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Protein Rotational Mobility and Lipid Fluidity
of Purified and Reconstituted Cytochrome c Oxidase

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SUMMARY

The rotational mobility of spin-labelled bovine heart mitochondrial cytochrome c oxidase in purified form, and incorporated into liposomes, was studied. A rigidly attached short-chain maleimide spin label permitted the measurement of the protein's overall rotational mobility by saturation transfer electron paramagnetic resonance. A long-chain maleimide spin label was used to detect the fluidity of the lipid hydrocarbon region adjacent to the protein by conventional EPR. The oxidase activity and cytochrome c binding were not affected by labeling. Reconstituted membrane vesicles, containing functionally incorporated enzyme (as indicated by high electron transport activity and respiratory control), were prepared by either sonication or cholate dialysis. One method of preparing the purified enzyme resulted in a high degree of protein rotational mobility at 4°C both in the purified detergent-solubilized enzyme (effective rotational correlation time of 100 ns) and in reconstituted membranes (correlation time of 40 μs). By contrast, another purification procedure resulted in little or no sub-millisecond protein rotational mobility both in purified form and in reconstituted membranes, suggesting the presence of large protein aggregates. Thus, the state of aggregation of cytochrome oxidase in membranes appears to depend on the state of aggregation prior to reconstitution. The mobile and immobile enzymes had the same high activity.
In both reconstituted preparations, the bulk of the lipid was quite fluid at 4°C, as probed by a free fatty acid spin label. The lipid hydrocarbon region adjacent to the protein, as probed by the long-chain maleimide spin label, was also quite fluid in the membranes containing mobile enzyme, but was strongly immobilized in the membranes containing immobile enzyme. Thus, the strong immobilization of lipid in our preparations of cytochrome oxidase is apparently caused by protein-protein interactions, not by rigidity at the protein-lipid boundary.
Information on macromolecular motion is important for understanding the physical mechanisms of membrane functions. This seems particularly evident for the electron transport chain of the mitochondrial inner membrane since its function involves the dynamic interaction of several membrane-bound enzyme complexes. The present study focuses on the rotational dynamics of the terminal component of the electron transport chain, cytochrome c oxidase (ferrocytochrome c:oxygen oxidoreductase, EC 1.9.3.1), a transmembrane and oligomeric enzyme consisting of six to ten polypeptides, two α-type hemes, two copper atoms, and lipid (1-4).

Freeze-fracture electron micrographs of isolated mitochondrial inner membranes, obtained by Wrigglesworth et al (5), showed the appearance of randomly dispersed intramembrane particles. These observations led to the suggestion that the electron transport complexes could possess considerable lateral and rotational mobility (6). Evidence for the lateral mobility of cytochrome oxidase in isolated mitochondrial inner membranes was obtained by Höchli and Hackenbrock (7). Their freeze-fracture electron micrographs indicated that rapid changes in the lateral distribution of intramembrane particles occur as a result of thermotropic phase transitions or the addition of antibodies monospecific for cytochrome oxidase.

By contrast, Sjöstrand and coworkers (8,9) have produced evidence that cristae membranes in intact mitochondria contain...
densely packed protein, suggesting that the inner membrane of mitochondria may be highly viscous. Junge and DeVault(10) have studied the rotational mobility of the cytochrome \( a_3 \) component of cytochrome oxidase by observing the time dependence of linear dichroism following flash photolysis of a \( CO-a_3 \) complex in intact mitochondria, and have interpreted their data to suggest that the rotational correlation time of the \( a_3 \) is at least 100 ms(11). Also, several laboratories have demonstrated that it is possible to obtain a 'membranous' preparation of cytochrome oxidase which is active(12,13), and in which the enzyme appears to be packed in an ordered array(14-16). These latter lines of evidence(8-13) suggest that cytochrome oxidase is densely packed within the mitochondrial membrane and rotationally immobilized by protein-protein interactions.

Apart from this controversy concerning the mobility of the cytochrome oxidase complex within the mitochondrial inner membrane, another question involves how lipid associates with protein within the complex. The mobility of lipid in 'membranous' cytochrome oxidase has been studied with nitroxide spin labels(17). The finding of an immobilized lipid component in these membranes has led to the proposal of an immobilized 'boundary' layer of lipid surrounding the enzyme. An alternative explanation is that lipids are immobilized by protein-protein interactions as found in other densely packed membranes(18,19).
In the present work, spin labels were used to study the motion of lipids and the rotational mobility of the spin-labeled protein (20) in various environments, including when it was functionally incorporated into the bilayer of a lipid vesicle. This approach, which has proven useful in the study of other membrane-bound proteins (18, 21) was used to assess the importance of lipid and protein mobility for enzyme function.
EXPERIMENTAL PROCEDURE

Materials- Asolectin containing 95% soy phosphatides was obtained from Associated Concentrates, Woodside, Long Island, N.Y., and was partially purified by acetone extraction (22). Spin label I, 4-maleimido-2,2,6,6-tetramethyl piperidinoxy was purchased from Syva. Dr. P. Devaux kindly provided us with spin label II, 2-(14-carboxyl tetramethyl-N-ethyl maleic ester)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy, and Dr. R. Cooke with spin label III, 2-(14-carboxytetradecyl-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy. The three spin labels used in this study are shown below.

Cholic and deoxycholic acids were from Aldrich and were recrystallized twice from ethanol (23). Tween 20 (polyoxyethylene sorbitan monolaurate), Tween 80 (polyoxyethylene sorbitan monooleate), Triton X-100, Triton X-114, phosphatidyl choline (egg), and cytochrome c (type VI) were from Sigma. Ficoll was obtained from Pharmacia and electrophoresis purity reagents were purchased from Bio-Rad.

Preparations- Beef heart mitochondria were prepared by the method of Blair (24). Cytochrome c oxidase was isolated from these mitochondria using Triton X-114 or deoxycholate as detergents, and we will refer to these preparations as Triton-oxidase (25) and DOC-oxidase (26). Enzyme preparations were stored
in liquid nitrogen in the form of small beads. This method allows the removal of small quantities of enzyme at a time and the activity remains constant for at least six months. Mitochondrial lipids were extracted with chloroform-methanol (27).

Incorporation and reconstitution of cytochrome oxidase into liposomes was attempted in two ways. The first method involved using pure neutral lipid according to the procedure of Karlsson et al (28) in a 50 mM borate buffer, pH 7.7, containing 150 mM NaCl. Phosphatidylcholine (egg) was suspended in the above borate buffer to a concentration of 25 mg/ml and sonicated under a water-saturated argon stream for 30 min using a conical centrifuge tube in an ice bath. After centrifugation of this suspension at 14,000 x g for 60 min, 1 ml of the supernatant was removed and mixed with 0.2 ml of cytochrome oxidase (aa3) to obtain a final concentration of 30 μM aa3. In the case of the Triton-oxidase (2-6% lipid by weight) the enzyme was incubated with the liposomes for 60 min at 0°C prior to sonication, since immediate sonication led to inactivation of the oxidase. The mixture was then sonicated 1 min on/1 min off/1 min on at 0°C.

The second method involved preparing liposomes using partially pure asolectin or mitochondrial lipids either by cholate dialysis (29) or by a modification of the sonication procedure of Racker (30). For the sonication procedure,
phospholipids (50 mg) were dried under a stream of nitrogen
redissolved twice in a small volume of ether and dried again
under nitrogen 10-20 min. To this dried preparation 2 ml of
50 mM KH$_2$PO$_4$, pH 7.5 was added and sonication of this suspension
was performed in a bath-type sonifier under argon for 15 min
at 22°C. Immediately after sonication, 2.4 mg of cytochrome
oxidase were added to 1.2 ml of the phospholipid suspension, and
the mixture was incubated at 22°C for 30 min. At the end of
the incubation period, the mixture was sonicated for 5 min under
argon. Concentration of both types of vesicle suspension was
achieved by centrifugation at 180,000 x g for 90 min. The
resulting pellet was resuspended to a final concentration
of 50 μM.

In order to immobilize the membrane vesicles, they were
placed in an EPR capillary which was inserted into a specially
designed Lucite tube and centrifuged at 48,000 x g for 24 hr.

Spin Labeling- To study protein rotational mobility,
cytochrome oxidase was spin labeled with spin label I (MSL),
using 2 moles of label per mole of Triton-oxidase, and 5 moles
per mole of DOC-oxidase. Preparations were incubated 10 min at
22°C and the non-covalently attached label was eliminated by
Sephadex G-25 chromatography in 50 mM KH$_2$PO$_4$, pH 7.5 and 0.25%
Tween 20. In the case of both the Triton-oxidase and DOC-oxidase
it was necessary to treat the spin-labeled oxidase with 1 mM
cysteine and 10 mM potassium ferricyanide for 60 min in order to minimize the weakly immobilized signal. At the end of the incubation period, the sample was diluted ten-fold in the above buffer and precipitated with ammonium sulfate (0.35 saturation). In the case of the Triton-oxidase it was not essential to chromatograph the preparation; the cysteine and ferricyanide treatment was enough to eliminate both the unreacted and the weakly immobilized spin label. This pellet was brought up in either 0.25 M sucrose for incorporation into liposomes by the sonication procedure, or detergent-containing buffer (50 mM KH$_2$PO$_4$, pH 7.5, 0.25% Tween 20, 0.05% cholate) for incorporation by the cholate dialysis procedure.

Labeling of cytochrome c oxidase with spin label II, MSL (1,14), was performed according to Favre et al (18) at a spin label:oxidase ratio of 2:1. The amount of MSL (1,14) bound to the lipid in the cytochrome oxidase complex was determined after alkaline chloroform-methanol extraction of the enzyme (31).

**Ficoll gradients** - To determine the amount of cytochrome oxidase incorporated into lipid vesicles by the different reconstitution procedures, discontinuous Ficoll gradients were prepared according to Carroll and Racker (32) in 50 mM KH$_2$PO$_4$, pH 7.5 using steps of 5, 6, 8, 10, 15, and 30% Ficoll. After temperature equilibration of the gradients at 4°C for 4 hr, 1 ml aliquots of the cytochrome oxidase liposomal suspension were carefully applied to the top and centrifuged at 150,000 x g for
17 hr at 4°C. Each step of the gradient was analyzed for phospholipid content by a total phosphorous determination (32) of an alkaline chloroform:methanol (2:1) extract, and expressed as mg/mg protein. Protein was determined by the method of Lowry et al (33), using defatted bovine serum albumin (Sigma Chemicals) as a standard. Heme a content was determined from the reduced minus oxidized difference spectrum using a $\Delta \varepsilon_{605-630} = 13.1 \text{ mM}^{-1} \text{ cm}^{-1}$ (34). Heme a$_3$ concentration was determined from difference spectra of reduced minus reduced plus CO using a $\Delta \varepsilon_{428.5-445} = 148 \text{ mM}^{-1} \text{ cm}^{-1}$ (34).

**Enzyme activity** - Polarographic assays in 4.0 ml of buffer at 25°C were carried out either according to Nicholls et al (35) in the presence of 1% asolectin, 67 mM KH$_2$PO$_4$, pH 7.4 using 12.5 mM potassium ascorbate, 0.5 mM TMPD and 2.5 to 25 μM cytochrome c, or according to Ferguson-Miller et al (36) for the determination of the dissociation constant for high affinity binding using 25 mM Tris-acetate, pH 7.8 and 7 mM potassium ascorbate, 0.7 mM TMPD, and 0.004 to 4.0 μM cytochrome c with 50 nM cytochrome oxidase (final concentration). In the latter case monomeric ferrocytochrome c was obtained by gel filtration through G-75 Superfine (Pharmacia) in a column (0.7 x 30 cm) equilibrated in 50 mM Tris-acetate pH 7.5 (37).

Assays for the activity and orientation of oxidase incorporated into liposomes were performed at 20°C in 50 mM KH$_2$PO$_4$, pH 7.5 using 25 mM potassium ascorbate and 1 mg/ml cytochrome c (32).
Uncoupling was achieved by 0.5 µg/ml valinomycin and 2.5 µM FCCP, while functional orientation was determined in the presence of 3% Tween 80 (v/v). The respiratory control ratio (RCR) was determined as the ratio of the rate of O₂ uptake in the presence of valinomycin and FCCP to that measured in their absence. The baseline rate in the absence of cytochrome c is determined in all cases and subtracted from the rates at the various cytochrome c concentrations.

Gel filtration in sodium dodecyl sulfate-urea—Spin-labeled cytochrome c oxidase (20 mg) was dissolved in 1 ml of buffer containing 5% sodium dodecyl sulfate, 8M urea, and 0.05 M NaH₂PO₄, pH 7.2 by heating at 37°C for 30 min (38). Chromatography was carried out at 22°C in a column (1.5 x 85 cm) of Sephacryl S-200 Superfine equilibrated with 5 mM SDS, 8M urea, 0.05 M NaH₂PO₄, pH 7.2 at a flow rate of 5 cm/hr. Fractions were collected in three pools corresponding to elution peaks and lyophilized after desalting by dialysis against deionized water. After lyophilization, the samples were brought up in 0.4 ml of the elution buffer and the amount of spin label present was measured.

Gel electrophoresis—Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed in a Bio-Rad Model 200 dual vertical slab cell using 1.5 mm spacers and 20-well combs at 50 mA per gel for 90 min. Gels were made according to Laemmli (39) as follows: 5% stacking (acrylamide/bisacrylamide stock, 30/0.8%) and 13.5-16.0% gradient separation gels (acrylamide/
bisacrylamide stock, 30/1.6%). Samples were placed on the gels after treatment with 1% SDS and 1% mercaptoethanol in stacking buffer containing 12.5% glycerol and a trace of bromophenol blue tracking dye. The samples were not frozen prior to gel electrophoresis or heated in solubilization cocktail at 100°C in order to avoid the polypeptide aggregation which occurs as a result of these procedures. SDS-urea gel electrophoresis was performed according to Downer et al(38). Gels were fixed, stained with coomassie blue(40), and, for calibration of the relative mobilities corresponding to each of the enzyme's subunits, densitometric tracings were recorded at 545 nm using a Gilford Model 240 spectrophotometer (data not shown). The molecular weight profiles of both types of gels were calibrated using phosphorylase a, bovine serum albumin, ovalbumin, chymotrypsin A, myoglobin, lysozyme, cytochrome c, and bovine trypsin inhibitor.

**EPR** A Varian E-109E spectrometer was employed in the absorption mode for the 1st harmonic in phase (V1) and the 2nd harmonic out of phase (V2') spectra. A 1.0 mm inside diameter glass capillary cell was used together with a quartz dewar. Temperature was controlled with a Varian E4540 Variable Temperature Controller. The microwave field strength received by the sample was determined by calibration with a solution of Fremy's salt(41). Microwave power setting was 10 mW for the
$V_1$ spectra, corresponding to a field strength of 0.13 gauss. Conventional EPR spectra ($V_1$) were recorded with a 100 kHz field modulation (1 gauss peak to peak amplitude). Saturation transfer spectra ($V_2'$) were recorded with a field modulation of 50 kHz (6.3 gauss amplitude) and phase sensitive detection at 100 kHz (second harmonic), out of phase. The phase was set by minimizing the signal at low microwave power (0.5 mW). To record the $V_2'$ spectrum, the microwave power setting was then increased to 40 mW corresponding to a field strength of 0.25 gauss.
RESULTS

Characteristics of enzyme preparations- The preparations which were utilized for the determination of the rotational mobility of the soluble and membrane-bound cytochrome oxidase are described in Table I. Data for chymotrypsin-treated preparations is also presented for comparison with previous results (32). Triton-oxidase had the smallest amount of polypeptide contamination, while DOC-oxidase was much more contaminated as shown in Fig. 1. It should be noted that this discontinuous buffer gradient gel allows the resolution of many high molecular weight contaminants not detected on continuous buffer gels employing urea and SDS (38). These high molecular weight contaminants could be eliminated by further purification using cholate and ammonium sulfate (32) and gel filtration on Sepharose 4B (38). The nine polypeptides seen in Fig. 1 are the seven polypeptides of bovine heart cytochrome C oxidase plus the two contaminants Va and VIB described by Downer et al (38); these could not be entirely eliminated by incubation of the enzyme with chymotrypsin (10 µg/mg oxidase protein) at 25°C in 0.1 M NaH₂PO₄, pH 7.8, 0.5% cholate for two hours (32,42). In the case of the Triton-oxidase no reduction of the specific activity occurred as a result of the proteolytic digestion, whereas in the case of the DOC-oxidase the activity declined from 400 to 140 electrons/s per aa₃ unit.
Assays of the activity of spin-labeled Triton-oxidase and DOC-oxidase are shown in Fig. 2. No differences could be detected between the native and spin-labeled enzymes. As shown in Fig. 2a, only the lipid-poor Triton-oxidase preparation could be activated by the addition of lipid, as previously reported (28). The binding affinity constant ($K_d$) of cytochrome $c$ at the high affinity site is 0.03 μM (Fig. 2b) in good agreement with other results (36).

As can be seen from Table II approximately one MSL spin label per oxidase remained after spin-labeling and the cysteine and ferricyanide treatment. In order to determine whether the spin population was bound to one distinct subunit of the enzyme, the preparation was dissociated with 2% SDS and 8 M urea and chromatographed as detailed in the Methods section. EPR was used to determine the amount of spin associated with each fraction. SDS-urea gel electrophoresis was then performed as in (38), and it was found that spin was associated only with fractions containing subunits II and III (MW 22,000 and 20,500); these were not resolved further because of spin loss which occurred during the chromatographic procedure. Kornblatt et al (43) have previously demonstrated that ($^{14}$C)-NEM and MSL both react with a subunit of MW 25,000 at pH 8.5, but subunits II and III were not resolved in their electrophoretic study. Recently, these investigators have produced evidence that ($^{14}$C)-NEM reacts with subunits III and VI at pH 7.0 with the labe-
ling of subunit III being predominant (44).

Reconstitution using phosphatidyl choline- Following spin labeling and chromatography the DOC-oxidase eluted always from the column as a turbid suspension, whereas the Triton-oxidase remained optically clear. The turbid suspension had the opalescent appearance characteristic of a vesicle suspension. An electron micrograph of a negatively-stained preparation of DOC-oxidase after chromatography (Fig. 3), demonstrates that it consists of a very heterogeneous population of vesicles with diameters ranging from 500 to 2500 Å; some multilamellar vesicles were also detected, but we found no respiratory control in these preparations.

Since saturation transfer EPR experiments require spin-label concentrations on the order of 10 μM or higher, a reconstitution procedure developed by Karlsson et al (28) was used at a low lipid to protein ratio (1:1, by weight), permitting convenient concentration of the enzyme to 50 μM. Negatively-stained preparations of DOC-oxidase reconstituted in this manner, showed vesicles of mean diameter 1020 ± 100 Å (Fig. 3), but with no detectable respiratory control; an analysis of these samples on a discontinuous Ficoll gradient (Fig. 4), demonstrated little or no enzyme incorporation in the phosphatidyl choline vesicles, in agreement with previous results (45). Using partially purified asolectin we were able to obtain 20% incorporation of cytochrome oxidase by this technique (as detected by Ficoll gradient analysis). However, with 80% of the enzy-
me unincorporated, the respiratory control ratio was very low
$(RCR = 1.3 \pm 0.2)$. Preparations with much greater respiratory control ratios
were obtained by different reconstitution procedures using much
higher lipid to protein ratios.

Reconstitution with high respiratory control—

These cytochrome oxidase vesicles were prepared by a modification of
Racker's sonication technique (30), and cholate dialysis tech-
nique (29), using partially purified asolectin or mitochondrial
lipids. Only Triton-oxidase was used, which after spin-labe-
ling and chromatography can be shown to be in a disaggregated
form (see later Results).

The complete incorporation of Triton-oxidase into parti-
ally purified asolectin vesicles is shown in Fig. 4. Similar
results were obtained with mitochondrial lipids. Electron
micrographs of these negatively-stained preparations are shown
at the bottom of Fig. 3 (mean vesicle diameter $420 \pm 30 \text{ A}$).

EPR spectra of the various preparations—

Determination of rotational motion of proteins by the technique of saturation
transfer EPR requires that a spin-label be bound to a macromo-
lecule such that the population of weakly immobilized spins is
minimized as detected on the $V_1$ spectrum. The molar ratio of
MSL to cytochrome c oxidase, the incubation time and the tempe-
rature were adjusted to minimize this weakly immobilized popu-
alation, but in the case of both the DOC- and Triton-oxidase
additional treatment with cysteine and ferricyanide was required. This treatment affected neither the activity of the enzyme nor the $K_d$ for high-affinity binding of cytochrome $c$ measured at low ionic strength. Both DOC- and Triton-oxidase preparations possess nearly identical $V_1$ spectra at $4^\circ C$ (Fig. 5), but when the Triton-oxidase is reconstituted by the cholate dialysis or sonication (our modification of (30)) procedures, a small weakly immobilized population of spins (at arrows) can be seen at $22^\circ C$ but not at $4^\circ C$. To avoid ambiguities all the spectra were recorded at $4^\circ C$.

**Lipid versus protein mobility**  As described by Jost et al. (46,47), when lipid (including some spin-labeled hydrocarbon chains) is added to oxidase which has been partially phospholipid depleted, the spin labels become strongly immobilized.

The DOC-oxidase was clearly in a membranous form (Fig. 3). As shown in Fig. 6, the fatty-acid spin-label FASL(1,14), becomes immobilized ($V_1$ spectrum) when it is added to this preparation, in agreement with results obtained with similar oxidase preparations (46).

However, Fig. 6 also shows that the rotational correlation time of the MSL spin-labeled DOC-oxidase is $>1$ ms ($V_2$ spectrum).

**Rotational correlation times of purified and reconstituted Triton-oxidase**  A preparation of Triton-oxidase was spin-labeled and either (a) chromatographed and then treated with cysteine and ferricyanide or (b) directly treated without chroma-
Both preparations were then precipitated at 0.35 saturation of ammonium sulfate and redissolved in detergent-containing buffer. These two types of spin-labeled Triton-oxidase were then reconstituted using partially purified asolectin or mitochondrial lipids by the cholate dialysis procedure. Similar results were obtained by our modification of the sonication procedure (30).

Saturation transfer spectra of the four types of preparations are shown in Fig. 7; indicated are the parameters \( \text{L, L'}, \text{C and C'} \), used to estimate the rotational correlation times. The Triton-oxidase, which was chromatographed following spin-labeling, possessed an effective rotational correlation time \( (\tau_2) \) of about 100 ns, as determined from the above parameters of its spectrum (see Fig. 8). This is the correlation time predicted \(^3\) for a sphere with a molecular weight of \( \sim 200,000 \) at \( 40^\circ\text{C} \). This preparation contains only 4% lipid (by weight) and the protein's estimated MW is 140,000 (4); it appears therefore that the chromatographed Triton-oxidase exists in a disaggregated form, in agreement with analytical ultra-centrifugation studies in Triton X-100 (48) and Emasol 1130 (49).

The Triton-oxidase that was not chromatographed prior to cysteine and ferricyanide treatment displayed a rotational correlation time of \( \geq 1 \text{ ms} \), indicating protein aggregation. Since a large part of this preparation aggregated on the Sephadex G-25 gel filtration column when chromatographed, and could
only be solubilized by continue elution, it appears that the enzyme exists in a heterogeneous mixture of particle sizes and that gel filtration in detergent slowly eliminates the higher molecular weight aggregates from the population.

As can be seen in Fig. 7, the Triton-oxidase which was mobile in the chromatographed form remained relatively mobile when reconstituted ($\tau_2 \sim 40 \mu s$), while the immobilized unchromatographed preparation remained immobilized when reconstituted. The vesicles made from the immobile and the mobile preparations were quite similar in size with diameters of $410 \pm 30 \AA$ and $420 \pm 30 \AA$ respectively (Fig. 3). Both preparations were fully active ($V_{\text{max}} = 400$ electrons/s) and possessed high respiratory control ratios (in some cases close to 5), indicating that most of the enzyme was functionally incorporated (32).

The rotational time of reconstituted cytochrome oxidase appeared to be independent of vesicle mobility, since centrifugation of the vesicle suspension to form a compact gelatinous pellet did not alter the $V_2$ spectra.

Treatment of the mobile Triton-oxidase in reconstituted vesicles, at low oxidase concentration (0.38 mg/ml) with the bifunctional imidoester, dimethyl suberimidate, at a final concentration of 10 mM does not result in a further immobilized spectrum. Electron micrographs of these vesicle preparations indicate that no intervesicular cross-linking occurs under these conditions, whereas SDS gel electrophoresis demonstrates exten-
sive polypeptide coupling of all the oxidase subunits, except for subunit I (MW 35,500) (4). Since the MSL spin-probe is bound to either subunit II or III, our cross-linking results indicate that we are not observing the independent rotational motion of these subunits. Under these conditions no inter-cytochrome oxidase cross-linking is believed to occur (4).

Glutaraldehyde treatment (40 mM final concentration) of concentrated vesicle suspensions (5 mg/ml) does result in an immobilized $V_2^*$ spectrum of the mobile preparation ($T_2$ increases from 40 $\mu$s to $\geq$ 1 ms); glutaraldehyde treatment of the immobile reconstituted oxidase did not appreciably alter the $V_2^*$ spectrum ($L''/L$ increased from 1.20 to 1.25).

A summary of the saturation transfer data obtained for the various preparations of cytochrome oxidase used in this study (Fig. 8), shows how effective correlation times were estimated (50). The spectral parameter $C'/C$ is especially sensitive in the $10^{-6}$ to $10^{-8}$ s range, while $L''/L$ is sensitive in the range of $10^{-3}$ to $10^{-6}$ s.

**Lipid mobility correlates with protein mobility**—Since the reconstituted Triton-oxidase vesicle preparations contained a lipid to protein ratio of $> 3000:1$, it is virtually impossible to selectively study the lipid adjacent to the protein using free spin-probes of the lipid phase. Therefore a lipid probe was used that was covalently attached to the protein, MSL(1,14). The nitroxide group of this probe is attached near
one end of the acyl chain and the other end reacts with the sulfhydryl group at pH 7.0 through the N-ethyl maleimide linkage. This probe has been used to study the lipid environment of rhodopsin in retinal disk membranes (18). MSL(1,14) was added at a molar ratio of 2:1 to aa3. Determinations of the total spin bound to the enzyme, following elimination of the non-covalently bound MSL(1,14), were performed as in (18) and lipid was then extracted by the alkaline chloroform-methanol procedure described in Methods. These results indicate that 90 ± 5% of the total spin was bound to the protein portion of the enzyme. As can be seen in Fig. 9, MSL(1,14) is immobilized on both the chromatographed (mobile protein) and the non-chromatographed (immobile protein) purified Triton-oxidase, but a greater proportion is immobilized on the non-chromatographed preparations. When these two types of Triton-oxidase were reconstituted, the preparation containing mobile protein displayed no evidence of a strongly immobilized MSL(1,14) (Fig. 9, center right), whereas the preparation containing immobile protein showed a large amount of immobilized MSL(1,14) (Fig. 9, center left). The spectra of added FASL(1,14) (free fatty acid) to these preparations (molar ratio of FASL(1,14) : oxidase was 1:1) are depicted at the bottom of Fig. 9. As expected, neither Triton-vesicle type immobilized a significant fraction of this free fatty-acid label at these high lipid to protein ratios. It is interesting to note that the spectrum of the MSL(1,14)
in the mobile Triton-oxidase (Fig. 9, center right) is slightly broader than that of the FASL(1,14) - MSL(1,14) difference spectrum in the same preparation (Fig. 9, bottom right). Thus, although the adjacent hydrocarbon chains are not strongly immobilized by the protein, they are slightly less mobile than the lipid chains far from the protein. The hyperfine splitting of the covalently labeled MSL(1,14) differs at most by 0.2 gauss from that of FASL(1,14) in the reconstituted systems indicating that the two probes are probably in similar hydrophobic environments.

DISCUSSION

The rotational mobility of isolated and reconstituted cytochrome c oxidase from beef heart mitochondria has been studied and correlated with the fluidity of the hydrophobic environment adjacent to the protein. Prior to chromatography, the spin-labeled complex never displays rotational motion in the sub-millisecond range of correlation times (Fig. 7). Chromatographed DOC-oxidase, which contains considerably more lipid and contaminant polypeptides (Fig. 1) than the Triton-oxidase preparation, has a tendency to vesiculate and/or form enzyme sheets even in detergent, and shows no sub-millisecond rotational motion. However, chromatographed preparations of spin-labeled Triton-oxidase, in the presence of detergent, display
rotational correlation times in the 100 ns range (Fig. 7), indicating that the enzyme is probably disaggregated. These results suggest that, as isolated, cytochrome oxidase is probably in the form of aggregated patches (as previously observed, Ref. 17), and that chromatography in the presence of detergent can, under certain conditions, disaggregate these patches.

Functional incorporation of cytochrome oxidase into lipid vesicles was achieved at high lipid to protein ratios (12.5:1 by weight), by the cholate dialysis or sonication procedures, but this did not seem to modify the aggregation state of the oxidase. Following the incorporation of non-chromatographed samples of cytochrome oxidase into liposomes, the rotational correlation time of the protein remains ≥ 1 ms; incorporation of chromatographed oxidase into liposomes results in a rotational correlation time in the 40 μs range, indicating that the oxidase probably remains in a disaggregated form in the membrane.

The activity of the complex in the reconstituted preparations does not seem to depend on its rotational correlation time; the respiratory control ratios are also very similar in the two kinds of reconstituted preparations and so are the lipid to protein ratios of the various populations of vesicles obtained in the Ficoll gradients. These results are independent of whether mitochondrial or partially purified asolectin is used for reconstitution. Therefore, sub-millisecond rotation
onal mobility of cytochrome c oxidase is not required for electron transfer from cytochrome c (its natural donor) to oxygen. Aggregated patches of the complex will efficiently transfer electrons from cytochrome c to O$_2$ and may in fact be present in the inner membrane of mitochondria, as previously suggested (51).

In contrast to non-chromatographed oxidase, both Ca$^{++}$-ATPase (MW ≈ 100,000) from sarcoplasmic reticulum (21,50) and rhodopsin (MW ≈ 20,000) (52) display a high degree of sub-millisecond rotational mobility both in native and reconstituted membranes (Fig. 8). In addition, when the mobility of the Ca$^{++}$-ATPase is inhibited, the enzyme activity is also inhibited. Like the immobile cytochrome oxidase preparations, spin-labeled recombinant acetyl choline receptors have no sub-millisecond rotational mobility, indicating protein aggregation (53).

The results with reconstituted cytochrome oxidase covalently labeled with MSL(1,14) at a lipid to protein ratio of 12.5:1 (by weight) indicate that the hydrophobic environment of the oxidase is very viscous in the aggregated form, and very fluid in the disaggregated form (Figs. 8 & 9). It is difficult to identify precisely the region around the oxidase sampled with this probe; the maximum distance from the maleimide moiety to the nitroxide radical is about 30 Å and therefore no more than the first three layers of lipid around the enzyme are sampled by the probe. It is not surprising that, in the aggregated form, the immediate environment of the oxidase should be so
viscous; in this case only a few lipid molecules are expected to be trapped between the oxidase complexes with their fatty acid chains in a constrained environment.

Earlier EPR studies with spin-labeled fatty acid chains and lipids in cytochrome oxidase preparations have shown a high degree of immobilization of lipids at low lipid to protein ratios (17,46,54); the results obtained with DOC-oxidase and the fatty acid spin-probe FASL(1,14) (Fig. 6) have confirmed this. In the previous studies (17,46,54), the amount of strongly immobilized lipid was shown to be proportional to the amount of protein, and the results were interpreted to mean that each protein independently immobilized a constant number of lipid molecules. This was visualized as a boundary layer of strongly immobilized lipid. An equally probable explanation for these results could have been the immobilization of lipid trapped within protein aggregates which do not disaggregate at high lipid concentrations. The latter explanation is supported by the results of the present investigation.

Furthermore, in the disaggregated form of the reconstituted enzyme, the fluidity of the hydrophobic environment around the oxidase measured with the MSL(1,14) probe is only slightly less than the fluidity of the bulk lipid hydrophobic environment measured with the FASL(1,14) probe (Fig. 9).

Theoretical considerations (55) and NMR data (56-59) suggest that, if a boundary layer of lipids exists around
proteins then, compared to the bulk lipids, its fatty acid environment may be more fluid below the bulk lipid phase transition and less fluid above the phase transition. The protein may act like an impurity or defect in the lipid lattice, altering somewhat the ordering of the fatty-acid chains near its hydrophobic environment. Dahlquist and coworkers (60,61) have performed deuterium NMR experiments on a DOC-purified oxidase preparation. They found evidence for two distinct populations of lipid hydrocarbon chains, one strongly immobilized and one approximately as mobile as pure lipid in the absence of protein. Under similar conditions of preparation, the DOC-oxidase used in the present investigation is aggregated. Oldfield et al (57) performed analogous NMR experiments on a cholate-purified cytochrome oxidase preparation and have not detected an immobilized phospholipid population. If this latter preparation contained disaggregated enzyme, then the discrepancy between those two NMR studies may be explained by our analysis; that is, a distinct strongly immobilized population of hydrocarbon chains exists only when the enzyme is aggregated.

A similar situation arises in rhodopsin-containing membranes (18), where the immobilization of the protein and lipid occur only at low lipid to protein ratios.

Acknowledgement- We wish to thank L. Packer, in whose laboratory most of this work was conducted, for helpful dis-
cussions throughout the course of this work.

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This work was supported by the Energy and Environment Division of the Lawrence Berkeley Laboratory, U.S. Department of Energy under contract No. W-7405-ENG-48. For a portion of this work M.S. was the recipient of a Chancellor's Patent Fund Grant from the University of California. D.D.T. was supported by a Helen Hay Whitney Postdoctoral Fellowship.

1 The abbreviations employed are: EPR, electron paramagnetic resonance; \( \text{aa}_3 \), cytochrome c oxidase; Triton-oxidase, cytochrome oxidase purified with Triton X-114 according to (25); DOC-oxidase, cytochrome oxidase purified with deoxycholate according to (26); MSL, 4-maleimido-2,2,6,6-tetramethylpiperidinoxyxl; MSL(1,14), 2-(14-carboxyltetradecyl-N-ethyl maleic ester)-2-ethyl-4,4-dimethyl-3-oxazolidinylxoyl; FASL(1,14), 2-(14-carboxyltetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinylxoyl; RCR, respiratory control ratio; TMPD, N,N,N',N'-tetramethylphenylenediamine; NMR, nuclear magnetic resonance; MW, molecular weight; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

2 Swanson, M.S., Petersen, L. and Packer, L., manuscript in preparation.

3 Using the formula: \( \tau_2 = (V_r/kT) \), where \( V = \text{cm}^3/\text{molecule} = 0.73 \text{ cm}^3/\text{g.(MW/}N_0) \). \( V \) is the molecular partial specific volume,
n the medium viscosity, k Plank's constant, T the absolute temperature, N₀ Avogadro's number and MW the molecular weight.
REFERENCES


### TABLE I

**Characteristics of the cytochrome oxidase preparations**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Purity ( \text{nmoles heme/ mg protein} )</th>
<th>Activity ( \text{activity electrons/ s per aa} )</th>
<th>Lipid Content ( \text{mg lipid/ mg protein} )</th>
<th>SH Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOC-oxidase</td>
<td>9.0-10.5</td>
<td>400</td>
<td>0.35-0.42</td>
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</tr>
<tr>
<td>Triton-oxidase</td>
<td>10.0-11.8</td>
<td>400</td>
<td>0.02-0.06</td>
<td>7</td>
</tr>
<tr>
<td>Chymotrypsin DOC-oxidase</td>
<td>10.0-11.7</td>
<td>140</td>
<td>ND(^{c})</td>
<td>7</td>
</tr>
<tr>
<td>Chymotrypsin Triton-oxidase</td>
<td>10.5-12.0</td>
<td>400</td>
<td>ND(^{c})</td>
<td>7</td>
</tr>
</tbody>
</table>

\(^{a}\) Activity is expressed as \( V_{\text{max}} \) value in 67mM \( \text{KH}_2\text{PO}_4 \), pH 7.4, 0.5% Tween 80 in the presence of 1% asolectin at 25°C.

\(^{b}\) Determined according to (62), in 0.1% SDS. Values, the mean of at least three determinations, were rounded to the nearest whole number.

\(^{c}\) Not determined.
TABLE II

Reduction of the covalently bound MSL spin probe

<table>
<thead>
<tr>
<th>Preparation</th>
<th># spin labels added/aa₃</th>
<th># spin labels reacted/aa₃</th>
<th># spin labels not reduced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triton-oxidase</td>
<td>2</td>
<td>1.5</td>
<td>1.1</td>
</tr>
<tr>
<td>DOC-oxidase</td>
<td>5</td>
<td>3.2</td>
<td>1.4</td>
</tr>
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</table>

a Based on the number of SH groups available for DTNB reaction (60) before and after spin-labelling (mean of 5 determinations).

b Calculated by double integration of spectra taken before and after cysteine and ferricyanide treatment (mean of 5 determinations).
Fig. 1. SDS gel electrophoresis of the cytochrome oxidase preparations used in this study. The numbers at the bottom of the gel profile refer to the number of μg loaded per channel. The stacking gel was removed for convenience, and did not contain any stained material.

Fig. 2. Polarographic activity assays of spin-labeled cytochrome c oxidase.

2a. Lineweaver-Burk plot of steady-state O₂ consumption.

2b. Eadie-Hofstee-Scatchard plot showing high affinity binding kinetics of cytochrome c.

All points represent mean averages of at least five separate determinations. Activity assays of native and spin-labeled cytochrome oxidase yielded identical results.

Fig. 3. Electron micrographs of preparations negatively-stained with 1% phosphotungstate (pH 7.0) followed by 1% uranyl acetate.

Fig. 4. A Ficoll discontinuous gradient analysis of protein incorporation into lipid vesicles. The sonication procedure of (28) was employed using preformed phosphatidyl choline vesicles and DOC-oxidase, and the cholate dialysis procedure of (29) was used employing either mitochondrial lipids or partially-purified asolectin and the Triton-oxidase, either chromatographed
or not. The use of mitochondrial lipids in the cholate dialysis procedure did not change our results in any detectable fashion.

Fig. 5. Conventional (V₁) spectra of the purified (in detergent) and reconstituted cytochrome oxidase (50 μM) preparations spin-labelled with MSL. The arrows designate the weakly immobilized signal present at 22°C in the Triton-oxidase preparations reconstituted by the cholate dialysis procedure.

Fig. 6. A conventional (V₁) spectrum of added FASL (1, 14) and second harmonic (V₂') spectrum of covalently bound MSL to purified DOC-oxidase (50 μM) in 50 mM KPi, pH 7.5, 0.25% Tween 20, prior to lipid addition.

Fig. 7. Second harmonic spectra (V₂') of purified and reconstituted Triton-oxidase preparations which were either not chromatographed or chromatographed after spin labelling. The spectral parameters L, L", C, C' are used to calculate effective rotational correlation times as described in Methods.

Fig. 8. Plots of the spectral parameters C'/C and L"/L and their relationship to the rotational correlation time, τ₂, illustrating the procedure used to determine effective correlation times for nitrooxide spin label saturation transfer EPR spectra (V₂'). The curves were obtained from theoretical
studies and model system experiments on isotropic rotational diffusion (41). Purified Triton-oxidase (in detergent) after chromatography (●●); Purified DOC-oxidase (in detergent) after chromatography (〇〇); Triton-oxidase, chromatographed and then reconstituted (△△); Triton-oxidase, not chromatographed and then reconstituted (■■); rhodopsin, as determined by (18) (▲▲); Ca\(^{++}\)-ATPase, native lipids (△△); Ca\(^{++}\)-ATPase, native lipids replaced with dipalmitoyl lecithin (◇◇). Both of the of Ca\(^{++}\)-ATPase values were determined by (49).

Fig. 9. Conventional (V\(_1\)) spectra of MSL(1,14) covalently bound to Triton-oxidase. The difference spectra of FASL(1,14)-MSL(1,14) at the bottom of the figure were obtained by computer subtraction using a PDP 11/34 computer attached to the EPR recorder. Purified aa\(_3\) concentration was 100 μM in 50 mM KH\(_2\)PO\(_4\), pH 7.5, 0.25% Tween 20, 0.05% cholate. Reconstituted aa\(_3\) concentration was 50μM in 50 mM KH\(_2\)PO\(_4\), pH 7.5.
CYTOCHROME OXIDASE

FIGURE 1
<table>
<thead>
<tr>
<th>Preparation</th>
<th>$K_m \times 10^{-8} M$</th>
<th>$K_m \times 10^{-6} M$</th>
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<tr>
<td>TRITON-oxidase</td>
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</table>

**FIGURE 2b**
FIGURE 3

DOC-oxidase

DOC-oxidase & lipid

TRITON-oxidase:
reconstituted & immobile

TRITON-oxidase:
reconstituted & mobile

XBB 796-7812
### SONICATION: PHOSPHATIDYLCHOLINE DOC- oxidase

<table>
<thead>
<tr>
<th>% FICOLL</th>
<th>PERCENT OF TOTAL</th>
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<th>RCR</th>
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<tr>
<td>5</td>
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<td></td>
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<td>15</td>
<td>68.8</td>
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### CHOLATE DIALYSIS: ASOLECTIN TRITON- oxidase

<table>
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</tr>
</tbody>
</table>

**FIGURE 4**
\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6}
\caption{Figure 6}
\end{figure}
FIGURE 9

IMMOBILE

MOBILE

Purified

Reconstituted

Difference Spectra
FASL(1,14)-MSL(1,14)

XBL796-3493
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