BROWNIAN ROTATION OF THE CYTOCHROME OXIDASE IN THE MITOCHONDRIAL INNER MEMBRANE

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1. Introduction

The mobility of enzyme molecules in biological membranes is subject to intensive studies [1–5]. These studies are important for an understanding of the mechanisms of active transport across membranes and of reactions in the plane of the membrane, as well. Depending on the mobility of cytochromes in the inner membranes of mitochondria two different types of mechanisms have been proposed for the electron transfer in the respiratory chain by Chance and coworkers [6]: i) tunnelling of electrons between translationally and rotationally locked cytochromes, ii) diffusion controlled reactions between molecules moving rapidly in the membrane. From the symmetry of the reaction velocities of the asymmetric carrier cytochrome *c* with cytochrome c_1 on one side and cytochrome *a* on the other one, these authors have considered a reaction mechanism from the locked situation as improbable [6].

This paper reports on the results of direct studies on the rotational mobility of the cytochrome oxidase in the inner mitochondrial membrane. Evidence is presented that this large protein (M.W.: ⩾ 70,000 per heme) rotates in the membrane with relaxation times shorter than 100 μsec. This rotation is at least partly hindered by fixation with glutaraldehyde or by high viscosity media. These results have been obtained by polarization spectroscopy of the cytochrome a_3-heme in aqueous suspensions of isolated mitochondria. The difficulty that random orientation of mitochondria in the suspension randomizes the orientation of the chromophores has been overcome by selective photolysis of spatially oriented cytochrome a_3–CO complexes with a linearly polarized laser pulse.

2. Experimental

Pigeon heart mitochondria were prepared according to the method of Chance and Hagihara [7]. Unless otherwise indicated they were suspended to an average protein concentration of 1 mg/ml in the following medium: 0.3 M sucrose; 4×10^{-2} M morpholinopropanesulphonate-NaOH, pH 7.4; 5×10^{-3} M succinate. The experiments were carried out at room temperature. The suspension was filled into a closed absorption cell (1 cm optical path), made anaerobic by the addition of succinate or dithionite, and then an amount of carbon monoxide was added as CO-saturated buffer, sufficient for quantitative formation of cytochrome a_3–CO complexes.

The absorption cell was mounted into a rapid kinetic spectrophotometer. An exciting laser beam and the measuring beam were impinging on the sample perpendicularly to each other. Except for minor modifications, the photometer was the same as that described by Chance and Erecinska [8]. The cytochrome a_3–CO complexes were photolyzed by a short pulse from a rhodamine 6G liquid dye laser at a wavelength of 585 nm. This resulted in a positive absorption change at 445 nm due to the appearance of reduced CO-free cytochrome a_3-hemes. This absorption change was recorded on a Tektronix storage oscilloscope. (For the spectral properties of the complexed and uncomplexed cytochrome a_3 heme, see [8 and 9].)

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The laser output was linearly polarized by means of a pair of glass slides mounted inside the resonant cavity at the Brewster angle. This produced a polarized output intensity ratio I↑/I→ greater than 25 at energies below 100 mJ per flash. The depolarization of the laser beam by light scattering in the turbid suspension was kept low, due to the low protein concentration in the sample. At 585 nm, the polarization ratio in direct transmission detected with an aperture of 1 was still greater than 20.

The measuring beam was polarized, too. Its electric vector was oriented either parallel or perpendicular to the electric vector of the exciting laser beam. The two types of absorption changes resulting from these two orientations are denoted $\Delta A_{\parallel}$ and $\Delta A_{\perp}$. The polarizer in the measuring beam was placed in front of the absorption cell to avoid different laser flash artifacts for the two polarizations. The polarization effects due to the monochromator grating have been corrected for by regulation of the measuring beam light source. The spectral bandwidth of the monochromator was 3 nm.

3. Results

When exciting the sample with linearly polarized light one would expect to photolyse by preference those cytochrome a_3–CO complexes with their transition moments parallel to the electric vector of the exciting light. This preference is valid only for non-saturating excitation intensities, since at saturating ones even the unfavorably oriented chromophores will be photolysed. When there is no resonant energy transfer before photolysis, the absorption changes due to the reappearance of CO-free cytochrome a_3 hemes will result from the same set of selected oscillators. For the sake of simplicity, I will first assume that the exciting and the measuring light interact with the same linear oscillator in the cytochrome a_3 hemes. If the very many oscillators in the sample were distributed isotropically and if they were fixed with respect to the laboratory system, then one would expect dichroic absorption changes with a theoretical maximum of $\Delta A_{\parallel}/\Delta A_{\perp} = 3$ [10]. However, energy transfer between oscillators of different orientations or rotation of oscillators will decrease this dichroic ratio. Thus, the dichroic ratio is an indicator of re-

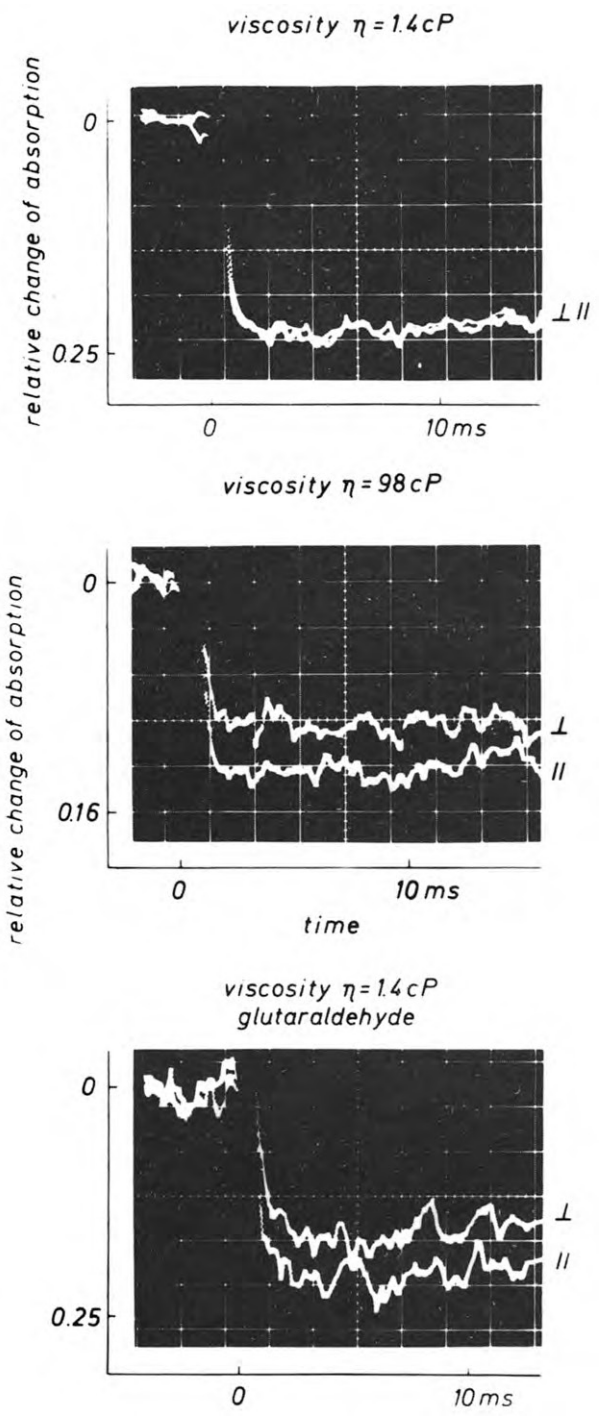

Fig. 1. Time course of the absorption changes at 445 nm on excitation of mitochondria with a laser flash at time $t = 0$. Laser beam and measuring beam perpendicular to each other, both linearly polarized, either in parallel (∥) or perpendicular (⊥).

sonant energy transfer or rotational freedom of the chromophore.

In this paper I will not go into the implications of the dichroic ratio for the as yet unknown symmetry properties of the electronic states of the cytochrome a_3 heme. In the context of this message, it is sufficient to know that these oscillators will not be spherical, because the heme is not. Then any dichroism observed, grossly speaking, indicates that there is no resonant energy transfer and no full rotational freedom of the cytochrome a_3 heme with respect to the laboratory system. The results of three experiments with pigeon heart mitochondria are depicted in fig. 1. Each trace represents the time course of the absorption change at 445 nm. At time $t = 0$ the sample has been excited by a laser flash. An amplitude value of 0.25 indicates stimulation of only 25% of the maximum absorption change due to nonsaturating flash energy (6 mJ). No dichroism was detected from mitochondria suspended in low viscosity isotonic buffer, as seen from the overlap of the two traces in fig. 1, left. The same experiment repeated with a higher time resolution has led to the same result which is documented in fig. 2. There, each polarization as well as the baseline has been run twice. Now, the traces in the middle of fig. 1 demonstrate that dichroism is induced, when mitochondria are suspended in a high viscosity dextran medium. A similar result was obtained with glutaraldehyde fixed mitochondria (2%, 1/2 hr incubation) in low viscosity buffer, as shown in fig. 1, right.

4. Discussion

It has to be questioned whether the absence of any dichroism in mitochondria suspended in isotonic buffer at low viscosity indicates resonant energy transfer or rotation of the chromophore. Resonance energy transfer is affected by three parameters: the distance of the transition dipoles, their relative orientations and the refractive index of the surrounding medium [11]. Both agents inducing dichroism in the above experiments, glutaraldehyde and dextran, may act on these parameters. I will leave out of consideration any drastic change of the refractive index, which is improbable. It is conceivable that glutaraldehyde, known to crosslink proteins by hydrogen bonds, may alter the distance and/or the orientation of the hemes However, it is rather improbable that dextran, in osmotically inactive concentrations, does the same.

So very probably resonance energy transfer is not the reason for the absence of dichroism in the low viscosity experiment. Instead, rotation of the chromophore with respect to the laboratory system has to be considered. This may consist of three contribution

1. Rotation of the mitochondrion in the solution.

Mitochondria can be idealized as prolate spheroids with a small axis of at least 5000 Å units [13]. Thus, their rotational relaxation time will exceed that of a sphere of the same diameter. This can be calculated from Perrin's formula: $\tau = \eta\, 2\pi r^3/3\,kT$ [14]. With $\eta = 1.4$ for 0.3 M sucrose solution at room temperature the sphere relaxation time becomes: $\tau = 25$ msec, a lower limit for the relaxation times of mitochondria Thus, in experiments with a time resolution of about 1 msec (fig. 1) or even 100 μsec (fig. 2) rotation of mitochondria should be negligible. Moreover, the occurrence of dichroism on mitochondria fixed with glutaraldehyde is hard to explain by a volume reducing action factor of about 1000 of this compound. This excludes the possibility that rotation of whole mitochondria is responsible for the absence of dichroism in the low viscosity experiment.

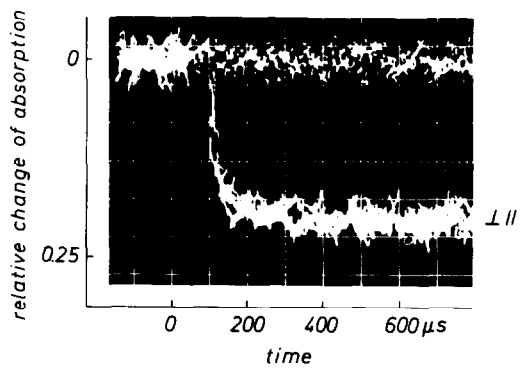

Fig. 2. Time course of the absorption changes at 445 nm from mitochondria suspended in low viscosity buffer ($\eta = 1.4$ cP). Superposition of two baselines and four other traces, two of which indicate absorption changes polarized in parallel and the two other perpendicularly to the polarization of the exciting laser beam. (Artifact duration about 100 μsec, instrument time 5 μsec.)

2. Rotation of the cytochrome oxidase in the membrane.

Whenever the cytochrome oxidase protrudes into the outer phase of the inner mitochondrial membrane one would expect that its rotational relaxation time in the membrane depends on the viscosity of the outer medium. On the other hand, crosslinking of different proteins by glutaraldehyde can be expected to reduce the rotational frequencies or even to block rotational degrees of freedom. Both expectations agree with the experimental observations. So, this component may be responsible for the absence of dichroism in the low viscosity experiment.

3. Rotation of the chromophore in the protein.

This possibility is hard to cope with, as the heme protein linkages of cytochrome a_3 are unknown. By analogy to the linkages in cytochrome *c* [15] it becomes probable that there will be several bonds between the heme and the protein which can be expected to hinder its rotation in the protein. So the most probable interpretation of the absence of dichroism in the photoselection experiment with mitochondria suspended in low viscosity buffer is the rotation of the cytochrome oxidase in the mitochondrial membrane. However, it has to be questioned whether this rotation is due to Brownian motion or if it is due to a recoil as a consequence of the photochemical cytochrome a_3 · CO complex dissociation. If, as is very probable, the heme is linked by several bonds to the protein in the electronic ground state, which has been monitored, then it is unlikely that a sufficient amount of rotational energy could be transferred to the protein, to cause sufficiently rapid rotation. Another kinetic argument against photochemical recoil causing the observed tumbling is that the reaction time constants of cytochrome *a* and cytochrome *c* are independent of whether the respiratory chain has been stimulated by an oxygen pulse or photochemically unlocked by the cytochrome a_3 · CO complex dissociation [8].

So the most probable reason for the observed tumbling of the cytochrome oxidase in the mitochondrial membrane is Brownian motion. With less than 100 μsec, the characteristic rotational relaxation time is shorter than the reaction time of cytochrome *a* and cytochrome *c*. Since the oxidase is larger than cytochrome *c* and cytochrome c_1, these components can be expected to rotate even more rapidly in the membrane. So, rotation will be important for adjusting the right conformation for the redox reactions between these components of the respiratory chain.

The rotation of the cytochrome oxidase reported herein corresponds to the rotation of rhodopsin, recently reported for the inner disk membranes of retinal rods [4, 5]. These findings give further support for the general concept of biological membranes being rather in a fluid than in a solid state [16].

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