

Cytochrome Oxidase Rotates in the Inner Membrane of Intact Mitochondria and Submitochondrial Particles*

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A transient dichroism is detected after photolysis by a linearly polarized laser flash of the cytochrome oxidase·CO complex in bovine heart mitochondria, rat heart mitochondria, and bovine heart submitochondrial particles. A decay in the absorption anisotropy is characterized by a time constant of about 300 to 400 μ s in both mitochondria and submitochondrial particles. Since vesicle tumbling in the time range less than 5 ms can be excluded in these experiments, we conclude that cytochrome oxidase rotates in the mitochondrial membrane with a relaxation time of several hundred microseconds. However, it is likely that only about one-half of cytochrome oxidase contributes to the observed decay, the remainder being relatively immobile.

Cytochrome oxidase, an integral transmembrane protein (1-3), is the terminal enzyme of the respiratory chain in the mitochondrial inner membrane (4). As judged by freeze-fracture electron microscopy of mitochondrial inner membrane (5), integral proteins are randomly dispersed rather than organized into a lattice. Höchli and Hackenbrock (6, 7) have observed rapid changes in the lateral distribution of intramembrane particles, which occur as a result of a thermotropic phase transition or the addition of antibodies monospecific for cytochrome oxidase. They conclude that cytochrome oxidase and other integral proteins are able to undergo lateral diffusion in the plane of the membrane.

In contrast, Junge and Devault (8) observed no detectable decay of flash-induced linear dichroism of the cytochrome oxidase·CO complex in mitochondria for times up to 5 ms. Taking into account subsequent studies of the orientation of the heme a_3 chromophore (9-11), this implies that cytochrome oxidase is completely immobilized in the mitochondrial membrane in the above time range.

We have made similar transient dichroism measurements using signal-averaging to improve the signal to noise ratio and found that a decay in the absorption anisotropy does occur. The data imply that both mobile and relatively immobile populations of cytochrome oxidase are present in the mitochondrial membrane.

EXPERIMENTAL PROCEDURES

Bovine heart mitochondria were prepared according to Hatefi and Lester (12) as modified by Bock and Fleischer (13) and stored frozen

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at -80°C . Rat heart mitochondria were isolated by the procedure of Crompton *et al.* (14). During both procedures, care was taken to avoid any contamination with blood. SMP¹ were prepared according to the method of Krebs *et al.* (15) from bovine heart mitochondria and stored at -80°C . For the transient dichroism experiments, mitochondria and SMP were diluted into buffer containing 50 mM Hepes, pH 7.4, and varying amounts of sucrose to a final concentration of 0.8 to 1.6 mg of protein/ml. Mitochondria and SMP were reduced by 1.3 mM ascorbate and 25 μ M TMPD and slowly bubbled for 1 min with CO.

The flash photolysis apparatus used for rotational diffusion measurements is described in detail elsewhere (16). Briefly, the sample was excited at 590 nm by a vertically polarized flash of duration 1 to 2 μ s from a dye laser (rhodamine 6G in methanol). Absorbance changes due to photolysis of the heme a_3 ·CO complex were measured at 446 nm. The signals were analyzed by calculating the absorption anisotropy, $r(t)$, given by

$$r(t) = \frac{A_V(t) - A_H(t)}{A_V(t) + 2A_H(t)} \quad (1)$$

where $A_V(t)$ and $A_H(t)$ are, respectively, the absorbance changes for vertical and horizontal polarization at time t after the flash. In each experiment, 64 signals were averaged using a Datalab DL 102A signal averager. A further improvement in signal to noise was achieved by averaging data from a number of experiments.

Samples were measured in sucrose solution in order to decrease the rate of vesicle tumbling and to reduce light scattering. Under the present condition, the effect of light scattering on $r(t)$ curves was negligible.

RESULTS AND DISCUSSION

Fig. 1 shows representative absorption signals, $A_V(t)$ and $A_H(t)$ for bovine heart mitochondria at 37°C in 50% sucrose and illustrates the good signal to noise level obtained after signal averaging. Plots of $r(t)$ for mitochondria and SMP measured under different conditions are shown in Figs. 2 and 3. Data for mitochondria were collected over a time range of 2 ms, during which time the anisotropy reached an essentially constant level. In the case of SMP, it was possible to collect data over a 5-ms time range, because of the better signal to noise ratio in the sample.

We first analyzed the $r(t)$ curves by fitting the data to the double exponential approximation:

$$r(t) = A_1 \exp(-t/\alpha_1) + A_2 \exp(-t/\alpha_2) \quad (2)$$

In this way, we obtained $\alpha_1 = 300 \pm 110 \mu\text{s}$, $\alpha_2 > 30 \text{ ms}$ at 37°C for mitochondria, and $\alpha_1 = 400 \pm 35 \mu\text{s}$, $\alpha_2 = 20 \pm 5 \text{ ms}$ at 22°C for SMP. Fractions of each component are $A_1/r(0) \approx 0.44$, $A_2/r(0) \approx 0.56$ for mitochondria, and $A_1/r(0) \approx 0.34$, $A_2/r(0) \approx 0.66$ for SMP. There were no pronounced differences in these decay times between bovine heart and rat heart mitochondria, or between fresh and frozen mitochondria.

The diameters of mitochondria and SMP were determined from electron micrographs of negatively stained samples. The diameter of mitochondria was about 0.4 to 4 μm implying a rotational correlation time of the whole mitochondria in 50% sucrose at 37°C greater than 60 ms. The SMP had diameters in the range of 400 to 1500 Å. From the vesicle size distribution, it was deduced that tumbling of SMP in 60% sucrose at 22°C only becomes significant for correlation times greater than 5 ms. Thus, we can exclude the possibility that vesicle tumbling

¹ The abbreviations used are: SMP, submitochondrial particles; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TMPD, N,N,N',N' -tetramethyl-*p*-phenylenediamine.

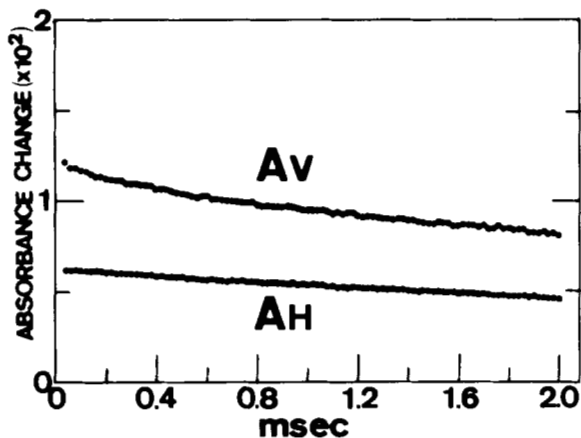


FIG. 1. Absorbance changes at 446 nm following flash photolysis of the cytochrome oxidase-CO complex in mitochondria. Bovine heart mitochondria (1.4 mg of protein/ml) in 50% sucrose at 37°C were photolysed with a vertically polarized laser flash at 590 nm, and absorbance changes were recorded as described under "Experimental Procedures."

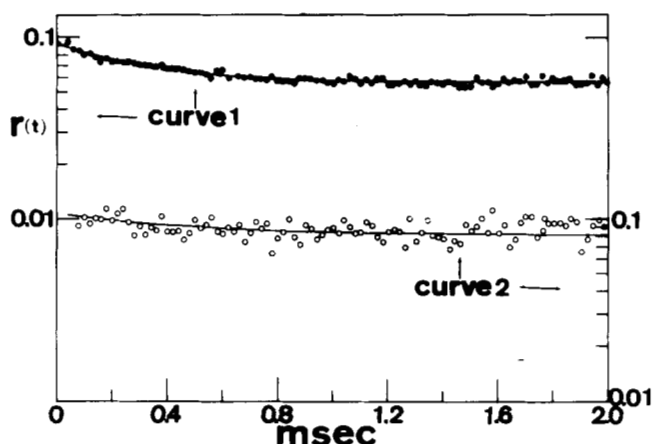


FIG. 2. Time-dependent absorption anisotropy of bovine heart mitochondria in the presence of 50% sucrose. Experimental conditions were the same as described in the legend to Fig. 1 with temperature of 37°C (Curve 1, left side scale, viscosity about 8 centipoise) and -6°C (Curve 2, right side scale). The absorption anisotropy was calculated as described under "Experimental Procedures" from signals similar to those shown on Fig. 1. The solid line and dashed line were obtained by fitting the data to Equations 2 and 4, respectively.

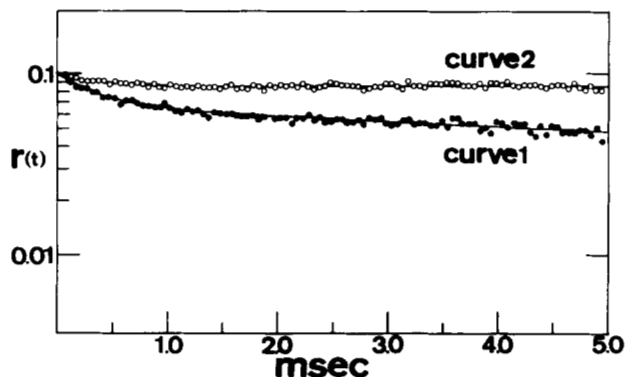


FIG. 3. Time-dependent absorption anisotropy of bovine heart SMP in the presence of 60% sucrose. Curve 1 was measured at 22°C (viscosity about 55 centipoise). In Curve 2, SMP was fixed with 2% glutaraldehyde in the presence of 60% sucrose and measured at 12°C. The solid lines were obtained by fitting the data to Equation 2. The concentration of SMP was 1.5 mg/ml.

contributes to the rapidly decreasing phase of $r(t)$ in either mitochondria or SMP.

Cytochrome oxidase is the only protein in the mitochondrial membrane known to react with CO; however, it is conceivable that our signals could contain a contribution from contaminating hemoglobin. This is unlikely since we did not detect any hemoglobin spectrophotometrically in our samples. As a further control, we performed flash photolysis experiments with mitochondria containing added human hemoglobin (spectral properties of bovine and human hemoglobin are identical (17)). Addition of hemoglobin decreased $r(0)$ to below 0.02 because of the very rapid rotation of hemoglobin faster than 1 μ s in 50% sucrose at 37°C (about 8 centipoise). Furthermore, hemoglobin contributed a rapid decay to the absorption transient ($A(t) = A_V(t) + 2A_H(t)$) resulting in a biphasic curve. Only the slow and monoexponential decay characteristic of cytochrome oxidase was observed in the absence of added hemoglobin. The possibility that chromophores other than heme a_3 contribute to the decay in $r(t)$ can thus be excluded.

We therefore conclude that the decay characterized by the time constant α_1 is due to rotational motion of cytochrome oxidase in the membrane. This conclusion is further substantiated by observing that the decay of $r(t)$ is strongly reduced by cooling to low temperature (Curve 2, Fig. 2) and by fixation with glutaraldehyde (Curve 2, Fig. 3).

In comparing our results to those of Junge and Devault (8), we note that the absorption anisotropy is more sensitive to rotational motion than is the dichroic ratio A_V/A_H used to evaluate their data. A simple calculation shows that a 40% decrease in $r(t)$ corresponds to a 10% decrease in the dichroic ratio when $r(0)$ is about 0.1. The decay of $r(t)$ in mitochondria was about 40 to 50% over 2 ms; it is conceivable that the corresponding smaller decay in the dichroic ratio might not have been detectable at the signal to noise level of their experiments. Their experiments were performed with pigeon heart mitochondria and bovine heart SMP, so that species differences could not entirely explain the differences between their conclusion and those of the present investigation.

Cytochrome oxidase maintains a fixed orientation (1, 2) with respect to the sidedness of the membrane, implying that rotation occurs around the normal to the plane of the membrane. A theoretical treatment of this case for heme proteins² shows that the expected form of $r(t)$ is given by

$$r(t)/r(0) = r(t)/0.1 = 3 \sin^2 \Theta_N \cos^2 \Theta_N \exp(-t/\phi_{\parallel}) + \frac{3}{4} \sin^4 \Theta_N \exp(-4t/\phi_{\parallel}) + \frac{1}{4} (3 \cos^2 \Theta_N - 1)^2 \quad (3)$$

where ϕ_{\parallel} is the rotational relaxation time about the membrane normal axis and Θ_N is the angle between this axis and the normal to the heme plane ($\phi_{\parallel} = 1/D_{\parallel}$ where D_{\parallel} is the rotational diffusion coefficient). Equation 3 expresses the rotational diffusion of a single rotating species and assumes 4-fold symmetry of the heme plane (10), in which case the limiting anisotropy $r(0)$ is 0.1. Our experimental $r(0)$ at 446 nm is close to 0.1 (range 0.106 to 0.092), so that the departure from 4-fold symmetry will be small at this wavelength, even if the 4-fold symmetry of the heme a_3 plane is broken (18). When different rotating species of cytochrome oxidase such as monomer, dimer, etc. are present, the measured $r(t)$ is the weighted sum of the individual $r(t)$ values with different ϕ_{\parallel} . It should be noted that the time-independent residual anisotropy $r(\infty) = \frac{1}{40} (3 \cos^2 \Theta_N - 1)^2$ is not changed.

Since the experimental data are not sufficiently accurate to provide a critical test of the validity of Equation 3, we used

² S. Kawato, K. Kinoshita, Jr., and R. J. Cherry, manuscript in preparation.

the more general Equation 2 for an initial evaluation of the results. Nevertheless, it is apparent that in the case of mitochondria, the anisotropy does reach a time-independent value as predicted by Equation 3. We therefore made a further analysis of the $r(t)$ curves of mitochondria with the following equation:

$$r(t) = r_1 \exp(-t/\phi_1) + r_2 \exp(-4t/\phi_1) + r_3 \quad (4)$$

In this way, we obtained $\phi_1 = 350 \pm 20 \mu\text{s}$, $r_1/r(0) = 0.31 \pm 0.09$, $r_2/r(0) = 0.15 \pm 0.05$, $r_3/r(0) = 0.54 \pm 0.05$, at 22°C .

Optical and ESR experiments with oriented membranes have indicated that the heme a_3 plane of cytochrome oxidase is approximately perpendicular to the plane of the membrane (9–11). If Θ_N is between 60 and 90° and all cytochrome oxidase is rotating, Equation 3 predicts that $r(\infty)/r(0)$ should be lower than 0.25 . The measured values of $r_3/r(0)$ in mitochondria were around 0.5 to 0.6 . The failure of the $r(t)$ curves to fall to the predicted value therefore indicates the existence of a population of cytochrome oxidase molecules which is very slowly rotating or immobile ($\phi_{\parallel} > 30$ ms) on the time scale of the experiments. The fraction of mobile cytochrome oxidase ($\phi_{\parallel} \approx 350 \mu\text{s}$) may be calculated to be around 60% , if Θ_N is close to 90° . In the case of SMP, the $r(t)$ curves do in fact exhibit a slowly decaying phase but it is difficult to decide whether this represents slowly rotating cytochrome oxidase species or vesicle tumbling, since the value of α_2 is in the range of the correlation time of vesicle tumbling of SMP. The value of $r(5 \text{ ms})/r(0)$ in SMP was around 0.5 . This indicates that at least 30% of cytochrome oxidase is very slowly rotating ($\phi_{\parallel} > 30$ ms).

The above analysis in terms of different populations of cytochrome oxidase in mitochondria and SMP is strongly supported by similar experiments in reconstituted membranes.³ The $r(t)$ curves were measured for cytochrome oxidase in phosphatidylcholine-phosphatidylethanolamine-cardiolipin vesicles. It was found that the anisotropy decayed within 2 ms to a constant value $r(\infty)/r(0) \approx 0.25$ as predicted by Equation 3 when $\Theta_N \approx 90^\circ$ and all cytochrome oxidase is mobile.

The relatively immobile species of cytochrome oxidase in mitochondrial membranes might be either oligomeric cytochrome oxidase or cytochrome oxidase complexed with other membrane proteins. The former possibility is supported by observations of self-association of cytochrome oxidase in reconstituted systems (19). The co-existence of different states of aggregation of other membrane proteins has also been proposed; for example, Band 3 proteins in erythrocyte membranes (20), $(\text{Ca}^{2+}\text{-Mg}^{2+})\text{ATPase}$ in sarcoplasmic reticulum (21), and cytochrome P-450 in microsomal membranes (22).

³ E. Sigel, S. Kawato, E. Carafoli, and R. J. Cherry, manuscript in preparation.

In conclusion, the present experiments help to resolve previous conflicting observations of the mobility of cytochrome oxidase. The demonstration of mobility adds further weight to the possibility that lateral diffusion may play a role in electron transfer between components of the respiratory chain. However, it appears that a relatively immobile fraction of cytochrome oxidase is also present in the membrane. The functional significance of these different states of cytochrome oxidase requires further investigation.

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