Asp-193 and Glu-218 of subunit II are involved in the Mn^{2+}-binding of *Paracoccus denitrificans* cytochrome c oxidase

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Abstract Cytochrome c oxidase contains a binding site for a non-redox-active metal at the interface of subunits I and II, usually a magnesium ion. In *Paracoccus denitrificans* oxidase, typically 20% may be replaced by manganese, using standard growth media. Site-directed mutants were constructed in subunit II (D193N and E218Q), and the isolated enzymes analyzed by total-reflection X-ray fluorescence spectrometry and EPR. Both mutants show a strong reduction of the manganese stoichiometry and a diminished electron transfer activity, demonstrating that D193 and E218 are involved in the binding of a manganese/magnesium ion in this site.
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Key words: Terminal oxidase; Magnesium-binding site; Electron transfer; Non-redox-active metal

1. Introduction

Cytochrome c oxidase (haem aa_3) (EC 1.9.3.1) is the terminal enzyme in the mitochondrial and many bacterial respiratory chains (for reviews see [1–3]). In *Paracoccus denitrificans*, two more terminal oxidases, an alternative cytochrome oxidase (cbb_3) [4] and a quinol oxidase (ba_3) [5], are members of a branched respiratory chain. Its cytochrome aa_3 consists of 4 subunits: subunits I–III are homologous to the mitochondrially encoded subunits of eukaryotic cytochrome oxidases [3,6]. Subunit I contains haem a and the binuclear centre (haem a_3·Cu_B) where oxygen reduction takes place. Subunit II comprises the 2 copper ions of the Cu_A centre which is the entry point for electrons donated from cytochrome c [7]. The role of subunit IV remains unknown since its deletion has no detrimental effects on its functional properties [8].

In addition to the redox-active metals, cytochrome c oxidases contain a non-redox-active metal ion not observed in quinol oxidases ([9]; and Gennis, personal communication). In the mitochondrial oxidase a magnesium ion is found consistently in stoichiometric amounts [10] while in bacterial oxidases manganese replaces the magnesium in substoichiometric amounts. The function of this non-redox-active metal ion is not known.

In the *Paracoccus* oxidase manganese occupies this site to 20–30%, and it has been shown that it acts as a reporter group for a redox-linked conformational change of the enzyme [11–13]. In many enzymes manganese can substitute for magnesium without loss of activity probably due to similar coordination properties of both ions. As magnesium is EPR silent, manganese can be used as a spectroscopic probe to investigate the occupancy of this site.

In cytochrome c oxidase the binding site for the manganese seems to be located in an acidic cluster between subunits I and II [12,14]. The crystal structures from bovine heart and *P. denitrificans* reveal Asp-399, Asp-404 and one propionate side chain of haem a_3 from subunit I to be located in this cluster together with Glu-218 and Asp-193 from subunit II [15,16]. In *Rhodobacter sphaeroides*, His-403, situated in the centre of the cluster, together with Asp-404 (subunit I; *Paracoccus* numbering) have been shown as binding ligands of the manganese [14].

Glu-218 of subunit II ligands Cu_A via the peptide carbonyl group and at the same time, seems a likely metal-binding partner via its carboxylate group, as can be seen in the crystal structure of the bovine enzyme [15]. The location of the manganese in the *Paracoccus* enzyme structure has not been detected due to low occupancy [16]. However, the crystal structure shows that Asp-193 is in close vicinity of this site though it may not directly be in contact with the manganese.

In this study, Glu-218 and Asp-193 were mutated to Gln and Asn respectively, by site-directed mutagenesis. The individual mutants were analysed by EPR and the metal content determined by TXRF. The results show that both mutants have lost their capability to bind manganese, demonstrating that both residues have a functional role in the binding of the metal ion.

2. Materials and methods

2.1. Strains, mutagenesis and enzyme preparation

Site-directed mutagenesis on the cloned *cta*C-E operon was performed according to the altered site mutagenesis protocol (Promega, Heidelberg). Complementation of the oxidase-deficient deletion mutant ST4 was performed as described previously [17]. Mutant strains were grown on succinate medium (containing manganese in the trace element solution) [18] including streptomycin sulfate (25 μg/ml). Membranes were isolated according to [19] and solubilized with dodecyl-β-D-maltoside. The protein was purified as described earlier [20] with all buffers containing 1 mM EDTA. SDS-PAGE, Western blotting and cytochrome c oxidase difference spectra were carried out as outlined previously [17].

2.2. Steady-state kinetics

Activity was assayed spectroscopically in 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 1 mM EDTA and 0.2 g/l dodecyl maltoside at 25°C as described earlier [17].

2.3. TXRF

The metal content was determined using an EXTRA II A (Atomika Instruments, Oberschleißheim) as described earlier [21]. The final buffer was changed by gel filtration to 100 mM Tris-acetate, 1 mM

EDTA, 0.2 g/l dodecyl maltoside pH 8 to avoid any interference of the sulphur determination by phosphate or chloride. The initial enzyme concentration was 50 μM. After addition of an internal standard (Rb; 100 ppm), 5 μl of the oxidase solution was dried on the sample carrier to a thin film before measurement.

2.4. EPR spectra

A Bruker ESP300E X-band spectrometer with Bruker dual mode cavity in perpendicular mode and an Oxford ESR910 helium flow cryostat were used for the EPR experiments. The reading of the temperature controller was calibrated against temperature measurements with an internal probe.

3. Results and discussion

To investigate the contribution of subunit II residues to the manganese-binding site of cytochrome c oxidase in P. denitrificans, two individual mutants were analyzed. Glutamate 218 was originally assumed to be involved in cytochrome c binding [17] and had previously been changed to glutamine. However, the crystal structure revealed that the carbonyl oxygen of this glutamate is one of the Cu_A ligands, and at the same time, its carboxylate side group is located within an acidic cluster. The second residue, aspartate 193, was changed to asparagine by site-directed mutagenesis, and both mutant oxidases were purified.

The manganese content of the mutants and wild type was determined by TXRF (see Table 1). While the manganese occupancy of the wild type was about 20% which is comparable to other investigations [10–12], the manganese stoichiometry of the mutants was reduced to below 5%. Clearly, the ability of both mutants to bind manganese was decreased. In eukaryotic oxidases the metal ion-binding site is occupied by magnesium while in the case of P. denitrificans and R. sphaeroides, the two metal ions (Mg^{2+}/Mn^{2+}) obviously compete for this binding site. For Rhodobacter, it was shown that varying the Mg/Mn ratio in the medium changed the ratio of bound manganese to the oxidase without any loss of activity [13].

The EPR visibility of the manganese makes it an ideally suited spectroscopic probe for the investigation of metal ion-binding sites. EPR spectra were recorded of wild type and mutants (see Fig. 1). The spectrum of the wild type can be attributed to tightly bound manganese [11] while the spectra of D193 and E218, within their noise level, show no presence of manganese. The spectra of the mutants exhibit the typical Cu_A signal. This is also observed for the Paracoccus wild-type enzyme when the strain was grown on a medium with low manganese content [11,22], while under normal growth conditions the manganese signal overlays the one of Cu_A. Together with the TXRF results, the EPR spectra dem-
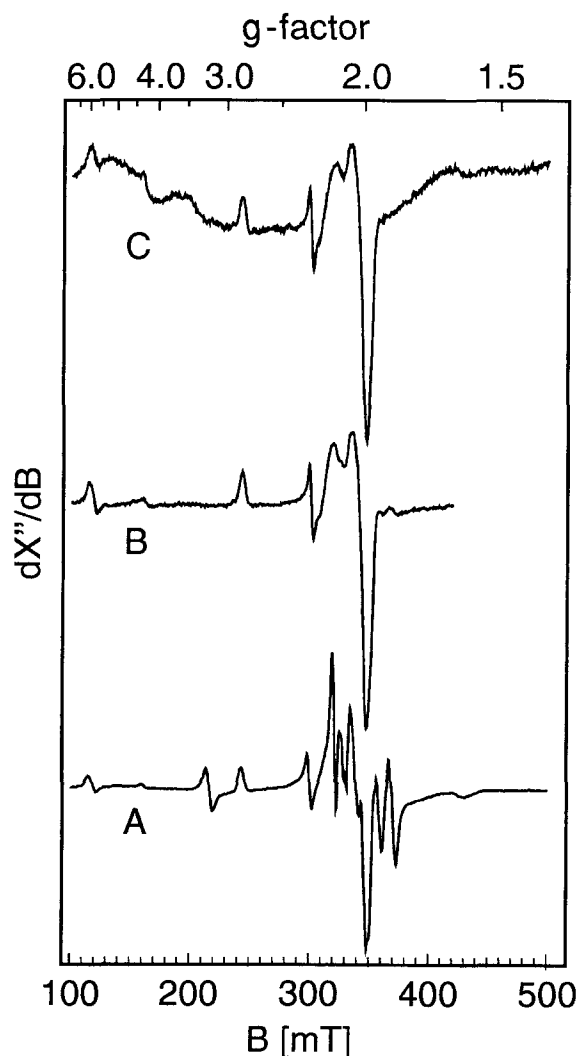

Fig. 1. Comparison of EPR spectra for: A: wild type; B: E218Q; and C: D193N. Spectra were taken at 10 K at a microwave frequency of 9.6471 (A), 9.6552 (B), and 9.6401 GHz (C); microwave power 100 μW (A), 200 μW (B), 1 mW (C); modulation amplitude 1.1 (A), 1.2 (B), and 1 mT (C); modulation frequency 100 kHz (A–C). Enzyme concentration was 60 μM.

onstrate that D193N and E218Q have lost their capability to bind manganese.

In addition, comparison of the mutant spectra to wild-type copper spectra (see above) shows that the Cu_A centre of the mutants remains undisturbed. It was demonstrated before that upon reduction of the oxidase the EPR signal of the manganese changes which is triggered by the reduction of Cu_A and may reflect a conformational change of the oxidase [12]. E218 is one of the ligands of the Cu_A centre via its carbonyl oxygen [16]. Removing the negatively charged carboxylate group by exchanging the glutamate against glutamine leads to the loss of manganese. By this structural role, E218 is the direct link between the Cu_A centre and the manganese-binding site.

Whereas the proton pumping capacity is identical for wild type and for both mutants (not shown), their turnover numbers are lowered to about 70–80% (see Table 1). As the reduction of Cu_A is sensed by the manganese as can be seen in the EPR spectrum, loss of the manganese-(II) in turn could

Table 1
Manganese occupancy (%) of the oxidase determined by TXRF, and the turnover number of the purified enzymes measured at 20 μM cytochrome c

	Mn^{2+} Occupancy[a] (%)	Turnover number (s^{-1})
wild type	21	459
D193N	4.2	318
E218Q	4.5	358

[a]Mean of 3 independent measurements; the oxidase concentration (haem aa_3) was calculated by dividing the sulphur concentration by the number of the sulphur containing amino acids, methionine and cysteine.

influence the electron flow at Cu_A via E218. A mutual cross-talk of both metal ions via E218 cannot be excluded.

The acidic cluster can be regarded as a sandwich-like structure of two negatively charged surfaces provided by subunits I and II. The positively charged manganese/magnesium ion probably acts as a charge compensation bracing the two surfaces together. Removal of this ion would loosen the structure due to repulsion of the negative charges. This in turn could impede the direct electron transport between Cu_A and haem *a* by increasing the inter-subunit distance.

The results of the TXRF and EPR clearly indicate that D193 and E218 are essential for the binding of manganese. The crystal structure suggests that E218 together with the two residues identified for subunit I [14] provide at least part of the metal ion-binding site in cytochrome *c* oxidase. However, according to the crystal structures ([15]; and C. Ostermeier, A. Harrenga and H. Michel, unpublished), the distance between D193 to the metal ion is too large to act as a direct ligand. Two explanations appear feasible at the moment: (i) either D193 is involved in metal binding via a water molecule; involvement of a water molecule has already been suggested earlier by Haltia [12]; (ii) alternatively, the negative charge is required during the assembly process and the insertion of the metal ion.

Combining mutant data for subunit I [14] and for subunit II (this study), 4 ligands for this binding site are now defined. At present, however, we cannot decide on the question of coordination number, which will only be answered conclusively by a high-resolution structure of oxidase.

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