Biochimica et Biophysica Acta, 501 (1978) 53—62

BBA 47432

STUDIES ON THE ORIENTATIONS OF THE MITOCHONDRIAL REDOX CARRIERS

I. ORIENTATION OF THE HEMES OF CYTOCHROME *c* OXIDASE WITH RESPECT TO THE PLANE OF A CYTOCHROME OXIDASE-LIPID MODEL MEMBRANE

M. ERECIŃSKA *, D.F. WILSON and J.K. BLASIE

Department of Biochemistry and Biophysics, School of Medicine, University of Pennsylvania, Philadelphia, Pa. 19104 (U.S.A.)

(Received May 3rd, 1977)

Summary

The liganded derivatives of mitochondrial cytochrome *c* oxidase have been prepared in hydrated oriented multilayers of membranous cytochrome *c* oxidase. The optical spectra of the liganded derivatives recorded at an angle of 45° between the incident light beam and the normal to the planes of the membranes in the multilayers show dichroic ratios of almost 2 in the visible region and 1.2—1.4 in the Soret region. The dichroic ratios were found to be similar for both cytochromes *a* and a_3. Electron paramagnetic resonance spectra of the azide, sulfide, and formate complexes of cytochrome *c* oxidase obtained as a function of the orientation of the applied magnetic field relative to the planes of the membranes in the multilayer confirm the optical data and demonstrate that both hemes of cytochrome *c* oxidase are oriented such that the angle between the heme normal and the membrane normal is approximately 90°.

Introduction

According to the classic view of cytochrome *c* oxidase [1,2], cytochrome *a* and a_3 are structurally and functionally distinct. Cytochrome *a* has a hemochromogen structure, with protein ligands occupying the 5th and 6th iron coordination positions while cytochrome a_3 resembles myoglobin or hemoglobin in that it has a weak or no ligand at the 6th coordination site. Modifications of

* To whom correspondence should be directed.

this model invoking strong heme-heme interactions between cytochrome *a* and a_3 have been proposed [3,4] to account for the optical and EPR properties of the enzyme and new models have been offered [5] in which cytochromes *a* and a_3 were considered structurally identical and the apparent differences in their reactivity were suggested to result from a strong negative cooperativity between the two hemes. Common to all these models is the idea that in the inhibited aerobic steady-state only one of the two hemes binds the ligand (the heme of cytochrome a_3) and thus the two hemes behave under these conditions as distinct entities.

Several ligands are available for cytochrome *c* oxidase (azide, cyanide, sulfide, formate, CO, and NO) which inhibit the enzyme activity and form spectrally identifiable heme compounds. The reactivity of the oxidase towards those ligands as well as the properties of the derivatives have been discussed at length in a number of original papers and review articles [1—7] and the reader is referred to those for pertinent information. It has been shown in the accompanying paper [8] that "membranous" cytochrome *c* oxidase [9] forms, during slow partial dehydration at 4°C, oriented multilayers in which the oxidized heme(s) of the enzyme are oriented in such a way that the normal to the heme plane lies in the plane of the membrane. Since in the fully oxidized state distinction cannot be made between the two hemes of the oxidase, in this work the liganded states of the enzyme are used to determine the orientation of cytochrome *a* and a_3 hemes with respect to the plane of this membrane.

Methods

Membranous cytochrome oxidase was isolated from pigeon breast mitochrondria by a modification of the method of Sun et al. [9] described previously [8]. The technique used to form partially dehydrated oriented multilayers of the oxidase and the experimental set-up used to obtain the optical and EPR spectra is given in detail in the accompanying paper [8].

Preparation of the derivatives. The azide and formate complexes of cytochrome *c* oxidase were made by placing a drop of 0.5 M ascorbate/0.5 M formate (or azide) mixture, pH 6.8, on the surface of the partially dehydrated oriented multilayers of the oxidase for 5 min and then gently removing the excess of liquid. The samples for the optical measurements were enclosed immediately thereafter in the special cuvettes described previously [8]; the partially dehydrated oriented multilayers of the oxidase for the EPR measurements were incubated for 20—30 min at room temperature in the EPR tubes and frozen by immersion of the tubes in liquid nitrogen.

The sulfide derivative for the EPR measurements was obtained in a similar way by placing a drop of 50 mM sodium sulfide in 1 : 1 (v/v) mixture of 0.33 M NaH_2PO_4 and 1 M ascorbate (pH 6.8). The incubation time was 30 min.

The CO derivative for the optical measurements was formed by placing a drop of 1 M ascorbate at pH 6.8, which had been previously saturated with CO gas at 4°C, on the surface of the oxidase multilayers for 2 min and then gently flowing the CO gas over the wet multilayer surface for 2 min before removing the excess liquid and enclosing the sample inside the cuvette.

Results

General considerations regarding the formation of the liganded derivatives of cytochrome c oxidase in oriented oxidase multilayers

The preparation of liganded derivatives of the oxidase in partially dehydrated oriented multilayers encounters difficulties which are not commonly observed in aqueous solutions of the enzyme. Firstly, penetration of the reducing agent and the ligand into the multilayers is a necessary prerequisite for the reaction, i.e. the reducing agent and the ligand must reach their reaction sites reasonably rapidly. Secondly, the reducing agent and the ligand are applied as aqueous solutions and penetration of water through the oriented multilayer may decrease the degree of orientation in the preformed multilayers. Thirdly, reaction or decomposition products of some reducing agents or ligands may be strongly alkaline or acidic which in turn may lead to structural changes in the protein and again result in a decrease in the degree of orientation in the multilayer.

The above-noted difficulties were found to be minimal when reagents were present in high concentrations (0.1—1.0 M) and in well-buffered media of high osmolarity. The high concentration facilitates diffusion into the multilayer since the rate of diffusion is proportional to the concentration gradient while the high osmolarity prevents swelling due to extensive water movement into the intermembrane spaces. Ligands such as azide or formate may be added to the suspending medium during formation of the multilayer in order to secure their presence between the membranes in the multilayer, but this does not appreciably affect either the rate or extent of formation of the liganded derivatives.

Optical spectra of liganded derivatives of the oxidase in hydrated oriented multilayers

The optical absorption spectra of the oxidase and its CO and formate derivatives recorded at an angle of 45° between the incident beam and the normal to the plane of the glass support of the oriented multilayer in the spectral region from 400 to 660 nm are shown in Figs. 1—3. Reduced cytochrome *c* oxidase in oriented multilayers exhibits characteristic absorption properties with its Soret maximum at 444 nm and the visible absorption maximum at 603 nm. A shoulder on the short wavelength side of the Soret absorption maximum is due to residual oxidized enzyme. It can be seen that the optical absorption obtained with horizontally polarized light (in relation to the laboratory frame) is greater than or equal to that obtained with vertically polarized light throughout the entire spectral region. The difference spectrum shown in the dotted line is that for reduced enzyme. The dichroic ratio (plotted as A_H/A_E) was calculated taking as the approximate baseline the broken straight line shown in the figure. These dichroic ratios were about 2 in the visible region decreasing to about 1.2—1.4 in the Soret region.

The optical spectrum obtained in the presence of CO (Fig. 2) is characteristic of reduced cytochrome *a* and reduced cytochrome a_3-CO compound; it has a maximum at 444 nm (reduced cytochrome aa_3) with a shoulder at 430 nm (assigned to the cytochrome a_3^{2+}-CO compound), and a broad visible absorption peak with a maximum at 603 nm.

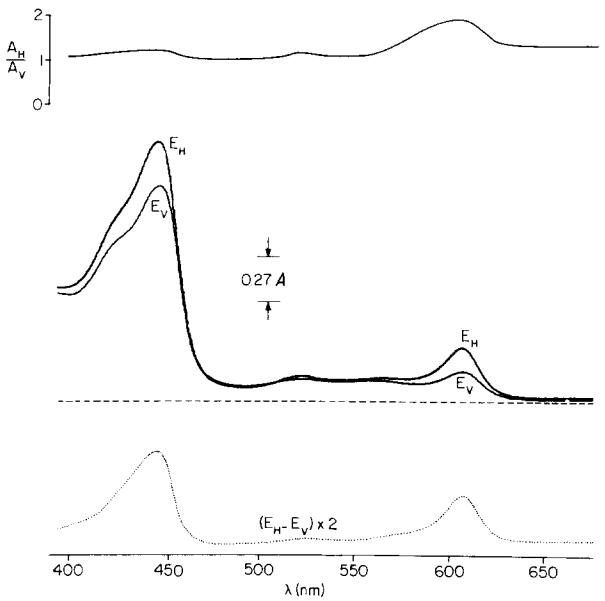

Fig. 1. Polarized absorption spectra of reduced cytochrome *c* oxidase in hydrated oriented membrane multilayers. The spectra were recorded with light polarized vertically (E_V) and horizontally (E_H) at an angle of 45° between the incident beam and normal to the planes of the membranes in the oriented multilayers. The 0.5 cm diameter multilayer was formed from 0.52 mg protein (6.7 nmol heme *a*) of "membranous" cytochrome oxidase and partially dehydrated at 4°C for 24 h at 90% relative humidity [8,9]. The sample was reduced by placing a drop of 1 M ascorbate solution, pH 6.8, on the surface of the multilayer and incubating for 10 min at room temperature. The broken line represents the approximate baseline against which the polarization ratio (A_H/A_V) was calculated. The dotted line is the difference spectrum.

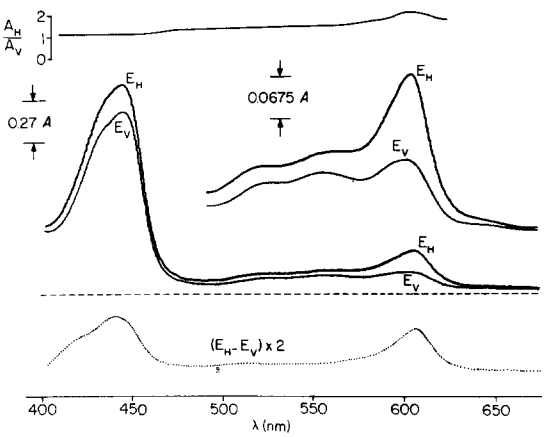

Fig. 2. Polarized absorption spectra of cytochrome a^{2+}-cytochrome $a_3{}^{2+}$-CO complex in hydrated oriented oxidase multilayers at an angle of 45°. The multilayer was formed and treated as described in the legend of Fig. 1. A drop of 1 M ascorbate solution saturated at 4°C with CO gas was placed on its surface and incubated for 2 min. The CO gas was blown gently over the surface for 2 min before enclosing the multilayer inside the cuvette.

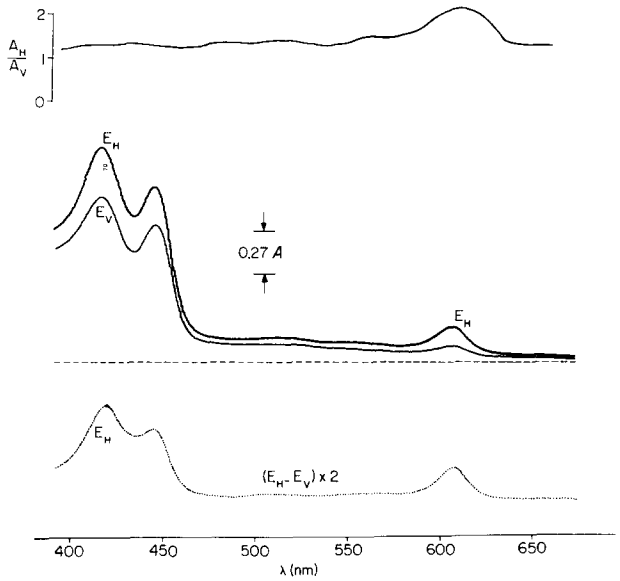

Fig. 3. Polarized absorption spectra of cytochrome a^{2+}-cytochrome $a_3{}^{3+}$-formate complex in hydrated oriented oxidase multilayers at an angle of 45°. The multilayer was formed and treated as described in the legend of Fig. 1. The formate derivative was produced by placing a drop of 0.5 M ascorbate/0.5 M formate mixture, pH 6.8, on the surface of the multilayer and incubating for 10 min at room temperature.

The CO compound exhibits the same optical polarization properties as the reduced cytochrome *a:* the dichroic ratio is essentially the same at 430 nm as at 445 nm.

Addition of formate to the aerobic oxidase in the presence of the reducing agent causes reduction of cytochrome *a* while cytochrome a_3 remains oxidized and liganded with formate [10]. The two absorption maxima, that of reduced cytochrome *a* (λ_{max} 444 nm) and that of cytochrome $a_3{}^{3+}$-formate (λ_{max} = 420 nm) remain well separated in the Soret region. The spectra shown in Fig. 3 demonstrate that the dichroic ratio is the same for these two maxima within experimental error.

The spectra of the oxidase and its liganded derivatives recorded at an angle of 0° between the incident light beam and the normal to the multilayer's glass support with horizontally and vertically polarized light beam were identical as they should be for reasons discussed previously [8] and are not shown here.

EPR spectra of the liganded derivatives of the oxidase in frozen oriented multilayers

A wide scan EPR spectrum of the oxidized oxidase is shown in Fig. 4. In agreement with the previous results [8], the low spin g = 3 resonance of the oxidized heme is most prominent when the plane of the mylar support of the multilayer is oriented parallel to the applied magnetic field. Conversely the g = 2.2 resonance is maximal when the plane of the support is oriented normal to the applied field. In addition, there is a small amount of a high spin g = 6 resonance signal which is more prominent when the plane of the mylar support is

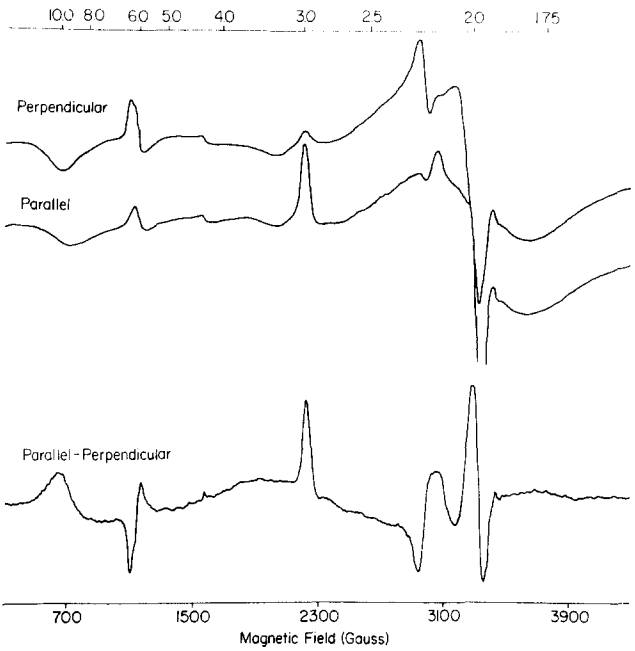

Fig. 4. EPR spectra of the oxidized cytochrome *c* oxidase in frozen oriented membrane multilayers where the planes of the membranes in the multilayer are parallel and normal (perpendicular) to the magnetic field. The oriented 1 cm diameter multilayer was formed 7 mg protein (90 nmol heme *a*) of "membranous" cytochrome oxidase and the sample partially dehydrated for 48 h at 4°C and 90% relative humidity. The partially dehydrated multilayer with its supporting mylar was cut into 2-mm strips which were inserted inside the EPR capillary and frozen and immersion in liquid N_2. EPR frequency was 9.148 GHz; power, 40 mW; sample temperature, 8.8°K. Scanning time, 3 min; time constant, 0.125 s. Modulation amplitude, 10 G.

oriented normal to the magnetic field. This resonance may arise from a small quantity of the oxidase being produced by the endogenous reducing equivalents in agreement with the finding that the magnitude of the $g = 6$ resonance signal is greatest in the half-reduced enzyme [11,12]. The $g = 10$ region contains a resonance which is maximal when the mylar support is oriented normal to the applied field. On the other hand, very little orientation dependence is seen in the $g = 2$ region where oxidized copper exhibits its characteristic EPR signal [13].

It has been shown using optical, EPR, and magnetic CD spectroscopy that the azide and sulfide-cytochrome a_3 compounds are low spin [14,15], while the formate-cytochrome a_3 complex is high spin. The cytochrome a_3-azide compound is characterized by g values at 2.9, 2.2, and 1.67 [14], while the cytochrome a_3-sulfide complex exhibits two split resonances with g values in the regions of 2.2, 2.5 and 1.87 [15]. The high spin cytochrome a_3-formate complex would be expected to exhibit an EPR resonance at $g = 6$.

The EPR spectra of the three derivatives of cytochrome oxidase in the frozen oriented multilayers are shown in Figs. 4—6. It can be seen that the conversion to the liganded state is incomplete; in addition to the resonance characteristic

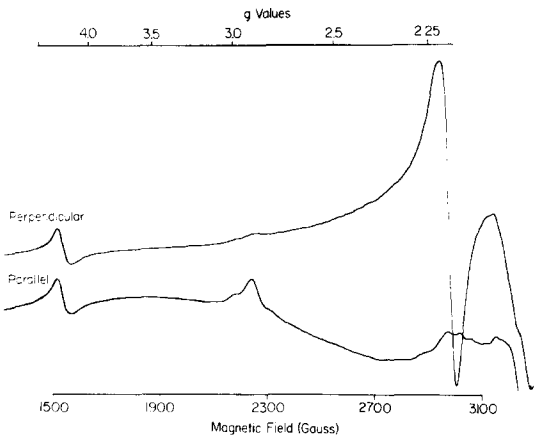

Fig. 5. EPR spectra of cytochrome a^{2+}-cytochrome $a_3{}^{3+}$-azide complex in frozen oriented membrane multilayers where the planes of the membranes are parallel and normal (perpendicular) to the magnetic field. The oriented multilayers were formed and treated as described in the legend of Fig. 4. A drop of 0.5 M ascorbate/0.5 M azide, pH 6.8, mixture was placed on the surface of the multilayers, and two 2-mm wide wet strips were inserted inside the EPR capillary. The sample was incubated at room temperature for 40 min and frozen by immersion in liquid N_2. EPR frequency, 9.148 GHz; power, 10 mW; sample temperature, 12.5°K. Scanning time, 2 min; time constant, 0.064 s. Modulation amplitude, 10 G.

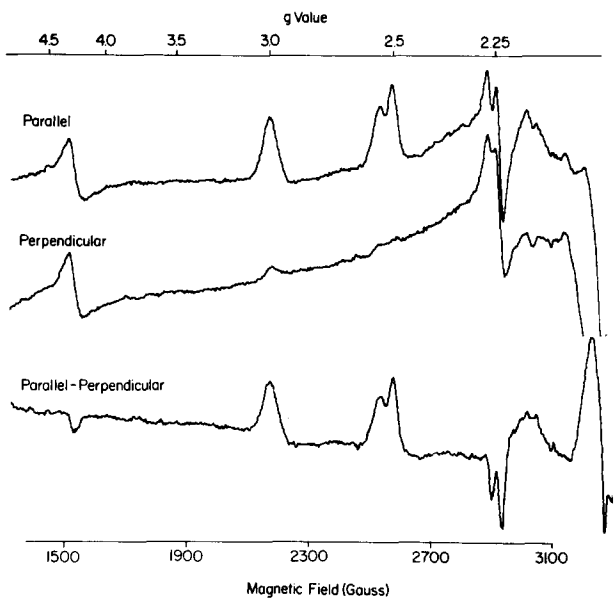

Fig. 6. EPR spectra of cytochrome a^{2+}-cytochrome $a_3{}^{3+}$-sulfide complex in frozen oriented membrane multilayers where the planes of the membranes are parallel and normal (perpendicular) with respect to the magnetic field. The oriented multilayer was formed and treated as described in the legend of Fig. 4. A drop of 50 mM sodium sulfide in 1 : 1 (v/v) mixture of 0.33 M NaH_2PO_4 and 1 M ascorbate was placed on the surface of the multilayer and two 2-mm wide wet strips were inserted inside the EPR capillary. The sample was incubated for 45 min at room temperature and frozen by immersion in liquid N_2. EPR frequency, 9.148 GHz; power, 5 mW; sample temperature, 9.5°K. Scanning time, 2 min; time constant, 0.064 s. Modulation amplitude, 10 G.

of the liganded forms (g = 2.9 for azide, g = 2.5 for sulfide, and g = 6 for formate) there is in each case a certain amount of the low spin g = 3 resonance characteristic of the oxidized, non-liganded heme of the oxidase [11—13]. It is also clearly shown that the g = 3 resonance signal as well as g = 2.9 resonance and the split resonance in the g = 2.5 region are maximal when the plane of the mylar supporting the multilayers is parallel to the applied magnetic field while they are minimal in the samples where the support plane is normal to the magnetic field.

The g = 2.2 resonance of the azide complex is most prominent when the plane of the mylar support is normal to the applied magnetic field. In the case of sulfide, the g = 2 region is more difficult to evaluate because there are at least three resonance signals (one from the oxidized heme and a split resonance from the oxidized heme-sulfide complex) which overlap in this region. The difference spectrum shows, however, that their orientation must be opposite to that of the g = 3 and g = 2.5 signals.

The high spin g = 6 resonance of the cytochrome a_3-formate complex is maximal (Fig. 7) when the plane of the mylar support is normal to the applied magnetic field. Since there is usually a certain, albeit small, amount of high spin resonance present in the oxidized oxidase [11—13], this contributes also to the resonance amplitude in the presence of formate. This high-spin contaminant would appear to be randomly oriented in the multilayers which may be a reason for the presence of a relatively large g = 6 resonance for the formate complex when the plane of the mylar support is oriented parallel to the magnetic field.

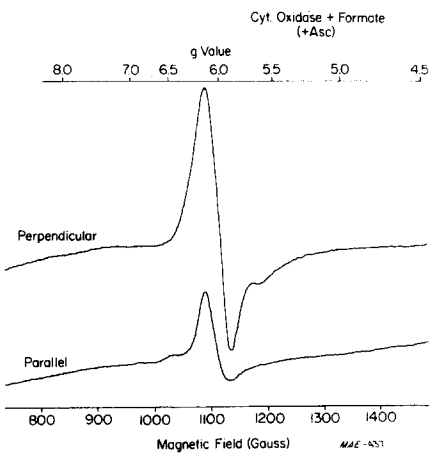

Fig. 7. EPR spectra of cytochrome a^{2+}-cytochrome a_3-formate complex in frozen oriented membrane multilayers where the planes of the membranes are parallel and normal (perpendicular) to the magnetic field. The oriented multilayer was formed and partially dehydrated as described in the legend of Fig. 5. The formate complex was made by placing a drop of 0.5 M formate, pH 6.8, mixture on the surface of the multilayer and incubating for 45 min at room temperature. The sample was frozen by immersion in liquid N_2. EPR frequency, 9.108 GHz; power, 20 mW; sample temperature, 9.5°K. Scanning time, 1 min; time constant, 0.032 s. Modulation amplitude, 10 G.

Discussion

The results of the optical and EPR studies on cytochrome *c* oxidase in various liganded states, within hydrated oriented membrane multilayers allow us to determine the orientation of both hemes of the enzyme, *a* and a_3 relative to the plane of the membrane. The theoretical basis for the interpretation of the optical and EPR spectra obtained from oriented membrane multilayers is presented in the first paper of the series [8] in which we showed that the average orientation of the planes of the membranes in membranous cytochrome oxidase multilayers was parallel to the plane of its support. In this work the same analyses are used to evaluate the experimental results.

The lack of dichroism in the optical spectra obtained at an angle of 0° between the incident light beam and the normal to the planes of the membranes for the reduced oxidase and for the cytochrome a^{2+}—$a_3{}^{2+}$-CO and cytochrome a^{2+}—$a_3{}^{3+}$-formate complexes indicates that the heme planes of both cytochrome *a* and a_3 are randomly oriented about any axis normal to the plane of the membrane. On the other hand, high dichroic ratios (A_H/A_E) observed with the incident light beam directed at an angle of 45° with respect to the normal to the planes of the membranes demonstrate that the hemes of the oxidase are highly oriented about any axis parallel to the plane of the membrane. In fact, we have shown [8] that such dichroic ratios for the Soret and visible absorption bands of the oxidized cytochrome oxidase in oriented membrane multilayers indicate that the angle between the heme normal and the membrane normal approaches 90°. Moreover, since the absorption bands of the liganded oxidase, which exhibit well separated maxima for cytochromes *a* and a_3 in the Soret region, have the same optical polarization properties (dichroic ratio), the conclusion can be drawn that both hemes *a* and a_3 have similar orientation with respect to the plane of the membrane.

This particular orientation of the hemes of the oxidase relative to the membrane plane is seen even more clearly from the EPR studies. The $g = 2.9$ resonance signal of the low spin cytochrome $a_3{}^{3+}$-azide complex and the split resonance in the $g = 2.5$ region for the cytochrome $a_3{}^{3+}$-sulfide complex (these *g* values correspond to the *z* component of the *g* tensor of their respective heme groups, i.e. the *g* tensor component, approximately normal to the heme plane [16]), are maximal when the planes of the membranes are parallel to the applied magnetic field and are virtually absent when the planes of the membranes are normal to the magnetic field. The opposite is true for $g = 2.2$ resonances (these *g* values correspond to the *y* component of the *g* tensor of the respective hemes, i.e. a principal component of the *g* tensor lying in the heme plane [16]) which are maximal when the magnetic field is normal to the planes of the membranes. Moreover, the $g = 6$ resonance signal of the high spin cytochrome $a_3{}^{3+}$-formate complex is maximal when the planes of the membranes are normal to the magnetic field (this *g* value corresponds to the equivalent *x* and *y* components of the *g* tensor, i.e. the two principal components of the *g* tensor in the heme plane [17]).

Since the low spin g_z heme resonance at $g = 3$ present in fully oxidized oxidase (generally assumed to arise from cytochrome *a*) [11—13] and the g_z heme resonances of the oxidized liganded cytochrome a_3 are all maximal when the

applied magnetic field is parallel to the planes of the membranes in the oriented multilayers while the g_y and g_x resonances of the non-liganded oxidized cytochrome *a* and liganded oxidized cytochrome a_3 show the opposite field orientation dependence, it can be conjectured that both the *a* and a_3 hemes must have similar orientations relative to the plane of the membrane. These orientations are such that the angle between the hemes' normals and the membrane normal is approx. 90°, in agreement with the conclusions drawn from the optical data described above. The particular line-shapes of the various heme resonances contain further information relating to the nature of the heme orientational averaging phenomena present in these multilayers and will be the subject of a subsequent publication.

It is also interesting to note that the various data indicate that the average orientations of the hemes are qualitatively similar in the oxidized and the reduced cytochrome *c* oxidase and in the reduced enzyme in the presence of CO which indicates that the two oxidase hemes have similar orientations irrespective of their redox state.

The identity of the chromophore responsible for the $g = 10$ resonance signal in cytochrome *c* oxidase is unknown. The observation that its EPR absorption shows an applied magnetic field orientation dependence very similar to that of the heme suggests that the chromophore may be a heme, perhaps a spin-coupled heme dimer which arises as an artifact of isolation.

Acknowledgements

This work was supported by an N.I.H. grant HL 18708 to M.E. and J.K.B. M.E. is an Established Investigator of the American Heart Association.

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