Mutations in the Ca^{2+} binding site of the *Paracoccus denitrificans* cytochrome *c* oxidase

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Abstract Recent structure determinations suggested a new binding site for a non-redox active metal ion in subunit I of cytochrome *c* oxidase both of mitochondrial and of bacterial origin. We analyzed the relevant metal composition of the bovine and the *Paracoccus denitrificans* enzyme and of bacterial site-directed mutants in several residues presumably liganding this ion. Unlike the mitochondrial enzyme where a low, substoichiometric content of Ca^{2+} was found, the bacterial wild-type (WT) oxidase showed a stoichiometry of one Ca per enzyme monomer. Mutants in Asp-477 (in immediate vicinity of this site) were clearly diminished in their Ca content and the isolated mutant enzyme revealed a spectral shift in the heme *a* visible absorption upon Ca addition, which was reversed by Na ions. This spectral behavior, largely comparable to that of the mitochondrial enzyme, was not observed for the bacterial WT oxidase. Further structure refinement revealed a tightly bound water molecule as an additional Ca^{2+} ligand.

Key words: Heme-copper oxidase; Heme *a* propionate; Non-redox active metal; Total-reflection X-ray fluorescence spectrometry; Site-directed mutagenesis; Structure refinement

1. Introduction

The heme aa_3-type cytochrome *c* oxidase (COX) is a membrane-bound terminal enzyme of the respiratory chain in mitochondria and many bacteria. It catalyzes the reduction of molecular oxygen and couples this reaction to proton translocation across the membrane [1–5]. While the bacterial and the mitochondrial enzymes differ strikingly in subunit complexity, structural data reveal close homologies in their mechanistically relevant subunits I and II [6–8]. During catalytic turnover, electrons are donated by cytochrome *c* to the bis-copper Cu_A center located in subunit II of oxidase and are subsequently transferred to the metal centers of subunit I, a low-spin heme *a* and the binuclear heme a_3/Cu_B center where water formation takes place.

A new binding site for a non-redox active metal ion has recently been deduced from the structure determination of both the mitochondrial [9] and the *Paracoccus* enzyme [10] located near the periplasmic end of the first transmembrane helix of subunit I and is assumed to be occupied by either a Ca or a Na ion.

Ca^{2+} binding by COX was first proposed by Wikström and collaborators [11,12], who described a spectral shift of heme *a* induced by Ca^{2+} in the reduced mammalian enzyme. The authors reported that no other metal cation could exert this spectral shift but that protons compete with Ca^{2+} and postulated electrostatic binding of Ca^{2+} to a heme *a* propionate. Subsequent experiments of the Moscow group [13–15] showed that the Ca^{2+} and proton-induced spectral shifts of heme *a* are reversed competitively by Na, but not by K ions. With similar ionic radii for Ca^{2+} and Na^+, these authors proposed a coordination-type cation binding site in COX accomodating a Ca^{2+}, Na^+ or possibly a hydronium ion. Surprisingly, Ca^{2+} showed no effect on the absorption spectra of the bacterial (*Paracoccus denitrificans* or *Rhodobacter sphaeroides*) and the yeast enzyme [15], pointing at species-specific differences in the binding site(s).

In this study, we analyze both the relevant metal ion content and the spectral shift upon Ca binding for the wild-type (WT) *P. denitrificans* enzyme and for mutants in amino acids liganding the metal ion. We show conclusively that in contrast to the mitochondrial enzyme, Ca^{2+} occupies this site in the isolated bacterial WT enzyme stoichiometrically. On replacing one of the ligands, Ca^{2+} is lost from the enzyme complex, but may be titrated back leading to perturbation of the spectrum of heme *a*, analogous to that observed for the bovine COX. Further structural refinement of the *P. denitrificans* COX reveals a tightly bound water as one of the oxygenous ligands to the Ca ion. Preliminary results of these studies were presented at the Marburg International Colloquium on Cell Respiration in September 1998.

2. Materials and methods

Specific mutations were introduced by site-directed mutagenesis as described earlier [16].

For protein expression, the mutated *cta*DII gene encoding subunit I of the aa_3-type cytochrome *c* oxidase of *P. denitrificans* was cloned into a derivative of the broad host-range vector pBBR1MCS [17]. Besides the streptomycin resistance gene [18] as a selection marker, the promoter region preceding the *cta*C-G operon [19] was cloned

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Abbreviations: TXRF, total-reflection X-ray fluorescence spectrometry; COX, heme aa_3-type cytochrome *c* oxidase; WT, wild-type

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upstream of the *cta*DII gene, leading to WT expression levels for cytochrome *c* oxidase in the recipient, AO1. This *P. denitrificans* strain carries a deletion of both the *cta*DI and *cta*DII genes encoding subunit I [20], as well as the insertion of the gentamycin resistance gene [21,22] in the operon encoding the alternative cbb_3-type cytochrome *c* oxidase [23].

The four-subunit COX was purified after solubilization of bacterial membranes with *n*-dodecyl-β-D-maltoside (Biomol, Hamburg, Germany) and binding to the specific F_v antibody fragment [24] carrying the streptavidine tag for affinity purification [25]. Excess antibody fragments were removed by gel filtration. Cytochrome *c* oxidase mutants were characterized for their subunit composition by SDS gel electrophoresis and Western blotting, for electron transfer [16] and proton translocation activities [22,26,27], and by optical spectroscopy in the visible region.

The mitochondrial oxidase was purified from beef heart as published [28,29]. In this procedure, deoxycholate and cholate as well as phosphate buffers are omitted. The cytochrome *c* oxidase is solubilized instead and chromatographed in dodecyl maltoside. This procedure leads to an enzyme preparation which is pulsed and shows fast and monophasic cyanide binding kinetics [29].

Metal ion contents of the purified enzyme complexes were determined by total-reflection X-ray fluorescence spectrometry (TXRF) measurements in a Tris-acetate buffer containing 0.2 mg/ml dodecyl maltoside and 1 mM EDTA, essentially as published [30], and metal to protein stoichiometries calculated on the basis of its known content of sulfur-containing amino acids.

Measurements of the spectral shifts induced by Ca^{2+} and Na^+ in the purified enzymes were performed as described earlier [13,15] in an SLM-Aminco dual-wavelength/split beam spectrophotometer. Further details are specified in the legend of Fig. 2.

Crystallographic refinement of the two-subunit cytochrome *c* oxidase complexed with an antibody F_v fragment was carried out starting with the published coordinates and structure factors [10] with the program CNS [31], version 0.3, using the Engh and Huber set of stereochemical parameters [32]. The resulting crystallographic *R*-factor is 19.9% (R_{free} = 23.3%).

3. Results and discussion

3.1. Ca occupancy in the isolated mitochondrial and bacterial WT and mutant enzymes as determined by TXRF

When the isolated bacterial COX and the bovine enzyme were analyzed for their Ca^{2+} content (Table 1), a striking difference in the molar ratio of Ca/enzyme monomer was noted. With both other redox active metals determined in their expected stoichiometries (3 Cu and 2 heme-Fe) for the bacterial and the mitochondrial complex, the *P. denitrificans* enzyme consistently yielded a stoichiometric calcium value, while the bovine COX only showed a low Ca occupancy. Both enzymes were treated identically in their final chromatographic preparation step for TXRF, consisting of a gel filtration chromatography in the presence of 1 mM EDTA for buffer exchange (see Section 2). TXRF allows for the simultaneous quantification of several elements of specific relevance

Table 1
Stoichiometries of selected metal ions in isolated COX complex monomers as determined by TXRF

Enzyme/mutant	Cu/ oxidase	Fe/ oxidase	Cu/ Fe	Ca/ oxidase
Bovine COX	3.2	2.3	1.36	0.23
P. denitrificans COX, WT	2.9	2.0	1.45	1.0
E56A	2.7	2.1	1.29	1.4
Q63A	2.9	2.1	1.38	1.1
E56A/Q63A	2.9	2.2	1.32	1.2
D477A	2.7	2.0	1.36	0.3
E56A/Q63A/D477A	2.4	1.8	1.33	0.2
D477R	3.2	2.2	1.44	0.2

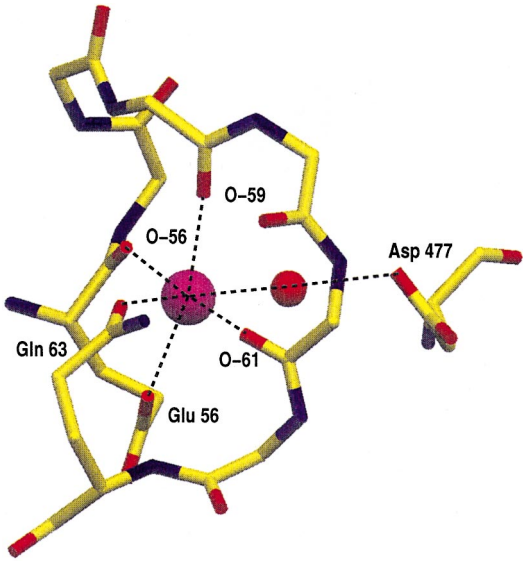
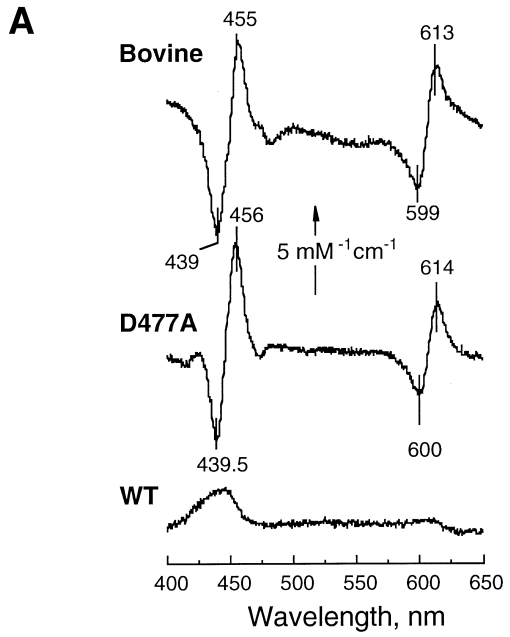


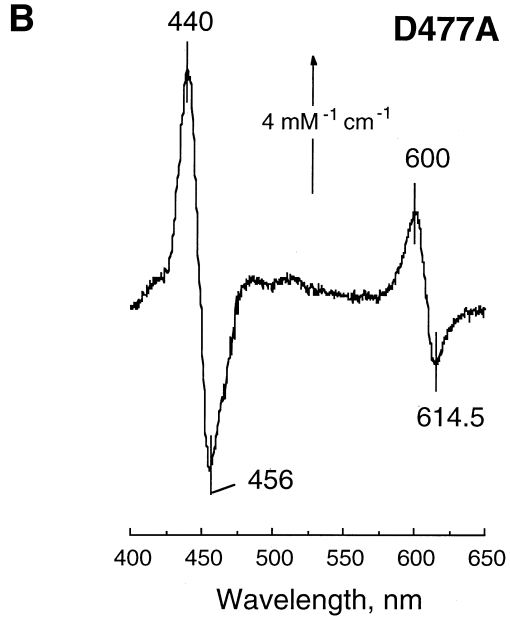
Fig. 1. Schematic representation of the Ca^{2+} binding site in the *P. denitrificans* cytochrome *c* oxidase. The figure was prepared using the program SETOR [33]. For further details, see Section 2.

to oxidase (Fe, Cu, Mn, Zn and Ca: [30]), their internal ratios and their stoichiometries per monomer complex when based on the sulfur value derived from the same determination. However, due to its relatively low sensitivity for Ca, experimental values in the range of 0.2 (see also below) run close to background in our assay system, thereby rather representing an upper limit value. We conclude that one Ca ion is firmly bound to the bacterial COX, despite prolonged exposure to chelating agents, while the bovine enzyme under comparable standard isolation procedures only shows a residual Ca content.

While the suggested metal binding sites in the two known COX structures [9,10] are not strictly conserved and actually are located in a region of subunit I of rather massive divergence, the bacterial structure [10] places the metal ion into an octahedral ligand field consisting of three backbone oxygen atoms (E56, H59, G61), two side-chain oxygens (E56 and Q63) and a water molecule in contact with a carboxylate oxygen of D477 (see Fig. 1 and discussion below). To test the influence of the latter three positions on Ca binding, three individual site-directed mutants were constructed, along with a double and the triple mutant. In all cases, alanine was chosen for a substitute, causing a loss of negative charge in most mutants. For the 477 position, a further replacement was devised, D477R (see Table 1), leading to a charge reversal. All mutant enzymes were expressed in the homologous *Paracoccus* host, purified to homogeneity using the F_v affinity tag system and assayed for correct subunit and heme composition (see Section 2). The purified complexes exhibited electron transfer activities in the range of 50–100% compared to the WT value and the triple mutant was further tested for proton pumping in the whole-cell assay [22], confirming its full functionality relative to the WT enzyme (not shown).

When assayed for their Ca content by TXRF (Table 1), none of the mutants in the 56/63 position revealed an altered Ca content, while any of the mutants comprising the 477 residue showed a considerable loss of Ca, with stoichiometries nominally in the 20–30% range at the most (see discussion above). This finding came as a surprise for several reasons:





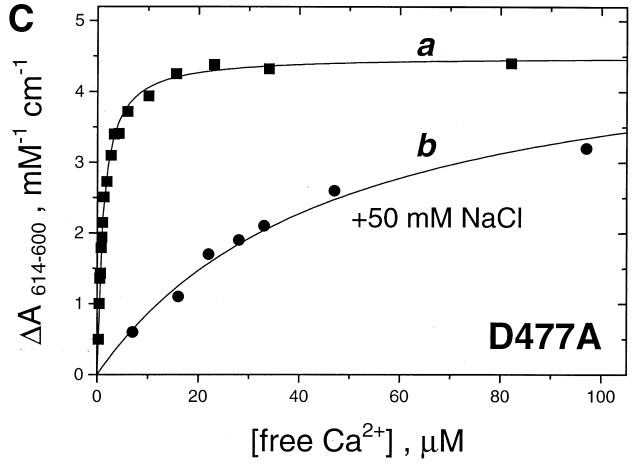


Fig. 2. Heme *a* spectral shift upon metal ion binding to isolated WT and mutant oxidase complexes. (A) Ca^{2+}-induced spectral shift. The cuvette contained 1.5 μM cytochrome *c* oxidase (*P. denitrificans* or bovine) in 50 mM Tris-MES buffer, pH 8.0, with 0.045% *n*-dodecyl-β-D-maltoside and 100 μM EGTA. The enzyme was pre-incubated for 5 min with 5 mM ascorbate and 100 μM TMPD in the presence of 2 mM KCN to convert COX to the mixed-valence state with heme *a* reduced ($a^{2+}a_3^{3+}$-CN) and the spectrum of this state recorded as baseline. Difference spectra were obtained 2 min after the addition of 1 mM $CaCl_2$. (B) Reversal of the Ca^{2+}-induced spectral shift by Na ions in the D477A mutant. 300 μM $CaCl_2$ was added to induce the spectral shift of heme *a*. The resulting spectrum was recorded as baseline. The difference spectrum shows the effect of 300 mM NaCl. Other conditions as in (A). (C) Determination of Ca affinity for the *P. denitrificans* COX mutant D477A. The enzyme (1.5 μM) was pre-incubated in the basic buffer with ascorbate, TMPD and cyanide as described in (A). Trace (a): in the absence of added Na^+, the effective K_D for Ca^{2+} is 1.1 μM, below the concentration of the enzyme in the cell. Therefore, the Ca^{2+}-induced absorption changes of heme *a* were titrated in the presence of a 10 mM nitrile-triacetate Ca^{2+}-buffering system (see [15]). Trace (b): in the presence of 50 mM NaCl, the effective K_D for Ca^{2+} increases to 48 μM, high enough to allow for accurate direct titrations of the absorption changes by added Ca^{2+}.
←

(a) With the loss of any of the immediate side-chain oxygen ligands (E56A, Q63A) and the concomitant charge loss (E56A) in any single and even more so in the double mutant(s), a severe disturbance in the ligand arrangement was to be expected. (b) The published coordinates [10] place the closest D477 side-chain oxygen at a 3.4 Å distance to the metal ion, making the prominent effect of mutating this residue difficult to explain. Energy minimization calculations (not shown) based on the previously published structure suggested a more favorable D447 position in which its side-chain carboxylate would move considerably (< 1 Å) closer to the Ca ion.

3.2. *Refined structure of the Ca site in the P. denitrificans oxidase*

However, further crystallographic refinement of the two-subunit cytochrome *c* oxidase [10] resulted in a defined positive difference electron density (F_o-F_c difference Fourier maps) on opposite sites of the published Ca ion position. The Ca ion is located further towards the ligand Q63 and an essential water molecule acts as a direct ligand and as a charge relay between the Ca ion and residue D477 (Fig. 1). Its carboxylate group functioning as a direct ligand can be ruled out, because it is well-defined in an unbiased electron density map ('simulated annealing omit electron density map'; [34]) too far away from the Ca ion.

The previously defined three backbone and two side-chain oxygen atoms (see above) are now all in a 2.3–2.4 Å distance to the central Ca ion and the water molecule is observed as the sixth coordination site in between the Ca^{2+} (2.5 Å) and the D477 oxygen (2.7 Å), with a direct distance Ca-D477 oxygen of 4.6 Å. Together with the above mutant data on Ca occupancy, we can only speculate that this water molecule must represent an important structural feature of the binding site. Its indirect perturbation by D477 mutation surpasses any mutational effects on the two other residues in the 56/63 positions.

3.3. Ca^{2+}/Na^{+}-induced spectral shifts in the bacterial mutant enzyme

As reported earlier [15], Ca^{2+} does not induce a spectral shift of heme *a* in the WT *P. denitrificans* COX (Fig. 2A, bottom trace). On the other hand, addition of excess Ca^{2+} to EGTA-treated COX from the D477A mutant perturbs the spectrum of the enzyme in much the same way as in the bovine COX, bringing about a small red shift of both the α- and Soret absorption bands of heme *a* (Fig. 2A, top and center trace). This effect of Ca^{2+} on the absorption spectrum of the D477A COX mutant is reversed by excess EGTA or other Ca^{2+} chelators (not shown) and by Na^{+} ions (Fig. 2B), but not by K^{+} (not shown). Titration with Ca^{2+} yields a hyperbolic curve corresponding to an apparent K_D of about 1 μM (Fig. 2C, trace a), close to the K_D measured for bovine COX [15]. The presence of Na ions greatly increases the K_D of Ca^{2+} required for induction of the red shift of heme *a* in the bacterial mutant enzyme (Fig. 2C, trace b) to around 50 μM, indicating competition between the two ions as in the bovine enzyme [14,15].

Taken together, the lack of a spectral shift in the bacterial enzyme can be rationalized by the fact that in WT *P. denitrificans* COX, a tightly bound Ca ion is located in a high affinity site and is unresponsive to externally added (competing) ions or chelators. Only when a mutation in a sensitive ligand (D477 and its connected water molecule) decreases its binding affinity, the mutant COX does no longer retain its bound Ca^{2+} and, consistent with this finding, addition of external Ca^{2+} produces a spectral shift, thus mimicking the situation in bovine COX. In addition, also the effect of Na^{+} is much the same as in the mammalian enzyme.

The role of the bound cation remains to be established. Wikström [11,12] originally proposed reversible binding of Ca^{2+} to propionates of heme *a* located at the bottom of an exit proton well in the middle of the mitochondrial membrane, which has not been confirmed by the structure. However, as emphasized earlier [15], the metal cation site in bovine COX appears to be closely associated with an opening of the 'H' proton channel (pore C in [9]) and a species-specific cation ligand S441 in bovine COX is very close to the D51 residue (bovine numbering) that was reported to undergo a redox-dependent conformational change relevant to proton pumping [9]. However, no significant effect of Ca^{2+}/Na^{+} on the activity of the mitochondrial COX or on proton pumping has been found for the mitochondrial enzyme (Kirichenko, unpublished data). Accordingly, activity measurements for the *P. denitrificans* enzyme do not reveal any significant difference between WT and calcium site mutant forms, nor does a mutation comprising the D477 position (accompanied by a loss of Ca binding) affect the proton pumping capacity of the bacterial enzyme (see above).

Bound Ca^{2+} is present in the structure of several hemoproteins undergoing a transition of the heme iron to higher oxidation states, like in peroxidases ([35–39] and references cited therein) where Ca^{2+} binding is either required for maintaining enzymatic activity or is discussed in terms of overall structure stabilization. At present, this latter role may be envisaged for the Ca binding site in COX as long as no functional defects are detectable upon Ca^{2+} removal.

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