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THE ORIENTATION OF IRON-SULFUR CLUSTERS AND A SPIN-COUPLED UBIQUINONE PAIR IN THE MITOCHONDRIAL MEMBRANE

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Summary

Oriented multilayers made from beef heart and yeast mitochondria and sub-mitochondrial particles were studied using electron paramagnetic resonance. EPR signals from membrane-bound iron-sulfur clusters and from a spin-coupled ubiquinone pair are highly orientation dependent, implying that these redox centers are fixed in the membrane at definite angles relative to the membrane plane. Typically the iron-iron axis (g_z) of the binuclear iron-sulfur clusters is in the membrane plane. This finding is discussed in terms of the protein structure. the tetranuclear iron-sulfur clusters can have their g_z axis either perpendicular or parallel to the membrane plane, but intermediate orientation was not observed.

Introduction

A variety of electron and hydrogen carriers function in the respiratory chain of mitochondria. These include hemes, flavins, ubiquinones, and iron-sulfur centers. The last components contain either two or four iron atoms in each cluster, and occur in NADH, succinate, and electron transfer flavoprotein dehydrogenases and in the cytochrome b-c_1 region.

Recently, oriented multilayers prepared from membraneous biological structures such as mitochondria, reconstituted cytochrome oxidase vesicles,

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Abbreviation: HiPIP, high potential iron-sulfur protein.

and chromatophores have been studied using optical and EPR techniques [1—6]. These specimens generally have order along a single axis, a property which has made it possible to obtain information describing the orientation of the hemes of cytochrome oxidase, cytochromes *b*, and c_1, cytochromes *c*-551 and *c*-555, and cytochrome *P*-450 in mitochondrial and chromatophore membranes [1—6].

The effect of disorder on the spectra of chromatophores in oriented multilayers has been studied by Blum et al. [7]; through computer simulation a procedure was devised for determining orientation and estimating disorder. Both stacking disorder and chromophore disorder (non-rigid orientation of the chromatophore in its protein and of the protein in the membrane) were found to contribute to disorientation (mosaic spread).

In order to relate the orientation of the EPR signals to the structural information, some knowledge of the electronic configuration of the chromophore is needed. A model proposed by Gibson et al. [8] to account for the EPR spectrum of binuclear iron-sulfur proteins (spinach ferredoxin classes) has received considerable experimental support [9,10]. Unfortunately, no comparable model [11—15] exists for tetranuclear iron-sulfur proteins (either HiPIP or bacterial ferredoxin classes).

The small *g* value anisotropy of flavin and ubiquinone radicals is of limited usefulness in determining orientation. However, the anisotropy introduced by spin-spin interactions between electron carriers can be used to obtain important information on the placement of electron carriers in the membrane.

In this paper, we describe the orientation of various iron-sulfur centers in oriented multilayers of mitochondrial membranes. We also present improved data on the orientation of a spin-coupled ubiquinone pair [16,17].

Materials and Methods

Beef heart mitochondria were prepared by the method of Löw and Vallin [18]. Cells of *Saccharomyces carlsbergensis* were grown as described by Ohnishi et al. [19] and mitochondria were prepared by the combined method of cell wall digestion and mechanical disruption [20]. The preparation of oriented multilayers was carried out as previously described [2,3].

The partially dehydrated multilayers were sliced into rectangular strips to fit into the sample holder. Reduction of the multilayers was usually accomplished by pipetting drops of a freshly prepared saturated solution of dithionite or the more physiological reductant, succinate. After incubation at room temperature for 1—5 min, the excess fluid was removed by blotting with a tissue and the multilayer was mounted in its holder and quickly brought to the temperature needed for observation of the EPR spectra. This treatment prevented the distruction of orientation. The samples could be repeatedly removed from the holder, brought back to room temperature and further reduced or oxidized. Oxidation was similarly accomplished with ferricyanide.

The oriented multilayers were inserted in a quartz sample holder for protection and ease in manipulation as illustrated in Fig. 1. Angles between the direction of Zeeman field and the plane of the multilayer samples were measured

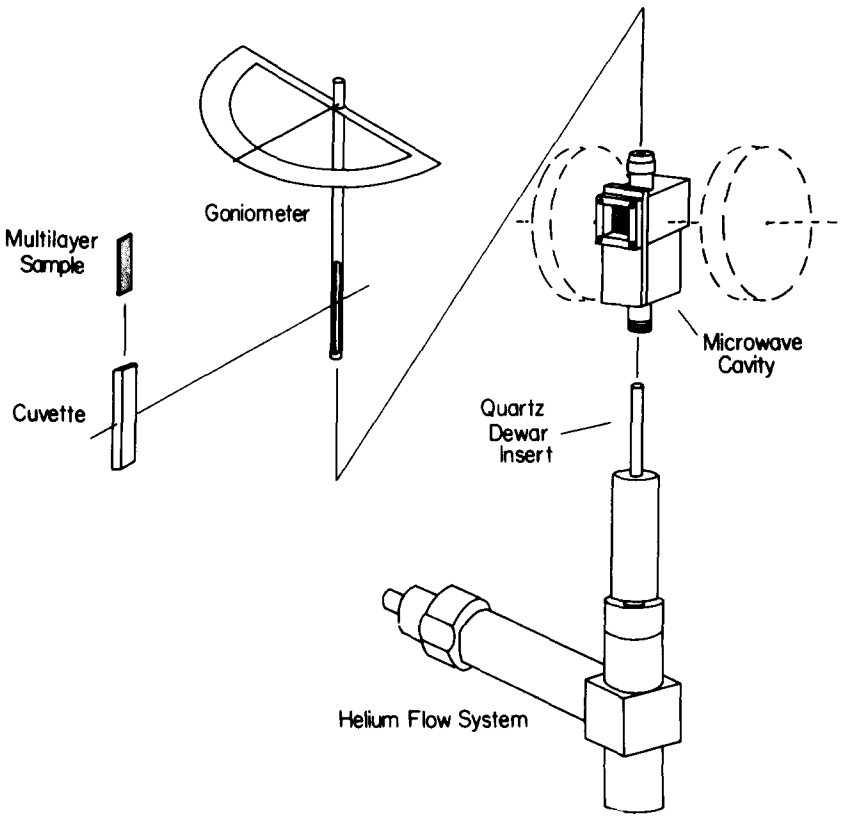

Fig. 1. Schematic diagram of the device for orientation and rotation of the multilayer sample. The helium flow system is Air Products Model LTD-3-110.

using a goniometer which was mounted on top of the quartz dewar insert (Fig. 1).

EPR spectra were obtained using either a Varian E-104 or E-109 microwave spectrometer. Sample cooling and temperature control have been described [2].

The data were analyzed using a computer program which has been previously described [7]. This program assumes ordering in the normal direction only and models deviations from perfect order using a Gaussian distribution of chromophore orientations.

In addition, a program which models dipolar coupling between radicals was written. Since the species to be modeled are nearly isotropic, the splitting was approximated as:

$$\Delta H \approx \mu_{eff}\, r^{-3}(1 - 3\cos^2\psi) = D(1 - 3\cos^2\psi)$$

since the g tensor of the radical pair could be taken as effectively parallel. r is the distance between the spins, ψ is the angle between the external magnetic field and the dipole axis and μ_{eff} is the magnetic moment of one of the interacting spins.

Results

Succinate dehydrogenase

Addition of succinate or a small amount of dithionite to oriented multilayers (as described in Materials and Methods) evolves '$g = 1.94$' EPR signals associated with binuclear iron-sulfur clusters in succinate dehydrogenase. If the reduction is done with dithionite it is important to limit the time of exposure of the multilayer to dithionite to prevent reduction of the NADH dehydrogenase components as well. As described in Materials and Methods, it is possible to repeatedly freeze and thaw the multilayers, enabling us to monitor their state of reduction with time. Fig. 2 shows EPR spectra, recorded at 20 K, of partially reduced multilayers prepared from beef heart mitochondria with the plane of the multilayers oriented parallel and perpendicular to the Zeeman field.

A signal at $g = 1.91$ can be tentatively assigned to Center S-1 in succinate dehydrogenase. This peak is maximal when the magnetic field is aligned with the normal to the multilayer. The signal at $g = 1.93$ is expected to be largely due to Center S-1; it is maximized when the field is parallel to the plane of the multilayer. The spectrum of Rieske's iron-sulfur center is also orientation dependent; the EPR signal at $g = 1.90$ is maximal when the magnetic field is parallel to the plane of the multilayer and the signal at $g = 1.80$ is maximal when the magnetic field is along the normal to the multilayer.

The g_z signals of Rieske's center and Center S-1 are visible near $g = 2.025$. This region is heavily overlapped by the Cu(II) spectrum from cytochrome oxidase, which is difficult to fully reduce in oriented preparations due to poor equilibration between the layers of the sample. It is clear, however, that the g_z signal of binuclear iron-sulfurs is maximal when the field is parallel to the plane of the multilayer.

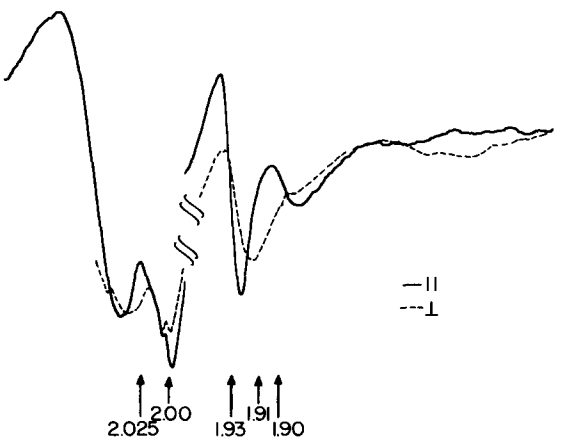

Fig. 2. EPR spectra recorded at 20 K of oriented multilayers of beef herat mitochondria which were partially reduced by succinate plus dithionite. EPR-operating conditions: microwave power, 5 mW; modulation amplitude, 10 gauss; scan speed, 250 gauss/min; time constant, 1 s. ⊥, the orientation in which the Zeeman field lies along the normal to the membrane phase; ||, the field is in the plane of the multilayer.

For purposes of analysis, computer simulations were made of individual centers. Fig. 3 shows one set of simulations of S-1 in the parallel and perpendicular orientations for various degrees of disorder. As the amount of mosaic spread increases the relative amplitudes of the g_x, g_y, g_z signals change as does change in amplitude of each signal with angle. The line positions stabilize for all mosaic spreads greater than 20°.

It is difficult to compare these simulations in detail with the overlapped data. However, a number of features can be picked out. In order to suppress the g_y signal to the extent seen in Fig. 2, the mosaic spread is about 20°—30°. This is in agreement with the other signal amplitudes and positions. The g_y and g_z signals have maximum amplitude when the magnetic field is parallel to the

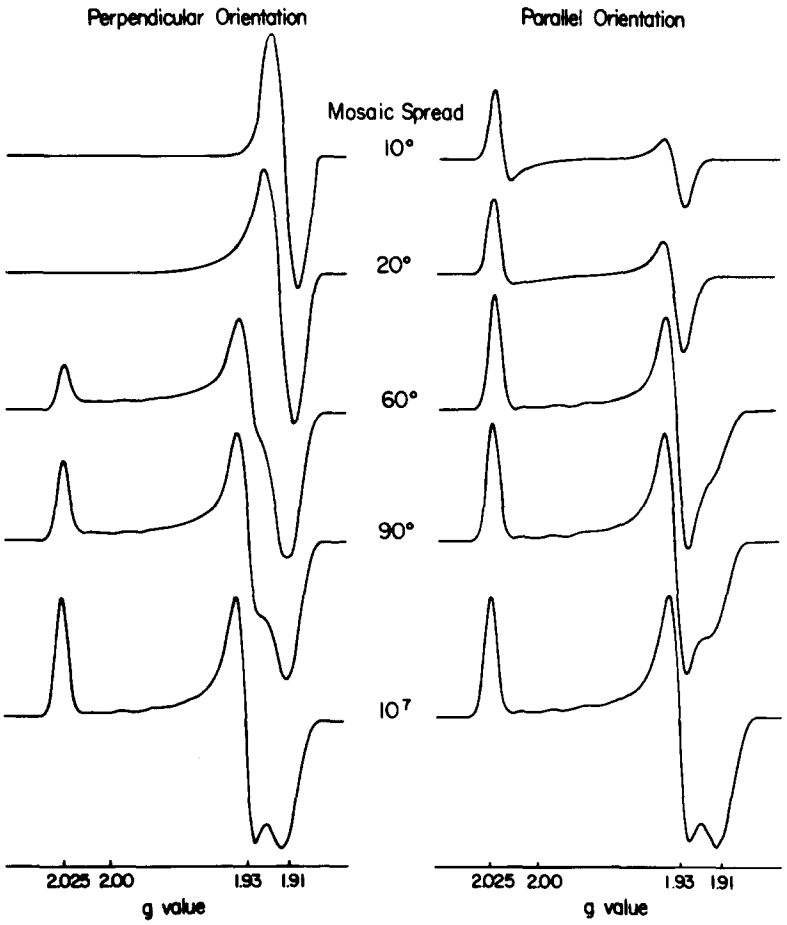

Fig. 3. Computer simulation of Center S-1 with different degrees of disorder. Two orientations of magnetic field relative to the multilayer plane are shown. The center is assumed to have g_y and g_z in the plane of the multilayer and g_x perpendicular to it. The mosaic spread parameter is the half-width of a Gaussian probability distribution for disorder. The assumed linewidth of Center S-1 varies with field according to $\Delta H^2 = \Delta H_x^2 l_x^2 + \Delta H_y^2 l_y^2 + \Delta H_z^2 l_z^2$, where l_x, l_y, l_z are the direction cosines of the principal g axes g_x, g_y, g_z, respectively, with the applied magnetic field and ΔH_x, ΔH_y, ΔH_z are Gaussian linewidth parameters. The values assumed in these simulations are ΔH_x = 13 G, ΔH_y = 9 G, ΔH_z = 6 G. With mosaic spread of 10^7 one gets a powder pattern which does not change with angle. It was compared with unoriented samples in order to set the linewidth parameters.

multilayer plane and the g_x signal is maximum when the field is perpendicular to the plane.

The orientation dependence of these iron-sulfur signals is summarized in Figs. 4 and 5. It is evident that the g_x directions correspond to the normal to the multilayer plane and the g_y and g_z directions are in the plane.

Fig. 6 shows EPR spectra of multilayers made from *S. carlsbergensis* mitochondria. These mitochondria contain no NADH dehydrogenase; therefore, we can eliminate the NADH dehydrogenase iron-sulfur centers from consideration. The orientation dependence of the signals is similar to that of the signals shown in Fig. 2, although somewhat better orientation of Center S-1 was obtained in the beef system.

Oriented multilayers prepared from mitochondria and submitochondrial particles show signals from cytochromes, Cu(II) and HiPIP-type iron-sulfur centers when oxidized. The former two have been previously described [2,5]. Fig. 7 shows the spectra of Center S-3 in oriented multilayers prepared from beef heart submitochondrial particles with the field parallel and perpendicular to the plane of the membrane. It is clear that the spectra are highly orientation dependent, enabling the g values to be partially resolved. From computer simulations we find that the data are consistent with g_z (2.02) axis perpendicular to the membrane plane and the g_x and g_y axes in the plane. Unfortunately, lack of knowledge about the relationship of the EPR spectra of HiPIPs to their structure precludes drawing structural conclusions at this time.

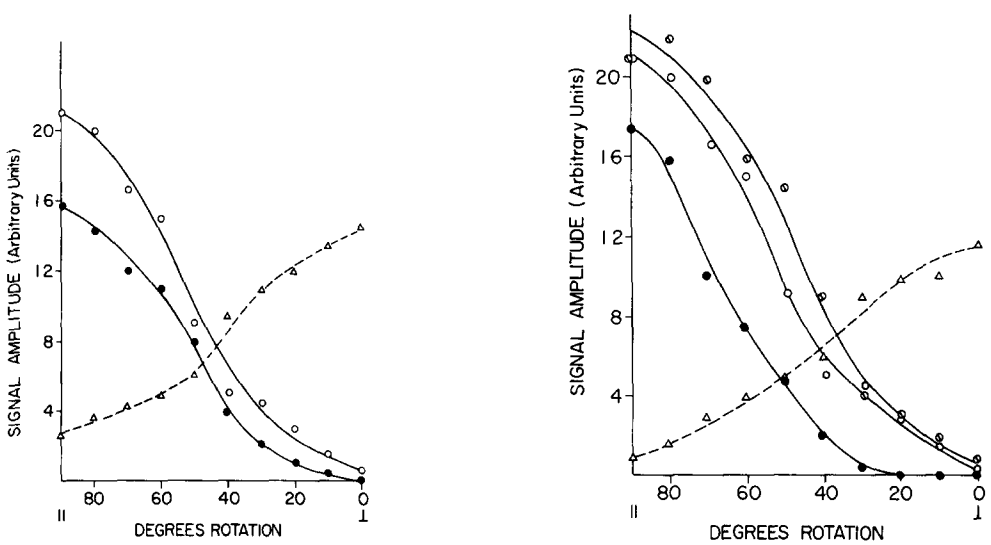

Fig. 4. Amplitudes of the Center S-1 EPR signals versus multilayer orientation. The signal at 1.91 (△- - - - -△), g_x, was measured from the high field baseline to the negative peak; that at 2.025 (○- - - - -○), g_z, was measured relative to the low field baseline just before the Cu^{2+} signal interferes, the 1.93 signal (●- - - - -●), g_y, was measured from the high field baseline to the positive peak to avoid interference from the g_x signal. Experimental conditions are as described in Fig. 2.

Fig. 5. Amplitude of the Rieske's center EPR signals versus multilayer orientation. Experimental conditions as in Fig. 2. Data taken as in Fig. 4 with: △- - - - -△, g_x = 1.82; ●———●, g_y = 1.90, and ○———○, g_z = 2.025 at 25 K or (Φ———Φ) 15 K.

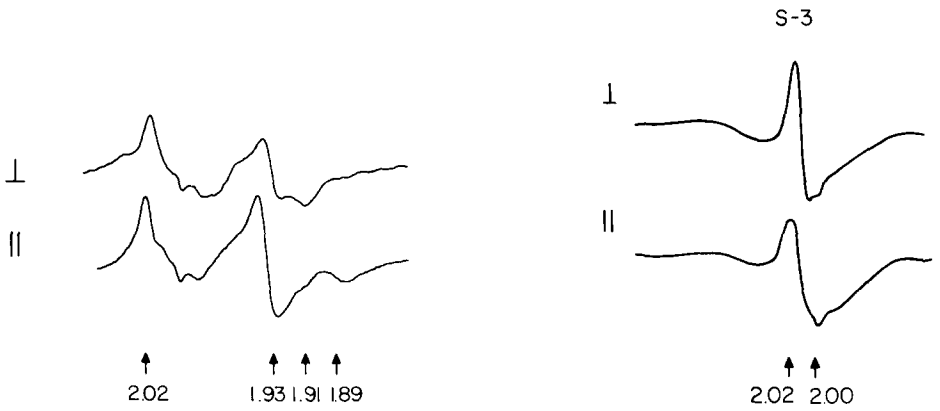

Fig. 6. EPR spectra of multilayers prepared from *S. carlsbergensis* mitochondria. Conditions were as described in Fig. 2 except 10 mW microwave power, and sample temperature of 30 K.

Fig. 7. EPR spectra of oriented multilayers prepared from beef heart submitochondrial particles oxidized with ferricyanide. Spectra were recorded at 11 K with 20 mW of microwave power; other conditions were as in Fig. 2.

NADH dehydrogenase

Fig. 8 shows the EPR spectra of dithionite-reduced multilayers with various orientations relative to the Zeeman field, recorded at 13 K. With the sample in the parallel orientation (Zeeman field in the plane of the multilayer) we see the g_z peak from Center N-4 at $g = 2.10$. As the multilayer is rotated towards the perpendicular orientation, this peak gradually decreases in amplitude. Conversely, the corresponding peak from Center N-2 at $g = 2.05$ is maximal with perpendicular orientation; overlapped with it is the g_z peak from N-3 at $g = 2.035$ in the parallel orientation.

At higher field, the resonances at 1.88 and 1.86 corresponding to the g_x of Centers N-4 and N-3, respectively, are maximal in the parallel direction [21]..

The orientation dependence of signals from tetranuclear iron-sulfur clusters in the NADH dehydrogenase segment is summarized in Fig. 9. All of the peaks have maximum intensity at 0° or 90°. It is interesting to note that, as predicted by computer simulations, the peak positions remain at the principal g values and do not shift with multilayer orientation.

It is also of interest to note that although the sample is highly ordered, with apparent 'dichroic ratios' of over 10 : 1 (ratio of maximum signal to minimum signal as the orientation is varied *), the signals at $g = 2.08$ from the electron-transferring flavoprotein iron-sulfur centers show no ordering (see Fig. 10). This implies that the center is only loosely associated with the membrane, rather than rigidly bound. It also provides us with a useful internal standard; since these signals are invariant to rotation we can eliminate the possibility of sensitivity changes during rotation of the sample or with time.

Further reduction of the multilayer by extended soaking in dithionite solu-

* We note that although this is not a true dichroic ratio in the sense that it does not correspond to what an optical measurement would yield. It is still a measure of order.

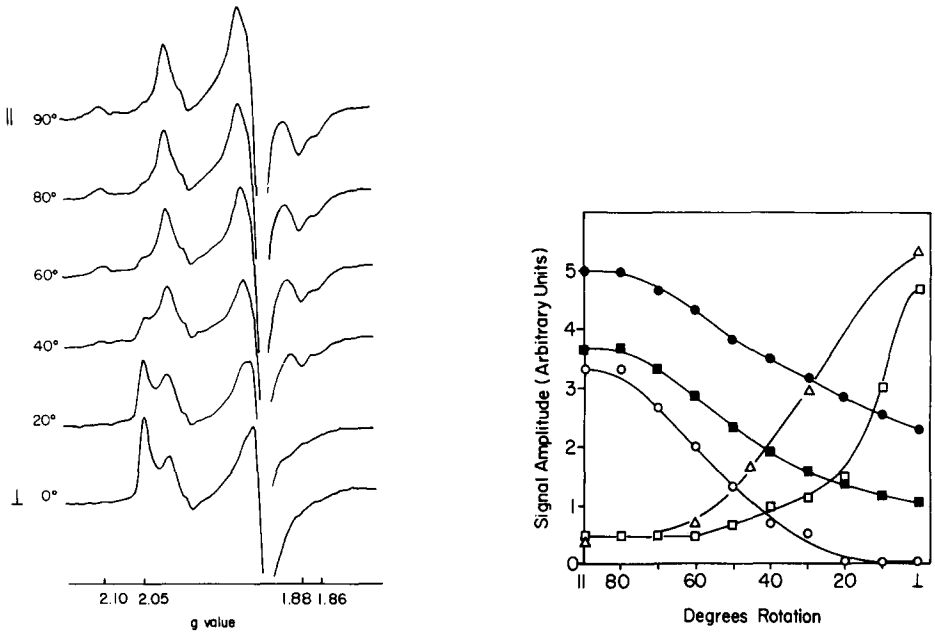

Fig. 8. EPR spectra recorded at 13 K of multilayers reduced with dithionite. EPR conditions were as in Fig. 2.

Fig. 9. Orientation dependence of EPR signals of various iron-sulfur centers in oriented multilayered beef heart submitochondrial particles. The g = 2.10 (○), 2.05 (□), and 1.88 (●) signals were recorded at 13 K and 5 mW, while g = 1.86 (■) and 2.035 (△) signals were at 11 K and 100 mW in order to reduce interference from other centers.

tion increases the intensity of the signals at g = 1.94 but causes loss of order. In the samples where Centers N-3 and N-4 (E_m = −240 mV) were significantly reduced the g = 2.03 and 1.91 signals were intensified in the perpendicular orientation. This suggests that Center N-1b (E_m = −240 mV) is at least partially reduced and is similarly oriented as Rieske's center and S-1. It is not clear whether additional signals include contribution from Center S-2; further experi-

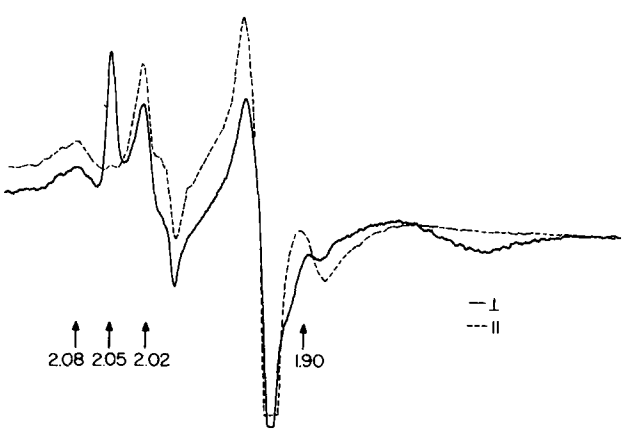

Fig. 10. EPR spectra of oriented multilayers of beef heart mitochondria at 19 K. Other conditions were same as in Fig. 2.

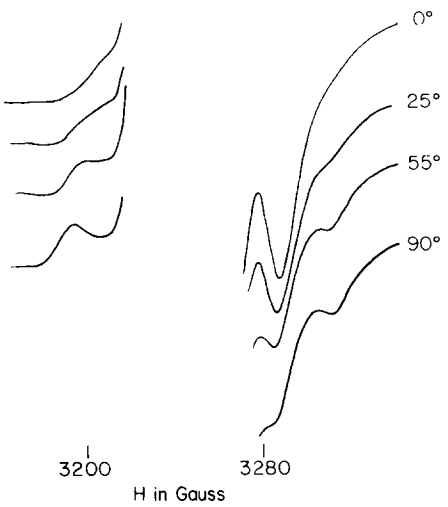

Fig. 11. EPR spectra recorded at 12 K of oriented multilayers prepared from beef heart mitochondria. The multilayers were prepared in the presence of 100 μM antimycin; after drying, they were dipped in a mixture of 10 mM fumarate and 20 mM ascorbate, inserted in a quartz sample tube and frozen in liquid N_2. The signals in the wings are due to the spin-coupled quinone pair; the large central peak is due to iron-sulfur Center S-3.

ments will be conducted to clarify this point using purified systems, such as, oriented liposomes with incorporated NADH-Q reductase.

Orientation of dipolar-coupled ubisemiquinone pairs

When dipolar coupling between electron carriers exists, oriented samples can be used to determine vectors between these electron carriers in the membrane. The simplest example is the dipole-coupled ubiquinone pair which is believed to function as electron acceptor of succinate dehydrogenase [22].

The nearly isotropic semiquinone species give rise to a quartet with the inner pair displaced by $\pm D$ and the outer pair displaced by $\pm 2D$ about the center. The angular dependence of the dipolar interaction is $(1 - 3\cos^2\psi)$ where ψ is the angle between the Zeeman field and the vector connecting the two spins.

The EPR spectrum of the dipolar-coupled ubisemiquinone pair can be evoked by addition of ferricyanide and succinate or fumarate and ascorbate, especially in the presence of antimycin [23]. This is particularly useful in oriented multilayers, since poising a sample at a given E_h is difficult at best.

In Fig. 11, the spectra of a multilayer prepared from beef heart mitochondria and treated with fumarate and ascorbate are shown at various orientations in the Zeeman field. The outer pair are maximal when the Zeeman field is along the normal to the membrane stack; one of the inner pair is overlapped by signals from iron sulfur Center S-3, but the other is maximal with the field in the plane of the multilayer.

Discussion

Interpretation in terms of Gibson's model

We have seen that the g_z signals from S-1, Rieske's center and at least one of

the N-1 type centers in NADH dehydrogenase are maximal with the Zeeman field in the plane of the membrane. Computer simulation indicates that the z direction in each of these centers is within 10° of the plane.

The model proposed by Gibson and coworkers [8—10] for binuclear ('plant ferredoxin type') iron-sulfur centers (see Fig. 12a) utilized two antiferromagnetically coupled high spin iron atoms. In the reduced state, one iron atom is formally ferrous and the other ferric, yielding a net spin of 1/2.

Admixture of the d_{xz} and d_{yz} orbitals of the high spin ferrous iron into the ground state causes g_x and g_y to fall below 2.00. The g_z value is relatively unaffected by the spacings of the d orbitals and lies slightly above 2.0. The z direction corresponds to the iron-iron axis.

Therefore, the Fe-Fe axis of all three binuclear Fe-S clusters which have been studied in oriented multilayers lies in the plane of the membrane. While this may be coincidental, it may tell us something about the architecture of membrane-bound iron-sulfur proteins. Soluble binuclear iron-sulfur proteins share the property of having segments with amino acid sequence cysteine-X-X-cysteine, where X is any other amino acid. If the polypeptide chains of the membrane bound iron-sulfur proteins are arranged in random coils perpendicular to the membrane, as has been proposed for cytochrome oxidase [24], such an amino acid sequence would lead to the carrying of the iron-sulfur cluster between adjacent coils. Thus the Fe-Fe axis would lie approximately in the membrane plane.

The tetranuclear iron-sulfur clusters are of two types: 'bacterial ferredoxin type' which shuttles between the net valency states of —2 and —3 which can be formally expressed as Fe(II)Fe(II)Fe(III)Fe(III) and Fe(II)Fe(II)Fe(II)Fe(III) redox states and 'HiPIP type' which shuttles between —1 and —2 net valency states. These share the cubane structure illustrated in Fig. 12b. Unfortunately,

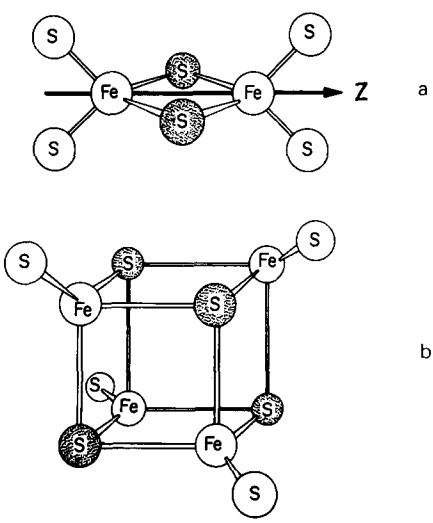

Fig. 12. Structures of two classes of iron-sulfur clusters: (a) binuclear center (plant ferredoxin type) contains two Fe and two acid-labile sulfur (shaded S); (b) tetranuclear center (bacterial ferredoxin and HiPIP types) contains four Fe and four acid-labile sulfurs.

no model exists which relates the magnetic anisotropy to the structure of these iron-sulfur clusters.

It is of interest, however, to note that these centers are fixed in the membrane. From simulation, we estimate the overall disorder parameter to be about 30°, of which about 20° is probably due to mosaic spread. The remainder includes inhomogeneity of the orientation of the proteins in the membrane and of the chromophores in the proteins. The disorder of all the iron-sulfur centers is similar in any one sample, with the exception, as noted, of the electron transferring flavoprotein iron-sulfur center. No general pattern emerges for the orientation of the tetra-nuclear iron-sulfur centers, which is perhaps not surprising in view of their cubane structure, which on casual inspection reveals little basis for a preferred orientation.

Orientation of dipolar-coupled pairs

Previously, we reported that the quinone pair was arranged so as to span the membrane [16,17]. The vector connecting the radicals was roughly perpendicular to the multilayer plane. The present orientation data are somewhat improved; computer modeling of the variation of signal with angle suggests that the vector lies within 15° of the normal to the multilayer, about half the disorder parameter necessary to fit the spectra.

The iron-sulfur Centers S-1 and S-2 in succinate dehydrogenase are spin coupled [25]. Unfortunately, we have not yet been able to reduce both centers to a great enough extent to observe the coupling in multilayers without destroying the order.

We know that g_x of Center S-1 lies along the normal to the membrane plane. The spectrum of the coupled (S-1)-(S-2) pair has been simulated [26]; the intercluster vector lies about 60° from the z axis and 45° from the y and x axes. This suggests that the vector between S-1 and S-2 lies about 50° from the normal. However, it would be useful to check this hypothesis by measurements on a fully reduced system containing no NADH dehydrogenase, such as succinate cytochrome *c* reductase vesicles.

Acknowledgments

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