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ROTATIONAL MOTION OF CYTOCHROME c OXIDASE IN PHOSPHOLIPID VESICLES

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SUMMARY Beef-heart cytochrome c oxidase was spin-labeled with 4-maleimido-2,2,6,-tetramethylpiperidinooxyl at subunit III. Electron spin resonance (ESR) second-harmonic 90° out-of-phase measurements of the enzyme in reconstituted functional phospholipid vesicles indicate that cytochrome c oxidase rotates rather rapidly with a correlation time of 34 μsec at 4° C.

INTRODUCTION

Information on the rotational mobility of cytochrome c oxidase (cytochrome c-oxygen oxidoreductase EC 1.9.3.1.) in a functional reconstituted system similar to that which operates in the mitochondrial cristae membrane would help to understand the role of this enzyme in both its functional properties i.e. electron transfer and proton translocation.

The study of Junge and DeVault (1) concerning the mobility of cytochrome c oxidase left opened the question whether cytochrome c oxidase is totally immobilized in the membrane, or it carries out only limited rotational diffusion around a single axis coinciding with the symmetry axis of heme a_3. By the technique of second harmonic out-of-phase saturation transfer ESR (2,3,4), which is sensitive to correlation times between 10^{-9} and 10^{-3} sec, it is possible to evaluate the rotational motion of macromolecules having a spin label covalently attached (2,5). Bovine cytochrome c oxidase is an oligomer containing 7 subunits (6). Subunit III, mol.wt.

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21'000, was shown to react with the n-ethylmaleimide spin label to a much larger extent than with any other subunits (7).

In the present investigation cytochrome c oxidase molecular motion was studied both with the enzyme free in a buffer-Triton-X-100 medium or bound to asolectin vesicles (8,9).

MATERIALS AND METHODS

Cytochrome c oxidase was prepared from bovine heart by the method of Yu et al (10) which gives a lipid depleted enzyme, it was stored at -70° C in 0.25 M sucrose/0.05 M sodium phosphate buffer, pH 7.4. The preparations had a heme/protein ratio between 10-11 nmoles/mg. The heme was determined using the extinction coefficient $\varepsilon = 12\ mM^{-1}cm^{-1}$ for dithionite-reduced minus oxidized enzyme at the wavelength couple 605 minus 630 nm and the protein by the biuret method (11) using bovine serum albumin as a standard.

For the study of rotational mobility of the enzyme in vesicles, cytochrome c oxidase was spin-labeled with 4-maleimido-2,2,6,6,-tetramethylpiperidinooxyl (Syva, Palo Alto, California) using 2 nmol of label per nmol of the enzyme for 15 minutes at room temperature. The non-covalently attached label was eliminated by passing the enzyme through a column of Sephadex G-25 coarse, (10 cm x 1.5 cm), equilibrated with 50 mM KH_2PO_4, pH 7.4 and 0.5 % Triton-X-100 at 4° C (13). Fractions of 0.5 ml were collected and concentrated by using dialysis tubing covered with dry Sephadex G-25 for 10 to 14 hours at 4° C. The concentration of the solutions was then measured spectrophotometrically.

Reconstituted cytochrome c oxidase-containing vesicles were prepared by a modification of the method of Hinkle et al. (9). A suspension of 40 mg of soya bean phospholipids/ml in 24.5 mM potassium cholate/100 mM-Hepes, pH 7.2, was sonicated to clarity at 6 μ peak-to-peak in an MSE sonicator. Cytochrome c oxidase was added to give a final concentration of 15 μM. The suspension was dialysed for 4 h against 100 vol. of 100 mM-Hepes, pH 7.2, then for a further 4 h against 200 vol. of 10 mM-Hepes/27 mM-KCl/73 mM-sucrose, pH 7.2, and finally for 12 h against 200 vol. of 1 mM-Hepes/30 mM-KCl/79 mM-sucrose, pH 7.2. All solutions were adjusted to the indicated pH with KOH. The procedure was carried out at about 4° C.

Measurements were made with a Varian E-104 X-band (9.5 GHz) spectrometer with a 200 μl flat-quartz cuvette. Temperature of the microwave cavity was controlled with an accuracy of ± 1° C by circulation of cooled nitrogen gas introduced from a modified variable temperature housing assembly holding an electric temperature sensor, without a Dewar insert. Conventional ESR spectra were recorded with a 100 KHz field modulation frequency of 5 Gauss. Saturation transfer spectra

were recorded with a field modulation of 50 KHz ($\omega M/2\pi$) and detection at 100 KHz, 90° out-of-phase; the incident microwave power was 70 mW on the dial. The phase was adjusted to minimize the second harmonic signal at 1 mW power.

Hepes and soya-bean phospholipids (recrystallized by the method of Kagawa and Racker (12)), were obtained from Sigma Chemical Co., cholic acid was recrystallized from sodium cholate obtained from Fluka AG, Buchs, Switzerland, Triton-X-100, L(+)-ascorbic acid and Tween 80 ("gas chromatography" grade) were obtained from Merck. All other reagents were of analytical grade.

RESULTS AND DISCUSSION

Using the method of Thomas, Dalton and Hyde (2,3) of second harmonic out-of-phase ESR, the spectra of spin-labeled

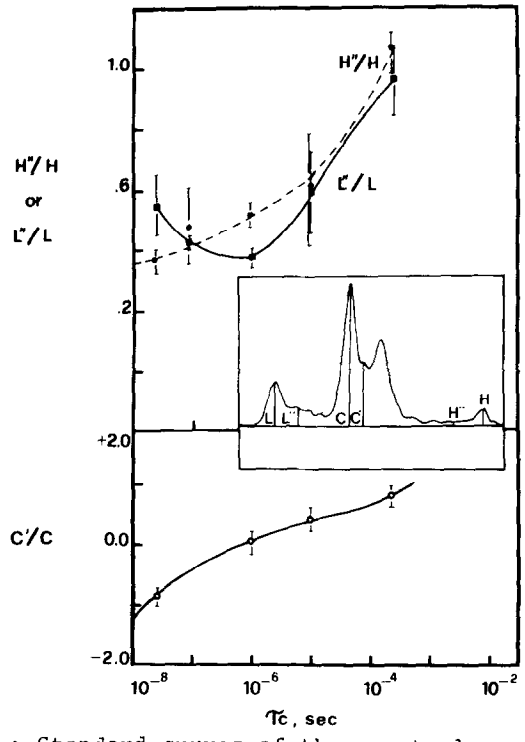

Fig. 1 : Standard curves of the spectral parameters as a function of τ_c.
The peak height ratios H''/H, L''/L and C'/C are obtained from the second harmonic out-of-phase spectra of spin-labeled hemoglobin, labeled with 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl at different viscosities (13). It is used as a model system for rotational diffusion (2) at 4° C. The inset shows the saturation transfer spectrum of the labeled hemoglobin in 80 % glycerol at 5° ± 1° C. The points in the graphs refer to averages and the bars to the ± standard deviations of four different experiments.

hemoglobin were recorded at different viscosities. Standard curves were thus obtained which were subsequently used to calibrate the ESR spectra of spin-labeled cytochrome c oxidase. The spectral parameters of hemoglobin (2,14) Figure 1, are plotted against the calculated rotational times of the molecule from the Stokes-Einstein equation using the radius of 29 Å and the viscosity values given for the H_2O-glycerol mixtures (15). These measurements can provide a good estimate of the motion of cytochrome c oxidase, since the saturation transfer spectra are sensitive to correlation times as long as 1 msec.

In the inset of Figure 1, the peaks of the ESR spectra utilized for the measurements of the rotational motion are indicated. In Figure 2A an ESR spectrum of cytochrome c oxidase in solution in the presence of 0.5 % Triton-X-100 is shown. From the spectral parameters used before and the calibration curve obtained for hemoglobin, a correlation time at 4° C of approximately 25 nsec can be calculated from the low field lines. The central and high field lines indicated similar values (25 and 56 nsec respectively).

When cytochrome c oxidase is incorporated into phospholipid vesicles, Figure 2B, the mobility of the enzyme is decreased and its rotational correlation time becomes 34 μsec at 4° C, as measured from the low and high field lines. The central line, however, indicated a rotational correlation time of 0.2 μsec. The higher values are consistent with the higher viscosity of the phospholipid membrane (approximately 10 poise) with respect to the buffer. From the Stokes-Einstein

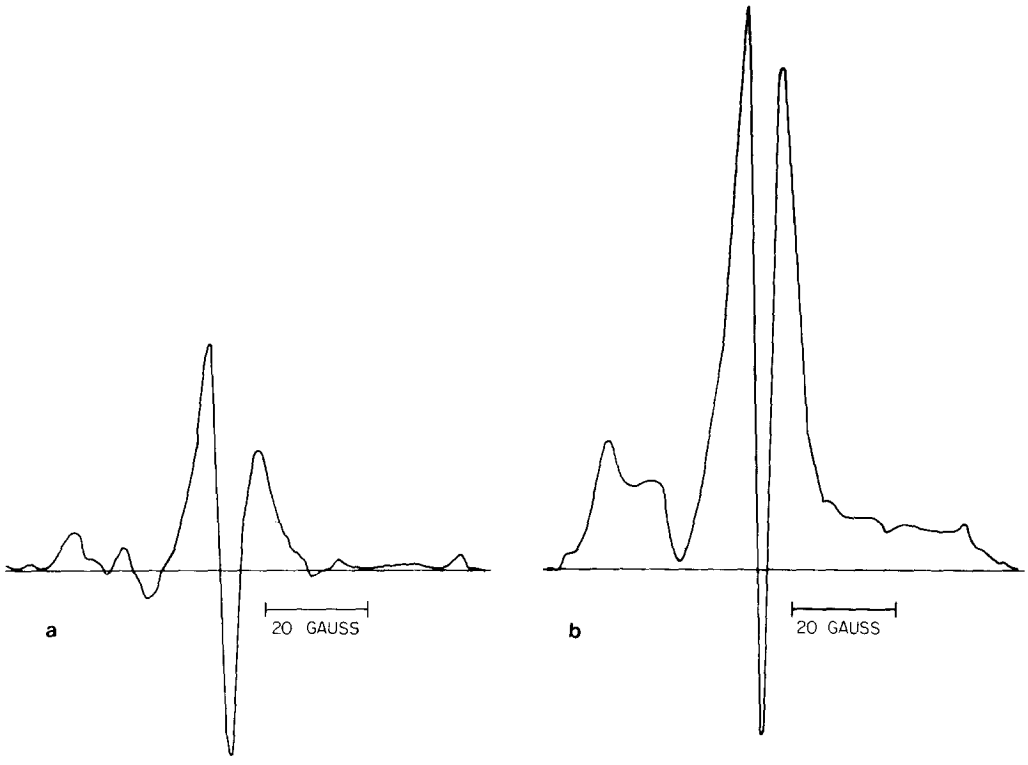

Fig. 2A: Second harmonic 90° out-of-phase spectrum of spin-labeled cytochrome c oxidase.
The spectrum was obtained at 4° C in a buffer containing 50 mM phosphate buffer, pH 7.4 and 0.5 % Triton-X-100 and 60 μM cytochrome c oxidase.

Fig. 2B: Second harmonic 90° out-of-phase spectrum of spin-labeled cytochrome c oxidase incorporated into vesicles.
The spectrum was obtained as in Figure 2A, and the concentration of cytochrome c oxidase was 15 μM.

equation $\tau_c = 4\pi r^3 \eta/3kT$, the rotational correlation time of 52 nsec for the free enzyme, and of 52 μsec for the membrane bound one, assuming a viscosity of 10 poise, can be calculated at 4° C. A radius of 37 Å was assumed for cytochrome c oxidase.

The results appear to be in fair agreement with the values predicted by the calculations. The consideration that the higher viscosity of the membrane is applied only upon a part of the molecule (16), a large portion of it being instead

exposed to the water phase, justifies the slightly slower motion calculated theoretically (52 μsec) with respect to that inferred from the high and low field lines (34 μsec). The lack of agreement for the time inferred from the central spectral line may be due to the non-spherical shape of cytochrome c oxidase and consequently to its non-complete isotropic motion (5,20).

The conclusion which can be drawn from the present experiments is consistent with the idea of a relatively rapid motion of the enzyme in the membrane which could not therefore be, in this case, in form of large aggregates (17).

The rotational motion of cytochrome c oxidase is in the time range of its catalytic activity. In fact the first order rate constant for the oxidation of the cytochrome c oxidase by molecular oxygen is of 3000 sec^{-1} (18). Thus several rotational events are allowed during the catalytic process. The enzyme, as studied above, was fully active after reconstitution. This indicates that, if large aggregates of the oxidase are present in the native membrane (19) they are not a necessary prerequisite for enzymatic activity.

The finding of Junge and DeVault (1) of no dichroic relaxation of cytochrome c oxidase which could be attributed to the rotational diffusion of the enzyme is not in disagreement without present findings. In fact, no dichroic relaxation should be found in the case of even rapid rotation of the enzyme around one single axis parallel to the symmetry axis of the a_3 heme.

The data reported above, in conjunction with the finding of Junge and DeVault (1) indicate that the cytochrome c oxi-

dase can rotate rapidly, although on a single preferential axis. During the course of this work we became aware that similar results were independently obtained by M. Swanson et al (21).

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