Zinc ions inhibit oxidation of cytochrome *c* oxidase by oxygen

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Abstract Cytochrome *c* oxidase is a membrane-bound enzyme that catalyses the reduction of O_2 to H_2O and uses part of the energy released in this reaction to pump protons across the membrane. We have investigated the effect of addition of Zn^{2+} on the kinetics of two reaction steps in cytochrome *c* oxidase that are associated with proton pumping; the peroxy to oxo-ferryl ($P_r \rightarrow F$) and the oxo-ferryl to oxidised ($F \rightarrow O$) transitions. The Zn^{2+} binding resulted in a decrease of the $F \rightarrow O$ rate from 820 s^{-1} (no Zn^{2+}) to a saturating value of $\sim$360 s^{-1} with an apparent K_D of $\sim$2.6 μM. The $P_r \rightarrow F$ rate ($\sim 10^4$ s^{-1} before addition of Zn^{2+}) decreased more slowly with increasing Zn^{2+} concentration and a K_D of $\sim$120 μM was observed. The effects on both kinetic phases were fully reversible upon addition of EDTA. Since both the $P_r \rightarrow F$ and $F \rightarrow O$ transitions are associated with proton uptake through the D-pathway, a Zn^{2+}-binding site is likely to be located at the entry point of this pathway, where several carboxylates and histidine residues are found that may co-ordinate Zn^{2+}.

Key words: Electron transfer; Flow-flash; Proton pumping; Cytochrome aa_3; Flash photolysis; *Rhodobacter sphaeroides*

1. Introduction

Cytochrome *c* oxidase belongs to a group of enzymes called terminal oxidases, which have in common a binuclear centre consisting of haem a_3 and Cu_B at which the four-electron reduction of dioxygen to water is catalysed. The electron donor is a water-soluble cytochrome *c*, which first donates electrons to a copper site, copper A (Cu_A). The electrons are then transferred intramolecularly to haem *a* and to the binuclear centre, both located within the membrane-spanning part of the enzyme. The protons used for reduction of O_2 to water (substrate protons) are taken up specifically from one side of the membrane and therefore the enzyme must provide proton-transfer pathways that conduct protons from the bulk solution to the binuclear centre. In addition, the enzyme uses part of the free energy released during reduction of O_2 to water to pump protons across the membrane, which also requires proton-conducting pathways spanning the entire membrane (for reviews on the structure and function of terminal oxidases, see [1–4]).

Two proton-transfer pathways have been identified [5,6] and shown to be involved in proton transfer (for review see [7]). In the *Rhodobacter sphaeroides* cytochrome *c* oxidase one of these pathways contains a highly conserved lysine residue (K(I-362)) and leads from the proton-input side towards the binuclear centre. The other pathway starts with a highly conserved aspartate (D(I-132)) and continues via an other highly conserved residue, glutamic acid of subunit I at position 286 (E(I-286)) towards the binuclear centre, or the output side of the enzyme. A specific output pathway for pumped protons has been suggested for the bovine enzyme [8] but the residues being part of this pathway are not conserved in the bacterial enzymes investigated to date. In the *R. sphaeroides* and *Paracoccus denitrificans* enzymes the corresponding region of the protein is not used for proton ejection [9,10]. Thus, it is presently not clear if specific pathways are used to transfer protons beyond the binuclear centre towards the proton-output side (see [11]).

Recent studies have shown that in photosynthetic reaction centres metal ions such as Zn^{2+} or Cd^{2+} inhibit proton uptake from the bulk solution to the Q_B site [12,13]. The metal-binding site was found to be located near the entry point of a proton-transfer pathway where a cluster of two histidines and an aspartate bind the Zn^{2+} or Cd^{2+} [14]. A similar effect was observed with the bc_1 complex from bovine heart, where Zn^{2+} was proposed to bind near a protonatable group of a proton-transfer pathway, slowing proton transfer through the pathway [15]. A zinc ion has also been found in the bovine cytochrome *c* oxidase [16] where it binds to the nuclear-coded subunit Vb [17]. Since the removal of the Zn^{2+} ion from the enzyme does not affect the turnover activity [18], this tightly bound Zn^{2+} presumably does not play a regulatory role in the bovine enzyme. A tightly bound zinc ion has not been found in the structural model of the *R. sphaeroides* cytochrome *c* oxidase (Svensson Ek et al., unpublished) and no such site has been identified in the *P. denitrificans* cytochrome *c* oxidase [6].

In this study we have investigated the effect of addition of exogenous Zn^{2+} ions ($ZnSO_4$) on the reaction of fully reduced cytochrome *c* oxidase with O_2. Using the flow-flash technique we were able to unravel the effect of Zn^{2+} binding on the kinetics of specific reaction steps of the catalytic cycle, especially those that are coupled to proton uptake from the bulk

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Abbreviations: Cu_A, copper A; Cu_B, copper B; binuclear centre, Cu_B and haem a_3; O, fully oxidised enzyme[1]; P_r, peroxy intermediate (formed upon reaction of the fully reduced enzyme with O_2)[1]; F, oxo-ferryl intermediate[1]; E(I-286), glutamic acid of subunit I at position 286[2]

[1] Reduction states of the enzyme.
[2] Amino acid residue nomenclature. If not otherwise indicated, amino acid residues are numbered according to the *R. sphaeroides* sequence.

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solution and proton translocation across the membrane (proton pumping).

2. Materials and methods

Bacteria were grown and the enzyme was purified as described earlier [19]. After elution of the enzyme from the Ni^{2+}-column, 20 mM EDTA was added to the solution to remove traces of metals. The EDTA was removed by repetitive dilution and re-concentration of the enzyme solution (using centricon (Amicon) tubes) in 0.1 M HEPES–KOH, pH 7.4, 0.1% β-D-dodecyl maltoside (in mQ water). When the concentration of EDTA was smaller than 2 μM the enzyme was concentrated to 70 μM and frozen in liquid nitrogen, in which it was also stored until use. Prior to use, the syringes of the mixing device (see below) were washed with EDTA and mQ water. All other glassware were washed with hydrochloric acid and mQ water. All buffers were prepared using mQ water.

For a detailed description of the preparation of the fully reduced enzyme and the experimental set-up, see [20]. Briefly, the enzyme solution in 50 mM HEPES–KOH, pH 7.5 and 0.1% β-D-dodecyl maltoside was evacuated using a vacuum line and air was exchanged for N_2 after which the enzyme was reduced using 2 mM sodium ascorbate and 0.7 μM PMS (phenazine methosulphate). After formation of the fully reduced enzyme, N_2 was exchanged for CO. The flow-flash experiments were performed using a locally modified rapid-mixing device with an asymmetric mixing ratio of 5:1 (Applied Photophysics, DX-17MV). The anaerobic enzyme solution was transferred to the smaller of the drive syringes (500 μl total volume) and the other syringe (2500 μl total volume) was filled with an O_2-saturated buffer solution. About 100 ms after mixing CO was dissociated with a 10 ns, ~50 mJ laser flash at 532 nm (Nd-YAG laser from Spectra Physics) which initiated the reaction of the fully reduced enzyme with O_2. After mixing, the enzyme concentration was ~3 μM (with <0.1 μM EDTA). The cuvette path length was 1.00 cm. The Zn^{2+} ($ZnSO_4$ from Sigma, the purest grade available) solution was added to the larger syringe prior to mixing. To ascertain that during the mixing time Zn^{2+} was bound to the enzyme, control experiments were done in which the enzyme was incubated with Zn^{2+} prior to mixing.

The catalytic activity of cytochrome *c* oxidase (0.2 nM) was measured as the rate of oxidation of reduced cytochrome *c* (initial change in absorbance at 550 nm, 40 μM cytochrome *c*) in a buffer consisting of 10 mM HEPES–KOH, pH 7.5, 80 mM KCl, 0.1% β-D-dodecyl maltoside. Horse-heart cytochrome *c* (Sigma, type VI) was reduced to more than 95% by hydrogen gas using platinum black (Aldrich, WI, USA) as a catalyst. Metal ion solutions were prepared from $CdSO_4$, $CuSO_4$, $MgSO_4$ (purest grades available).

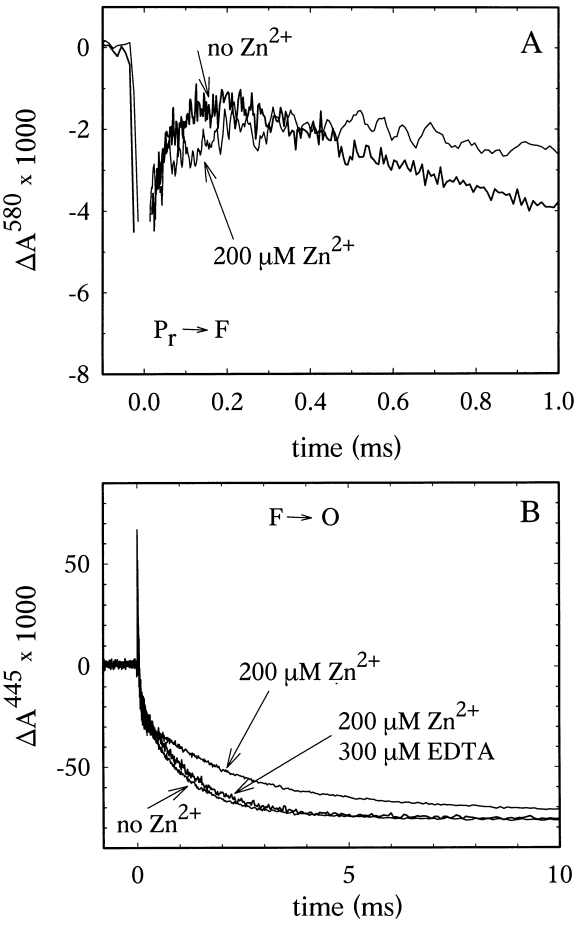

Fig. 1. Absorbance changes at 580 nm (A) and 445 nm (B), associated with reaction of the fully reduced *R. sphaeroides* cytochrome *c* oxidase with oxygen after flash photolysis (at $t=0$) of CO. The effect of the addition of Zn^{2+} or Zn^{2+}/EDTA to the solution is shown. Experimental conditions after mixing, 3 μM enzyme (the traces have been scaled to 1 μM reacting enzyme), 50 mM HEPES–KOH, pH 7.5, 0.1% β-D-dodecyl maltoside, 170 μM CO, 1 mM O_2, $T=22$°C. The concentrations of Zn^{2+} and EDTA are indicated in the graph. The enzyme solution was reduced using 2 mM sodium ascorbate and 5 μM PMS (prior to mixing).

3. Results

The effect of the addition of Zn^{2+}, Cd^{2+}, Cu^{2+} and Mg^{2+} on the turnover rate of *R. sphaeroides* cytochrome *c* oxidase was investigated at pH 7.5 (1000 s^{-1} before addition of the metal ions). The addition of Zn^{2+}, Cd^{2+} and Cu^{2+} resulted in a decrease in the rate, while no effect was observed with Mg^{2+}. The largest effect was observed with Zn^{2+} where the rate decreased by a factor ~7 to reach a saturating level. Since in these experiments no information was obtained about the reaction step(s) in which inhibition occurs, we used the flow-flash technique to investigate the effect of metal binding to cytochrome *c* oxidase on the kinetics of specific reaction steps during reaction of the fully reduced enzyme with dioxygen. The reduced enzyme with CO bound to haem a_3 at the binuclear centre was mixed with an oxygen-saturated solution containing various amounts of Zn^{2+}. About 100 ms after mixing, the CO ligand was flashed off using a short laser flash. The following reaction of the *R. sphaeroides* enzyme with O_2 has been investigated in detail previously [20]. First, O_2 binds with a rate constant of 1.2×10^5 s^{-1} at 1 mM O_2. The O_2 binding is followed by electron transfer from haem *a* to the binuclear centre forming the so-called peroxy intermediate (P_r) with a rate constant of $\sim2\times10^4$ s^{-1}. Non of these reactions is associated with proton uptake from the bulk solution and addition of 200 μM Zn^{2+} did not have any effect on the kinetics of these first two reaction phases (not shown).

The decay of P_r is associated with formation of the oxoferryl intermediate (F) with a rate constant of 7.4×10^3 s^{-1}, accompanied by proton uptake from the bulk solution. Finally, the oxidised enzyme (O) is formed with a rate constant of ~750 s^{-1} [20], also accompanied by proton uptake from the bulk solution.

Fig. 1 shows absorbance changes at 580 and 445 nm after flash photolysis of CO from the fully reduced enzyme in the presence of O_2. At 580 nm the increase in absorbance ($\tau\cong135$ μs) is associated with formation of F, i.e. the $P_r\rightarrow F$ transition (Fig. 1A). The slower decrease in absorbance at 445 nm with a rate constant of 820 s^{-1} ($\tau\cong1.2$ ms) is associated with the $F\rightarrow O$ transition (Fig. 1B).

As shown in Fig. 1, the rates of both transitions decreased upon addition of Zn^{2+}. The Zn^{2+} concentration was varied in

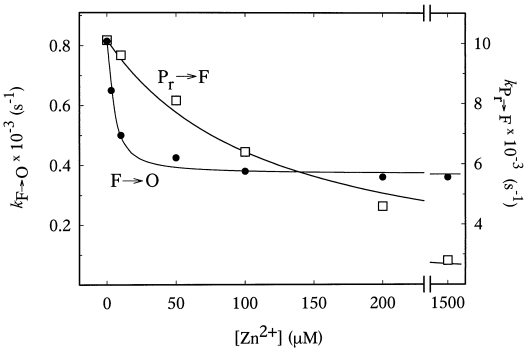

Fig. 2. The $P_r \rightarrow F$ (□, increase in absorbance with $\tau \cong 100$ μs at 580 nm, see Fig. 1A) and $F \rightarrow O$ (●, decrease in absorbance with $\tau \cong 1$ ms at 445 nm, see Fig. 1B) transition rates as a function of the Zn^{2+} concentration added to the solution. The solid lines are fits of the data with an Equation derived from $CCO+Zn^{2+} \leftrightarrow CCO–Zn^{2+}$ (CCO is cytochrome *c* oxidase), from which the K_D values were determined ($K_D(F \rightarrow O) = 2.6 \pm 0.5$ μM, $K_D(P_r \rightarrow F) = 120 \pm 20$ μM). Conditions were as in Fig. 1.

a range up to 1.5 mM and the dependencies of the $P_r \rightarrow F$ and $F \rightarrow O$ rates on the Zn^{2+} concentration are shown in Fig. 2. As seen in Fig. 2, the saturation behaviour was different for the two reaction steps. The $F \rightarrow O$ transition rate decreased from 820 s^{-1} to ~360 s^{-1} with an apparent K_D of 2.6 ± 0.5 μM. The $P_r \rightarrow F$ transition rate decreased much slower with increasing Zn^{2+} concentration ($K_D = 120 \pm 20$ μM) and reached a limiting value of ~3000 s^{-1} at high Zn^{2+} concentrations. Addition of EDTA resulted in full restoration of the kinetics to those observed in the absence of Zn^{2+}.

Slightly faster $P_r \rightarrow F$ and $F \rightarrow O$ rates were found in this study as compared to our earlier studies. This difference is due to the removal of residual metal ions by washing the enzyme solution in EDTA prior to the experiment.

Above ~10 μM Zn^{2+}, biphasic $F \rightarrow O$ kinetics were observed at 445 nm, while monophasic kinetics were found at 580 nm and 590 nm, where the average rates at 445 nm (single exponential fits) were slightly slower than those obtained at 580/590 nm (see Section 4).

4. Discussion

We have investigated the effect of binding of Zn^{2+} ions to cytochrome *c* oxidase on the kinetics of specific reaction steps during reaction of the fully reduced enzyme with oxygen. In a recent investigation it was found that binding of Zn^{2+} and Cd^{2+} ions to bacterial photosynthetic reaction centres from *R. sphaeroides* results in inhibition of proton uptake that follows light-induced charge separation [12,13]. In the reaction centres the metal ions were found to be co-ordinated to one aspartate and two histidine residues at the entry point of a proton-transfer pathway leading from the bulk solution to the Q_B-binding site [14].

The identification of the Zn^{2+}-binding site(s) in cytochrome *c* oxidase is more difficult than in the photosynthetic reaction centres because the investigated reaction steps ($P_r \rightarrow F$ and $F \rightarrow O$) are associated with the uptake of substrate protons as well as proton pumping (i.e. the uptake and release of pumped protons). All protons used during the $P_r \rightarrow F$ and $F \rightarrow O$ transitions are taken up through the D-pathway, but not through the K-pathway (for review see [7]). Consequently, in analogy with the scenario observed with the photosynthetic reaction centres, it is likely that in cytochrome *c* oxidase Zn^{2+} binds near the entry point of the D-pathway at which a cluster of histidine residues and carboxylates is found (see Fig. 3 and [21,22]).

We have shown previously that during the $P_r \rightarrow F$ transition, proton transfer to the binuclear centre takes place in two steps; The proton is transferred internally from E(I-286) to the binuclear centre forming F, followed by rapid re-protonation of E(I-286) from the bulk solution [22,23]. Thus, even though the $P_r \rightarrow F$ transition is about 10 times faster than the $F \rightarrow O$ transition, a smaller effect of Zn^{2+} at low Zn^{2+} concentrations was observed for the $P_r \rightarrow F$ than for the $F \rightarrow O$ transition because the former is rate-limited by an internal proton transfer and not by proton uptake from the bulk solution.

E(I-286) is also involved in proton transfer during the $F \rightarrow O$ transition, but the transition rate depends more strongly on the bulk solution pH (unpublished data) and a large (~7) kinetic isotope effect was observed for the transition [22]. In addition, the same kinetic isotope effect was found even if the electron transfer to the binuclear centre was slowed by two orders of magnitude by increasing the redox potential of Cu_A (the electron donor to the binuclear centre during the $F \rightarrow O$ transition) [24]. This observation indicates that there is a tight coupling between the electron and

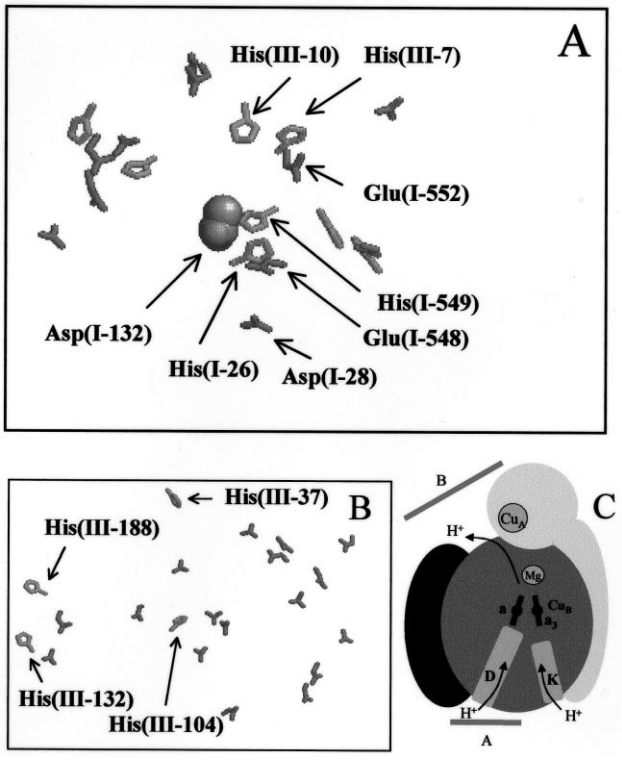

Fig. 3. The structure of cytochrome *c* oxidase (from *R. sphaeroides*, Svensson Ek et al., unpublished) around the entry point to the D-pathway (A) and from the top (B). In (C) a schematic representation of cytochrome *c* oxidase is shown indicating the protein portions that are illustrated in A and B. In A, the position of Asp-(I-132), at the entry point of the D-pathway, is indicated. Potential Zn^{2+}-binding sites are indicated: surface-exposed Asp and Glu residues and His residues are shown. Residues within 20 Å from D-(I-132) are shown and residues within 10 Å from D(I-132) are numbered. Residues 1–13 of subunit I are not included.

proton transfer in this reaction step and that both reactions are slowed when proton uptake from the bulk solution is slowed.

The observation of different K_D values for the $P_r \rightarrow F$ and $F \rightarrow O$ transitions indicates that there are two Zn^{2+}-binding sites. These sites may be found at different locations or they may be located adjacent to each other (one low- and one high-affinity site), affecting proton transfer through the same pathway. Recently, Mills and Ferguson-Miller (personal communication) found that Zn^{2+} may inhibit cytochrome *c* oxidase from the proton-output side of the enzyme, which suggests that one binding site may be found on the outside of the protein (see Fig. 3). An output pathway for pumped protons, if any, has not been identified and therefore it is not clear exactly where pumped protons are released (see [11]). The binding of Zn^{2+} on this side may provide an electrostatic barrier for the release of pumped protons. Alternatively, binding of Zn^{2+} near Cu_A may result in an increase in the redox potential of the site, which results in a slowed $F \rightarrow O$ transition.

Above $\sim$10 μM Zn^{2+}, biphasic kinetics were observed for the $F \rightarrow O$ transition at 445 nm. A similar behaviour was observed previously with the bovine cytochrome *c* oxidase [20,25] where both kinetic phases are associated with a transition between the same intermediates ($F \rightarrow O$).

A future determination of the three-dimensional structure of the *R. sphaeroides* cytochrome *c* oxidase with bound Zn^{2+} may identify the binding site(s) in the enzyme.

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