ORIENTATION OF THE HEMES OF CYTOCHROME *c* OXIDASE AND CYTOCHROME *c* IN MITOCHONDRIA

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1. Introduction

The key enzyme in mitochondrial respiratory chain is cytochrome *c* oxidase which catalyzes the transfer of electrons from reduced cytochrome *c* to molecular oxygen with the formation of water and concomitant synthesis of one molecule of ATP for each two electrons transferred. The enzyme is large (80 × 70 × 90 Å [1] equivalent mol. wt. of ∿125 000 [2]) and is tightly bound to the mitochondrial inner membrane from which it can be removed only with the help of detergents.

In a preliminary communication [3] we have reported that 'membranous' cytochrome *c* oxidase [4] forms upon slow partial dehydration at 4°C oriented multilayers in which the bundles of α helices occurring within the enzyme molecule have an average orientation normal to the plane of the membrane. Moreover, electron paramagnetic resonance (EPR) studies of these oriented multilayers of membranous cytochrome oxidase showed that the low spin form of the oxidized heme(s) of the enzyme is oriented such that the normal to the heme plane lies in the plane of the membrane. Polarized optical spectra of these oriented multilayers confirm the EPR results regarding orientation of the oxidase heme(s) for both the oxidized and reduced forms of the enzyme.

In this communication[+] we describe the preparation of oriented multilayers of mitochondrial membranes and present experimental evidence which directly demonstrates that cytochrome *c* oxidase and cytochrome *c* are specifically oriented within the membrane. The average orientation of the hemes of cytochrome oxidase is such that the normal to the plane of the hemes lies in the plane of the mitochondrial membrane.

[+]A detailed theoretical treatment of the polarized optical spectroscopy and EPR spectroscopy of hemes in oriented multilayers of membranous cytochrome oxidase will be presented elsewhere (manuscript in preparation).

2. Materials and methods

Pigeon breast mitochondria were prepared as described by Erecińska et al. [5] and kept frozen at −50°C. Aliquots of rapidly thawed mitochondria (approximately 3–6 mg prot.) were swollen in 2 mM phosphate buffer, pH 7.2 and centrifuged onto glass or thin Mylar sheets at 70 000 × *g* for 1 h obtain 0.5–1 cm diameter, 0.5–1 mm thick pellets. The pellets supported on the glass or Mylar substrates were partially dehydrated at 4°C for 24–48 h in an inert atmosphere maintained at 90% relative humidity.

The optical spectra were measured using a scanning dual wavelength spectrophotometer equipped with a computer memory [5] designed and constructed in the laboratories of the Johnson Foundation. The partially dehydrated pellet on its glass substrate was enclosed in a tightly sealed cuvette which maintained the relative humidity and had windows for the passage of the light beam. The cuvette was mounted on a movable shaft which allowed changes in the angle and position of the cuvette with respect to the light source. A schematic drawing of the experimental set-up is shown in fig.1. The vertical square plate represents the plane of the supporting glass substrate of the pellet.

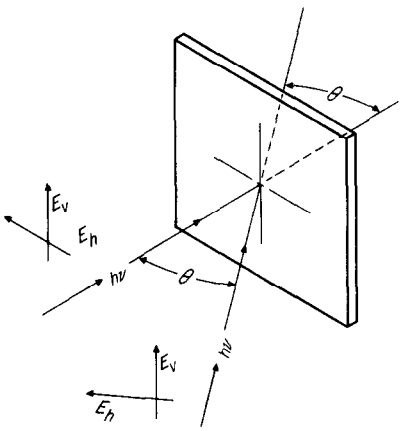

Fig.1. Schematic representation of the experimental set-up used to record the optical absorption spectra. Explanatory remarks are presented in the methods section.

The sample is illuminated with monochromatic light ($h\nu$) and absorption spectra measured with light polarized either vertically or horizontally. The sample is then rotated with respect to the light source by an angle θ and the spectra are recorded again. Because the light which leaves the monochromators exhibits certain degree of wavelength-dependent polarization each spectrum was recorded as the difference spectrum against a base-line obtained in the absence of a mitochondrial pellet (but with the glass substrate) and with polarizer in the appropriate position. The samples were reduced by placing a drop of 1 M sodium ascorbate (pH 6.8) on the surface of the pellet and then removing the excess liquid before enclosing the sample inside the cuvette.

For the EPR measurements, the partially dehydrated pellet (together with its supporting Mylar substrate) was cut into strips (~2 X 10 mm) which were inserted inside a 3 mm internal diameter EPR-tube in such a way that 2–4 strips were in place and the planes of the supporting Mylar were all parallel. The samples were frozen by immersion of the quartz tubes in liquid nitrogen. The EPR spectra were measured using a Varian E-109 spectrometer equipped with an Air Products LTD-3-110 liquid helium cryostat and a PDP-11 computer. The detailed experimental condition during the measurement are given in the figure legends.

3. Results

3.1. *Optical absorption spectra of oriented multilayers of mitochondrial membranes*

The Soret region of the optical absorption spectrum of oxidized cytochromes in the mitochondrial membrane pellet is shown in fig.2. The upper spectra (A) were obtained with the incident light beam falling at an angle of 0° with respect to the normal to the plane of the glass substrate, while the lower ones (B) were recorded when the angle between the normal to the plane of the substrate and the incident light beam was 45°. Both spectra A and B exhibit absorption bands characteristic of a mixture of oxidized cytochromes *a*, *b* and *c*. When the incident light beam falls at θ equal to 0°, the spectra obtained with vertically and horizontally polarized light are equal while when θ equals 45°, the spectrum with horizontally polarized light exhibits higher absorption intensity than that observed for vertically polarized light. Because the absorption bands of the cytochromes in the Soret region overlap considerably both in the oxidized and the reduced state, it is of advantage

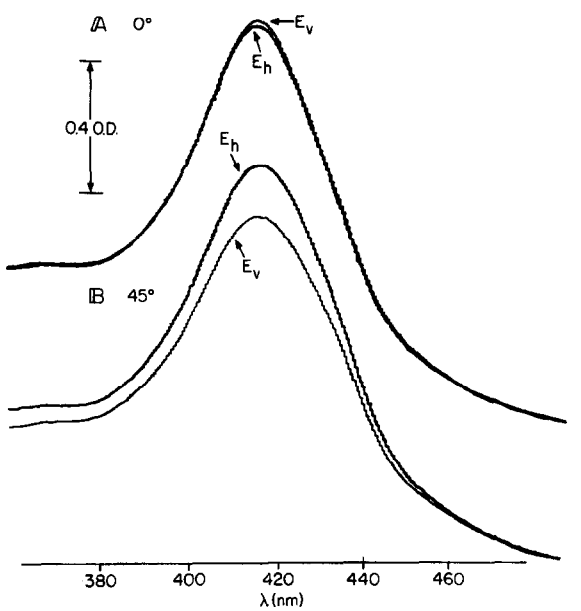

Fig.2. Soret absorption spectra of the oxidized chromophores in the oriented mitochondrial multilayers. Preparation of the sample and the instrument are described in the methods section.

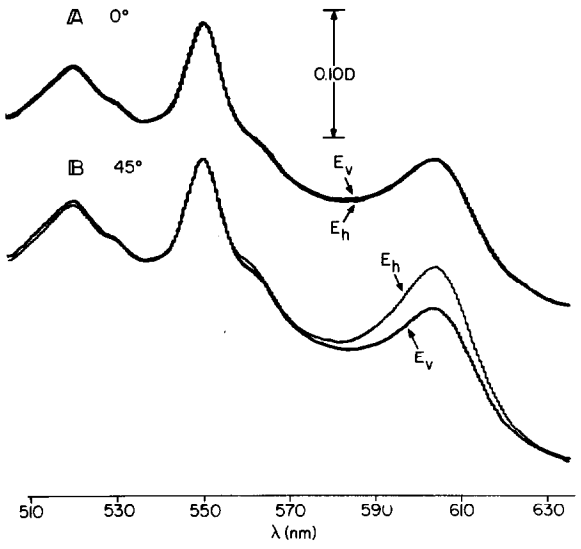

Fig.3. Visible absorption spectra of the reduced chromophores in the oriented mitochondrial multilayers. The partially dehydrated sample was reduced by the addition of 1 M ascorbate as described in the methods section.

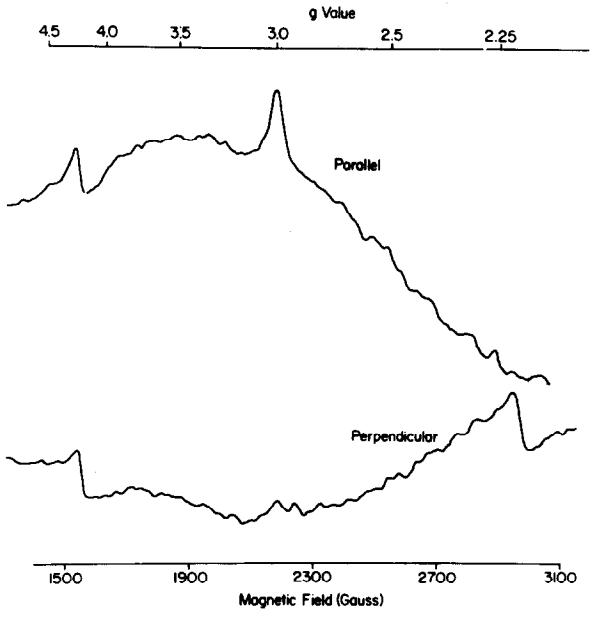

Fig.4. EPR Absorption spectra of the oxidized oriented mitochondrial multilayers with the magnetic field parallel and perpendicular to the plane of the membrane. EPR Frequency 9.158 GHz, temperature 8.8°K, microwave power 10 mW. Scanning time 2 min, time constant 0.12 s.

to examine the optical properties of the reduced cytochromes in the visible region where their absorption bands are much sharper and the position of their maxima more separated. The absorption spectra of the mitochondrial samples reduced with ascorbate are shown in fig.3. As with the oxidized sample, the spectra recorded at θ equal to 0° both with vertically and horizontally polarized light are equal. At θ equal to 45°, the absorption of cytochrome oxidase (λ_{max} = 605 nm) is almost 2-fold greater with light polarized horizontally than it is with the light beam polarized vertically. Small differences are also seen in the region of cytochromes *b* absorption (a shoulder at the long wavelength side of the cytochrome *c* peak) while there is practically no difference at the absorption maximum of cytochrome *c* (λ_{max} = 550 nm).

3.2. *EPR absorption spectra of oriented multilayers of mitochondrial membranes*

EPR Spectra of the oriented mitochondrial membrane pellet are shown in fig.4. It can be seen that when the plane of the supporting Mylar substrate of the pellet is parallel to the magnetic field a prominent g = 3 signal characteristic of a low spin oxidized heme of cytochrome *c* oxidase [6] is observed. This signal is virtually absent when the substrate plane is oriented perpendicular to the magnetic field. In contrast a g = 2.2 signal is present in the sample in which the plane of the substrate is oriented perpendicular to the magnetic field but is essentially absent in the sample in which the plane of the substrate is oriented parallel to the magnetic field. No difference in the behavior of g = 4.3 signal is observed.

4. Discussion

Assuming that the swollen mitochondria form oriented membrane multilayers similar to those formed from the vesicular forms of many natural and model membranes using identical methods, it can be conjectured that the planes of the mitochondrial membranes lie predominantly parallel to the pellet's substrate. Hence, the experimental results presented in this work show that cytochrome *c* oxidase in situ

is highly oriented with respect to the plane of mitochondrial membrane. This fact, perhaps, is not unexpected on the basis of numerous observations which indicate that mitochondrial membranes are highly organized structures. The demonstration of the orientation of the redox-components with respect to the membrane provides us with a new experimental approach to the study of these enzymes and brings us one step closer to understanding of the mechanism of their action.

The evidence for the orientation of cytochrome *c* oxidase in mitochondrial membranes in situ rests on two types of experimental approaches, discussed briefly† below: optical spectroscopy and EPR spectroscopy.

4.1. *Optical spectroscopy*

Molecular absorption spectra of cytochromes like those of other heme proteins can be described as a first approximation in terms of a model derived from molecular orbital theory in which the $\pi \rightarrow \pi^*$ transitions reasonably account for the optical spectra of simple porphyrins [7–9]. For metalloporphyrin complexes with an effective four-fold axis of symmetry (to which the iron porphyrins belong) these transitions are predicted to be polarized in the plane of the molecule (i.e., x–y polarized) and doubly-degenerate (the x and y molecular axes being equivalent). This was implicit in the determination of the orientation of the plane of the porphyrin relative to the crystal axes in myoglobin [10], hemoglobin [11,12] and cytochrome *c* [13] using optical absorption measurements with polarized light and can be used to interpret the experimental results presented above.

The lack of dichroism in the optical spectrum of the oxidized and reduced cytochrome oxidase at an angle of 0° between the incident beam and the normal to the plane of the membrane indicates that the heme planes of this hemeprotein are randomly oriented about any axis normal to the plane of the membrane. In contrast, the high dichroic ratio (A_H/A_V) observed with the incident light at an angle of 45° with respect to the normal to the plane of the membrane indicates that oxidase molecules are ordered in such a way that while the planes of their hemes are randomly oriented about the normal to the plane of the membrane, they are not randomly oriented about any axis parallel to the membrane plane.

In fact, the dichroic ratio (A_H/A_V) observed for the α-band of the reduced cytochrome oxidase (fig.3) would indicate in a qualitative sense that the average orientation of the normal to the plane of the oxidase hemes lies near the membrane plane. The lower dichroic ratio in the Soret region relative to the α-band can arise from the presence of multiple components with overlapping absorbance and different orientations as well as from deviations from four-fold symmetry of the heme (i.e., x–y inequivalency) [13].

4.2. *Electron paramagnetic resonance measurements*

EPR Spectroscopy of single crystals of myoglobin [14,15], hemoglobin [14] and cytochrome *c* [16] have been employed successfully to determine the orientation of the heme planes with respect to the crystal axes and to each other. Thus, EPR measurements can be used to provide us with information on the orientation of the heme with respect to the plane of the membrane.

The low spin heme signal of oxidized cytochrome *c* oxidase is characterized by the three principal *g*-values where $g = 3$ is the value of the *g*-tensor parallel to the effective four-fold symmetry axis (g_z), while $g = 2.2$ and $g = 1.5$ are *g*-tensor values perpendicular to this axis (g_y and g_x, respectively). Thus, the $g = 3.0$ signal will be maximal when the magnetic field is parallel to the normal to the heme plane while the $g = 2.2$ and $g = 1.5$ signals will be maximal when the magnetic field is parallel to the heme plane itself. The measured intensity of $g = 3.0$ signal is maximal when the magnetic field is parallel to the plane of the mitochondrial membrane. Thus, the EPR data are in agreement with the polarized optical spectra and confirm the finding that the normal to the plane of the low spin heme(s) of oxidized cytochrome oxidase lies in the plane of mitochondrial membrane (i.e., the heme plane is 90° to the plane of the membrane). This orientation for the hemes is further substantiated by the observation of the resonances at the g_z, g_x and g_y principal values.

The heme plane of cytochrome *c* is apparently oriented differently with respect to the plane of the mitochondrial membrane than are the hemes of cytochrome oxidase. The dichroic ratio of less than 1.1 determined by the polarized optical spectroscopy suggests that (a) the heme plane of cytochrome *c* is either less oriented in general than the heme plane of cytochrome oxidase or oriented such that its heme

plane is closer to the plane of the membrane or that (b) a large fraction of cytochrome *c* is oriented completely randomly in this experimental situation.

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