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SYMMETRY, ORIENTATION AND ROTATIONAL MOBILITY IN THE a_3 HEME OF CYTOCHROME c OXIDASE IN THE INNER MEMBRANE OF MITOCHONDRIA

WOLFGANG JUNGE* and DON DeVAULT

Johnson Research Foundation, University of Pennsylvania, School of Medicine, Philadelphia, Pa. 19174 (U.S.A.)

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SUMMARY

The photoinduced linear dichroism of absorption changes resulting from photolysis of the complex between heme a_3 of the cytochrome oxidase and CO is studied. The experiments started from isotropic solutions or suspensions of the enzyme both in its isolated form and in mitochondria. The anisotropy responsible for the linear dichroism was induced by excitation with a flash of linearly polarized light. The dichroic ratios observed with various systems; polymerized enzyme in solution, enzyme in mitochondria and in submitochondrial particles (at 20 °C as well as at liquid N₂-temperature) all approached a value of 4/3 which characterizes a chromophore which is circularly degenerate. Therefrom we conclude that the interaction of heme a_3 with its microenvironment within the protein does not break its four-fold symmetry.

The experiments with mitochondria and submitochondrial particles suspended in aqueous buffer revealed similarly high dichroic ratios without any dichroic relaxation other than a rather slow one which could be attributed to the rotation of the whole organelle in the suspending medium. Therefrom we conclude that the cytochrome oxidase either is totally immobilized in the membrane, or that it carries out only limited rotational diffusion around a single axis coinciding with the symmetry axis of heme a_3 . In the light of independent evidence for a transmembrane arrangement of the oxidase and for the general fluidity of the inner mitochondrial membrane we consider anisotropic mobility of the cytochrome oxidase around an axis normal to the plane of the membrane as the most likely interpretation. Then our experimental results imply that the plane of heme a_3 is coplanar to the membrane.

* Permanent address and address for reprint requests: Max-Volmer-Institut für Physikalische Chemie und Molekularbiologie, Technische Universität Berlin, D-1000 Berlin 12, Str. 17, Juni 13 5, Germany.

INTRODUCTION

Cytochrome *c* oxidase is bifunctional in the inner membrane of mitochondria. It mediates the terminal electron transfer step in the respiratory chain and in doing so conserves part of its energy for the synthesis of ATP. The general interest in this enzyme focuses on two major items, the mechanism by which it collects and transfers electrons from cytochrome *c* to oxygen and how, in the course of conserving the energy, it produces the vectorial result of an electrochemical potential difference of the proton across the cristae membrane of mitochondria. (For a recent review, see ref. 1). While the first mechanism mainly involves the internal structure of the enzyme, the second one is related to its interaction with the membrane. This paper bears on both aspects. We investigate the orientation of one of the two hemes within the molecule and also the rotational mobility of the oxidase in the cristae membrane.

The internal structure of the oxidase is still under discussion. It is characterized by at least two subunits [2], each containing one heme and probably one copper. Its total molecular weight is at least 140 000 [3], higher figures being possibly due to tightly bound lipids. The shape of the molecule is only vaguely known. There is some indication [4] for an oblong structure with axial dimensions 68×135 Å, perhaps resulting from the aggregation of two subunits of approximately 50 Å [5]. The two hemes in the oxidase are functionally distinct, one, heme a_3 , reacting with oxygen and liganding CO, the other one, heme *a*, reacting with cytochrome *c*. Based on the absence of any detectable difference in the redox potentials of the unliganded hemes it was argued that the two hemes and their microenvironments are identical [6], the spectroscopic and thermodynamic differences being induced by ligand binding only. However, this is not compatible with the observation that only one of the hemes is EPR-“visible” even in the absence of any ligand [1]. It was shown that the two hemes interact with each other [7, 8]. The arrangement of the functional groups in the protein is unknown. It has been argued that there is a topological sequence a -Cu-Cu- a_3 with the largest gap being between heme *a* and the coppers [1]. Both hemes seem to be shielded from the aqueous environment [9].

The activity of the oxidase was shown to depend on its interaction with detergents [10] or lipids [5, 11]. Their absence causes formation of inactive polymers. Studies on the interaction of the enzyme with artificial lipid vesicles revealed that it readily dissolves in their membranes. When in solution it immobilizes approximately one ring of lipid molecules, while the fluidity of the wider lipid environment is practically unaffected [12].

The location of the oxidase in the cristae membrane is subject to controversy. Two independent lines of evidence from different laboratories favour a transmembrane arrangement, with the enzyme extruding into both aqueous phases. From H-D-exchange data it was concluded that about 60 % of the enzyme is exposed to an aqueous environment [13]. Moreover, the enzyme was reported to be accessible to antibodies [14] and selective surface agents [15] from both sides of the membrane. The assignment of access from either side was based on the inversion between mitochondria and sub-mitochondrial particles. This inversion is experimentally based on the following experiments: electron microscopic studies on the location of the coupling factor F1 (e.g. ref. 16), studies revealing the inversion of the electric potential [24, 25] and studies on the access of externally added cytochrome *c* to the oxidase (e.g. ref. 17).

Indirect evidence for a transmembrane arrangement of the oxidase came from studies on the proton uptake associated with the reduction of dioxygen by cytochrome *c* via the oxidase. It was concluded that proton binding occurs from the aqueous phase opposite to the one where cytochrome *c* reacted with the oxidase [18, 19]. The above cited references visualize the oxidase as transmembrane with the a_3 heme being nearer to the matrix side and the a heme nearer to the C-side of the membrane. This structural model, however, is opposed by another one which represents all the hemes between cytochrome c_1 and a_3 to be on the matrix side of the membrane [20]. Studies on the influence of an electrochemical potential difference of the proton on the redox potentials of the various hemes were interpretable by a transmembrane arrangement of the cytochrome oxidase, too. However, as pointed out in ref. 21, these results by themselves do not prove such a configuration.

Lipid vesicles doped with cytochrome *c* oxidase generate an electric potential difference [22] as well as a pH difference [23] if electrons flow from cytochrome *c* to oxygen. Since it was shown that an electrochemical potential difference of the proton is generated during electron flux through the respiratory chain [18, 19, 24, 25], it is most probable that the enzyme in situ contributes to the electrochemical potential generation. A transmembrane orientation with the hemes and the coppers lined up to form an electron well across the membrane is a favorable configuration for this function.

To detect orientation of the a_3 heme within the protein and possibly rotational mobility of the whole enzyme in the cristae membrane, we employ the method of linear dichroism by photoselection.

MATERIALS AND METHODS

The principle of photoselection experiments is illustrated in Fig. 1. We excited an isotropic suspension of mitochondria (or solution of cytochrome *c* oxidase) with

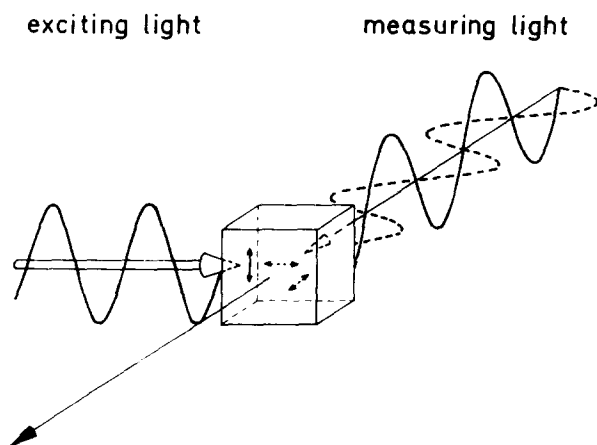


Fig. 1. Geometry in photoselection experiments with isotropic suspensions. The waves indicate the E-vector polarization of the measuring and the exciting light. The vertically polarized exciting light interacts by preference with those chromophores with their transition moments oriented in parallel to the polarization of the exciting light.

a linearly polarized flash of light. The wavelength of the light is tuned to photolyse the complex formed between the a_3 heme and carbon monoxide. Photolysis causes rapid absorption changes of the a_3 heme which relax within several 100 ms depending on the CO concentration. Since the probability for light absorption depends on the relative orientation of a molecule towards the electric vector of the exciting light, the photolysed ensemble of a_3 hemes will be preferentially oriented with respect to the polarization of the exciting light. This in turn implies that the extent of the absorption changes will depend on whether they are measured by light polarized parallel or perpendicular to the exciting light (i.e. linear dichroism). The relaxation of this photo-induced dichroism reflects the rotational diffusion of the chromophores with respect to the lab system.

Pigeon heart mitochondria were prepared according to the method of Chance and Hagiwara [27]. Beef heart submitochondrial particles were kindly provided by Dr. C. P. Lee (see ref. 16). French press particles were prepared according to ref. 28 (courtesy of Dr. K. Fairns and Dr. D. Wilson). Isolated cytochrome oxidase was prepared according to Yonetani [29] (courtesy of Dr. R. Colonna and Dr. T. Yonetani). Mitochondria were suspended in the following reaction medium (if not otherwise indicated): mitochondrial protein, 0.3 mg/ml, morpholinopropane sulfonate (MOPS) buffer, 50 mM, pH 7.4; tetramethylphenylenediamine (TMPD), 10 μ M; ascorbate, 3 mM; KCl, 50 mM. After being inserted into the optical absorption cell (1 cm \times 1 cm \times 4.5 cm high) the sample was supplied with a small volume (100 μ l) of buffer saturated with carbon monoxide. After injection of CO the absorption cell was sealed.

The low protein concentration was chosen to reduce the effect of light scattering on the polarization of the exciting and of the interrogating light, although these low concentrations were unfavorable for obtaining optimum signal-to-noise ratios [30]. Submitochondrial particles, being less turbid, were used at higher concentrations (0.5 mg/ml) with the result of better signal-to-noise ratio.

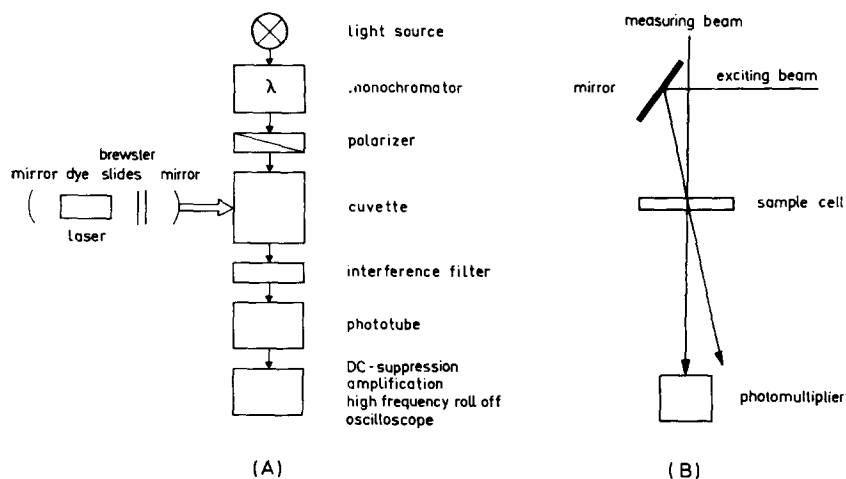


Fig. 2(A). Block-diagram of the laser spectrophotometer. The beam geometry is as in Fig. 1. This geometry underlies Figs 3–6. (B) Beam geometry in the experiments at liquid nitrogen temperatures. This geometry was chosen to at least partially overcome the effects of polarization dependent reflection at cracks in the glycerol glass on the apparent dichroic ratio (see text).

The 1 cm absorption cell was mounted in a spectrophotometer [33, 34] (see Fig. 2A) The sample was excited by a linearly polarized light pulse from a rhodamin-6G laser at 585 nm. The laser energy at the approximately 1 cm² aperture of the absorption cell was varied between 1 and 15 mJ, which was nonsaturating as required for photoselection to occur. The percent saturation indicated in Figs 4–6 was calculated by comparing the absorption change obtained with that expected (based on prior experiments [26]) for saturating excitation of samples having the same protein concentration. The half-maximum duration of the laser flash was 0.3 μ s. The laser beam was linearly polarized by glass plates, placed within the laser cavity at Brewster's angle. The polarization ratio of its output was greater than 15 : 1 at the highest energy in the experiments as determined with the aid of a Glan-Thompson prism.

The principle of the optical and the electrical set-up is illustrated in Fig. 2. The absorption changes of the cytochrome *a*₃ heme were observed at a wavelength of 445 nm except for the low temperature experiments where the spectrum was scanned. The bandwidth of the monochromator was set to 3.3 nm. The intensity of the measuring beam was 2 mW/cm² at 445 nm. It was derived from a high pressure, water-cooled mercury arc operated at 900 V and 1.0 A.

The measuring light for the experiments shown in Figs 5 and 6 consisted of a 100 Watt W-I₂ incandescent lamp operated at about 7.0 A filtered with a 445 nm interference filter (4 nm bandwidth) and then focused onto the cuvette. The intensity measured at the cuvette was 0.8 mW cm⁻². The transient output current of the photomultiplier was amplified and passed through a high frequency roll-off-filter [34]. The filter setting was such as to provide a filter relaxation time equal to 0.5 % of the oscilloscope sweep width.

Low temperature experiments

Those were carried out by suspending French press particles from pigeon mitochondrial in glycerol in order to obtain a glass clear enough to avoid severe reflection artifacts in polarization experiments. Out of 20 glasses prepared there was only one justifying polarization experiments. It was impossible to obtain glasses which were totally free of cracks. To overcome polarization artifacts the photo-selected linear dichroism was measured in a configuration dissimilar to the one illustrated in Fig. 1. As shown in Fig. 2B, the exciting and the measuring beam were directed almost parallel to each other. The photoinduced linear dichroism was then determined from two sets of experiments: (1) excitation polarized vertical, measuring light polarized vertical and horizontal; (2) excitation polarized horizontal, measuring light polarized vertical and horizontal. The dichroic ratios given were averages of these recordings.

Glycerol samples were prepared as follows: glycerol was bubbled for 4 h with CO, then debubbled by centrifugation. Mitochondrial particles were spun down at 100 000 g for 20 min in the above medium and then resuspended in glycerol under an N₂ atmosphere. The sample filled into a 1.5 cm \times 1.5 cm \times 1 mm absorption cell [34] was then rapidly cooled by dipping into liquid nitrogen. The measurement was carried out after partial evaporation of the liquid nitrogen.

Check-up for artifacts

Since the experimental results reported in this paper differed from those one of us (WJ) reported earlier [26], a careful check for possible artifacts in photoselection experiments was made. The control experiments follow those applied in photoselection experiments with chloroplasts [32].

Photomultiplier linearity and prepolarization by the monochromator

The monochromator prepolarized the measuring light intensity by a ratio of $1.2 = I_{\uparrow}/I_{\downarrow}$. This was corrected for to yield equal DC-output of the photomultiplier by readjustment of the high voltage of the tube. This was supposed to make the changes in the output voltage after photolysis for both polarizations of the measuring beam (see Fig. 1) directly comparable to each other. We checked the linearity of the photomultiplier tube (Telefunken XP 1080, S 11, 4 stages) in the voltage range between 600 and 800 V by means of calibrated neutral gray filters and found no significant deviations. Moreover, the linearity becomes evident from the two traces in the right of Fig. 3. The polarization of the exciting beam in this experiment was chosen such that no photoinduced dichroism was to be expected. The absence of linear dichroism under these conditions proves that eventual nonlinearities of the photomultiplier did not come into play.

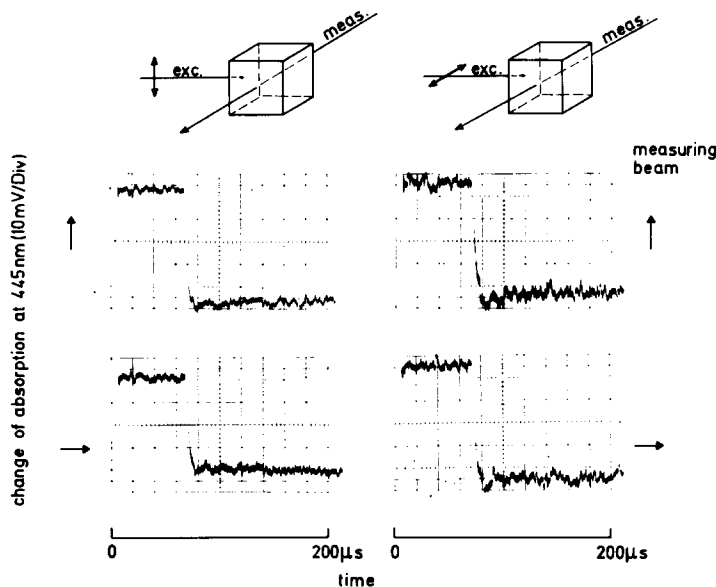


Fig. 3. Control experiment with a suspension of mitochondria. Above: beam geometry and E-vector polarization of excitation; right margin: E-vector polarization of the measuring light; left traces: the measuring beam polarized in parallel (above) and perpendicular (below) to the exciting one; right traces: the E-vector of the exciting beam is directed parallel to the propagation vector of the measuring one. Thus it is perpendicular to both polarizations of the measuring beam. The absence of linear dichroism under these conditions excludes certain artifacts (see text).

Reproducibility of the laser energy in subsequent flashes

The absorption traces at vertical and at horizontal polarization of the mea-

suring beam were taken from successive laser shots. Since nonsaturating energy of the laser was a prerequisite for photoselection to occur, any variation in the laser energy between successive shots would have caused an apparent dichroism of the signals. The absence of any significant variation of the laser energy is demonstrated by comparison of the two traces at the right of Fig. 3. The laser high voltage had to be turned down and up again between subsequent pairs of shots. This was not too reproducible. In consequence the laser energy varied between subsequent pairs, which explains the variance in the degree of saturation between these pairs.

Depolarization of the polarized light in a turbid suspension

Depolarization by light scattering in a turbid suspension of mitochondria decreases the dichroic difference in photoselection experiments. Scattering is more pronounced at shorter wavelength than at longer ones. The influence of the protein concentration and hence the turbidity on the apparent dichroic ratios is illustrated in Table I. It is evident that too high a protein concentration in the absorption cell almost eliminated the apparent linear dichroism in our experimental set-up. At the standard protein concentrations used with mitochondria, the effect of scattering was only slight; it was even less with the less turbid submitochondrial particles and almost negligible with the isolated enzymes.

TABLE I

Dependence of the dichroic ratio at 445 nm ($\Delta A_{||}/\Delta A_{\perp}$) on the protein concentration in a suspension of pigeon heart mitochondria. Optical pathlength, 1 cm; % saturation at 0.3 mg/ml protein, 30 %. The observed decrease in the apparent dichroic ratio with increasing protein concentration is due to depolarization by light scattering in the turbid suspension.

Protein concentration (mg/ml)	$\Delta A_{ }/\Delta A_{\perp}$
0.3	1.25
0.65	1.08
1.2	1.00

Bursts of scattered flash light

Since we placed no polarizer between the absorption cell and the cathode of the photomultiplier (see Fig. 2A), the contribution of eventual artifacts from scattered flash light to the apparent absorption changes for both polarizations of the measuring beam should be approximately equal. Nevertheless, we tried to minimize flash burst artifacts by the following filter combination placed on the cathode of the photomultiplier: interference-filter, Dr. Anders 445 nm (4 nm); bandfilters, Wratten 47B and 34.

Preselection by exposure of the sample to the measuring beam

For the sake of a high signal-to-noise ratio, the intensity of the measuring beam was chosen rather high (2 or 0.8 mW/cm²). Since, at the given concentration, the recombination of the photolysed heme · CO complex was slow one might expect that the measuring light caused partial photolysis and, because of its linear polari-

zation, preselects an anisotropic ensemble. To avoid this the measuring light was switched to the sample only about 10 ms prior to the laser flash and was shut off after completion of an oscilloscope run. Hence, the amount of light energy presented to the sample prior to the laser flash (at least 1 mJ) was, at most, about $2 \text{ mW} \times 1 \text{ cm}^2 \times 10 \text{ ms}$ which is two orders of magnitude below the energy of the laser flash and so negligible.

Photoselection due to sieve effect was shown to be negligible in experiments with chloroplasts [32]. Since it is the more pronounced the higher the pigmentation of an organelle, sieving will not be important in experiments with mitochondria with their much lower pigment concentration compared to chloroplasts.

RESULTS

Isolated hemoproteins

The method and the experimental set-up was first tested in experiments with isolated hemoproteins. Fig. 4 shows the absorption changes at 445 nm arising from excitation of the respective heme · CO complex in hemoglobin (right) and the cytochrome oxidase in solution. We observed no dichroism for hemoglobin A down to a time resolution of $1 \mu\text{s}$, almost no dichroism if the cytochrome oxidase was suspended in buffer (phosphate, 100 mM, pH 7.4) in the presence of the detergent Tween-80 (1 %) and a dichroic ratio of 1.3 if the oxidase was suspended in buffer only, with no detergent.

These results could be interpreted as follows: hemoglobin is known not to polymerize in solution. According to Tao's rule of thumb for the rotational relaxation of spherically approximated globular proteins in solution [35], its rotational relax-

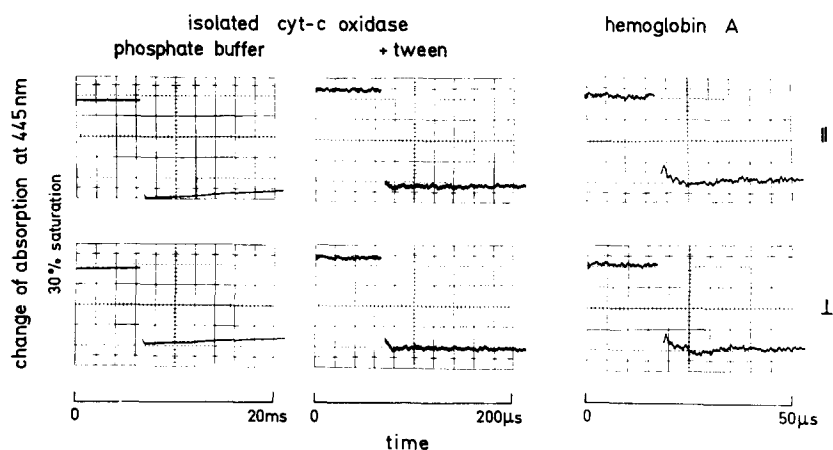


Fig. 4. Photoselection experiments with isolated hemoproteins. The signs || and \perp , stand for the relative polarizations of the measuring and the exciting light respectively. The isolated enzymes were reduced by dithionite, CO saturated and suspended in the following respective media at an OD ($432 \text{ nm} = 0.6$). Left: phosphate buffer, pH 7.4, 0.1 M; middle: phosphate buffer, pH 7.4, 0.1 M; detergent TWEEN 80, 1 %; right: phosphate buffer, pH 7.4, 0.1 M. (Suspension of the isolated cytochrome-oxidase in phosphate buffer in the presence of sodium cholate, 2 % plus EDTA, 2 mM instead of TWEEN as detergent yielded no dichroism as in the presence of TWEEN.)

ation time should be: $\tau = \eta (M_r/3.69) \text{ ps} = 20 \text{ ns}$, where η is the viscosity of the medium. Hence one expects that rotational diffusion of hemoglobin dissipates any information on photoselection three orders of magnitude more rapid than resolvable in our photometer. The cytochrome oxidase is larger, and rotates less rapidly, but still fast enough to be unresolved in our experiments. The rotational relaxation times should increase only if the enzyme polymerizes, which it does in the absence of detergents [29]. In fact, we observed a dichroic ratio of 1.3 (see left traces in Fig. 4) under these conditions. The absence of any dichroic relaxation in the time range of 20 ms implies that the clusters of polymeric cytochrome oxidase formed were at least 5000 Å in diameter. The absence of any dichroism under conditions where it should not appear proves the absence of artifactual apparent dichroism under our experimental conditions.

The large size of the clusters of polymerized oxidase is somewhat surprising and so is the strong immobilization within these clusters. This immobilization becomes evident from the fact that the observed dichroic ratio comes very close to the expected maximum for a circularly degenerate "immobilized" chromophore (see below)*. In order to find out whether higher dichroic ratios were to be obtained on complete immobilization of the oxidase, we carried out experiments with French-press particles frozen to liquid nitrogen temperature.

Low temperature experiments

Photoselection experiments at liquid nitrogen temperature with French-press particles in glycerol were complicated by two factors:

- (1) the appearance of cracks in the glycerol glass (see experimental),
- (2) the appearance of a signal component even at room temperature, relaxing within 20 μs , revealing a difference spectrum at variance from the one observed for the slower component which had a spectrum that one usually observes for the photolysis of the $a_3 \cdot \text{CO}$ complex in aqueous solution. Instead of peaking negatively at 445 nm and positively at 430 nm, the rapid component peaked negatively at both these wavelengths with a zero crossover at 337 nm. To avoid confusion with this as yet unidentified signal, the dichroic ratios were read out only 200 μs after excitation, hence after the decay of the fast component.

The influence of the cracks was at least partially eliminated by recording the linear dichroism for different settings of the polarization of the exciting light (see Experimental). The values for the dichroic ratios varied by about ± 0.1 between these settings and by the same amount between different glasses. The average value for the dichroic ratio $\Delta A_{\parallel}/\Delta A_{\perp}$ is 1.35 ± 0.1 . Because of the ambiguities due to selective reflection at the cracks and to different effects of turbidity in the imperfectly homogenized glasses, the above dichroic ratio can be taken as semiquantitative, only. The fact that the dichroic ratio is not considerably higher than 4/3, the theoretical maximum for a circularly degenerate chromophore [31], suggests that the symmetry of the a_3 heme (fourfold) is not broken by its interaction with the protein. Therefore, we can say that the polymerized oxidase in solution must be almost completely "immobilized".

* As further specified in the Discussion section, the "immobilization" need not be complete and the above results can be understood in terms of motion around one single axis, the symmetry axis of the heme.

Experiments with mitochondria and submitochondrial particles

In photoselection experiments with pigeon heart mitochondria in buffer solution we observed dichroic ratios of $A_{||}/A_{\perp} = 1.27 \pm 0.03$ (20 experiments with 5 different preparations). As obvious from a comparison of the upper with the respective lower traces in Fig. 5, there is no evidence for any dichroic relaxation either at a

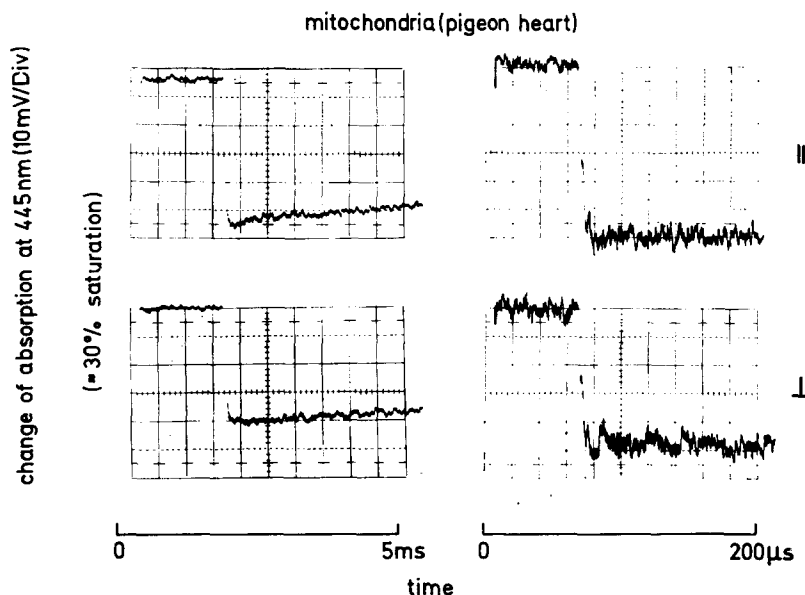


Fig. 5. Photoinduced linear dichroism with suspensions of pigeon heart mitochondria. The dichroic ratios of the upper with their lower traces are around 1.27. There is no pronounced dichroic relaxation neither at a time resolution of $20 \mu\text{s}$ nor at 5 ms.

$20 \mu\text{s}$ or a 5 ms time scale. A relaxation of the transient photodichroism could be expected to result from the rotation of whole mitochondria in the suspension. Instead of the seconds time scale, we preferred to detect this rotation for submitochondrial particles at a 10 ms time-scale, where the stability of the measuring beam was higher. Submitochondrial particles of the kind we used in these experiments have a size distribution ranging [16] from 500 to 2000 Å in diameter. According to Perrin's formula [36] for the rotational relaxation time of spheres in viscous media:

$$\tau = \frac{3 \cdot \eta \cdot V}{RT}$$

where η is the viscosity, V the volume of the sphere and RT is as usual, this implies tumbling times ranging between 0.8 and 50 ms at a viscosity of 1 cP.

A partly dichroic relaxation is in fact obvious from a comparison of the two traces in Fig. 6 (left side). The halftime of the relaxation is about 1 ms in low viscosity (≈ 1 cP) buffer. It is evident that the extent of the absorption change recorded for perpendicular polarization of the exciting and the measuring beam increases while the one for parallel polarizations decreases. As theoretically expected, the increase of

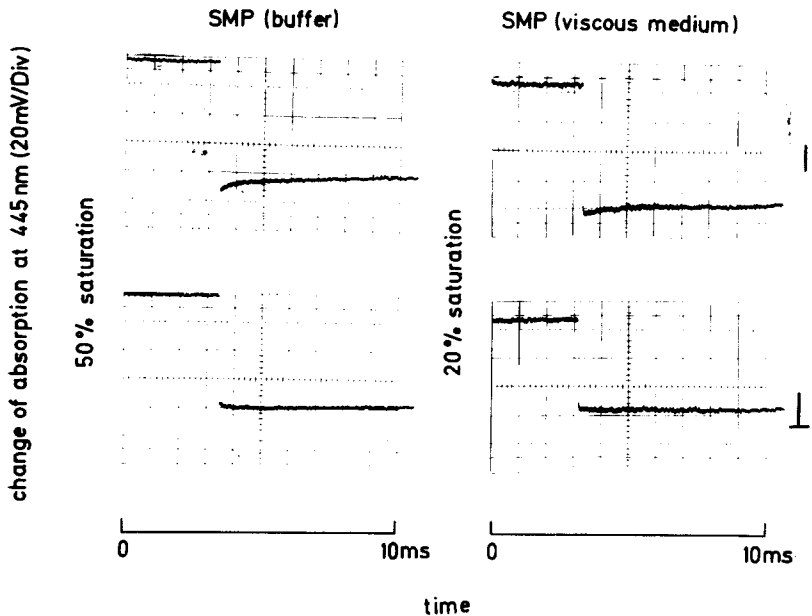


Fig. 6. Photoinduced linear dichroism with suspensions of submitochondrial particles from beef heart. Left: A partial dichroic relaxation with a half-time of about 1 ms is apparent. The particles were suspended in the standard buffer solution with an approximate viscosity of 1 cP. Right: The dichroic relaxation is slowed down. The particles were suspended in the same buffer but under addition of the polysaccharide dextran to yield a viscosity of about 8cP. The difference in saturation between the left and the right traces resulted from readjustment of the laser high voltage between subsequent pairs of shots. (The turning off of the laser voltage during intermissions was technically necessary. The reproducibility of the voltage dialling was low.)

the perpendicular component accounts for only half of the losses of the parallel component.

Almost all the dichroism relaxed within 10 ms which again is compatible with the above cited size distribution. In order to confirm experimentally that the observed dichroic relaxation was entirely due to rotation of submitochondrial particles in the suspension but not to any rotational relaxation of the enzyme within the membrane, we increased the viscosity of the suspending medium from about 1 cP to about 8 cP by adding the highmolecular-weight polysaccharide dextran. This, as obvious from the traces in the right of Fig. 6, slowed down the dichroic relaxation.

It is improbable that such a slight increase of the medium's viscosity influences the rotational relaxation time of an enzyme embedded in the membrane. This relaxation time should be dominated by the viscosity of the membrane (which for several biological and artificial membranes was found to range between 1 and 10 P) rather than by the much lower viscosity of the surrounding aqueous phases. Hence, the observed dichroic relaxation should be attributed to the rotation of the organelles rather than to any rotation of the enzyme within the membrane. The dichroic ratio obtained with submitochondrial particles at less than 30 % saturation was 1.26 ± 0.3 .

DISCUSSION

Symmetry of the cytochrome a_3 heme

The above photoselection experiments with the isolated cytochrome oxidase when polymerized, as well as those on mitochondria and submitochondrial particles, revealed dichroic ratios in the order of 1.3. This comes close to the theoretical expectation for a circularly degenerate chromophore which is "immobilized" with respect to the lab system (for a theory of photoselection, see ref. 31). This expectation value is 4/3. Since this ratio was observed even for frozen systems we conclude to an almost perfect circular degeneracy of heme a_3 . This implies that the fourfold symmetry of the heme is not broken by its interaction with its microenvironment within the protein.

Circular degeneracy of the transition moments of the a_3 heme is suggested by the four-fold symmetry of the isolated heme. However, it is conceivable, although unlikely, that the interaction of the heme with the protein breaks this symmetry. That the symmetry at least of heme a is not broken finds independent support from photoselection studies on cytochrome c and comparative EPR studies on different hemo-proteins. Photoselection revealed a circular degeneracy of both the Soret and the α -band of cytochrome c [37]. EPR studies on the cytochrome oxidase revealed that the "visible" heme reveals much less rhombicity than the cytochrome c heme [38]. Thus, heme a can be expected to approximate a perfect circular degeneracy even better than cytochrome c .

The rotational mobility of the cytochrome a_3 heme in the inner mitochondrial membrane

The only relaxation of the photoinduced linear dichroism we observed for mitochondria and submitochondrial particles was attributable to the rotation of these organelles in the suspension. There was no dichroic relaxation which could be attributed to the rotational diffusion of the enzyme in the membrane. If we take into account the evidence of a circular degeneracy of the transition moments of the a hemes, two alternative conclusions can be derived therefrom:

(A) either the cytochrome oxidase is completely immobilized within the cristae membrane.

(B) or it rotates around one single axis, which is parallel to the symmetry axis of the a_3 heme, so that there is no change in the interaction of the already circularly degenerate heme with light.

Considering the above-cited evidence for a transmembrane orientation of the cytochrome oxidase (see Introduction) and the evidence for a fluid structure of the inner mitochondrial membrane [39], we would rather tend to favor the second interpretation (B) of our experiments.

If the cytochrome oxidase were transmembrane, the polarity distribution of the enzyme along its axis which is normal to the membrane should be: hydrophilic-hydrophobic-hydrophilic. If so, the probability for any rotational diffusion around an axis in the plane of the membrane will be low. Nevertheless, rotational motion around an axis perpendicular to the membrane could still be possible, since it does not require flipping of polar groups across a hydrophobic layer. So, one would like to visualize the oxidase tumbling around an axis perpendicular to the plane of the membrane, provided that it is embedded in a fluid matrix. The only evidence for a

fluid structure of the inner mitochondrial membrane comes from studies on the fluorescence depolarization by rotational diffusion of a Ca^{2+} ionophore which operates in this membrane [39]. A rotational relaxation time of about 10 ns was reported, which parallels the rotational relaxation time expected for a molecule of a similar size in an environment with a fluidity of some poise, but is incompatible with a quasi-crystalline membrane. If the cytochrome oxidase carries out such an anisotropic rotation, which is probable although not proven, our experimental results imply that the plane of the a_3 heme is parallel to the plane of the inner mitochondrial membrane. This configuration is illustrated in Fig. 7.

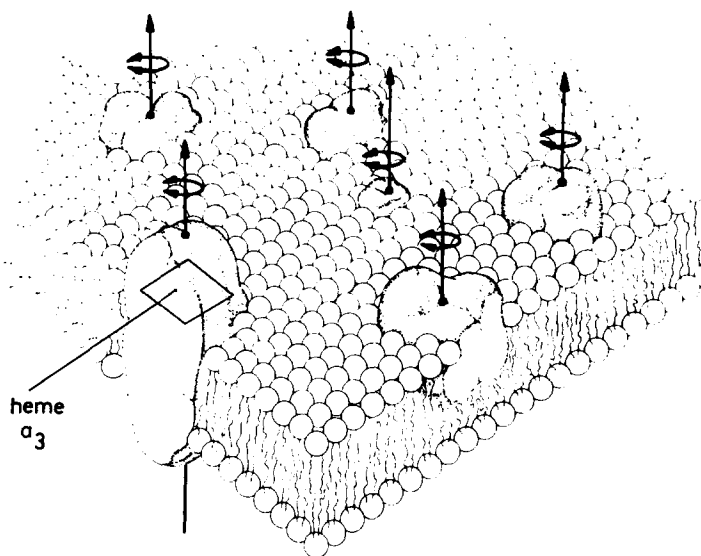


Fig. 7. Schematic representation of the most probable configuration of the cytochrome-oxidase in the inner membrane of mitochondria. (The drawing is an adaption from ref. 40.) The cytochrome oxidase is transmembrane with heme a_3 located nearer to the matrix side. It carries out anisotropic Brownian rotational diffusion around a single axis which is normal to the plane of the membrane. Heme a_3 , having four-fold symmetry, is oriented coplanar to the membrane.

Possible reasons for the variance between the results in comparison to previous findings

In a preceding paper [26] one of us (WJ) reported results at variance from the ones presented in this communication. It was shown that no photoinduced dichroism is observable for mitochondria suspended just in water unless these are fixed with glutaraldehyde or suspended in a high viscosity medium [26]. Since the oscilloscope traces revealing the absence of any dichroism were just on top of each other several times (see Fig. in ref. 26) without any intermediate data processing, there was certainly no interpretational artifact involved. However, in order to obtain a better signal-to-noise ratio in the experiments with mitochondria suspended in buffer only (for higher time resolution), a higher protein concentration was used than in the other experiments where no dichroic relaxation was to be expected. At the given protein concentration (1 mg/ml), however, according to Table I in this paper, the depolarization due to light scattering almost completely eliminates the apparent linear dichroism in photoselection.

In order to check whether or not this was the only reason for the apparent absence of linear dichroism in the earlier experiments, we tested to see if the suspending medium was of any influence. Neither low salt or high salt nor hypotonic or hypertonic conditions was found to affect the linear dichroism near 1.3 that we observed in this series of experiments. We also found no influence of the "age" of mitochondrial preparations.

Prospectives

The lack of dichroic relaxation in photoselection experiments with the complex between cytochrome oxidase and CO under conditions where the enzyme is bound to the membrane allows one to photoselect membrane patches having a favorable orientation to the electric vector of the exciting flash light. Hence, one can examine the respiratory chain in just those membrane patches which are selected from an originally isotropic ensemble. By carrying out flow-flash studies with oxygen and monitoring the linear dichroism of the other hemes in the respiratory chain, one might expect to get information on their relative orientation with respect to the a_3 heme and to their rotational mobility within the inner mitochondrial membrane.

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