ORIENTATION OF THE NO LIGAND OF CYTOCHROME a_3 IN NITROSYL CYTOCHROME *c* OXIDASE

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1. Introduction

Investigations of the site of oxygen reduction in cytochrome *c* oxidase have recently been accelerated by the development of techniques to orient 'membranous' cytochrome *c* oxidase [1,2], submitochondrial particles [3,4] and mitochondria [5,6] into hydrated multilayer arrays. The orientation of the heme prosthetic groups relative to the membrane plane has been determined for several oxidation and liganded states of cytochrome *c* oxidase and for other members of the mitochondrial electron transport chain [1–7]. This work extends these studies to the orientation of the dissociable NO ligand of cytochrome a_3 in nitrosyl cytochrome *c* oxidase. Preliminary account of this work has appeared already [8].

2. Materials and methods

'Membranous' cytochrome *c* oxidase was isolated from pigeon breast mitochondria by a modification [1] of the method in [9]. Aliquots of the oxidase (2–4 mg protein) were diluted in 2 mM phosphate buffer (pH 7.2) and centrifuged onto thin Mylar sheets at 70 000 × *g* for 60 min. The oriented multilayers were formed by slow partial dehydration at 4°C as in [1–3]. The partially dehydrated pellet together with its supporting Mylar was cut into 2 × 10 mm strips which were inserted inside a 3 mm i.d. quartz EPR tube in such a way that two strips were in place and the planes of the supporting Mylar were parallel. The EPR capillary was filled above the level containing the samples with dithionite-saturated solution of 1.2 M sucrose containing 0.1 M Tris (pH 8.5) and 0.1 M $NaNO_2$ (or Na $^{15}NO_2$) (Prochem. Summit, NJ) and incubated for 3–5 min at room temperature. The samples were frozen by immersion of the tubes in liquid nitrogen.

EPR spectra of the oriented nitrosyl cytochrome *c* oxidase multilayers were recorded on a Varian Model E-109 EPR Spectrometer at 100 kHz modulation frequency, a modulation amplitude of 2 G, 15 mW power and a frequency of 9.09 GHz. Sample temperature was maintained at 140 ± 5K using a JOEL constant temperature accessory utilizing liquid nitrogen boil off. The sample was positioned vertically in the EPR cavity for maximum signal. Spectra were then recorded for every 10° ± 5° increment of rotation as measured by a 3 in. protractor, from having the normal to the membrane plane parallel to the direction of the magnetic field until it was perpendicular to the direction of the magnetic field and generally 10° past each extreme.

3. Results

The EPR absorption spectra of oriented nitrosyl cytochrome *c* oxidase multilayer with ^{14}NO and ^{15}NO recorded at 3 different angles with respect to the direction of the magnetic field are shown in fig.1,2. It can be seen that the magnitudes of the *g* tensor components and the hyperfine lines exhibit strong dependence on the direction of the applied magnetic field. When the multilayer is rotated through an angle of 120° (fig.3) the lowest field *g* resonance at $g=2.09$ is maximal when the magnetic field is parallel to the

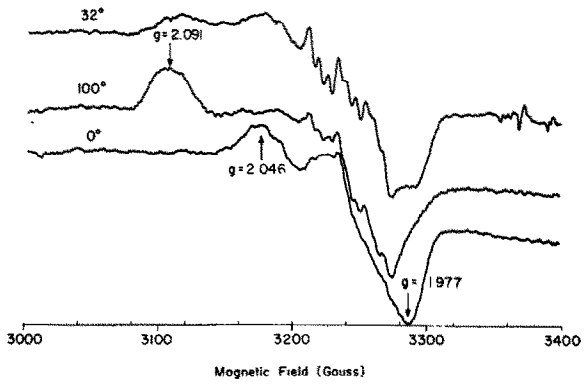

Fig.1. EPR spectra from a nitrosyl (^{14}NO) cytochrome *c* oxidase in oriented multilayers of the 'membranous' enzyme for 3 different angles between the magnetic field direction and the normal to the membrane plane. Experimental conditions are given in section 2.

planes of the membranes whereas it is minimal when the membranes are normal to the magnetic field. The resonances at g=2.046 and 1.977 exhibit opposite orientation with respect to the g=2.09 signal, i.e., they are maximal when the membranes are normal to the direction of the magnetic field and minimal when the membranes are parallel to the magnetic field. (Figure 3 presents averaged values from three independent experiments.)

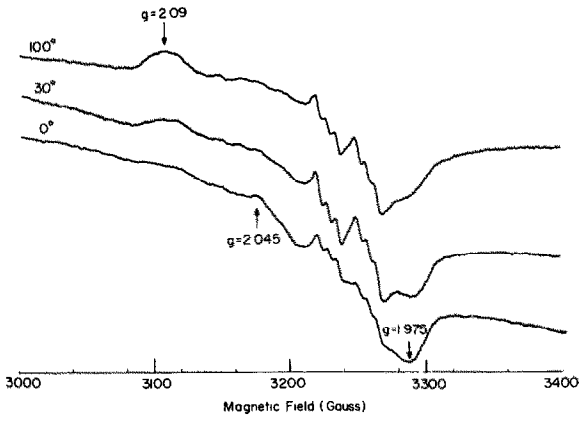

Fig.2. EPR spectra from a nitrosyl (^{15}NO) cytochrome *c* oxidase in oriented multilayers of the 'membranous' enzyme for 3 different angles between the magnetic field direction and the normal to the membrane plane. Experimental conditions are given in section 2.

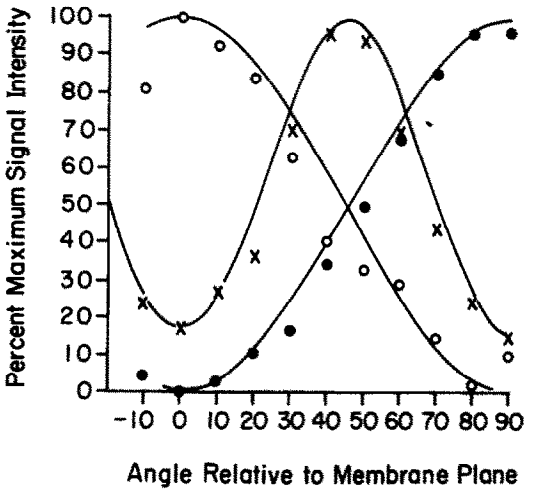

Fig.3. Plot of relative EPR signal intensities for nitrosyl cytochrome *c* oxidase membranes as a function of angle between the magnetic field direction and the normal to the membrane plane. The EPR spectra were recorded under the conditions in section 2. The g=1.97 resonance follows exactly the behaviour of the g=2.046 signal. Each point is the average value from 3 independent experiments. (○) g=2.046; (●) g=2.09; (X) hyperfine amplitude.

Hyperfine coupling is well resolved with coupling constant of 21.1 G for ^{14}NO and 29.5 G for ^{15}NO. The hyperfine pattern from NO is not a simple triplet for ^{14}NO and doublet for ^{15}NO but exhibits 9 lines for ^{14}NO and 6 lines for ^{15}NO with additional coupling constant of 6.8 G for the superhyperfine splitting. Maximum intensity of the hyperfine lines is observed at neither a parallel nor normal orientation of the membranes in the magnetic field but at an angle of 45°.

4. Discussion

Oriented multilamellar arrays provide a valuable means of obtaining structural information from membrane proteins which resist purification or crystallization. The oriented membranes formed from cytochrome oxidase have cylindrical symmetry with the unique axis being normal to the plane of the membrane [1]. In EPR studies of oriented membranes all angles are referenced to this unique direction. Studies of the orientation of the heme planes of cytochromes *a* and a_3 in these oriented membranes showed in fully

oxidized and reduced oxidase and in the presence of various ligands that both hemes were oriented with the normal to the heme plane being perpendicular to the normal to the membrane plane [1–3]. Moreover it was demonstrated that the orientation of the g resonances which correspond to the g_x and g_y axes of the g tensor were different in the low spin ferric heme in fully oxidized oxidase [3,4] and in half-reduced liganded oxidase [2,3]. This was used to support the contention that the resonances seen in the two different oxidase states do belong to two different hemes, those of cytochrome a and a_3 [3].

The EPR spectrum of the oriented nitrosyl cytochrome oxidase exhibits rhombic symmetry with g values at 2.09, 2.05 and 1.97 assigned to g_x, g_z and g_y, respectively [10]. Of the principal g values the g=2.005 was assigned to g_z because it was closest to the free electron value and because it showed the hyperfine splitting of NO nitrogen [11]. The assignment of the g_x and g_y axes was made arbitrarily [10,11]. The results presented here provide further support for this assignment. The highest field g tensor at g=1.97 is maximal when the planes of the membranes are oriented normal to the direction of the magnetic field. Because the plane of the cytochrome a_3 heme is normal to the plane of the membrane [1–6] such behaviour is expected for a resonance arising from one of the g tensor components which lie in the plane of the heme (i.e., either g_x or g_y). The g=2.09 resonance is at 90° with respect to the g=1.97 signal which is in agreement with its assignment to the other in-plane component of the g-tensor. These results do not allow us, however, to assign the appropriate resonance to the invidual axes. This is because the geometry of the oriented multilayers permits the determination of the orientation only with respect to the plane of the membrane whereas the position of the g tensor axes with respect to the heme nitrogen atoms requires the knowledge of the orientation of the heme within the protein molecule.

The angular dependence of the g=2.005 is more difficult to assess because of the superimposed changes in the hyperfine structure. Inspection of fig.1,2 shows, however, that the g=2.005 resonance is maximal when the planes of the oriented membranes are parallel to the magnetic field (and minimal when they are normal to the magnetic field) which is in agreement with its assignment to the g_z axis of the g tensor. The g=2.046 resonance seen in the spectra (fig.1–3) has previously been observed [12] in the nitrosyl derivatives of myoglobin, catalase and cytochrome c and designated $g_?$ because of its unknown origin. Our results show that this resonance is also present in the highly purified preparation of cytochrome c oxidase [1,9] and is highly oriented with respect to the planes of the multilayers.

The hyperfine distribution is typical for covalently-bound NO with an unprotonated nitrogenous *trans* ligand on the heme [10,11]. The shift in hyperfine coupling constant from 21.1 to 29.5 G and number of hyperfine lines from 9 to 6 upon replacing ^{14}NO with ^{15}NO is consistent with the shift in magnetogyric ratio and nuclear spin of ^{14}N (I=1 and V_n = 1.934) and ^{15}N (I=1/2 and V_n = 2.712) (12). The smaller hyperfine coupling constant of 6.8 G is in the 6.4–6.8 G range found for several other nitrosyl heme proteins [12].

The angle of the hyperfine tensors was used [13–15] to evaluate the Fe–N–O bond angle in single crystals of nitrosyl myoglobin and hemoglobins. The angular dependence of the hyperfine intensity, seen in fig.3, shows maximum intensity at 45° to the membrane normal and minimum intensity at 0° and 90°. The 45° angle between the direction of maximum hyperfine intensity and the membrane normal in nitrosyl cytochrome c oxidase membranes and the previous findings that the normal to the cytochrome a_3 hemes is perpendicular to the normal to the membrane plane suggest a Fe–N–O bond angle 135° in the direction of the normal to the membrane.

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