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Conformation of the c_{552}:aa_3 electron transfer complex in *Paracoccus denitrificans* studied by EPR on oriented samples

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Abstract The EPR spectral parameters of aa_3 oxidase and cyt c_{552} from *Paracoccus denitrificans* were studied in purified oxidase and enriched cyt c_{552}. The orientation of the *g*-tensors of hemes *a* and c_{552} were determined on partially ordered membranes, enriched cyt c_{552} and a c_{552}:aa_3 subcomplex. The known correlation of *g*-tensor to molecular axes in histidine/methionine ligated hemes permits us to position cyt c_{552} with respect to the parent membrane. Taken together with previous data on the interaction surface between aa_3 oxidase and cyt c_{552}, these results allow us to arrive at a single conformation for the c_{552}:aa_3 electron transfer complex.

Keywords: Cytochrome c_{552}; Electron transfer; Supercomplex; aa_3 oxidase; *Paracoccus denitrificans*

1. Introduction

In aerobically grown *Paracoccus denitrificans*, the cytochrome bc_1 complex and the aa_3-type cytochrome oxidase have been shown to be arranged in a stoichiometric supercomplex and can be purified as such [1,2]. Electron transfer between cyt c_1 and subunit II of the oxidase is mediated by a membrane-bound cytochrome c_{552} which is an integral part of this supercomplex. A soluble form of cyt c_{552} lacking the N-terminal transmembrane helix has been heterologously expressed in *Escherichia coli* and its structure has subsequently been solved by X-ray crystallography and by NMR in both redox states [3–5]. Site-directed mutagenesis analyses, NMR spectroscopy on a transient complex between the soluble domain of cyt c_{552} and a soluble fragment of cyt aa_3 subunit II [6–8] and in silico docking studies [9] have been performed to elucidate the conformation of the electron transfer complex. So far, no unambiguous solution was arrived at [9].

EPR spectroscopy on partially ordered membrane multilayers can yield information on the geometry of certain redox centres with respect to the membrane [10–14]. The situation is particularly advantageous in the case of histidine/methionine ligated *c*-type cytochromes for which a complete attribution of paramagnetic to molecular axes has been established via single crystal EPR studies on cytochrome *c* from equine heart [15]. This correspondence of axes was later shown to hold for other *c*-type hemes with his/met ligation [11].

In order to provide geometric constraints for modelling of the electron transfer complex between cyt c_{552} and its interaction partners, we have determined the orientation of this cytochrome in native cytoplasmic membranes as well as in a cyt c_{552}:aa_3 subcomplex.

2. Materials and methods

Wild-type *P. denitrificans* (from the Collection des Bactéries de l'Institut Pasteur, CIP 71.11) was grown according to Ludwig [2]. Cytoplasmic membranes were prepared by lysozyme treatment and osmotic lysis essentially as described in [16]. Cytochrome oxidase aa_3 was purified as described in [17] by subsequent chromatographies on DEAE-Sepharose CL-6B and Source 30 Q (Pharmacia) columns, and eluted at 280 mM NaCl in a 200 mL linear gradient of 250–350 mM NaCl. Fractions enriched in cytochrome c_{552} were obtained after the DEAE-Sepharose CL-6B step and eluted at 240 mM NaCl in a 500 mL linear gradient of 220–340 mM NaCl. Solubilised subcomplex containing both cytochrome c_{552} and aa_3 oxidase in about equal amounts, as determined spectroscopically (see below) were eluted after DEAE Sepharose CL-6B typically around 265 mM NaCl. Samples were concentrated and washed using an ultrafiltration cell (Ultrafree, Millipore), frozen in aliquots and stored at −70 °C.

Protein concentrations were measured using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). Concentrations of aa_3 oxidase and cyt c_{552} were determined spectroscopically (on a Shimadzu 1601 UV/Vis spectrometer) using extinction coefficients of 23.4 mM^{-1} cm^{-1} at 605 nm and of 20 mM^{-1} cm^{-1} at 552 nm, respectively. Reduced-minus-oxidized difference absorption spectra were recorded between 500 and 650 nm according to Haltia et al. [16]. Reduction and oxidation were achieved by addition of ascorbate and ferricyanide to sample buffered to pH 7.5 by 50 mM Tris/Cl.

Oriented multilayers were obtained by partial dehydration of membrane fragments or detergent solubilised sample onto mylar sheets as described by Rutherford and Setif [10]. EPR spectra were recorded on a Bruker ESP 300 X-band spectrometer fitted with an Oxford Instruments liquid helium cryostat and temperature control system.

The published 3D structures of cytochrome c_{552} and aa_3 oxidase from *P. denitrificans* (pdb-entries 1QL4 and 1QLE, respectively) were analysed and represented using the Swiss PdbViewer (version 3.7; http://www.expasy.ch/spdbv).

3. Results

3.1. Identification of aa_3's and cyt c_{552}'s EPR spectra

Almost 30 years ago, Erecinska et al. published an EPR study on ordered membrane multilayers from *P. denitrificans*

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[18]. In their work, only one low spin paramagnetic species was detected and attributed to heme *a*. Since both cyt c_{552} and the cytochrome bc_1 complex are now known to be present in significant proportions in *Paracoccus* membranes [1], we tried to identify the corresponding EPR spectra of these redox centres. Fig. 1 shows a comparison of the EPR signals resulting from low spin heme compounds in purified aa_3 oxidase (top spectrum) and enriched cyt c_{552} (bottom spectrum) to those of membrane fragments and a solubilised subcomplex containing both c_{552} and the aa_3 oxidase. From these spectra, all three *g*-values of both centres could be determined ($g_z = 2.96$, $g_y = 2.23$, $g_x = 1.51$ for cyt c_{552} and $g_z = 2.83$, $g_y = 2.29$, $g_x = 1.6$ for heme *a*).

The spectrum recorded on membrane fragments showed that the g_z-peak of cyt c_{552} largely dominated the signal arising from heme *a* which appears as a pronounced shoulder on the high field side of the g_z-line of cyt c_{552}. A comparison of the *g*-values described above to those published in [18] demonstrated that in the earlier work the g_z-peak of cyt c_{552} was misinterpreted as arising from heme *a*. The $g_y = 2.29$ signal, by contrast, was correctly attributed to heme *a*, whereas cyt c_{552}'s g_y-line escaped detection due to the strong Mn-signal present in the *Paracoccus* aa_3 oxidase [19]. The g_x-troughs were not picked up in the study by Erecinska et al.

3.2. Orientation of heme a's and cyt c_{552}'s g-tensor axes in native membranes and in the cyt c_{552}:aa_3 subcomplex

Partially ordered multilayers were produced both with membrane fragments and with the solubilised c_{552}:aa_3 subcomplex. Representative spectra recorded at selected magnetic field orientations with respect to the mylar sheet are shown in Fig. 2. Polar plot evaluations of signal intensities at all detectable *g*-values in these two samples are depicted in Fig. 3. The *g*-tensor directions of heme *a* were found to be oriented at 0° (g_z, filled squares), 40° (g_y, filled diamonds) and 50° (g_x, stars) with respect to the mylar sheet in the c_{552}:aa_3 subcomplex. The same

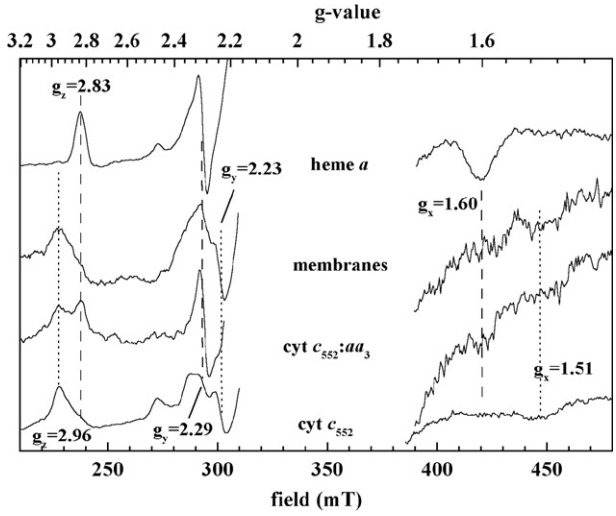

Fig. 1. Comparison of EPR spectra arising from low spin heme compounds in (from top to bottom) purified aa_3 oxidase, membrane fragments, the c_{552}:aa_3 subcomplex and a sample strongly enriched in cyt c_{552}, all from *Paracoccus denitrificans*. The high field part of the spectra is expanded by a factor of 4. EPR settings: microwave frequency, 9.44 GHz; temperature, 15 K; microwave power, 6.3 mW; modulation amplitude, 3.2 mT.

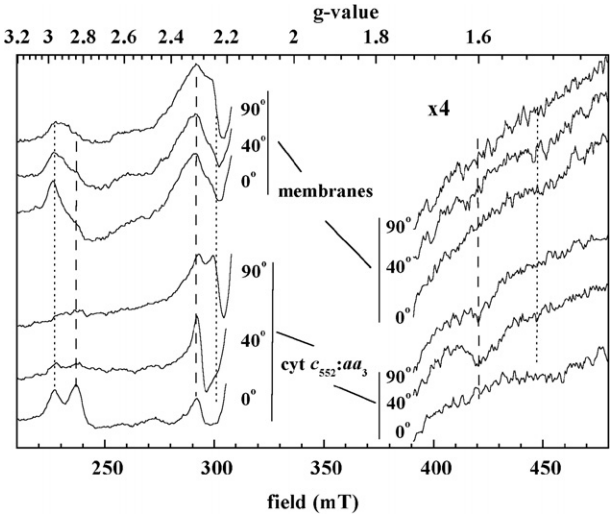

Fig. 2. EPR spectra recorded on partially oriented multilayers obtained from (top three spectra) membrane fragments and (bottom three spectra) the c_{552}:aa_3 subcomplex from *Paracoccus denitrificans*. For the sake of simplicity, only representative spectra at three orientations (0°, 40°, 90° angle between the magnetic field vector and the plane of the mylar sheets) are shown. Data points obtained at all orientations measured are shown in the polar plots of Fig. 3. As in Fig. 1, the high field part of the spectra is expanded by a factor of 4. EPR settings are as given in the legend to Fig. 1.

orientations were seen in the oriented membrane sample for the g_z and g_y directions (Fig. 3A, open symbols). The g_x-signal was too weak to be reliably evaluated. Membrane fragments dry down onto mylar sheets so that the membrane plane and the mylar sheets are coplanar and orientations of *g*-directions with respect to the sheets are thus equivalent to those with respect to the membrane [10]. Since g_z is roughly parallel to the heme normal [15], an orientation of 0° for heme *a*'s g_z-direction is in line with the crystallographically determined 3D structure in which the plane of heme *a* is perpendicular to the membrane.

The coincidence of heme *a*'s *g*-directions in membrane fragments and in the solubilised and ordered subcomplex demonstrates that the latter sample arranges itself in a geometry resembling that of the native membranes. Upon detergent-depletion, individual subcomplexes probably stick together at their hydrophobic (membrane-exposed) surfaces forming large sheets of aggregated protein which subsequently dry down on the mylar surface just as native membranes do. This phenomenon has been observed previously on several detergent solubilised complexes [10,12,13].

Fig. 3B shows the orientations of cyt c_{552}'s principal *g*-tensor directions. In the subcomplex, g_z was found to point parallel to the membrane indicating that c_{552}'s heme plane is roughly perpendicular to the membrane, similar to what is observed for heme *a*. The g_y-axis is perpendicular implying that g_x (which is of small amplitude) must be maximal when the field is parallel to the membrane.

The oriented membrane sample shows a globally similar orientation dependence for cyt c_{552}'s *g*-axes. For the g_z-direction, the polar plots obtained on membranes and on the subcomplex almost superimpose. By contrast, the polar plot of the g_y-signal in membranes is significantly less well defined in the membrane sample. Whereas the lobe still points towards 90°, more