid vesicles by the cholate dialysis method (Racker, 1972). A protein to the phospholipid ratio of 0.5 (w/w) was used. The enzyme complex in these protein–phospholipid vesicles should have an environment similar to that in the mitochondrial inner membrane. The formation of vesicles has been established by freeze–fracture electron microscopy (Gwak et al., 1985). The ubiquinol–cytochrome *c* reductase–phospholipid (1:30) vesicles have been shown to have properties similar to those of the mitochondrial membrane (Leung & Hinkle, 1975). The protein–phospholipid vesicle preparation prepared by the described conditions is capable of translocating protons across the membrane and catalyzing electron transfer from ubiquinol to cytochrome *c*. Although it has been shown (Leung & Hinkle, 1975) that a higher phospholipid to protein ratio is beneficial to proton gradient formation, the protein concentration needed for DSC experiments has prevented us from using high phospholipid to protein ratios.

If the two electron-transfer complexes have no specific interaction, no difference in DSC characteristics should be observed between phospholipid vesicles embedded with a mixture of two complexes and a mixture of phospholipid vesicles embedded with one or the other electron-transfer complex. Table II shows the enthalpy changes during thermodenaturation of various electron-transfer complexes embedded in phospholipid vesicles. There is a significant difference in the enthalpy change of exothermodenaturation of phospholipid vesicles embedded with a binary complex of ubiquinol–cytochrome *c* and succinate–ubiquinone reductases as compared to the mixture of phospholipid vesicles embedded individually with ubiquinole–cytochrome *c* or succinate–ubiquinone reductase. This suggests specific interaction between these two complexes. Although some interaction was also observed in the binary systems of ubiquinol–cytochrome *c* reductase and cytochrome *c* oxidase, and of succinate–ubiquinone reductase and cytochrome *c* oxidase, the differences in the enthalpy change of thermodenaturation in these two cases are too small, suggesting much weaker interactions exist, if any. There is no difference in the enthalpy changes of exothermodenaturation of the binary system of succinate–ubiquinone and NADH–ubiquinone reductases, and of NADH–ubiquinone and ubiquinol–cytochrome *c* reductases, regardless of whether they are incorporated into phospholipid vesicles in combination or individually, suggesting that no specific interaction exists between these complexes. The interaction between NADH–ubiquinone and ubiquinol–cytochrome *c* reductases has been studied by Poore et al. (1982) using saturation transfer (ST) EPR and found to be phospholipid concentration dependent. At lower phospholipid concentration, a stoichiometric interaction is observed, while at higher phospholipid concentration, little interaction is observed. The DSC data reported here (Table II) support the idea of little interaction between NADH–ubiquinone and ubiquinol–cytochrome *c* reductases.

As discussed earlier (Gwak et al., 1984, 1985; Yu et al., 1985), the energy for the exothermic transition of an electron-transfer complex embedded in phospholipid vesicles is derived from the collapse, upon thermodenaturation, of a strained interaction between the unsaturated fatty acyl groups of phospholipids and a protein surface in the electron-transfer complex which was exposed by the removal of the interacting or neighboring protein complex during isolation. Such a strained interaction occurs only when a vesicle is formed. That is why no exothermic transition is observed when the electron-transfer complex is in the dispersed form. The addition of detergent to the electron–transfer complex phospholipid vesicles also causes the disappearance of the exothermic transition upon thermodenaturation. Little exothermic transition was observed in mitochondrial or submitochondrial preparations because there is no such "artificially" exposed area in the native complex which will interact with phospholipids under strained conditions. When two interacting or neighboring complexes are mixed together before being embedded in phospholipid vesicles, the exposed area on the protein surface is greatly diminished. Thus, a less strained interaction occurs upon vesicle formation, and less enthalpy change of exothermodenaturation is observed.

It has been reported that thermodenaturation of the mitochondrial membrane under aerobic conditions is accompanied by a heat release of 20–25 mcal/mg of protein. Such a heat release was attributed to the autoxidation of the iron sulfur protein (Tsong & Knox, 1984). This explanation is difficult to apply to the present study because the same electron-transfer complex is involved in the thermodenaturation but, depending upon whether or not a protein–phospholipid vesicle is formed, shows endothermic or exothermic transitions. Furthermore, under our experimental conditions, the submitochondrial preparation showed only endothermic transitions in the DSC thermogram.

Saturation Transfer EPR Studies of Spin-Labeled Ubiquinol–Cytochrome c Reductase Embedded in Phospholipid Vesicles in the Presence and Absence of Other Electron-Transfer Complexes. Ubiquinol–cytochrome *c* reductase was labeled with 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy as described under Materials and Methods. This MSL ubiquinol–cytochrome *c* reductase, which is enzymatically active, was embedded in phospholipid vesicles alone or together with succinate–ubiquinone reductase, NADH–ubiquinone reduc-

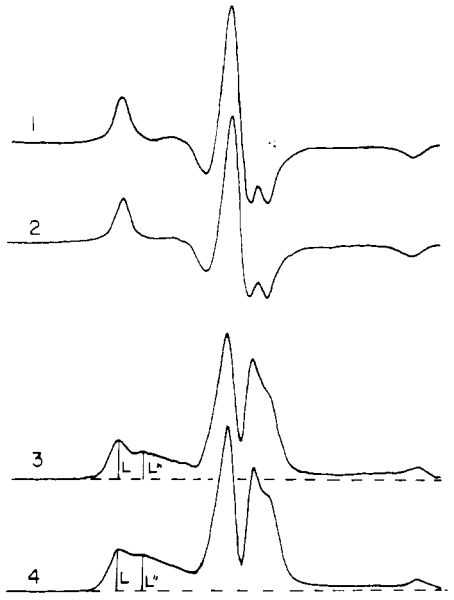

FIGURE 3: EPR spectra of spin-labeled ubiquinol–cytochrome *c* reductase in the presence and absence of succinate–ubiquinone reductase. Spectra 1 and 2 are of spin-labeled ubiquinol–cytochrome *c* reductase embedded in phospholipid vesicles in the absence and presence of succinate–ubiquinone reductase. Spectra 3 and 4 are the saturation transfer EPR spectra of the same samples. Protein concentrations were 13.2 and 4.5 mg/mL for the ubiquinol–cytochrome *c* and succinate–ubiquinone reductases, respectively. The instrument settings are given under Materials and Methods.

Table III: Effect of Other Electron-Transfer Complexes on the Rotational Correlation Times (τ_2) of Spin-Labeled Ubiquinol–Cytochrome *c* Reductase[a]

preparations	τ_2 (μs)	preparations	τ_2 (μs)
QCR × PL	28	(QCR + SQR) × PL	70
(QCR + Q) × PL	29	(QCR + SQR + O) × PL	71
(QCR + NQR) × PL	38	(QCR + CcO) × PL	41
(QCR + NQR + Q) × PL	39		

[a] The ratios of phospholipids to protein and the ratios of protein to protein used in the pairing complexes were the same as those described in Table II. A 10-fold molar excess of UQ_2 to cytochrome c_1 was used.

tase, or cytochrome *c* oxidase. The EPR measurements of these electron-transfer complex phospholipid vesicles show typical spin-immobilized spectra. The spectra are identical regardless of whether the protein–phospholipid vesicles contained one or more complexes (see spectra 1 and 2 of Figure 3). This suggests that the difference in mobility of the spin-label on ubiquinol–cytochrome *c* reductase in the presence of and absence of other complexes is too small to be measured by conventional EPR. Therefore, the protein rotational diffusion of the spin-labeled complex was measured by saturation transfer EPR. From the change of the ratio of two low-field signals (L''/L) (see spectra 3 and 4 of Figure 3), rotational correlation times (τ_2) can be calculated (Thomas et al., 1976; Poore et al., 1982). Table III shows the effect of other electron-transfer complexes on the rotational correlation time of spin-labeled ubiquinol–cytochrome *c* reductase. When mixed with succinate–ubiquinone reductase before being embedded in phospholipid vesicles, a significance increase in τ_2 was observed compared to that of spin-labeled ubiquinol–cytochrome *c* reductase embedded in phospholipid vesicles alone. An effect of cytochrome *c* oxidase or NADH–ubiquinone reductase on the τ_2 of ubiquinol–cytochrome *c* reductase is also observed, but it is much smaller. It is conceivable that at least part of the observed effect resulted from a change in the

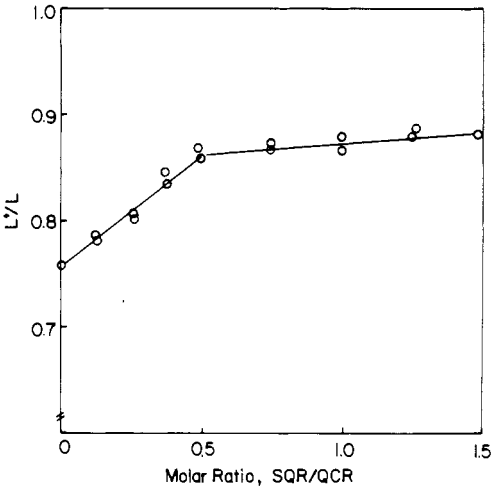

FIGURE 4: Titration of spin-labeled ubiquinol–cytochrome *c* reductase with succinate–ubiquinone reductase. Increasing amounts of succinate–ubiquinone reductase were added to a constant amount of spin-labeled ubiquinol–cytochrome *c* reductase. The solutions were incubated for 30 min at 3 °C before being embedded in phospholipid vesicles. Two milligrams of phospholipid per milligram protein was used in all samples. L''/L was calculated from the saturation transfer EPR spectra of each sample. The instrument settings are given under Materials and Methods.

fluidity of the membrane by inclusion of protein complexes other than ubiquinol–cytochrome *c* reductase. Also, it should be noted that the rotational correlation time obtained from saturation transfer EPR is only an approximate value; it is based on the calibration curve derived from the isotropic motion of the spin-label. The values obtained, however, agree with those obtained by other methods, such as flash photolysis (Cherry, 1979). Although in this study our main concern is with the relative τ_2, in the presence and absence of other electron-transfer complexes, the τ_2 values obtained are in good agreement with those reported for other systems (Poore et al., 1982; Quintanilha et al., 1982).

To ensure that the observed τ_2 increase upon mixing succinate–ubiquinone reductase with spin-labeled ubiquinol–cytochrome *c* reductase is indeed due to the specific interaction between these two complexes and the formation of a binary complex, succinate–cytochrome *c* reductase, and not due to the change of protein concentration or self-aggregation upon addition of succinate–ubiquinone reductase, a titration of spin-labeled ubiquinol–cytochrome *c* reductase with succinate–ubiquinone reductase was carried out. If a specific interaction between these two complexes exists, it is expected that a break point in the τ_2 will be obtained when the ratio of succinate–ubiquinone reductase to ubiquinol–cytochrome *c* reductase approaches 0.5, because in the isolated succinate–cytochrome *c* reductase and the mitochondrial inner membrane the ratio between succinate–ubiquinone reductanse and ubiquinol–cytochrome *c* reductase has been calculated to be 0.5. The titration data, shown in Figure 4, are exactly as predicted, suggesting that in the membrane, succinate–ubiquinone and ubiquinol–cytochrome *c* reductases are strongly associated.

Effect of Ubiquinone on the Interaction between Ubiquinol–Cytochrome c Reductase and Other Electron-Transfer Complexes. In the mitochondrial inner membrane, ubiquinone is present in 10–15 molar excess compared to cytochrome c_1. In the isolated, fully active succinate–cytochrome *c* reductase, however, the concentration of ubiquinone is approximately equal to that of cytochrome c_1. It is, therefore, of interest to see whether or not the interaction described in the preceding sections is affected by the presence of exogenous ubiquinone.

The isolated ubiquinol–cytochrome *c* reductase, with or without the spin-label, was mixed with a ubiquinone derivative (UQ_2) at a 10-fold molar excess to cytochrome c_1 prior to incorporation into phospholipid vesicles. The thermotropic properties (T_m and ΔH) of the ubiquinol–cytochrome *c* reductase–phospholipid vesicles formed with ubiquinone are similar to those of protein–phospholipid vesicles formed in the absence of ubiquinone, indicating that exogenous ubiquinone shows little effect on the thermotropic properties of ubiquinol–cytochrome *c* reductase. The effect of exogenous ubiquinone on the interaction between succinate-ubiquinone reductase and ubiquinol–cytochrome *c* reductase or between ubiquinol–cytochrome *c* reductase and other complexes is also negligible because the enthalpy changes of thermodenaturation of the protein–phospholipid vesicles containing two enzyme complexes and ubiquinone deviate very little from those enthalpy changes given in Table II.

When ST EPR was carried out with the spin-labeled ubiquinol–cytochrome *c* reductase in the presence of exogenous UQ_2, the rotational correlation time is similar to that observed in the absence of ubiquinone (Table III). These results suggest that the molar excess of the free form of ubiquinone in the mitochondrial inner membrane plays little role in the protein–protein interaction between succinate–ubiquinone and ubiquinol–cytochrome *c* reductases. This, however, does not preclude the pool function activity of free ubiquinone (Ragan & Cottingham, 1985; Gutman, 1985; Kroger & Klingenberg, 1973a,b) involved in the interaction between NADH–ubiquinone and ubiquinol–cytochrome *c* reductases (Poore et al., 1982) and in the interaction of the latter with the other ubiquinone reductases (Lenaz & Desantis, 1985). It seems quite convincing that the electron transfer between NADH dehydrogenase and ubiquinol–cytochrome *c* reductase is mediated through the free ubiquinone, whereas the electron transfer between succinate–ubiquinone reductase and ubiquinol–cytochrome *c* reductase is mainly involved with the protein-bound quinones. The protein-bound quinone may be capable of equilibrating with the free-form quinone.

From the results presented here, and the fact that separation of succinate–cytochrome *c* reductase into succinate–ubiquinone and ubiquinol–cytochrome *c* reductases requires stronger dissociation conditions than does the separation of other complexes, one can conclude that succinate–ubiquinone reductase and ubiquinol–cytochrome *c* reductase may indeed exist as a complex in the membrane. This conclusion differs from the general theme of the free diffusible model of electron-transfer complexes, supported by the results of studies of membrane fusing (Schneider et al., 1980) and the diffusion coefficient of each electron-transfer complex measured by fluorescence recovery after photobleaching (Gupte et al., 1984). The results obtained from the fluorescence recovery after photobleaching method would be more conclusive if the diffusion data were confirmed by using antibodies against different components of a given complex. The relative small difference in the diffusion coefficient obtained among the tested complexes (Gupte et al., 1984) and the use of a large excess of antibody specific for a whole electron-transfer complex leave room for future clarification. More recently, Hochman et al. (1985) have redetermined the diffusion coefficients of cytochrome oxidase, specifically modified cytochrome *c*, and phospholipid (PE) by FRAP, and the diffusion coefficients obtained for these components are quite close to those reported by Gupte et al. (1984). However, with slightly different assumptions on the concentration of cytochrome oxidase and the maximum electron-transfer rate, and considering the possible overestimation of the diffusion coefficient due to the reduction of the steric hindrance in the swollen mitochondria, they formulated a dynamic aggregate model for electron transfer. This model refers mainly to the electron transfer between the cytochrome b–c_1 complex and cytochrome *c* oxidase, and no assessment was made on the interaction between succinate–ubiquinone reductase and ubiquinol–cytochrome *c* reductase. The dynamic aggregate hypothesis of electron transfer between ubiquinol–cytochrome *c* reductase and cytochrome oxidase fits the DSC and ST EPR data quite well, as both studies show a partial interaction between these two complexes.

The strongest evidence used to support the idea that electron-transfer complexes exist individually in the membrane is when mitochondria were fused with phospholipid vesicles, a decrease in electron-transfer activity, e.g., succinate–cytochrome *c* and NADH–cytochrome *c* reductases, is observed, which can be restored by the addition of exogenous ubiquinone (Schneider et al., 1980). This phenomenon can, however, be explained by a decrease in the binding affinity of the protein-bound ubiquinone, resulting from the introduction of extra phospholipid during membrane fusion. Thus, the existence of a supermacromolecular complex in the succinate–cytochrome *c* reductase region of the respiratory chain is highly possible and should not be negated by results of a given type of experimentation. In fact, the DSC and ST EPR data suggest that some electron transfer proceeds by the random collision mechanism, such as NADH–ubiquinone reductase and ubiquinol–cytochrome *c* reductase; some proceeds via a partial aggregate mechanism, such as ubiquinol–cytochrome *c* reductase and cytochrome oxidase; and some form a stable complex, such as succinate–ubiquinone reductase and ubiquinol–cytochrome *c* reductase.

Registry No. Succinate–ubiquinone reductase, 9028-11-9; ubiquinol–cytochrome *c* reductase, 9027-03-6.

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Double-Inhibitor and Uncoupler–Inhibitor Titrations. 1. Analysis with a Linear Model of Chemiosmotic Energy Coupling

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ABSTRACT: The results of double-inhibitor and uncoupler–inhibitor titrations have been simulated and analyzed with a linear model of delocalized protonic coupling using linear nonequilibrium thermodynamics. A detailed analysis of the changes of the intermediate $\Delta\tilde{\mu}_H$ induced by different combinations of inhibitors of the proton pumps has been performed. It is shown that with linear flow–force relationships the published experimental results of uncoupler–inhibitor titrations are not necessarily inconsistent with, and those of double-inhibitor titrations are inconsistent with, a delocalized chemiosmotic model of energy coupling in the presence of a negligible leak. Also shown and discussed are how the results are affected by a nonnegligible leak and to what extent the shape of the titration curves can be used to discriminate between localized and delocalized mechanisms of energy coupling.

In the chemiosmotic view (Mitchell, 1966) energy transduction by oxidative phosphorylation in mitochondria and bacteria, as well as by photophosphorylation in chloroplasts, is accomplished by redox ($\Delta\tilde{\mu}_H$[1] generating, primary) and ATPase ($\Delta\tilde{\mu}_H$ utilizing, secondary) proton pumps coupled through the common intermediate $\Delta\tilde{\mu}_H$, which is the thermodynamically and kinetically competent driving force for ATP synthesis. Despite much supporting evidence for and wide acceptance of this delocalized protonic coupling mechanism, a series of reports in the past few years has raised doubts as to whether it provides a completely correct description of biological energy transduction (for reviews see: Westerhoff et al., 1984; Ferguson, 1985). Many of the re-

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[1] Abbreviations: $\Delta\tilde{\mu}_H$, transmembrane difference in the electrochemical potential of protons; ATPase, adenosinetriphosphatase; ATP, adenosine triphosphate.

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