Biochimica et Biophysica Acta, 501 (1978) 63—71
© Elsevier/North-Holland Biomedical Press

BBA 47433

STUDIES ON THE ORIENTATIONS OF THE MITOCHONDRIAL REDOX CARRIERS

II. ORIENTATION OF THE MITOCHONDRIAL CHROMOPHORES WITH RESPECT TO THE PLANE OF THE MEMBRANE IN HYDRATED, ORIENTED MITOCHONDRIAL MULTILAYERS

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

Orientations of the active site chromophores of the mitochondrial redox carriers have been investigated in hydrated, oriented multilayers of mitochondrial membranes using optical and EPR spectroscopy. The hemes of cytochrome *c* oxidase, cytochrome c_1, and cytochromes *b* were found to be oriented in a similar manner, with the normal to their heme planes lying approximately in the plane of the mitochondrial membrane. The heme of cytochrome *c* was either less oriented in general or was oriented at an angle closer to the plane of the mitochondrial membrane than were the hemes of the "tightly bound" mitochondrial cytochromes. EPR spectra of the azide, sulfide and formate complexes of cytochrome *c* oxidase in mitochondria in situ obtained as a function of the orientation of the applied magnetic field relative to the planes of the membrane multilayers showed that both hemes of the oxidase were oriented in such a way that the angle between the heme normal and the membrane normal was approx. 90°.

Introduction

Detailed knowledge of the orientation of membrane-bound enzymatic proteins and in particular of their active site with respect to the plane of the membrane is especially important for enzymes which may catalyze transmembrane processes such as movements of electrons, ions, and small molecules across the

* To whom the correspondence should be directed.

membrane. Recent developments in the techniques for the formation of oriented multilayers both of artificial [1–3] and natural membranes [4,5] have permitted the preparation of hydrated oriented multilayers of mitochondrial membranes [6]. The active sites of the respiratory chain carriers, the principal occupants of the mitochondrial inner membrane, are asymmetric chromophores and therefore, it is possible to determine spectroscopically their orientation with respect to the plane of the membrane. This information can subsequently be used to evaluate the structural and functional implications of the particular orientation that the individual chromophores assume in the native membranes.

Experimental results discussed in the first two papers of this series have established [7,3] that isolated cytochrome *c* oxidase is oriented in an artificial lipid-cytochrome oxidase membrane in such a way that the normals to the planes of the two hemes of the enzyme, *a* and a_3, both lie approximately in the membrane plane. It was also demonstrated [3] that the approximate orientation of the two hemes depends neither on their redox state nor on the liganded state of the heme iron.

In an earlier paper [6], we reported that the heme(s) of cytochrome *c* oxidase is similarly oriented with respect to the plane of the mitochondrial inner membrane. In this work we present data which establish the orientations of both hemes of cytochrome *c* oxidase in various redox and liganded states and of the hemes of other cytochromes of the mitochondrial respiratory chain. Moreover, we discuss some preliminary results concerning the orientations of mitochondrial iron-sulfur centers.

Methods

Hydrated oriented multilayers of mitochondrial membranes were formed from pigeon breast mitochondria [8] by the technique described previously [6, 7]. Usually about 0.7—1.0 mg of mitochondrial protein was used to prepare a 0.5 cm diameter multilayer for optical studies and 8—12 mg protein was used to obtain a 1 cm diameter multilayer for the EPR studies.

Fully reduced membrane multilayers were prepared in an analogous manner to that described previously [7] except that a 30% sucrose solution saturated with $Na_2S_2O_4$ was used to reduce the chromophores. The azide, formate, and sulfide derivatives of the oxidase were formed in a manner analogous to that described previously [3] except that incubations as long as 45—60 min were required to obtain sufficiently high concentrations of liganded derivatives in the multilayers. Detailed conditions for the preparation of various derivatives of the oxidase are given in the figure legends.

Results

Optical spectra of cytochromes in hydrated oriented mitochondrial multilayers

Polarized optical spectra of the oxidized and reduced cytochromes in hydrated oriented mitochondrial membrane multilayers recorded at an angle of 45° between the incident light beam and the normal to the planes of these membranes in the spectral region 400—600 nm are shown in Figs. 1 and 2. It can be seen that the optical absorption in both the oxidized and the reduced

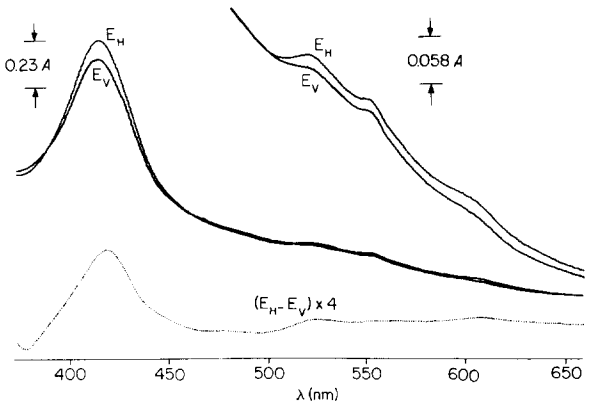

Fig. 1. Polarized optical absorption spectra of the oxidized chromophores in hydrated oriented mitochondrial membrane multilayers. The spectra were recorded with light beam polarized vertically (E_V) or horizontally (E_H) at an angle of 45° between the incident beam and normal to the plane of oriented multilayers (cf. ref. 7 for precise specimen-beam geometry). The 0.5 cm in diameter multilayer film was formed by centrifugation [3,6,7] from 0.6 mg mitochondrial protein and partially dehydrated for 24 h at 4°C and 90% relative humidity. Dotted line represents the difference spectrum.

samples with the light beam polarized horizontally with respect to the laboratory frame is greater than or equal to that with the light polarized vertically. The 45° angle of incidence was generated by rotation of the multilayer glass support about an axis in the plane of the multilayer which was vertical in relation to the laboratory frame (see ref. 7 for details). The dichroic ratios A_H/A_V are greater in the visible region of the spectrum than in the Soret region for probable reasons discussed previously [7]. Since the absorption maxima in the visible region of various cytochromes in their reduced state have greater extinction and the individual absorption bands overlap to a lesser degree than in the oxidized state, it is easier to analyze in detail the spectra of the reduced multilayer.

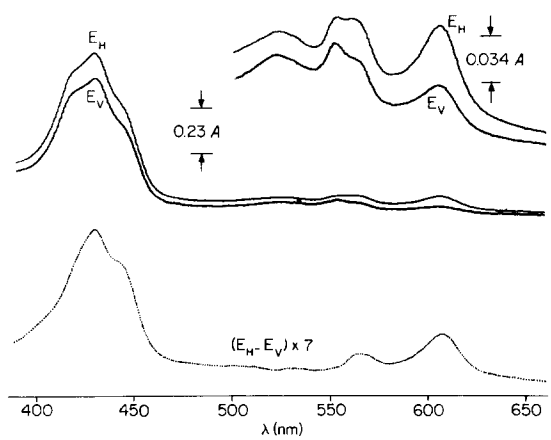

Fig. 2. Polarized optical absorption spectra of the reduced chromophores in hydrated oriented mitochondrial membrane multilayers at an angle of 45°. The multilayer was formed and dehydrated as described in the legend of Fig. 1. The specimen was reduced by placing a drop of dithionite-saturated 30% solution of sucrose on the surface of the multilayer and incubating for 60 min at room temperature.

The most pronounced dichroism is exhibited for the absorption maximum of cytochrome *a* (λ_{max} = 605 nm): the dichroic ratios are approx. 1.6—1.7, almost as high as in the hydrated oriented multilayers of membranous cytochrome oxidase. Dichroic ratios of 1.4—1.6 are also seen in the 560—564 nm region where cytochromes *b* absorb maximally, while the dichroic ratio is about 1.1 in the 550–553 nm region where cytochromes *c* + c_1 exhibit their characteristic absorption maxima. Although the dichroic ratios are generally lower for the Soret absorbance, greater dichroism is seen even in the latter region for the absorption maxima of cytochrome oxidase (445 nm) and cytochromes *b* (430 nm) than for the absorption maxima of the cytochromes *c* (415 nm). The difference spectrum represented by dotted lines shows this behavior even more clearly than do the individual spectra.

Because long (>10 min) exposure to dithionite solution was required to ensure reduction of cytochromes *b* in the multilayer, the possibility of disorientation of the oriented mitochondrial multilayers induced by this prolonged incubation had to be taken into account. However, the same overall pattern could be seen at shorter incubation times (not shown): the dichroic ratios were much higher for cytochrome oxidase and cytochromes *b* than for cytochromes *c*. The overall absorbance in the region where cytochromes *b* exhibit their characteristic maxima (560—564 nm) was, however, smaller due to their incomplete reduction.

EPR absorption spectra of the oxidized cytochromes in frozen oriented mitochondrial multilayer

It has been shown previously [6] that the amplitude of the low spin heme *g* = 3 resonance (g_z) of the oxidized cytochrome oxidase is maximal in the mitochondrial membrane multilayers when the plane of their mylar support is parallel to the applied magnetic field while it is virtually zero when the plane of the mylar support is normal to the magnetic field. The opposite is true for its *g* = 2.2 signal resonance (g_y). Closer inspection of the EPR spectra presented in Fig. 3 shows that the *g* = 3.78 (g_z) resonance due to cytochrome *b*-566 [9—12] the *g* = 3.41 (g_z) resonance due to cytochrome *b*-561 [9—12] and the *g* = 3.37 (g_z) resonance arising from cytochrome c_1 are all of maximal amplitude in multilayers for which the plane of the mylar support is oriented parallel to the magnetic field. Although the amplitudes of the individual resonances are small due to their relatively large linewidths, the difference spectrum leaves little doubt as to the nature of their dependence on the orientation of the applied magnetic field relative to the plane of the multilayer.

The relatively broad low spin heme resonance which arises from cytochrome *c* (*g* = 3.05) is probably hidden beneath the narrower *g* = 3.0 resonance of the oxidized cytochrome oxidase. But the unusual broadness of the *g* = 3 resonance in the multilayer whose plane is oriented normal to the applied magnetic field and a slight shift in the position of the resonance towards lower field strength suggests that cytochrome *c* also contributes to this resonance for that particular orientation of the multilayer. Difference spectra show only the relatively narrow *g* = 3.0 resonance of cytochrome oxidase indicating that the EPR of cytochrome *c* is independent of the direction of the applied magnetic field with respect to the plane of the multilayer.

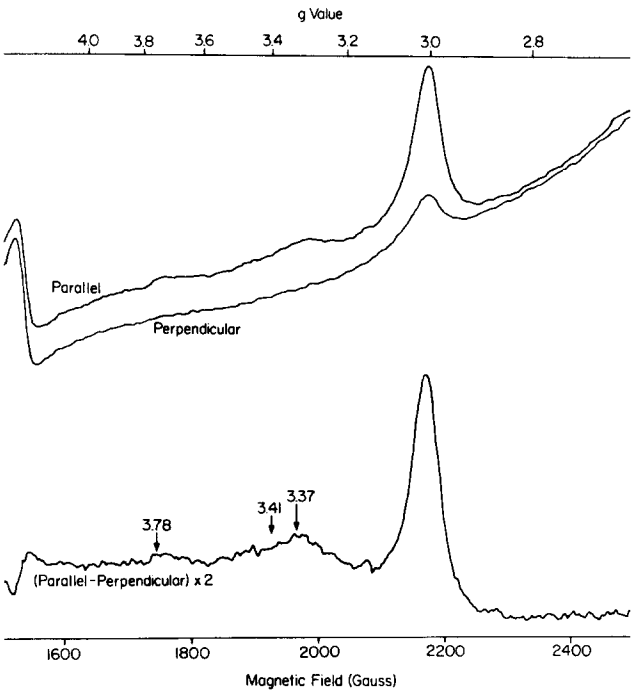

Fig. 3. The EPR spectra of the oxidized cytochromes in mitochrondrial frozen oriented membrane multilayers arranged parallel and normal (perpendicular) to the applied magnetic field. The oriented 1 cm diameter multilayer was formed from 8 mg protein of mitochondria, dehydrated for 56 h at 4°C and 90% constant relative humidity. The partially dehydrated specimen was cut into 2-mm strips, inserted inside an EPR quartz tube [3,7] and frozen by immersion in liquid N_2. EPR frequency was 9.148 GHz, power 5 mW, sample temperature 8.5°K. Scanning time, 2 min; time constant, 0.128 s. Modulation amplitude, 12.5 G.

EPR spectra of the reduced chromophores in frozen oriented mitochondrial membrane multilayers

Preliminary data on partially reduced (Fig. 4) and fully reduced oriented mitochondrial multilayers (Fig. 5) suggest that iron-sulfur centers may also be specifically oriented with respect to the plane of the mitochondrial membrane. The $g = 1.9$ resonance seen in Fig. 4 is of somewhat greater amplitude in the multilayers oriented with the plane of their mylar support parallel to the applied magnetic field and the same is true for $g = 1.93$ resonance (Fig. 5). Because the resonances of various iron-sulfur centers overlap to a significant extent and are saturated at different temperatures, systematic studies are required to determine their precise dependence on the orientation of the applied magnetic field relative to the plane of the multilayer (Salerno, J. and Ohnishi, T., unpublished).

EPR spectra of the cytochromes in frozen oriented mitochondrial multilayers in the presence of azide, sulfide, and formate

In the reduced aerobic azide, sulfide, or formate-inhibited steady-state all of the mitochondrial cytochromes (except cytochrome a_3) become reduced while cytochrome a_3 remains oxidized and bound to a ligand. The spectra of the azide,

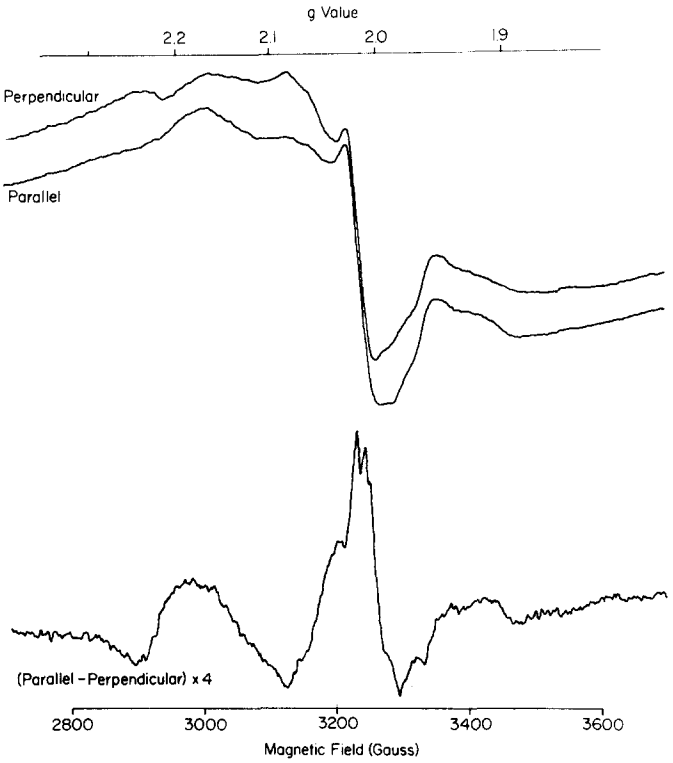

Fig. 4. EPR spectra of partially reduced chromophores in frozen, oriented mitochondrial membrane multilayers arranged parallel and normal (perpendicular) to the magnetic field. The partially dehydrated mitochondrial multilayer obtained as described in the legend of Fig. 3 was reduced by placing a drop of 0.3 M Tris · HCl, pH 8.0, saturated with dithionite on the surface of 2-mm strips of the membranes (cut with their mylar support) and incubated for 30 min at room temperature inside an EPR capillary. The sample was frozen by immersion in liquid N_2. EPR frequency, 9.101 GHz; microwave power, 5 mW; sample temperature, 24°K. Scanning time, 2 min; time constant, 0.128 s. Modulation amplitude, 16 G.

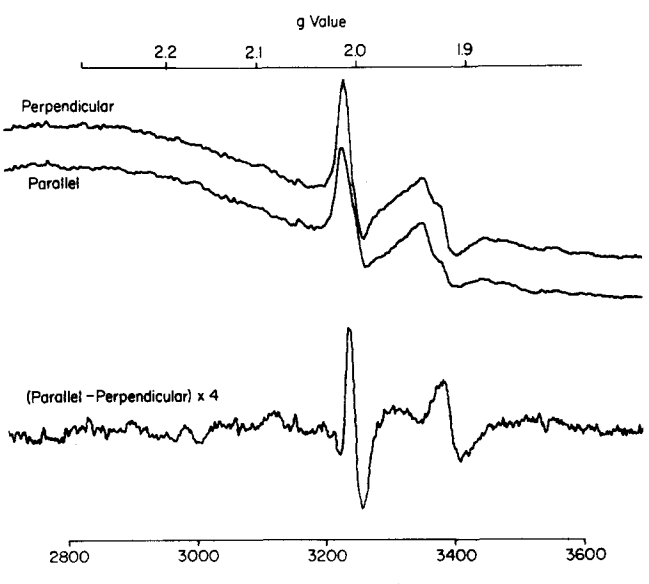

Fig. 5. EPR spectra of reduced chromophores in frozen, oriented mitochondrial membrane multilayers arranged parallel and normal (perpendicular) to the magnetic field. The partially dehydrated mitochondrial multilayer obtained as described in the legend of Fig. 3 was reduced by immersing the cut strip into 30% sucrose solution saturated with dithionite inside an EPR capillary. Incubation was 30 min at room temperature and the sample was frozen by immersion in liquid N_2. EPR frequency, 9.10 GHz;

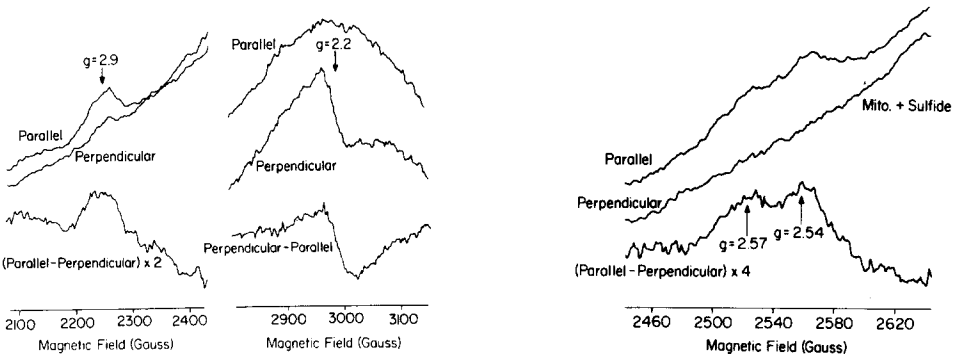

Fig. 6. EPR spectra of cytochrome $a_3{}^{3+}$-azide complex in frozen, oriented mitochondrial membrane multilayers arranged parallel and normal (perpendicular) to the magnetic field. The multilayer was formed and partially dehydrated as described in the legend of Fig. 3. A drop of 0.5 M ascorbate/0.5 M azide solution, pH 6.8, was placed on the surface of the multilayers and two 2-mm wide wet strips were inserted inside the EPR capillary with their mylar support parallel to each other. Incubation was 45 min at room temperature and the sample was frozen by immersion in liquid N_2. EPR frequency, 9.13 GHz; microwave power, 10 mW; and sample temperature, 13°K. Scanning time, 1 min; time constant, 0.064 s. Modulation amplitude, 12.5 G.

Fig. 7. EPR spectra of cytochrome a_3-sulfide complex in frozen oriented mitochondrial membrane multilayers arranged parallel and normal (perpendicular) to the magnetic field. The multilayer was formed and partially dehydrated as described in the legend of Fig. 3. 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, was placed on the surface of the multilayer and two 2-mm wide wet strips were placed inside an EPR capillary. The specimen was incubated for 45 min at room temperature and frozen by immersion in liquid N_2. EPR frequency, 9.098 GHz; microwave power, 2 mW; sample temperature, 12.2°K. Scanning time, 2 min; time constant, 0.128 s. Modulation amplitude, 12.5 G.

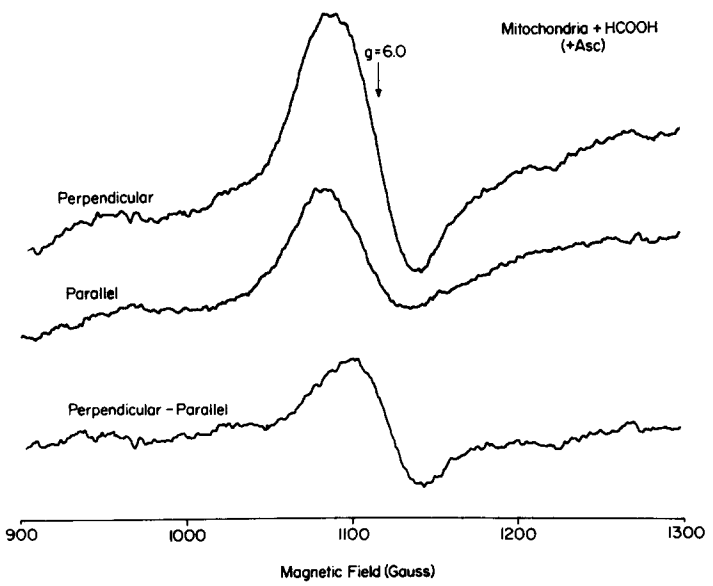

Fig. 8. EPR spectra of cytochrome a_3-formate complex in frozen, oriented mitochondrial membrane multilayers arranged parallel and perpendicular to the magnetic field. The multilayer was formed and partially dehydrated as described in the legend of Fig. 3. A drop of 0.5 M ascorbate/0.5 M formate, pH 6.8, mixture was placed on the surface of the multilayer and two 2-mm wide wet strips were inserted inside an EPR capillary. The specimen was incubated for 45 min at room temperature and frozen by immersion in liquid N_2. EPR frequency, 9.104 GHz; microwave power, 40 mW; sample temperature, 6.1°K. Scanning time, 2 min; time constant, 0.25 s. Modulation amplitude, 10 G.

sulfide, and formate complexes of cytochrome a_3 are shown in Figs. 6—8. The heme g_z resonance of the azide complex at $g_z = 2.9$ (and the split g_z resonances at $g = 2.57$ and $g = 2.54$ of the sulfide complex) appears almost exclusively in the multilayer oriented with its mylar support parallel to the applied magnetic field while heme g_y resonance at $g = 2.2$ is only apparent when the plane of the multilayer is oriented normal to the magnetic field. (This is very clearly seen in the difference spectra.)

The heme $g_{x,y}$ resonance ($g_x = g_y$) of the high spin formate complex of cytochrome $a_3{}^{3+}$ at $g = 6$ is maximal in multilayers whose plane is oriented normal to the magnetic field. Since in the mitochondrial sample, the high spin resonance may not be entirely due to the cytochrome $a_3{}^{3+}$-formate complex and may contain contributions from other less well oriented heme groups, the spectrum shows that a certain portion of the $g = 6$ resonance is also present in multilayers whose plane is parallel to the applied magnetic field.

Discussion

It was shown by our earlier spectroscopic studies [6] of hydrated oriented multilayers of mitochondrial membranes that the average orientation of the membrane planes was parallel to the plane of the glass or mylar support. Moreover, it was found that the planes of the hemes of mitochondrial cytochrome *c* oxidase in such multilayers were oriented at an angle very close to 90° between the heme normal and the membrane normal.

The experimental results presented in this paper show that in addition to both the *a* and a_3 hemes of cytochrome *c* oxidase, those of cytochrome c_1 and cytochromes *b* are all oriented in a similar manner, with the normal to their heme planes lying approximately in the plane of the mitochondrial membrane. Since phosphorylation at site III is associated with cytochrome *c* oxidase and phosphorylation at site II with the cytochrome *b*-c_1 complex, the similar orientation of the hemes of the two complexes with respect to the plane of the mitochondrial membrane may represent a structural requirement for energy coupling.

The various results on the oxidized cytochrome *c* oxidase as well as on its liganded derivatives in frozen, oriented mitochondrial membrane multilayers show that the hemes of cytochrome *a* and a_3, in mitochondria in situ are oriented similarly to those in hydrated oriented multilayers formed from isolated "membranous" cytochrome *c* oxidase. Furthermore, it appears that the average orientation of cytochrome *c* oxidase hemes and those of "tightly bound" cytochromes is qualitatively similar in both their oxidized and reduced states.

The heme plane of cytochrome *c* is either apparently less oriented with respect to the plane of the mitochondrial membrane than are the hemes of the "tightly bound" cytochromes or it is oriented such that its heme plane is closer to the plane of the membrane. This follows from the lack of dichroism of the α-band absorption of cytochrome *c* (dichroic ratio approx. 1) and the apparent lack of dependence of the cytochrome *c* heme resonances on the orientation of the applied magnetic field relative to the plane of the membranes in the multilayer. Experiments are in progress to distinguish these two possibilities.

Acknowledgements

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

References

1 Lesslauer, W. and Blasie, J.K. (1972) Biophys. J. 12, 175—190
2 Lesslauer, W., Cain, J. and Blasie, J.K. (1972) Proc. Natl. Acad. Sci. U.S. 69, 1499—1503
3 Erecińska, M., Wilson, D.F. and Blasie, J.K. (1978) Biochim. Biophys. Acta 501, 53—62
4 Santillan, G., Schwartz, S., Dratz, E. and Blasie, J.K. (1977) J. Mol. Biol., in the press
5 Herbette, L., Marguardt, Scarpa, A. and Blasie, J.K. (1977) Biophys. J., submitted
6 Erecińska, M., Blasie, J.K. and Wilson, D.F. (1977) FEBS Lett. 76, 235—239
7 Blasie, J.K., Erecińska, M., Samuels, S. and Leigh, Jr., J.S. (1978) Biochim. Biophys. Acta 501, 33—52
8 Erecińska, M., Oshino, R., Oshino, N. and Chance, B. (1973) Arch. Biochem. Biophys. 157, 431—445
9 Orme-Johnson, N.R., Hansen, R.E. and Beinert, H. (1971) Biochem. Biophys. Res. Commun. 53, 871—878
10 Wilson, D.F., Erecińska, M., Leigh, Jr., J.S. and Koppleman, M.C. (1972) Arch. Biochem. Biophys. 151, 112—121
11 Orme-Johnson, N.R., Hansen, R.E. and Beinert, H. (1974) J. Biol. Chem. 249, 1928—1939
12 Der Vartanian, D.V., Albrecht, S.P.J., Berden, J.A., van Gelder, B.F. and Slater, E.C. (1973) Biochim. Biophys. Acta 292, 496—501