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STUDIES OF THE ORIENTATION OF THE MITOCHONDRIAL REDOX CARRIERS

III. ORIENTATION OF THE g_x AND g_y AXES OF THE HEMES OF CYTOCHROME OXIDASE WITH RESPECT TO THE PLANE OF THE MEMBRANE IN ORIENTED MEMBRANE MULTILAYERS

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Summary

The EPR absorption properties of the hemes of cytochrome oxidase and their liganded derivatives were examined in oriented multilayers from isolated oxidase, mitochondrial membranes and membrane fragments of a bacterium, *Paracoccus denitrificans*. The hemes of the oxidase in all the systems investigated were oriented normal to the plane of the multilayers. The directions of the g signals corresponding to the g_x and g_y axes of the g tensor were found to be different in low-spin ferric heme in fully oxidized oxidase and in half-reduced liganded oxidase. It is suggested that this different orientation of g_x and g_y in fully oxidized oxidase and half-reduced liganded oxidase arises because the respective EPR signals belong to two different hemes, those of cytochrome a and a_3.

Cytochrome c oxidase, one of the most important enzymes of cellular metabolism, is responsible for both the reduction of molecular oxygen to water and energy transduction at the third site of mitochondrial oxidative phosphorylation. The enzyme consists of four oxidation-reduction components: two cytochromes (a and a_3) and two copper atoms (the 'visible' and the 'invisible' copper). Cytochromes a and a_3 strongly interact with one another (heme-heme interaction) such that the chemical properties of each of the cytochromes depend on the redox and/or liganding state of the other [1—5]. It is essential to the understanding of the mode of action of cytochrome oxidase to indetify each of the hemes and their properties and to determine changes in these properties that are associated with enzymatic function.

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Recent developments in the preparation of oriented multilayers of isolated membranous cytochrome oxidase [6,7] and of mitochondrial membranes [8,9] have made it possible to examine the orientation of heme chromophores relative to the plane of the membrane. It was found that both hemes of cytochrome oxidase were oriented with the normal to the plane of the heme lying in the plane of the multilayer [6—10]. It was further reported [10] that the EPR-detectable heme of oxidized cytochrome oxidase in submitochondrial particles had its heme plane oriented in such a way that the g_y and g_x axes of the g tensor formed angles of 30° and 60° to the membrane normal.

In the present paper the EPR absorption properties of the hemes of cytochrome oxidase and their derivatives are examined in oriented multilayers to determine if the orientations of the components of the g tensor in the heme plane can be used to identify the hemes of cytochromes a and a_3. Evidence will be presented that the orientations of the g resonances which correspond to the g_x and g_y axes of the g tensor are different in the ferric low-spin heme in fully oxidized oxidase and in half-reduced liganded oxidase. This information suggests that the EPR resonances observed in the two oxidase states belong to two different hemes, those of cytochromes a and a_3.

Materials and Methods

Mitochondria were isolated from pigeon breast muscle using bacterial proteinase (Nagarse) [11] and kept frozen at −25°C. 'Membranous' cytochrome oxidase was purified from pigeon breast mitochondria by a modification [6] of the method of Sun et al. [12].

Paracoccus denitrificans was grown aerobically at 30°C as described previously [13] and the membrane fragments containing the respiratory chain components were prepared by the method of Scholes and Smith [14].

Hydrated oriented multilayers of membranous cytochrome oxidase and mitochondrial membranes were formed by the technique described previously [6,8]. The hydrated oriented multilayers from *P. denitrificans* membranes were formed essentially the same way except the aliquots of the rapidly thawed membrane fragments were diluted in 50 mM phosphate buffer, pH 7.2, and centrifuged onto thin Mylar sheet at 70 000 × g for 2 h.

Preparation of the derivatives. The partially dehydrated oriented multilayers were cut together with the supporting Mylar into 2-mm wide strips and rapidly inserted inside a 3 mm internal diameter quartz EPR sample tube. The derivatives were formed after the specimens were placed inside the sample tube.

The azide derivatives were formed by immersing the oriented multilayers in 1.2 M sucrose/0.05 M ascorbate, pH 6.9/0.5 M azide. Incubation was 15 min at room temperature. The specimen was frozen by immersion of the sample tube in liquid nitrogen.

The sulfide derivatives were formed by immersing the specimens in 1.2 M sucrose/0.1 M phosphate buffer, pH 7.0/0.02 M sodium sulfide. The EPR capillary was tightly sealed and incubated for 30 min at room temperature prior to freezing in liquid nitrogen.

The EPR spectra were measured using a Varian E-109 spectrometer equipped with an Ait Products LTD-3-110 liquid helium kryostat and a PDP-11 com-

puter. The detailed conditions for the measurements are given in the figure legends. In all the plots, 0° is defined as the position in which the plane of the oriented multilayers is normal to the magnetic field.

Results

Orientations of the cytochrome oxidase hemes in hydrated multilayers of 'membranous' cytochrome c oxidase. The EPR spectra of the oriented multilayers of oxidized cytochrome *c* oxidase at various angles between the planes of the membranes and the magnetic field are shown in Fig. 1. It can be seen that the magnitudes of the *g* tensor exhibit strong dependence on the direc-

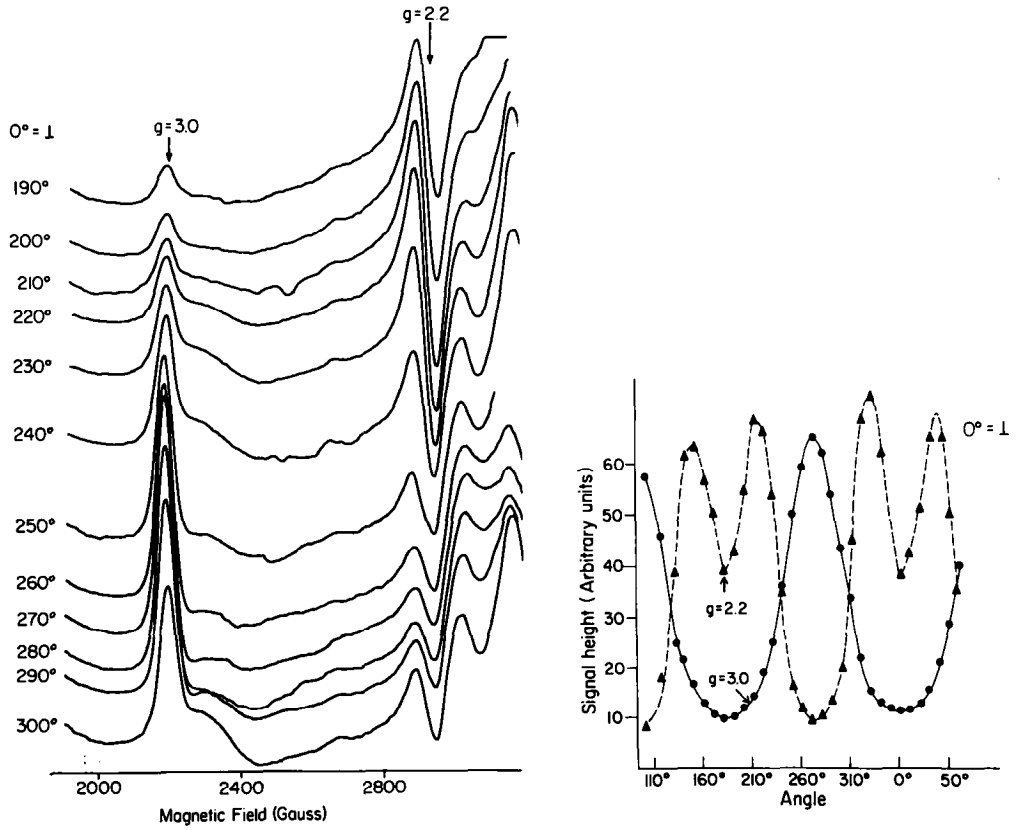

Fig. 1. EPR spectra of the oxidized cytochrome *c* oxidase in oriented multilayers of the 'membranous' enzyme at various angles between the direction of the magnetic field and the normal to the membrane plane. The oriented 1 cm diameter multilayer was formed from 5.2 mg protein (75.6 nmol heme *a*) of 'membranous' cytochrome oxidase and 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 by immersion in liquid nitrogen. EPR frequency, 9.106 GHz; microwave power, 5 mW; sample temperature, 11 K; scanning time, 2 min; time constant, 0.25 s; modulation amplitude, 12.5 g.

Fig. 2. Plot of relative EPR signal amplitudes for oxidized cytochrome oxidase in oriented multilayers of the 'membranous' enzyme as a function of angle between the magnetic field direction and the normal to the plane of the membrane. The EPR spectra were recorded under conditions specified in the legend to Fig. 1.

tion of the applied magnetic field. When the oriented multilayer is rotated through an angle of 360° the lowest field resonance at $g = 3.0$ (this signal corresponds to the z axis of the g tensor, i.e. the one which is approximately normal to the heme plane [15]) shows two maxima separated by 180°, i.e. the signal is maximal when the magnetic field is parallel to the plane of the membranes whereas it is minimal when the membranes are normal to the magnetic field (Fig. 2). In contrast, maximum intensity of the $g = 2.2$ signal (this signal correspond to one of the two axes of the g tensor which lie in the plane of the heme [15]) is observed at an angle of approximately 30° between the normal to the membrane and the direction of the magnetic field. Thus, as the sample is rotated through 360° with respect to the magnetic field four maxima are clearly observed at 30°, 150°, 210° and 330° (Fig. 2).

The angular dependences of the magnitudes of the resonances correponding to the three principal components of the g tensor of the azide and sulfide derivatives of membranous cytochrome oxidase are shown in the next four figures. The lowest field resonances ($g = 2.9$ in the azide complex and the double resonances around $g = 2.5$ in the sulfide complex) in both derivatives are maximal when the planes of the oriented multilayers are parallel to the direction of the magnetic field and minimal when the membranes are normal to the magnetic field.

The $g = 2.2$ signal of the azide complex is maximal when the planes of the multilayer are normal to the direction of the magnetic field and minimal when the planes of the membranes are parallel to the magnetic field (Fig. 4). The split resonances at around $g = 2.2$ for the sulfide complex (Figs. 5 and 6) are 90° out of phase with respect to the $g = 2.2$ signal of the azide complex, i.e. they are maximal when the plane of the membranes is normal to the magnetic field.

The highest field resonances of the azide complex at $g = 1.67$ and of the sulfide complex at $g = 1.87$ (these signals correspond to the other axis (g_x) of the g tensor which lies in the plane of the heme [15]) show angular depen-

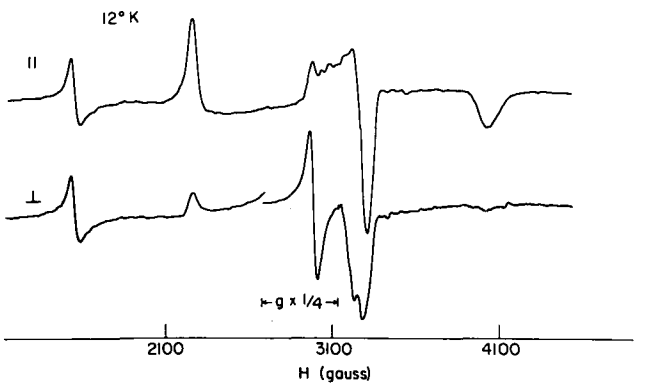

Fig. 3. EPR spectra of cytochrome a^{2+}-cytochrome a_3^{3+}-azide complex in frozen oriented membrane multilayers where the planes of the membranes are parallel and normal to the magnetic field. The oriented multilayer was formed and treated as described in the legend to Fig. 1. The conditions for the formation of the azide derivative are given in Materials and Methods. EPR frequency, 9.109 GHz; microwave power 5 mW; sample temperature, 12 K; scanning time, 2 min; time constant, 0.25 s; modulation amplitude, 10 G.

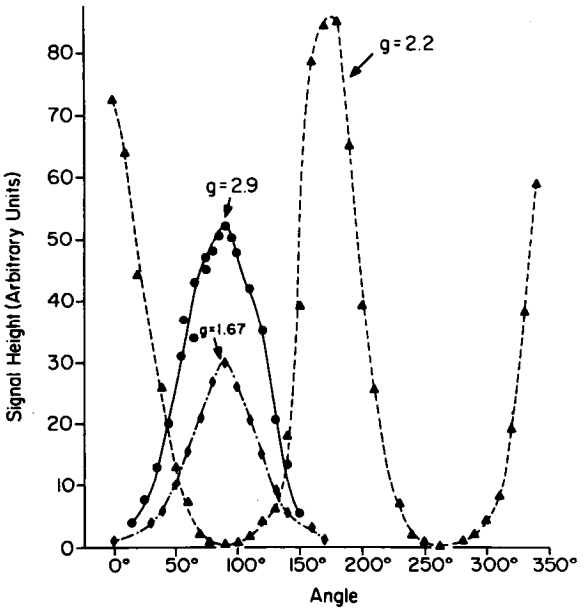

Fig. 4. Plot of relative EPR signal magnitudes for cytochrome a^{2+}-cytochrome a_3^{3+}-azide complex in frozen oriented multilayers of 'membranous' cytochrome oxidase as a function of angle between the direction of the magnetic field and the normal to the plane of the membrane. The EPR spectra were recorded under conditions specified in the legend to Fig. 3.

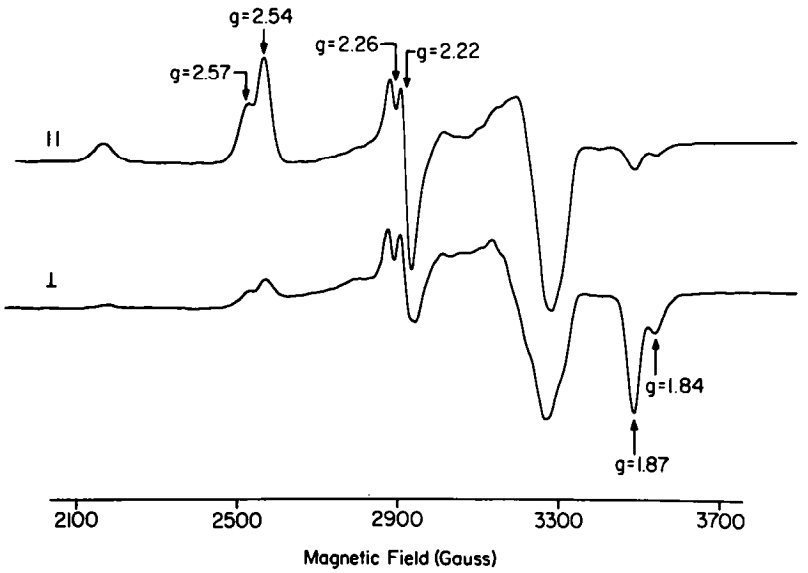

Fig. 5. EPR spectra of cytochrome a^{2+}-cytochrome a_3^{3+}-sulfide complex in frozen oriented membrane multilayers where the planes of the membranes are parallel and normal to the direction of the magnetic field. The oriented multilayer was formed and treated as described in the legend to Fig. 1. The condition for the formation of the sulfide derivative is given in Materials and Methods. EPR frequency, 9.106 GHz; microwave power, 5 mW; sample temperature, 11 K; scanning time, 2 min; time constant, 0.25 s; modulation amplitude, 12.5 G.

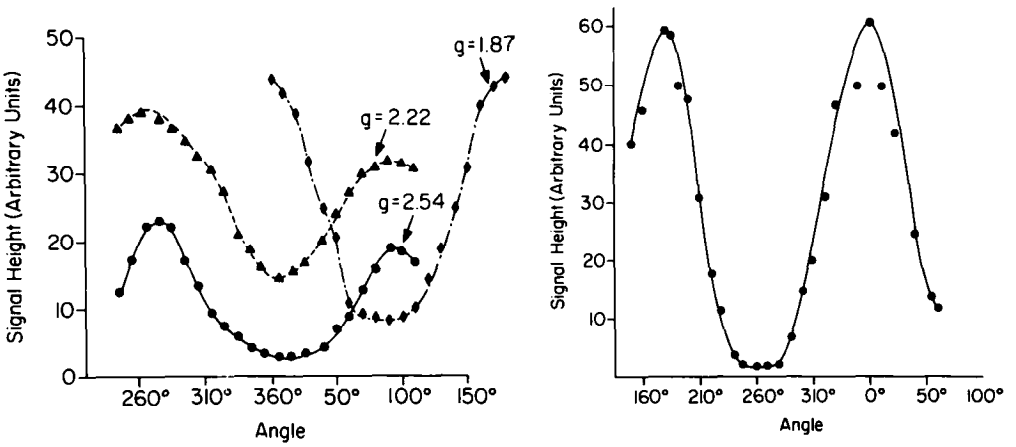

Fig. 6. Plot of relative EPR signal magnitudes for cytochrome a^{2+}-cytochrome a_3^{3+}-sulfide complex in frozen oriented multilayers of 'membranous' cytochrome oxidase as a function of angle between the direction of the magnetic field and the normal to the plane of the membrane. The EPR spectra were recorded under conditions specified in the legend to Fig. 5.

Fig. 7. Plot of relative magnitudes of the $g = 2.2$ signal for cytochrome a^{2+}-cytochrome a_3^{3+}-azide complex in frozen oriented multilayers of mitochondrial membranes as a function of angle between the direction of the magnetic field and normal to the plane of the membranes. The oriented 1 cm diameter multilayer was formed from 7.6 mg mitochondrial protein, dehydrated for 50 h at 4°C and 90% constant relative humidity. The specimen was further treated to obtain the azide complex as described in Materials and Methods. The EPR spectra were recorded under the following conditions: frequently, 9.107 GHz microwave power, 10 mW; sample temperature, 9.6 K; scanning time, 2 min; time constant, 0.25 s; modulation amplitude, 16 G.

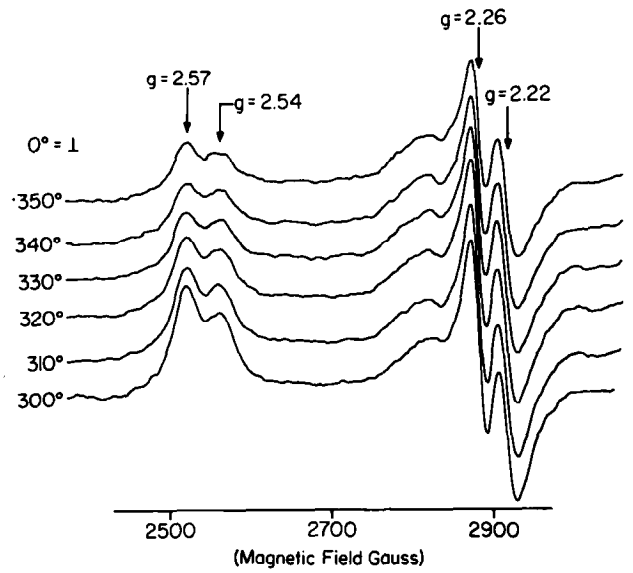

Fig. 8. EPR spectra of cytochrome a^{2+}-cytochrome a_3^{3+}-sulfide complex in frozen oriented multilayers of mitochondrial membranes at various angles between the direction of the magnetic field and normal to the plane of the membrane. The oriented multilayer was formed as described in the legend to Fig. 7. The conditions for the formation of the sulfide derivative are given in Materials and Methods. EPR frequency, 9.108 GHz; microwave power, 10 mW; sample temperature, 14.4 K; scanning time, 2 min; time constant, 0.25 s; modulation amplitude, 16 G.

dences on the direction of the magnetic field exactly opposite (i.e. 90° shifted) to those of their respective $g = 2.2$ signals. All of this information can easily be followed from the plots in Figs. 4 and 6.

Orientations of the cytochrome oxidase heme signals in hydrated multilayers of mitochondrial membranes. The angular dependences of the EPR signals of the oxidized oxidase and its azide and sulfide derivatives in oriented mitochondrial membranes were essentially the same as those in the oriented multilayers of membranous cytochrome oxidase. Two examples are presented in Fig. 7 (the angular dependence of the $g = 2.2$ signal in the azide complex) and Fig. 8 (the angular dependence of the signals of the sulfide complex).

Orientations of the hemes of cytochrome oxidase in hydrated oriented membrane multilayers from P. denitrificans. *P. denitrificans*, a Gram-negative bacterium, posseses a membrane-bound respiratory chain which closely resembles that of the mitochondrial inner membrane [16]. The terminal oxidase is cytochrome aa_3 which reacts with CO, NO and other ligands. The oxidized ferric heme signal characteristic of the oxidase exhibits g values at 2.87 and

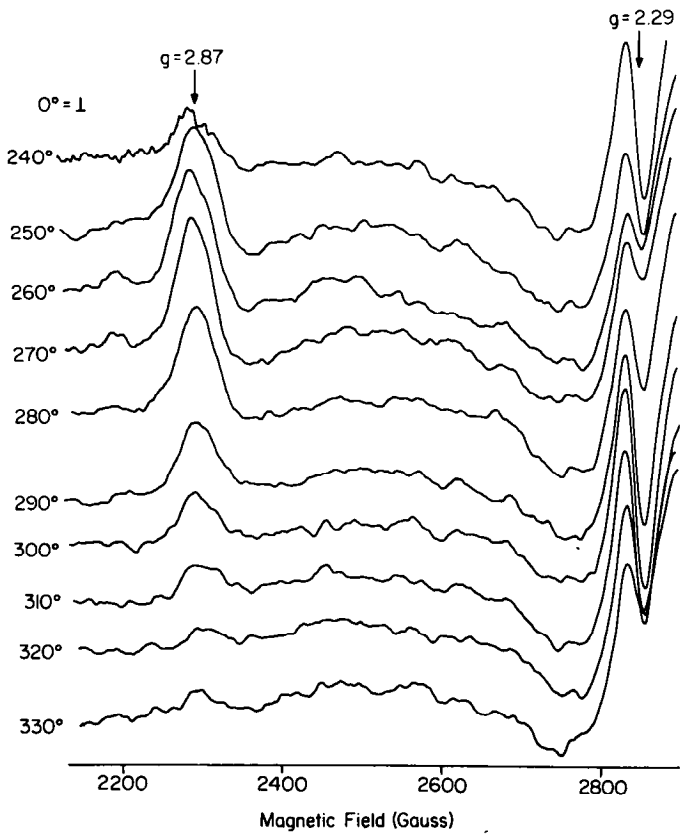

Fig. 9. EPR spectra of oxidized cytochrome oxidase in oriented multilayers of *P. denitrificans* membrane fragments at various angles between the direction of the magnetic field and normal to the plane of the membranes. The samples were prepared as described in Materials and Methods. EPR frequency, 9.108; GHz; microwave power, 5 mW; sample temperature, 12.5 K; scanning time, 2 min; time constant, 0.5 s; modulation amplitude, 20 G.

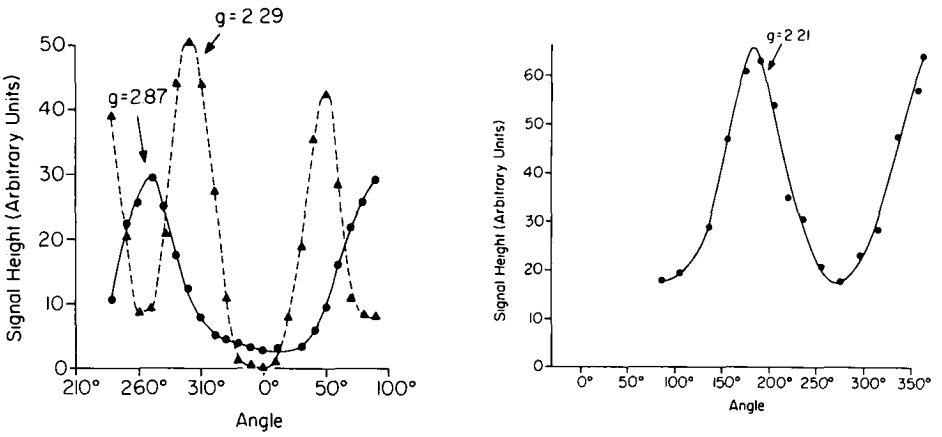

Fig. 10. Plot of relative magnitude of the EPR signals for oxidized oxidase in oriented multilayers of membrane fragments from *P. denitrificans* as a function of angle between the direction of the magnetic field and notmal to the plane of the membranes. The EPR spectra were recorded under conditions specified in the legend to Fig. 9.

Fig. 11. Plot of relative magnitudes of the $g = 2.2$ signal for cytochrome a^{2+}-cytochrome a_3^{3+}-azide complex in frozen oriented multialyers from *P. denitrificans* membrane fragments as a function of angle between the direction of the magnetic field and normal to the plane of the membrane. The azide complex was formed as described in Materials and Methods. EPR conditions; frequency, 9.106 GHz; microwave power, 10 mW; sample temperature, 12 K; scanning time, 2 min; modulation amplitude, 20 G.

2.29. The angular dependences of these resonances in the oxidized oriented membrane fragments are shown in Figs. 9 and 10. The lowest resonance at $g = 2.87$ is maximal when the membranes of the oriented multilayers are parallel to the magnetic field and minimal when the membranes are normal to the magnetic field. As the multilayers are rotated through 360°, two intensity maxima are observed 180° apart. In contrast, the $g = 2.29$ resonance is maximal at an

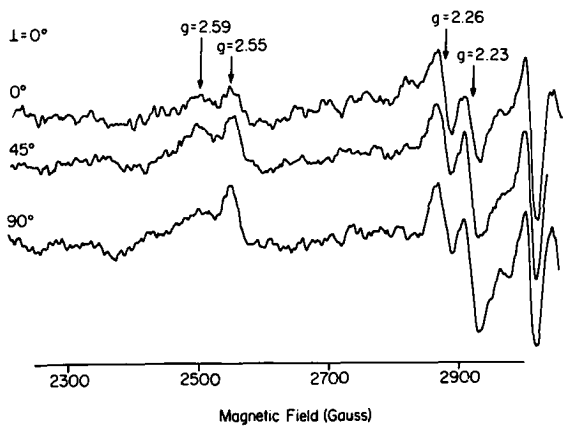

Fig. 12, EPR spectra of cytochrome oxidase-sulfide complex in oriented membrane fragments from *P. denitrificans* for three different angles between the magnetic field and the normal to the plane of the membrane. The sulfide derivative was formed as described in Materials and Methods. EPR frequency, 9.106 GHz; microwave power, 10 mW; sample temperature, 14 K; scanning time, 2 min; time constant, 0.25 s; modulation amplitude, 20 G.

TABLE I

ORIENTATION OF THE HEMES OF CYTOCHROME *c* OXIDASE

Oxidase	Cytochrome *	Condition	Orientation with respect to the membrane normal					
			g_z		g_y		g_x	
			g value	Angle (°)	*g* value	Angle (°)	*g* value	Angle (°)
Pigeon breast (cytochrome aa_3)	*a*	aerobic	3.0	90	2.25	30	1.46	60
	a_3	azide-inhibited	2.9	90	2.2	0	1.67	90
	a_3	sulfide-inhibited	2.57, 2, 54	90	2.26, 2,22	90	1.87, 1.84	0
P. dentrificans (cytochrome aa_3)	*a*	aerobic	2.87	90	2.29	50	n.d.	
	a_3	azide-inhibited	2.9	90	2.21	0	1.67	90
	a_3	sulfide-inhibited	2.59, 2.55	90	2.26, 2.23	90	n.d.	
T. pyriformis ** (cytochrome a_2 (d))	iron-chlorin	aerobic	2.96	90	2.26	0	n.d.	

* The cytochromes *a* and a_3 are identified as discussed in the text.

** From ref. 25.

angle of approximately 50° between the normal to the membrane and the direction of the magnetic field. Thus, when the membranes are rotated through 360° there are four intensity maxima: at 50° and 130° and at 220° and 310° (Fig. 10).

The angular dependences of the ferric heme signals in the azide and sulfide derivatives of cytochrome aa_3 of *P. denitrificans* are essentially the same as those of isolated oriented oxidase from pigeon breast mitochondria (Figs. 11 and 12 shows some representative results). The angles of orientation of the three principal g axes for the hemes of the oxidase isolated from pigeon breast mitochondria and membrane fragments from *P. denitrificans* are summarized in Table I.

Discussion

Analyses of EPR spectra of the low spin ferric heme complexes (see for example refs. 17 and 18) showed that their symmetry is not tetragonal (D_{4h}) but rhombic (D_{2h}) or lower. The anisotropies in the g tensor are brought about by a tetragonal distortion of the axial ligands ($g_z \neq g_y$, g_x) and by non-equivalent interactions of the d_{xz} and d_{yz} orbitals of the iron with its immediate environment ($g_x \neq g_y$).

The directions of the principal axes of the g tensor in relation to the plane of the heme can be determined from the analyses of single crystals where the orientation of the heme with respect to the crystal axes is known. It has been generalized [19] from the results obtained on crystals of metmyoglobin-azide [20], metmyoglobin-cyanide [21], metmyoglobin-imidazole [21] and horse-heart cytochrome *c* [22] that the axis of the g tensor giving rise to the largest g value (g_z) is directed within 15° of being normal to the plane of the heme. The pattern with respect to the in-plane components of the g tensor (g_y and g_x) is less well-defined. Measurements of low spin derivatives of myoglobin [19—22] showed that in all of them the g_y and g_x axes lie at approximately 30° ± 5° with respect to the N-Fe-N direction in the heme plane. In cytochrome *c*, however, the g_y and g_x axes lie at approximately 5° with respect to the N-Fe-N direction. Therefore, it seems that although the orientations of the g_y and g_x axes may be reasonably specific for a given hemeprotein, their absolute orientation cannot as yet be predicted from a priori considerations. In as much as suitable single crystals of cytochrome oxidase are not available, comparable information for the hemes of cytochrome oxidase does not exist. Thus, although the principal axes of the g tensor are perpendicular to each other and their angles with respect to the membrane plane are known, their positions with respect to the heme nitrogen atoms must await the availability of X-ray or electron diffraction analyses at a resolution sufficient to determine the heme orientation.

Our previous studies have shown [6—9] that hydrated oriented multilayers of membranous cytochrome oxidase are cylindrically symmetrical about the normal to the plane of the membranes. Hence, the optical and EPR absorption analyses show that the heme planes of the cytochrome *c* oxidase are randomly oriented about an axis normal to the membrane plane whereas they are highly oriented about any axis parallel to the plane of the membrane. Studies

described in this work provide experimental information on the orientations of the g tensor axes which lie in the plane of the heme (g_x and g_y) in various oxidation and liganding states of the oxidase with respect to the plane of the oriented multilayers. It should be kept in mind while evaluating the results that when the oriented multilayers are rotated through 360° in the magnetic field, the magnitudes of the signal corresponding to the g tensor axis which lies in the plane of the multilayer, or at 90° with respect to it, will exhibit two maxima, 180° apart. On the other hand, for those axes of the g tensor which are directed at any other angle, there will be four maxima during 360° rotation because for each maximum at an angle of x between 0° and 90° there are corresponding maxima at 180° ± x and 360° ± x (or 0° ± x).

It is essential in discussing cytochromes a and a_3 of cytochrome c oxidase to decide on the definition to be used in identifying these cytochromes. It is generally agreed that the cytochrome which reacts with molecular oxygen, carbon monoxide and NO and which, under aerobic steady-state conditions, reacts with the respiratory inhibitors sulfide, cyanide and azide should be called cytochrome a_3. Thus, under the conditions used in this paper it is reasonable to assign the measured low-spin ferric heme azide and sulfide signals to cytochrome a_3. On the other hand, the assignment of the low spin ferric heme signal in the fully oxidized oxidase to cytochrome a is more controversial (see, for review, ref. 5). Therefore, one may ask whether the possible differences in the orientations of the signals of cytochrome oxidase in various states of reduction and/or liganding could be used to help to identify the hemes of cytochromes a and a_3.

The $g = 2.9$ (azide compound) and $g = 2.5$ (sulfide compound) resonances are maximal when the magnetic field is parallel to the plane of the membranes. Thus, the g_z axis of the g tensor for the heme, which according to the definition above is that of cytochrome a_3, lies in the plane of the membrane [7]. The g tensor axes for the $g = 2.2$ (g_y) and $g < 2$ (g_x) signals lie at 90° and 0° respectively, to the plane of the membrane for the azide compound while they are at 0° and 90°, respectively, for the sulfide compound. This reversal of the two in-plane g values probably indicates that interactions of the d_{xz} and d_{yz} orbitals with ligand orbitals are different in the azide and sulfide derivatives and that the g tensor axes have rotated 90° in the heme plane rather than that the heme itself has rotated 90° in its own plane.

The low-spin ferric heme resonance at $g = 3.0$ in the fully oxidized cytochrome oxidase is, like the lowest field g resonance in the azide and sulfide derivatives, maximal when the planes of the membranes are parallel to the magnetic field. Thus the plane of the heme which gives rise to this resonance is also normal to the plane of the membranes. However, comparison of the sulfide and azide compounds with the low-spin ferric heme in fully oxidized cytochrome c oxidase shows marked differences in the orientation of the g tensor axes which lie in the plane of the heme. In the fully oxidized oxidase, the $g = 2.2$ resonance is maximal near 30° with respect to the membrane normal while the $g = 1.5$ resonance is maximal near 60° with respect to the membrane normal (90° with respect to the $g = 2.2$ signal). There are four possibilities to be considered: (1) The same heme is responsible for the low-spin ferric heme resonance in the oxidized oxidase and in the sulfide and derivatives but when the

latter compounds are formed the heme rotates in its own plane by approximately 30°. (2) The same heme is involved as in (1) but the *g* tensor axes have rotated 30° in the plane of the heme. (3) Different hemes, i.e. those of cytochrome *a* and a_3, are responsible for the signals in the fully oxidized oxidase and in the sulfide and azide derivatives, respectively, but the heme of cytochrome *a* is normally rotated within its own plane by approximately 30° with respect to the heme of cytochrome a_3. (4) Different hemes are responsible for the resonances in fully oxidized oxidase and in the sulfide and azide derivatives, respectively, but the heme of cytochrome *a* has its in-plane *g* tensor axes rotated 30° with respect to those of cytochrome *c*.

One possible approach to obtain additional information on the nature of cytochrome *a* and a_3 which may be pertinent to the present discussion is to compare the properties of cytochrome oxidases from higher organisms with those of genetically removed but functionally similar oxidases. If the orientation of the hemes of cytochrome oxidase is important to the mechanism of the reaction, similar behavior should be observed for cytochrome oxidase in oriented multilayers of membranes from *P. denitrificans* for which the respiratory chain is very similar to that of higher organisms [14,16] and for the functionally similar oxidase in oriented multilayers of *Tetrahymena pyriformis* oxidase [23,24].

Comparison of cytochrome oxidase from pigeon breast muscle with cytochrome c oxidase of P. denitrificans and T. pyriformis. In *P. denitrificans* the heme which reacts with sulfide and azide, i.e. according to the definition above this one which corresponds to the heme of cytochrome c_3 is normal to the plane of the membrane and the in-plane axes of the *g* tensor (g_y and g_x) are at 0° and 90° (and 90° and 0°) with respect to the membrane normal. Thus, the oxidized liganded heme in *P. denitrificans* has essentially the same orientation as does the reactive heme in the oxidase from pigeon heart muscle mitochondria.

The ferric heme in the fully oxidized oxidase from both *P. denitrificans* and *T. pyriformis* [25] is also oriented such that its heme plane is normal to the plane of the membrane. However, the in-plane components of the *g* tensor in the hemes of the two oxidases are rotated by different angles with respect to the normal to the membrane. The $g = 2.2$ signal (g_y) is at 50° in *P. denitrificans* and at 90° in *T. pyriformis*, whereas it is at 30° in cytochrome oxidase from pigeon breast muscle mitochondria. It is tempting to conclude that the heme which is responsible for the latter resonances in the various fully oxidized oxidases and shows a 'variability' from one species to the other either in its structure (directions of the g_y and g_x axes in the heme plane) or in the rotational orientation of its plane about an axis in the membrane plane does indeed belong to cytochrome *a*. Although this is our preferred interpretation of the results, the information presented in this work does not, in fact, allow us to draw such a conclusion with any certainty. Similarly, insufficient information on the chemistry of the oxidase prevents us from distinguishing between the possibilities (1)—(4) mentioned above.

The general conclusion which can be drawn from the data is that the operation of cytochrome oxidase probably requires that the hemes of the oxidase must be oriented with their planes normal to the plane of the membrane, since

such orientation is seen in all the species investigated. The elucidation of the geometry of the hemes, their identity and detailed relation with respect to the membrane (rotational angle) must await further studies.

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