Spin-Label Electron Paramagnetic Resonance and Differential Scanning Calorimetry Studies of the Interaction between Mitochondrial Cytochrome *c* Oxidase and Adenosine Triphosphate Synthase Complex†

Zhi-Hua Qiu, Linda Yu, and Chang-An Yu*
Department of Biochemistry, Oklahoma State University, Stillwater, Oklahoma 74078
Received August 13, 1991; Revised Manuscript Received November 15, 1991

ABSTRACT: The interaction between cytochrome *c* oxidase complex and adenosine triphosphate synthase (F_1F_0) complex in the purified, dispersed state and embedded in phospholipid vesicles was studied by differential scanning calorimetry and by spin-label electron paramagnetic resonance. The detergent-dispersed cytochrome oxidase and F_1F_0 complexes undergo endothermic thermodenaturation. However, when these complexes are embedded in phospholipid vesicles, they undergo exothermic thermodenaturation. The energy released is believed to result from the collapse of a strained interaction between unsaturated fatty acyl groups of phospholipids and an exposed area of the complex formed by the removal of interacting proteins. The exothermic enthalpy change of thermodenaturation of a protein–phospholipid vesicle containing both cytochrome oxidase complex and F_1F_0 was smaller than that of a mixture of protein–phospholipid vesicles formed from each individual electron transfer complex. This suggests specific interaction between cytochrome oxidase complex and F_1F_0 in the membrane. Further evidence for interaction between these two complexes is provided by saturation transfer EPR studies in which the rotational correlation time of spin-labeled cytochrome oxidase increases significantly when the complex is mixed with F_1F_0 prior to being embedded in phospholipid vesicles. From these results, it is concluded that at least a part of cytochrome oxidase and a part of F_1F_0 form a supermacromolecular complex in the inner mitochondrial membrane. No such supermacromolecular complex is detected between F_1F_0 and ubiquinol–cytochrome *c* reductase.

The mitochondrial respiratory (electron transfer) chain, which generates the proton gradient and membrane potential (Mitchell, 1966, 1976; Hatefi, 1985) for the synthesis of ATP via a multicomponent ATP synthase complex (F_1F_0 or complex V)[1] (Kagawa, 1978; Penefsky, 1979; Amzel & Pedersen, 1983; Walker et al., 1985; Senior, 1988), is composed of four multisubunit enzyme complexes: NADH–Q (NQR) (Hatefi et al., 1962), succinate–Q (SQR) (Ziegler & Doeg, 1962), and ubiquinol–cytochrome *c* reductases (QCR) (Rieske et al., 1964) and cytochrome *c* oxidase (CcO) (Griffiths & Wharton, 1961). They are commonly referred to as complexes I, II, III, and IV, respectively. The essential redox components, subunit structure, and topology of each electron transfer complex have been more or less established (Capaldi, 1979; Von Jagow & Sebald, 1980; Wikstrom et al., 1981; Hatefi, 1985). However, the detailed electron transfer mechanism, either within a complex or between complexes, as well as the interaction between these electron transfer complexes, remains to be fully elucidated. All these electron transfer complexes except SQR are capable of driving ATP synthesis when reconstituted into phospholipid vesicles in the presence of F_1F_0 complex. The physical relationships between each of them and between the electron transfer complexes and F_1F_0 are not well understood.

The high protein to phospholipid (PL) ratio (Blair, 1963) observed in the mitochondrial inner membrane and earlier results from electron microscope studies led investigators to suggest that electron transport complexes may exist as structural units, arranged as an order array arrangement (Klingenberg, 1976; Sjostrand & Cassel, 1978). However, the lack of unit stoichiometric relationships (Hochli & Hackenbrock, 1978) among the electron transfer complexes and the fact that large integral proteins are able to diffuse freely and independently over a considerable distance (Hochli & Hackenbrock, 1976, 1978; Schneider et al., 1980, 1982; Hackenbrock, 1981; Kawato et al., 1981; Sowers & Hockenbrock, 1981) led investigators to question the idea of an ordered macromolecular assembly (Lehninger, 1959; Blair et al., 1963; Erecinska et al., 1980) and to propose random collision (Schneider et al., 1980, 1982; Hackenbrock, 1981; Gupte et al., 1984; Hackenbrock et al., 1986) and dynamic (partial) aggregation (Hochman et al., 1982, 1983, 1985) models. Both models are based on similar data obtained from measuring diffusion coefficients of fluorescence-labeled antibodies against electron transfer complexes, using a fluorescence recovery after photobleaching technique (Gupte et al., 1984; Hochman et al., 1985). Other approaches, such as differential scanning calorimetry (DSC) and saturation transfer electron paramagnetic resonance (STEPR) (Gwak et al., 1986), used to study interaction between electron transfer complexes show that SQR and QCR may form supercomplexes in the native membrane state. Little information is available about the possible interaction between electron transfer complexes and the F_1F_0 complex. Recently, we used the DSC and STEPR to study the interaction between cytochrome *c* oxidase and F_1F_0 complex in an attempt to gain some insight into the energy coupling mechanism. Close physical contact between oxidase and F_1F_0 is necessary if the localized proton gradient (Beard & Dilley, 1988) is to be the driving force for ATP synthesis. On the other hand, if the bulk phase

† This work was supported in part by grants from OCAST and NIH (GM 30721).
* Author to whom correspondence should be addressed.

[1] Abbreviations: Complex V, ATP synthase complex; CcO, cytochrome *c* oxidase; DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; MSL, maleimide spin label (4-maleimido-2,2,6,6-tetramethyl-1-piperidinyl-*N*-oxy); QCR, ubiquinol–cytochrome *c* reductase; SQR, succinate–Q reductase; Q, ubiquinone; STEPR, saturation transfer electron paramagnetic resonance.

0006-2960/92/0431-3297$03.00/0

proton gradient is the main force, direct physical contact between electron transfer complexes and the F_1F_0 complex is not necessary. The results of DSC and STEPR indicate that CcO does indeed form a complex with F_1F_0 in phospholipid vesicles. The DSC study is based on the assumption that if two lipoprotein complexes exist separately in a 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. Differences in the thermodenaturation temperatures and enthalpy changes would suggest formation of a physical complex between CcO and F_1F_0. In the STEPR study, the formation of a physical complex between CcO and F_1F_0 complexes will be indicated by an increase of rotational correlation time of spin-labeled CcO. Herein we report experimental details and results of DSC and STEPR studies with CcO and F_1F_0 complexes embedded in phospholipid vesicles.

Materials and Methods

Materials. Cytochrome *c*, type III, and sodium cholate are from Sigma. Asolectin was obtained from Associated Concentrates, Inc., and purified according to the procedure reported by Kagawa (1971). Centricon-30 was obtained from Amicon. Other chemicals were of the highest purity commercially available.

Enzyme Preparations and Assays. Highly purified beef heart mitochondrial succinate–Q (Yu & Yu, 1982), succinate–cytochrome *c* (Yu & Yu, 1982), ubiquinol–cytochrome *c* (Yu & Yu, 1980) reductases, and cytochrome *c* oxidase (Yu et al., 1985) were prepared and assayed according to methods reported from this laboratory. Cytochrome *c* oxidase as prepared is in the delipidated form, it contains 7–8 μg of phospholipid/mg of protein. F_1F_0 was prepared according to Serrano et al. (1976), with modifications as described by Laird et al. (1986), and assayed spectrophotometrically by the method of Stigall et al. (1978). F_1F_0 as prepared is in the delipidated form, it contains 3–4 μg of phospholipid/mg of protein. F_0, the membrane sector of ATP synthase complex (complex V), was prepared according to Galante et al. (1981). Complex V was suspended in 50 mM Tris-acetate, pH 7.5, containing 0.25 M sucrose, 5 mM EDTA, and 5 mM dithiothreitol. The protein concentration was adjusted to 5 mg/mL, and solid urea was added to a final concentration of 4 M. After 2 h of incubation in ice, the mixture was centrifuged at 140000*g* for 1 h. The pellets were suspended in the same buffer, and the extraction procedure was repeated. The twice extracted pellets were suspended in 50 mM Tris-acetate buffer, pH 7.5, homogenized, and frozen. To solubilize F_0, the suspension was mixed with phospholipid to a protein to phospholipid ratio of 0.5. The mixture was then sonicated in ice four times, for 30 s each time, using a small probe of a Cell Disruptor of Heat-Systems-Ultrasonics, model W-220 F. Solubilized F_0 was recovered in the supernatant solution after 2 min of centrifugation in a Beckman microcentrifuge.

Preparation of Maleimide Spin-Labeled (MSL) Cytochrome c Oxidase. Cytochrome *c* oxidase, 30 mg/mL in 10 mM Tris-HCl, pH 7.4, containing 1 M KCl, 1 mM EDTA, 1% sucrose (TKS), and 0.5% Tween-80 was incubated with a 5 molar excess of 4-maleimide-2,2,6,6-tetramethyl-1-piperidinyl-*N*-oxyl (MSL) for 1 h at room temperature. The stock solution of MSL (10 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 buffer, pH 7.4, containing 0.5% Tween-80 and 1% sucrose. Fractions containing MSL–cytochrome *c* oxidase were pooled and concentrated by Centrico-30 to a protein concentration of approximately 20 mg/mL. MSL–cytochrome *c* oxidase obtained by this method contains no free spin-label. The absence of free spin-label in the preparation is confirmed by the conventional EPR spectra.

Preparation of CcO and F_1F_0 Complex–Phospholipid Vesicles. The protein–phospholipid vesicles were prepared by the cholate-dialysis method of Racker (1972). CcO complex, with or without MSL labeling, alone or in combination with F_1F_0, 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 7 mg/mL protein, 10 mg/mL sodium cholate, and 10.5 mg/mL asolectin. After incubation at 0 °C for 30 min, the solution was dialyzed overnight against 100 volumes of 50 mM phosphate buffer, pH 7.4, with four changes of buffer to form vesicles. In these vesicles, all the integral proteins of CcO and F_1F_0 are expected to be completely embedded in the vesicles because the phospholipid to protein ratio (1.5) used is much higher than that needed to form complete vesicles from CcO or F_1F_0. The minimum ratios of phospholipid to protein required for vesicles formation are 1.0 (Yu et al., 1985) and 0.5 (Laird et al., 1986) for CcO and F_1F_0, respectively. The protein–phospholipid vesicles formed were collected as precipitates by centrifugation at 80000*g* for 1 h and were resuspended in 50 mM phosphate buffer, pH 7.4, to a protein concentration of 20 mg/mL. The suspensions were used for the DSC and STEPR experiments.

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 0 °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 1 cm/min. All DSC scans reported in this paper were run at a rate of 2.5 K/min at a sensitivity of 0.1–0.5 mcal/s. After the first scan, the samples were cooled to the original temperature and rescanned. Since after the first scan the protein was completely and irreversibly denatured, no thermotransition peaks were observed in the second scan. Thus the second scan could be used as a baseline. 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 dipalmitoylphosphatidylcholine (DPPC)–H_2O suspension, assuming the enthalpy change of the DPPC phase transition to be 8.5 kcal/mol (Wilkinson & Nagle, 1980).

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; and scan rate, 100 G/min. Saturation transfer EPR spectra were recorded using the same instrument settings as those described by Thomas et al. (1976) and Poore et al. (1981). A field modulation of 5 G and microwave frequency of 9.5 GHz were

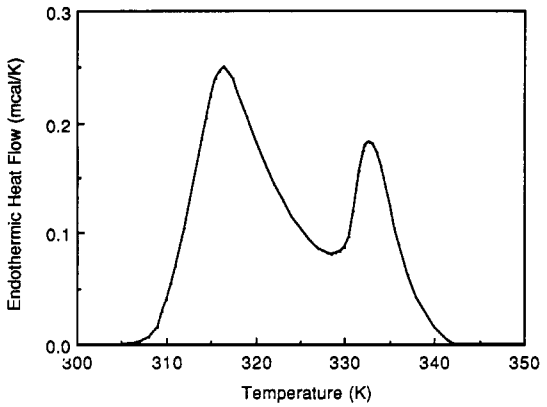

FIGURE 1: DSC thermogram of purified F_1F_0 complex. A 65-μL aliquot of purified F_1F_0 complex, 53.8 mg/mL, suspended in 10 mM Tris-SO_4, pH 7.7, buffer, containing 0.5 mM EDTA, 1 mM $MgSO_4$, 0.5 mM DTT, and 50 mM sucrose (buffer A), was placed into a large volume capsule, and the same amount of buffer A was used as reference. The recorder scanning speed was 1 cm/min. All scans were run at a rate of 2.5 K/min and a sensitivity of 0.5 mcal/s.

Table I: Thermotropic Properties of Isolated Cytochrome c Oxidase and F_1F_0 in Phospholipid Vesicles or in Detergent-Dispersed Form[a]

preparations	T_m (K)	ΔH (mcal/mg of protein)
CcO (dispersed)	330	2.54
F_1F_0 (dispersed)	316.5/332.5	1.29
CcO + F_1F_0 (dispersed)	330	2.07
CcO × PL (vesicles)	336	−19.00
F_1F_0 × PL (vesicles)	329/349.5	1.31/−2.62

[a] The molar ratio of cytochrome c oxidase to F_1F_0 complex is 1.

employed with phase-sensitive detection at 100 kHz (second harmonic) 90° out phase. Incident microwave power was 40 mW on the dial. The phase was adjusted to minimize the second harmonic signal. Approximate rotational correlation time (τ_2) were obtained from the ratio of the two 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.

Other Analytical Methods. Protein concentration was determined by the biuret method (Gornall et al., 1949), using bovine serum albumin as the standard. Absorption spectra were measured in a Cary spectrophotometer model 219. Phospholipid was determined by phosphorus content (Ames & Dubin, 1960), assuming an average molecular mass of 889 kDa.

Results and Discussion

Thermotropic Properties of Isolated CcO and F_1F_0 Complexes. Thermotropic properties of isolated CcO in the detergent dispersed form and in phospholipid vesicles have been described before (Yu et al., 1985). Purified CcO complex undergoes endothermic thermodenaturation but shows exothermic thermodenaturation when incorporated into phospholipid vesicles.

Figure 1 shows the differential scanning calorimetric thermogram of F_1F_0 in the detergent-dispersed form. Two endothermic transient peaks at 316.5 and 332.5 K were observed. The ΔH of thermodenaturation of F_1F_0 dispersed in detergent was calculated to be 1.29 mcal/mg of protein (Table I). When F_1F_0 was incorporated into phospholipid vesicles and subjected to DSC analysis, an endothermic peak at 329 K and an exothermic peak at 349.5 K were observed (Figure 2) with enthalpy changes of 1.31 and −2.62 mcal/mg of protein, respectively. The ratio of phospholipid (asolectin) to

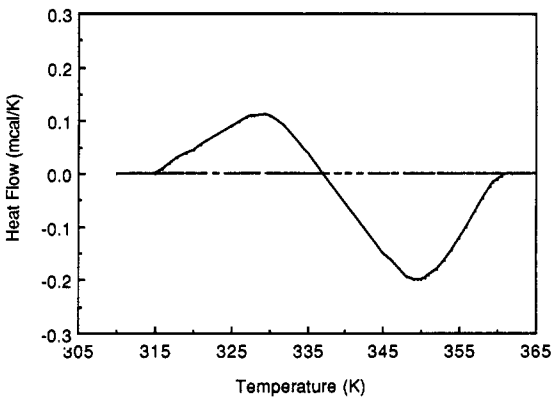

FIGURE 2: DSC thermogram of F_1F_0 complex embedded in phospholipid vesicles. The purified F_1F_0 complex (10 mg/mL) was mixed with an asolectin micellar solution [20 mg/mL in 50 mM sodium/potassium phosphate buffer, pH 7.4 (buffer A), containing 1% sodium cholate] at the weight ratio of 1:1.5. After an incubation at 0 °C for 30 min, the mixture was dialyzed against 100 volumes of buffer A overnight, with four changes of buffer. The precipitates were collected by centrifugation at 80000*g* for 1 h and resuspended in buffer A. The protein concentration was 26.1 mg/mL. The DSC instrument settings were as in Figure 1.

protein was 1.5. The endothermic ΔH change of purified F_1F_0 is comparable to that of electron transfer complexes, whereas the exothermic ΔH change of the F_1F_0 vesicle preparation is rather small compared to electron transfer complex vesicles. This is probably due to the contribution of F_1 portion of the complex because F_1 undergoes an endothermic transition. Under similar conditions, cytochrome c oxidase undergoes endothermic thermodenaturation at 330 K, with a ΔH of 2.54 mcal/mg of protein in the detergent-dispersed form and an exothermic thermodenaturation with a T_m of 336 K and a ΔH of −19 mcal/mg of protein, when reconstituted into phospholipid vesicles (see Table I).

When equal molar purified cytochrome c oxidase and F_1F_0 complexes were mixed and subjected to DSC analysis, the mixture showed an endothermic transition at 330 K with a ΔH of 2.07 mcal/mg of protein. The ΔH of the mixture of cytochrome c oxidase and F_1F_0 differs from the average ΔH value of 1.91 mcal/mg of protein of the two individual complexes. This suggests that some interaction between these two complexes exists. Since both complexes used were in the detergent-dispersed form, the difference observed in thermotropic properties cannot be solely attributed to the interaction between them. Other considerations should be made. For instance, the redistribution of detergent between the two complexes may affect the thermotropic behavior. It is also possible that the observation of an apparent change in ΔH stemmed from the artificial environment created during isolation of the complexes.

To confirm the existence of a specific interaction between cytochrome c oxidase and F_1F_0, studies were carried out with both 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 constant phospholipid to protein ratio of 1.5 was used. The ratio between cytochrome oxidase complex and F_1F_0 varies from 0.3 to 2.0. These enzyme complexes in protein–phospholipid vesicles should have an environment similar to that in the mitochondrial inner membrane. If the two lipoprotein complexes have no specific interaction, then no difference in DSC chracteristics 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 complex. The enthalpy change (Table II)

Table II: Exothermic Enthalpy Changes of Thermodenaturation of Various Electron Transfer Complexes and F_1F_o Complex Embedded in Phospholipid Vesicles[a]

preparations	T_m (K)	ΔH (mcal/mg of protein)
(CcO + F_1F_o) × PL	337	−3.46
(CcO × PL) + (F_1F_o × PL)	336	−6.32
(SQR + F_1F_o) × PL	337	−3.63
(SQR × PL) + (F_1F_o × PL)	338	−3.38
(QCR + F_1F_o) × PL	332	−8.60
(QCR × PL) + (F_1F_o × PL)	332	−7.60

[a] The molar ratio of electron transfer complexes to F_1F_o is 1, and the weight ratio of phospholipids to protein is 1.5 in all cases.

during exothermic denaturation of phospholipid vesicles embedded with a binary complex of cytochrome *c* oxidase and F_1F_0 is significantly different from that observed with a mixture of phospholipid vesicles embedded individually with cytochrome *c* oxidase or F_1F_0. This suggests specific interaction between these two complexes. No similar observation was made in the binary systems of ubiquinol–cytochrome *c* reductase or succinate–Q reductase and F_1F_0, indicating that no, or much weaker, interactions exist between these complexes. The maximum difference in ΔH between vesicles embedded with a mixture of cytochrome *c* oxidase and F_1F_0 and a mixture of vesicles embedded with cytochrome *c* oxidase or F_1F_0 was observed when the ratio of the two lipoprotein complexes is approximately one (see Figure 3). This suggests that the interaction between cytochrome *c* oxidase and F_1F_0 is specific. The accuracy of assessment of stoichiometry between the two complexes may have been compromised by uncertainty concerning the intactness of each complex.

As discussed earlier (Gwak et al., 1984, 1986; 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 unsaturated fatty acyl groups of phospholipids and a protein surface on the electron transfer or other lipoprotein complex which was exposed, by removal of an interacting protein from a complex or a complex from a supermacromolecular complex, during isolation. Such an interaction occurs only when a vesicle is formed; therefore, no exothermic transition is observed when cytochrome *c* oxidase or F_1F_0 is in the dispersed form. The addition of detergent to the electron transfer complex–phospholipid vesicles also causes the disappearance of the exothermic transition during thermodenaturation. Little exothermic transition was observed in mitochondrial or submitochondrial preparations because there is no such exposed area in the native complex or supercomplex to interact with phospholipids under strained conditions. When two interacting complexes are mixed together before being embedded in phospholipid vesicles, the exposed area on the protein surface is greatly diminished through the protein–protein interaction. Thus, less strained interaction occurs upon vesicle formation, and less enthalpy change of exothermic denaturation 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; this heat release was attributed to the autooxidation of iron sulfur protein (Tsong & Knox, 1984). This explanation is not applicable to the present study because there are no iron–sulfur proteins in cytochrome *c* oxidase and F_1F_0.

STEPR Studies of Spin-Labeled Cytochrome c Oxidase Embedded in Phospholipid Vesicles in the Presence and Absence of F_1F_0 Complex. Cytochrome *c* oxidase was labeled

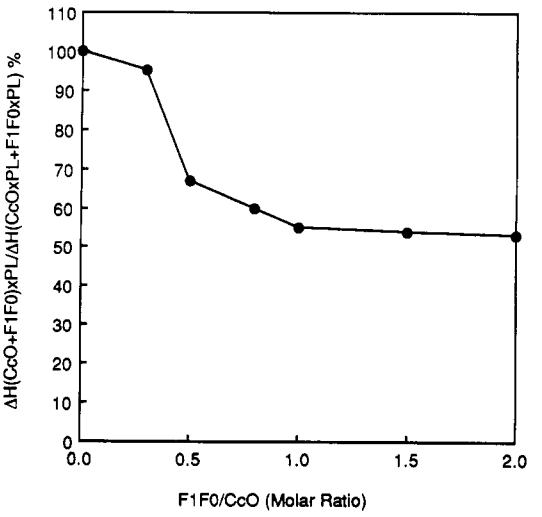

FIGURE 3: Comparison of thermodenaturation ethalpy changes of phospholipid vesicles formed with mixtures of cytochrome *c* oxidase and F_1F_0 complexes at various molar ratios and of mixtures of phospholipid vesicles of individual complexes. The molecular masses used in calculation of molar ratios were 500 000 and 200 000 daltons for cytochrome *c* oxidase and the F_1F_0 complex, respectively. The ratio of phospholipid to protein was 1.5 by weight in all cases.

with 4-maleimido-2,2,6,6-tetramethyl-1-piperidinyl-*N*-oxyl as described under Materials and Methods. This MSL–cytochrome *c* oxidase, which is enzymatically active, was embedded in phospholipid vesicles alone or together with F_1F_0. 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 only CcO or CcO and F_1F_0 complexes (see spectra 1 and 2 of Figure 4). This suggests that the difference in mobility of the spin-label on cytochrome *c* oxidase, in the presence and absence of F_1F_0, is too small to be measured by conventional EPR. Therefore, the protein rotational diffusion of the spin-labeled complex was measured by STEPR. From the change of the ratio of two low-field signals (L″/L) (see spectra 3 and 4 of Figure 4), rotational correlation times (τ_2) can be calculated (Thomas et al., 1976; Poore et al., 1981). The τ_2 of spin-labeled CcO was calculated to be 75 μs. This is higher than the τ_2 obtained by Ariano and Azzi (1980) (34 μs at 4 °C). The discrepancy can be explained by the presence of a different amounts of phospholipid in the vesicles. Ariano vesicles were made with a much higher phospholipid to protein ratio than were the vesicles used in our studies. As indicated in Figure 5, the rotational correlation time of spin-labeled cytochrome *c* oxidase is significantly affected by the presence of F_1F_0. The maximum τ_2 of 180 μs was obtained when the ratio of F_1F_0 to CcO approached 1. An increase in τ_2 was observed only when CcO was mixed with F_1F_0 before being embedded in phospholipid. The mixture of spin-labeled cytochrome *c* oxidase complex and F_1F_0 phospholipid vesicles showed the same τ_2 did of CcO phospholipid vesicles alone. Since F_0 is the membrane-anchoring part of F_1F_0 complex, the interaction of this complex with CcO is most likely through the F_0. This deduction is supported by the observation that the τ_2 of spin-labeled CcO increased when it was mixed with F_0 prior to the formation of vesicles (see Table III). The τ_2 of spin-labeled CcO was not affected by the addition of F_1 prior to the formation of vesicles (data not shown).

A similar effect of succinate–Q reductase on τ_2 of spin-labeled ubiquinol–cytochrome *c* reductase has been reported (Gwak et al., 1986). It is conceivable that at least part of the observed effect resulted from a change in the fluidity of the

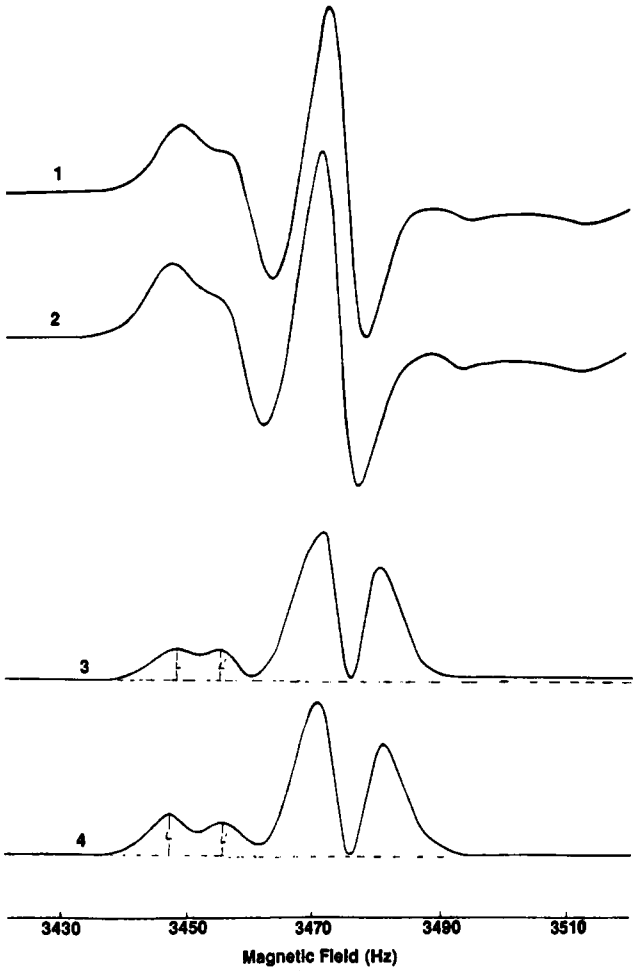

FIGURE 4: EPR spectra of spin-labeled cytochrome c oxidase in the presence and absence of F_1F_0 complex. Spectra 1 and 2 are conventional EPR spectra of spin-labeled cytochrome c oxidase embedded in phospholipid vesicles in the presence or absence of F_1F_0 complex. Spectra 3 and 4 are the saturation transfer EPR spectra of the same samples. The protein concentrations were 9.5 and 20.5 mg/mL for CcO and (CcO + F_1F_0) vesicles, respectively.

Table III: Effect of F_oF_1 Complex on the Rotational Correlation Times (τ_2) of Spin-Labeled Cytochrome c Oxidase[a]

preparations	L″/L	τ_2 (μs)
(MSL–CcO × PL)	0.73	75
[(MSL–CcO + F_1F_o) × PL]	1.02	180
[(MSL–CcO × PL) + (F_1F_o × PL)]	0.74	80
[(MSL–CcO + F_o) × PL]	0.96	160
[(MSL–CcO × PL) + (F_o × PL)]	0.65	75

[a] The molar ratio of CcO to F_1F_o used was 1:1. The molar ratio of CcO to F_o used was 1:5.

membrane by inclusion of protein complexes other than the spin-labeled complex. 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 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 the F_1F_0 complex, the τ_2 values obtained are in agreement with the DSC data.

From the results of DSC and STEPR experiments, we conclude that cytochrome c oxidase complex and F_1F_0 may exist as a supermacromolecule complex in the membrane. This conclusion differs significantly from the free diffusible model of electron transfer complexes derived from results of mem-

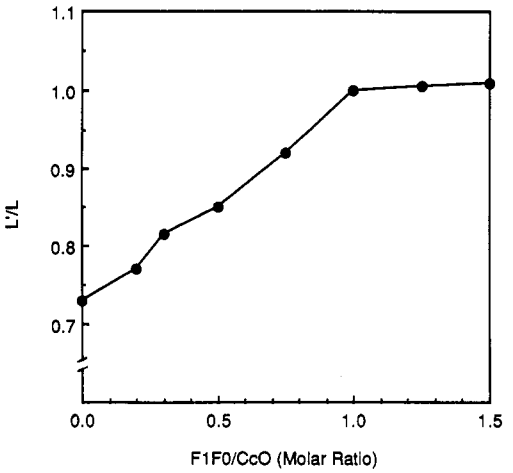

FIGURE 5: Effect of F_1F_0 complex on STEPR of spin-labeled cytochrome c oxidase. Increasing amounts of F_1F_0 complex were added to a constant amount of spin-labeled cytochrome c oxidase. The solutions were incubated for 30 min at 0 °C before being embedded in phospholipid vesicles. 1.5 mg of phospholipid/mg of protein was used. L″/L was calculated from the saturation transfer EPR spectra of each sample. Instrument settings are given under Materials and Methods.

brane fusing (Schneider et al., 1980) and fluorescence recovery, after photobleaching, measurements (Gupte et al., 1984). Measurement of the diffusion coefficients by the fluorescence recovery after photobleaching technique of F_1F_0 in mitochondrial membranes has not been reported. Since it has been clearly established that some electron transfer complexes form a supermacromolecule complex and others do not, it is likely that the formation of such complexes serve some regulatory function in the respiratory chain. Similarly, the formation of a supercomplex between F_1F_0 and cytochrome c oxidase, but not with other electron transfer complexes, may help control energy generation in mitochondria. The formation of supermacromolecule complexes from some electron transfer and ATP synthase complexes indicates that some of these mitochondrial complexes do not follow the random diffusion model, even though they are capable of doing so.

ACKNOWLEDGMENTS

Journal Article No. J6069 of the Agricultural Experiment Station, Oklahoma State University. We express our sincere thanks to Dr. Roger Koeppe for his critical review of this paper.

Registry No. *cis*-(CB)(Ph_2MeP)W(CO)$_4$, 138783-52-5; *cis*-(CB)(Ph_2PCH$_2$CH=CH$_2$)W(CO)$_4$, 138783-53-6; *cis*-(CB)(Ph_2P-(CH$_2$)$_2$CH=CH$_2$)W(CO)$_4$, 138812-89-2; *cis*-(CB)(Ph_2P-(CH$_2$)$_3$CH=CH$_2$)W(CO)$_4$, 138783-54-7; *cis*-(CB)(Ph_2P-(CH$_2$)$_4$CH=CH$_2$)W(CO)$_4$, 118772-55-7.

REFERENCES

Ames, B. N., & Dubin, D. T. (1960) *J. Biol. Chem. 235*, 769–775.

Amzel, L. M., & Pedersen, P. L. (1983) *Annu. Rev. Biochem. 52*, 801–824.

Ariano, B. H., & Azzi, A. (1980) *Biochem. Biophys. Res. Commun. 93*, 478–485.

Beard, W. A., & Dilley, R. A. (1988) *J. Bioenerg. Biomembr. 20*, 129–139.

Blair, P. V., Oda, T., Green, D. E., & Fernandez, M. H. (1963) *Biochemistry 2*, 756–764.

Capaldi, R. A. (1979) *Membrane Protein in Energy Transduction*, Marcel Dekker, New York.

Cherry, R. J. (1979) *Biochim. Biophys. Acta 559*, 289–327.

Colbeau, A., Nachbaur, J., & Vignais, P. M. (1971) *Biochim. Biophys. Acta 249*, 462–492.
Erecinska, M., Davis, J. S., & Wilson, D. F. (1980) *J. Biol. Chem. 255*, 9653–9658.
Galante, Y. W., Wong, S.-Y., & Hatefi, Y. (1981) *Arch. Biochem. Biophys. 15*, 643–651.
Gornall, A. G., Barwawill, C. J., & David, M. M. (1949) *J. Biol. Chem. 177*, 751–766.
Griffiths, D. E., & Wharton, D. C. (1961) *J. Biol. Chem. 236*, 1850–1856.
Gupte, S., Wu, E.-S., Hochli, L., Hochli, M., Kenneth, J., Sowers, A. E., & Hackenbrock, C. R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2606–2610.
Gwak, S.-H., Yu, L., & Yu, C. A. (1986) *Biochemistry 25*, 7675–7681.
Hackenbrock, C. R. (1981) *Trends Biochem. Sci. 6*, 151–154.
Hackenbrock, C. R., Chazotte, B., & Gupte, S. S. (1986) *J. Bioenerg. Biomembr. 18*, 331–368.
Hatefi, Y. (1985) *Annu. Rev. Biochem. 54*, 1015–1069.
Hatefi, Y., Haavik, A. G., & Griffiths, D. E. (1962) *J. Biol. Chem. 237*, 1676–1680.
Hochli, M., & Hackenbrock, C. R. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1636–1640.
Hochli, M., & Hackenbrock, C. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1236–1240.
Hochman, J., Schindler, M., Lee, J. G., & Ferguson-Miller, S. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6866–6870.
Hochman, J., Schindler, M., Lee, J. G., & Ferguson-Miller, S. (1983) in *Biochemistry of Metabolic Processes* (Lennon, D., Stratman, F., & Eahlten, R., Eds.) pp 441–450, Elsevier Science, New York.
Hochman, J., Ferguson-Miller, S., & Schindler, M. (1985) *Biochemistry 24*, 2509–2516.
Kagawa, Y. (1978) *Biochim. Biophys. Acta 505*, 45–93.
Kagawa, Y., & Racker, E. (1971) *J. Biol. Chem. 246*, 5477–5487.
Kawato, S., Siegel, E., Carafoli, E., & Cherry, R. J. (1981) *J. Biol. Chem. 256*, 7518–7527.
Klingenberrg, M. (1964) in *Biological Oxidation* (Singer, T. P., Ed.) p 3, Wiley, New York.
Laird, D. M., Eble, K. S., & Cunningham, C. (1986) *J. Biol. Chem. 261*, 14844–14850.
Lehninger, A. (1959) *Rev. Med. Phys. 31*, 136–146.
Mitchell, P. (1966) *Chemiosmotic coupling in oxidation and photosynthetic phosphorylation*, Glynn Research, Bodmin, Cornwall, U.K.
Mitchell, P. (1976) *J. Theor. Biol. 62*, 327–367.
Penefsky, H. S. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 49*, 223–280.
Poore, V. M., Fitzsimmon, J. T. R., & Ragan, C. I. (1981) *Biochim. Biophys. Acta 693*, 113–124.
Racker, E. (1972) *J. Membr. Biol. 10*, 221–235.
Rieske, J. S., Hansen, R. F., & Zaugg, W. S. (1964) *J. Biol. Chem. 239*, 3017–3023.
Schneider, H., Lemasters, J. J., Hochli, M., & Hackenbrock, C. R. (1980) *J. Biol. Chem. 255*, 3748–3756.
Schneider, H., Lemasters, J. J., & Hackenbrock, C. R. (1982) *J. Biol. Chem. 257*, 10789–10793.
Senior, A. E. (1988) *Physiol. Rev. 68*, 177–231.
Serrano, R., Kanner, B. I., & Racker, E. (1976) *J. Biol. Chem. 251*, 2453–2461.
Sjostrand, F. G., & Cassel, R. Z. (1978) *J. Ultrastruct. Res. 63*, 111–137.
Sowers, A. E. & Hackenbrock, C. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6246–6250.
Stigall, D. L., Gatante, Y. M., & Hatefi, Y. (1978) *J. Biol. Chem. 253*, 956–964.
Thomas, D. D., Dalton, L. R., & Hyde, J. S. (1976) *J. Chem. Phys. 65*, 3006–3024.
Tsong, T. Y., & Knox, B. E. (1984) *Biophys. J. 45*, 296a.
Von Jagow, G., & Sebald, W. (1980) *Annu. Rev. Biochem. 49*, 281–314.
Walker, J. E., Fernley, I. M., Gay, N. J., Gibson, B. W., Northrop, F. D., Powell, S. J., Runswick, M. J., Saraste, M., & Tybulewiez, V. L. (1985) *J. Mol. Biol. 184*, 677–701.
Wilkinson, D. A., & Nayle, J. H. (1980) *Biochemistry 19*, 187–192.
Wikstrom, M., Krab, K., & Saraste, M. (1981) *Annu. Rev. Biochem. 50*, 623–655.
Yu, C. A., & Yu, L. (1980) *Biochim. Biophys. Acta 591*, 409–420.
Yu, C. A., Gwak, S.-H., & Yu, L. (1985) *Biochim. Biophys. Acta 812*, 656–664.
Yu, L., & Yu, C. A. (1982) *J. Biol. Chem. 257*, 2016–2021.
Ziegler, D. M., & Doeg, K. A. (1962) *Arch. Biochem. Biophys. 97*, 41–50.