ELLIPTICITY OF CYTOCHROME a_3 AND ROTATIONAL MOBILITY OF CYTOCHROME *c*-OXIDASE IN THE CRISTAE MEMBRANE OF MITOCHONDRIA

Ulrich KUNZE and Wolfgang JUNGE

Max-Volmer-Institut für Physikalische Chemie und Molekularbiologie, Technische Universität Berlin, Strasse des 17. Juni 135, D-1000 Berlin 12, Germany

Received 20 June 1977

1. Introduction

Cytochrome *c*-oxidase mediates the terminal step in the respiratory chain. It accepts electrons one by one from cytochrome *c* and accumulates four reducing equivalents until it finally reduces oxygen. The reducing equivalents reside on two hemes (cytochrome *a* and cytochrome a_3, resp.) and on two coppers. There is good evidence that the electron transfer from cytochrome *c* to oxygen crosses the cristae membrane of mitochondria whereby it contributes to the electric potential generation (for a review, see ref. [1]).

The orientation of the two hemes relative to the cristae membrane and the rotational mobility of the whole enzyme is subject to current studies [2,3]. A previous paper [2] reported on the polarized flash photolysis of the complex formed between cytochrome a_3 and Co. The linear dichroism of the resulting absorption changes was analysed to yield information on heme symmetry and rotational mobility of the enzyme. At a given wavelength pair of excitation (585 nm) and of observation (445 nm), a dichroic ratio of about 4/3 was observed, which was interpreted as indicative of an 'effective' 4-fold symmetry of the π-electron system in cytochrome a_3. To account for the fact that the same dichroic ratio resulted for the isolated and immobilized enzyme as well as for the enzyme in mitochondria, two alternative interpretations were given: (a) Either cytochrome-c-oxidase is completely immobilized within the cristae membrane, or (b) it carries out only uniaxial Brownian rotation with the rotation axis in parallel to the symmetry axis of the heme (normal to the heme plane).

In this communication we extend these studies with an aim to resolve the ambiguity concerning the rotational mobility of the enzyme. The rational is that the 'effective' 4-fold symmetry of heme *a* should be only approximate. By scanning the photoinduced linear dichroism over the wavelength domain one should detect electronic transitions which are preferentially polarized along two mutually perpendicular axes within the heme plane.

We excited CO-complexed cytochrome *c*-oxidase in the α-band region (595 nm) and scanned the linear dichroism of the resulting absorption changes over the Soret band region. The spectrum of the dichroic ratio shows a complicated structure which can be quantitatively understood under the assumption that the *x* and the *y* polarized transitions of the CO-complexed as well as of the uncomplexed cytochrome a_3 are not really degenerate. Their respective absorption peaks are split by up to 13 nm. The absence of the previously assumed circular degeneracy of the heme implies that any rotational diffusion of cytochrome a_3 would affect the spectrum of the dichroic ratio in a very critical way (if occurring at times shorter than resolved in the photoselection experiment). The fact that we observed the same dichroic spectrum for the isolated, immobilized enzyme as with mitochondria leads us to conclude that cytochrome *c*-oxidase does not carry out Brownian diffusion around any axis in the cristae membrane. This holds for the time range up to 100 ms.

2. Materials and methods

Rat liver mitochondria were prepared according

to Vinogradov et al. [4]. For stabilization nupercaine (0.4 mM) was added to the storage medium (at 30–40 mg/ml of protein). Mitochondria were suspended at an average protein concentration of 0.6 mg/ml in the following standard reaction medium: morpholinopropane sulphonate, 50 mM (pH 7.4); KCl, 50 mM; tetraphenylendiamin, 10 μM; Na-ascorbate, 3 mM. To facilitate anaerobiosis the buffer medium was degassed before the addition of mitochondria. The suspension was filled into an absorption cell (2 cm thickness) which was sealed and allowed 15 min for anaerobiosis. Then a small amount of CO saturated buffer was added to yield a final CO concentration of about 100 μM. No special measure was taken to prevent Brownian rotation of mitochondria in the suspension, since previous experiments [2] have shown that this rotation is negligible in the time domain up to 100 ms.

Purified cytochrome *c*-oxidase was obtained from two sources. Dr Hanns Weiss kindly provided a sample from *Neurospora crassa* [5]. Some of the experiments were carried out with a preparation from beef heart according to the procedure by Yonetani [6] and furthermore purified on a sepharose column [7]. The concentrated solution of cytochrome *c*-oxidase was applied for about 1 h to a DEAE-Sephadex column, the contents of which was finally transferred into the optical absorption cell. Buffer medium was then added to yield the following average concentrations in the absorption cell: cytochrome *c*-oxidase, 0.5 mg/ml; Tris-acetate, 20 mM (pH 8); KCl, 50 mM; tetraphenylendiamin, 50 μM and Na-ascorbate, 5 mM. After anaerobiosis the sample was supplied with CO-saturated buffer to yield a final CO-concentration of 100 μM. The degree of immobilization of the enzyme by its attachment to the DEAE-Sephadex is assumed to be perfect, as there is no change in the photo-induced linear dichroism if the enzyme molecules are crosslinked by glutaraldehyde (0.1%), added before and during the loading of the DEAE-Sephadex column. The perfect immobilization parallels similar observations with photosystem I particles from green plants [8]. The principle of polarized photochemistry with cytochrome *c*-oxidase was described previously ([2], for a general review on photoselection, see Albrecht [9]). In brief: excitation of an isotropic ensemble of chromophores by linearly polarized light will select a subset with its transition moments oriented by preference in parallel to the E-vector of the exciting light. The linear dichroism of the absorption changes in consequence of photochemical events yields information on the relative orientation of the excited and the observed transition moments as well as on rotational motion of the chromophores.

The optical absorption cell was mounted into a rapid kinetic spectrophotometer with laser excitation as illustrated in fig.1. To increase the signal-to-noise ratio of the very small dichroic differences at certain wavelength three measures were taken: (1) The measuring light was set high (typical 50 $\mu W/cm^2$), but to avoid preexcitation of the sample it was on only during the measuring interval (100 ms). (2) Signals were induced repetitively and averaged on a Nicolet 1072 computer. (3) To eliminate the influence of fluctuations of the laser energy or of the measuring light intensity, which might cause error if the signals for the two mutually perpendicular signals are recorded shot after shot, both polarizations were recorded together. For this the polarization of the measuring beam was rotated at a frequency of 100 kHz by means of a Morvue photoelastic modulator (PEM3). The direct input into the Nicolet averaging computer then yielded the time average of the

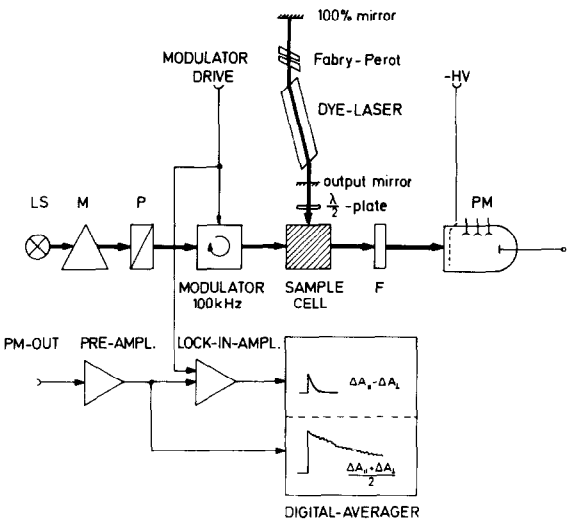

Fig.1. Spectrophotometer for measuring linear dichroism in photoselection. The polarization of the measuring beam is rapidly modulated at 100 kHz. (LS) Light source. (M) Monochromator. (P) Polarizer. (F) Guard filter.

signals for parallel and for perpendicular polarization $((\Delta A_{\parallel} + \Delta A_{\perp})/2)$ as the analogue-to-digital converter intergrated over the modulated signal input over an address time of 2 ms. Another fraction of the signal was passed through a lock-in amplifier to extract the modulated component. The output of the lock-in amplifier fed into a second input of the signal averager yielded the dichroic difference $(\Delta A_{\parallel} - \Delta A_{\perp})$.

The sample was excited by a 5 μs pulse from a rhodamin 6G liquid dye laser (energy attenuated to excite only 20% of the chromophores within each sample). The center wavelength of the laser emission was 595 nm at a halfwidth of 8 nm (FWHM). The monochromator in the measuring beam was calibrated, its slit was set for a half-width of 2 nm.

3. Results

The flash induced difference spectrum of isolated cytochrome *c*-oxidase complexed with CO is shown in the upper part of fig.2 (note that $\Delta I/I = -\Delta A \times 2.3$). It was measured for parallel polarization of the exciting flash and the measuring beam. The spectrum is almost identical with the one published by Yonetani [10]. With mitochondria a very similar difference spectrum was obtained, however, superimposed to it there was a minor component due to the photolysis of the CO-complex of other hemoproteins. This component, the spectrum of which is represented by a broken line in fig.2 (upper part), was readily separated from the absorption change due to cytochrome *c*-oxidase, because it relaxed within 5 ms in contrast to $\geqslant$ 100 ms of the oxidase. The rapidly relaxing component did not contribute to the photoinduced linear dichroism either. We attribute the rapid component to hemoglobin. After subtraction of this component the difference spectra from mitochondria and from the isolated oxidase were almost identical.

The points in the lower part of fig.2 give the average dichroic ratio of the absorption changes of cytochrome *c*-oxidase. Each point represents the average from between 5 and 15 experiments with different preparations of the isolated and immobilized enzyme as well as with mitochondria. The standard deviation is also indicated. It naturally increases in those regions where the absorption changes are small.

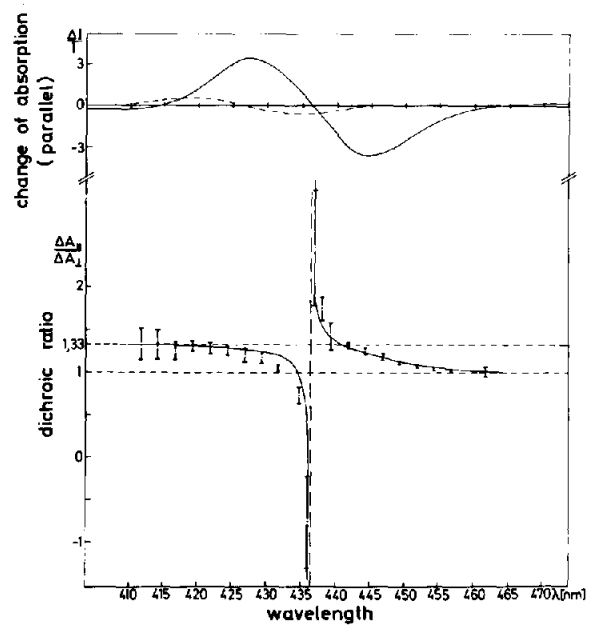

Fig.2. Upper part; Spectrum of the absorption changes resulting from the photolysis of the complex between cytochrome *c*-oxidase with CO. The solid line represents the spectrum obtained for a preparation from *Neurospora crassa*. The broken line represents the spectrum of rapidly relaxing absorption changes which are observed in mitochondria in addition to larger absorption changes of the cytochrome *c*-oxidase. (This component is subtracted before the linear dichroism is evaluated.) Fig.2. Lower part; Spectrum of the dichroic ratio of the flash-induced absorption changes $(\Delta A_{\parallel}/\Delta A_{\perp})$ of CO-complexed cytochrome *c*-oxidase. The points represent the average values from between 5 and 15 experiments with the isolated and immobilized enzyme and with mitochondria. The error bars give the standard deviation from the mean values. The line represents the theoretical simulation of the dichroic spectrum based on assumptions which are illustrated in fig.3.

The major features of the dichroic spectrum are as follows:

(a) There is an about antisymmetric pole of the dichroic ratio in the close neighbourhood of the isosbestic point of the transition cyt a_3-CO versus cyt a_3 at 436 nm.

(b) The dichroic ratio at the blue end of the absorption of cyt a_3-CO (415 nm) seems to approach a figure of 4/3.

(c) The dichroic ratio at the red end of the Soret band region of cytochrome a_3 approaches a fig of 1 (460 nm).

(d) The two branches of the dichroic spectrum 'cross-over' in a way that the branch which is higher at the pole becomes smaller at the fringes and vice versa.
(e) Within the error limits there was no difference detected between the dichroic spectrum of the isolated, immobilized enzyme and of the enzyme in mitochondria.

4. Discussion

The discussion will be based on two generally accepted assumptions: (1) There is no resonant transfer of electronic excitation from cytochrome *a* to cytochrome a_3. (This is evident from the different shapes of the action spectrum for the photolysis of the cyt a_3-CO complex by Castor and Chance [11] and the absorption spectrum of the CO-complexed oxidase (cyt a plus cyt a_3-CO) by Yonetani [10].) (2) There is no influence of the photolysis of the cytochrome a_3-CO complex on the absorption spectrum of cytochrome-a_3. (This is the accepted basis for any spectroscopic discrimination of the two hemes in the oxidase in the literature, e.g., ref. [13].)

A thorough evaluation of the dichroic spectrum to yield the location, magnitude and polarization of the various subbands of cytochrome *c*-oxidase is hampered by the fact that no unequivocal picture will result. Instead, as will be shown elsewhere [12], a whole field of configurations will explain the data likewise. Fortunately all allowed configurations share the following common properties:
(1) Cytochrome a_3 behaves like an elliptic oscillator, i.e., the *x* and the *y* polarized transitions within each absorption band are usually not degenerate.
(2) Ligation of CO into the 6th coordination site of the iron in cytochrome a_3 influences the ellipticity
(3) It may be that there is a reorientation of the heme plane within the protein after photolysis of the CO complex.

Parameters describing the field of configuration to mimic the data are then: the ellipticity at any wavelength of absorption and the tilt angles of the heme plane of cytochrome a_3 after photolysis of the CO-complex. Of the possible configurations we will here present only one for illustrative purposes. It is given in fig.3. The division of the respective bands of cyt-a_3 and cyt a_3-CO into *x* and *y* polarized subbands in fig.3 together with a tilt of the heme plane

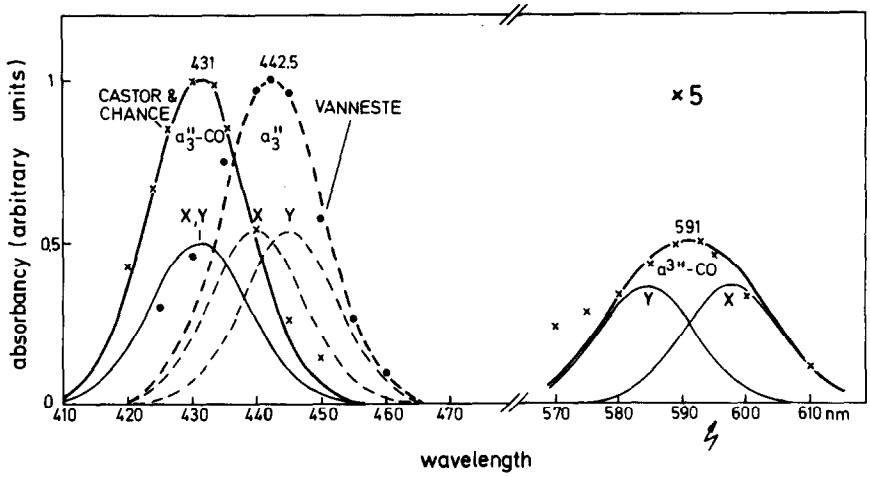

Fig.3. Analysis of the probable absorption spectra of the CO-complexed cytochrome *c*-oxidase and of the uncomplexed enzyme in terms of either *x* or *y* polarized Gaussian components. The composition given produces the theoretical dichroic spectrum which is illustrated in the lower part of fig.2 by a solid line. (Crosses) Experimental points from the action spectrum for the photolysis of the CO-complex from Castor and Chance [11]. (Points) Taken from the calculated absorption spectrum of reduced cytochrome a_3 after Vanneste [13]. (Fat line) Gaussian simulating the absorption of CO-complexed cyt a_3. (Thin lines) Gaussians composing the absorption of CO-complexed cyt a_3. (Fat broken line) Gaussian simulating the absorption of uncomplexed cyt a_3. (Thin broken lines) Gaussians composing the absorption of uncomplexed cyt a_3.

by 17° around the x axis gives rise to the theoretical expected spectrum of the dichroic ratio which is illustrated in fig.2 (lower part) by a line.

The fine structure of the absorption bands and the implications for the dichroic ratio shall be discussed in some detail. The thick solid curve in fig.3 represents a probable absorption spectrum of cytochrome a_3-CO fitted to the photochemical action spectrum by Castor and Chance [11] (see crosses). The thick broken line gives an absorption spectrum of cytochrome a_3 shaped according to data by Vanneste [13] (see the points in fig.3). The absorption bands of hemoproteins in the Soret region and some bands in the orange to red region result from $\pi \rightarrow \pi^*$ transitions. In the red region there may be $\pi \rightarrow d_{xz,yz}$ transitions involved, too. However, polarized photochemistry studies on crystals from various hemoproteins have shown that all these transitions are polarized within the heme plane (see [14]). In heme a the 4-fold symmetry of the porphyrin skeleton is broken by the vinyl in position 4 and by the formyl in position 8. Attachment of the heme to the protein may furthermore act on the symmetry. Therefore each absorption band of heme a may be more or less split into two sub-bands which are polarized along two mutually perpendicular axes within the heme plane (denoted x and y, without assignment to any direction in the ring system, so far). Depending on the wavelength split between the two sub-bands the chromophore will appear elliptical or, if there is no split, circular. When the wavelength is scanned across each composite band, the ellipticity will change from say preferentially x over circular to y. One of the possible composition of the absorption bands of cytochrome a_3 into x and y polarized sub-bands is illustrated in fig.3 by thin lines. The following bandwidth (FWHM) of the respective Gaussians and the following splits between the maxima (in parentheses) are assumed: cytochrome a_3-Soret, 17.3 nm (5 nm); cytochrome a_3-CO-Soret, 18.3 nm (0 nm); cytochrome a_3-CO-α-band, 18.3 nm (13.4 nm).

Qualitatively the observed dichroic spectrum correllates with the sub-bands as follows: At any wavelength the observed absorption changes are composite from contributions from cytochrome a_3 and from cytochrome a_3-CO. The observed dichroic ratio then is:

$$DR_{obs} = (\Delta A_{\parallel}(a_3) + \Delta A_{\parallel}(a_3\text{-CO}))/(\Delta A_{\perp}(a_3) + \Delta A_{\perp}(a_3\text{-CO}))$$

As a_3-CO disappears while a_3 appears the respective absorption changes are of opposite sign.

The observed dichroic spectrum can be explained only if it is assumed that the laser excitation hits into a noncircular absorber. The absorber should be elliptical with preference along say the x axis. At the pole the denominator of the dichroic ratio vanishes. At right of the pole, where the absorption of cytochrome a_3 exceeds the one of cytochrome a_3-CO, the dichroic ratio is positive. This implies that the dichroic ratio of cytochrome a_3 ($\Delta A_{\parallel}(a_3)/\Delta A_{\perp}(a_3)$) exceeds the one of cytochrome a_3-CO in the neighbourhood of the pole. It has to be concluded that the short-wavelength sub-band of a_3 is preferentially x, while the long-wavelength sub-band of a_3-CO is less x, possibly x,y or y. The dichroic ratio at the blue end is approaching 1.333. This implies that the Soret band of cytochrome a_3-CO might be circular. The dichroic ratio at the red end is 1, which implies ellipticity of the cytochrome a_3 long-wavelength part along y. A tilt of the heme plane around the x axis after the photolysis increases the curvature of the dichroic spectrum in the neighbourhood of the pole.

In conclusion we find that the spectrum of the dichroic ratio clearly shows the influence of heme ellipticity in cytochrome a_3. Additionally there is some indication that there might be a reorientation of the heme after photolysis of the CO-complex.

What are the implications for the rotational mobility of cytochrome c-oxidase in the cristae membrane of mitochondria? As there is no circular degeneracy of the heme in cytochrome a_3, any even limited rotational motion will affect the spectrum of the linear dichroism. Our observation that the same dichroic spectrum results with the immobilized enzyme and with mitochondria implies that the enzyme does not carry out any rotational diffusion at relaxation time shorter than 100 ms. (The alternative conclusion that the residual mobility in the cristae membrane is accidentially the same as on the DEAE-Sephadex column is considered as highly unlikely.). The structural basis for this immobilization of cytochrome c-oxidase in the cristae membrane has still to be elucidated.

These results support and extend the observations by Erescinska et al. [3], made with desiccated mitochondrial pellets, that the average heme *a* in the oxidase is oriented almost perpendicular to the cristae membrane. Rotational mobility around the normal to the membrane, however, was not to be detected in these studies.

Acknowledgements

The authors are very much indepted to Dr Hanns Weiss for a gift of cytochrome *c*-oxidase from *Neurospora crassa*. They are very grateful to Ms Allison McGeer for preparing the enzyme from beef heart and to Ilse Columbus for preparing the mitochondria. Financial support from the Deutsche Forschungsgemeinschaft is acknowledged.

References

[1] Malmström, B. G. (1973) Quart. Rev. Biophys. 6, 389–432.
[2] Junge, W. and DeVault, D. (1975) Biochim. Biophys. Acta 408, 200–214.
[3] Erescinska, M., Blasie, J. K. and Wilson, D. F. (1977) FEBS Lett. 76, 235–239.
[4] Vinogradov, A., Scarpa, A. and Chance, B. (1972) Arch. Biochem. Biophys. 152, 646–654.
[5] Weiss, H., Sebald, W. and Bücher, Th. (1971) Europ. J. Biochem. 22, 19–26.
[6] Yonetani, T. (1966) in: Biochemical Preparations (Machly, A. ed) pp. 14–20, John Wiley, New York.
[7] Kornblatt, J. A., Baroff, G. A. and Williams, G. R. (1973) Can. J. Biochem. 51, 1417–1427.
[8] Junge, W., Schaffernicht, H. and Nelson, N. (1977) FEBS Lett. in press.
[9] Albrecht, A. C. (1961) J. Molec. Spectrosc. 6, 84–108.
[10] Yonetani, T. (1960) J. Biol. Chem. 235, 845–852.
[11] Castor, L. N. and Chance, B. (1955) J. Biol. Chem. 217, 453–465.
[12] Kunze, U. and Junge, W. (1977) in preparation.
[13] Vanneste, W. H. (1966) Biochemistry 5, 838–848.
[14] Eaton, W. A. and Hochstrasser, R. M. (1968) J. Chem. Phys. 49, 985–995.