FEBS Letters 503 (2001) 142–146 FEBS 25127

# $Zn^{2+}$ binding to the cytoplasmic side of *Paracoccus denitrificans* cytochrome *c* oxidase selectively uncouples electron transfer and proton translocation[1]

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Received 4 July 2001; accepted 7 July 2001

First published online 30 July 2001

Edited by Richard Cogdell

**Abstract Using a combination of stopped-flow spectrophotometric proton pumping measurements and time-resolved potential measurements on black lipid membranes, we have investigated the effect of $Zn^{2+}$ ions on the proton transfer properties of *Paracoccus denitrificans* cytochrome *c* oxidase. When zinc was enclosed in the interior of cytochrome *c* oxidase containing liposomes, the *H/e* stoichiometry was found to gradually decrease with increasing $Zn^{2+}$ concentration. Half-inhibition of proton pumping was observed at $[Zn^{2+}]_i = 75$ μM corresponding to about 5–6 $Zn^{2+}$ ions per oxidase molecule. In addition, there was a significant increase in the respiratory control ratio of the proteoliposomes upon incorporation of $Zn^{2+}$. Time-resolved potential measurements on a black lipid membrane showed that the electrogenic phases slowed down in the presence of $Zn^{2+}$ correspond to phases that have been attributed to proton uptake from the cytoplasmic side and to proton pumping. We conclude that $Zn^{2+}$ ions bind close to or within the two proton transfer pathways of the bacterial cytochrome *c* oxidase.** © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

*Key words:* Cytochrome *c* oxidase; Proton pumping; $Zn^{2+}$ binding; Black lipid membrane; Proton slip

## 1. Introduction

Cytochrome *c* oxidase (COX, for recent reviews, see [1,2]), the terminal enzyme of the respiratory chain, catalyzes electron transfer from cytochrome *c* to molecular oxygen, reducing the latter to water. This redox reaction is coupled to proton translocation across the inner mitochondrial or bacterial membrane. In most COXs, the proton pumping stoichiometry is one proton per transferred electron or four protons per reduced oxygen, although lower ratios have also been reported [3,4].

Two proton transfer pathways have been identified in the crystal structure of *Paracoccus denitrificans* COX [5]. The so-called K-pathway leads from the cytoplasm via Lys-354 ('K') towards the binuclear center whereas the D-pathway proceeds from Asp-124 ('D') via Glu-278 to the region between the two hemes. Flow-flash [6,7] and charge translocation measurements [8,9] in combination with site-directed mutagenesis have shown that the K-pathway is involved in proton uptake associated with the transfer of the first two electrons to the oxidized enzyme whereas the other two substrate protons and all pumped protons are taken up via the D-pathway.

$Zn^{2+}$ ions have been shown to have a functional, catalytic or regulatory role for more than 300 proteins [10,11]. In many cases, zinc is tightly bound to the protein by interaction with sulfhydryl groups of cysteines and/or imidazole groups of histidines. These tightly bound $Zn^{2+}$ ions cannot be removed by chelators such as EDTA. Bovine-heart cytochrome *c* oxidase, for example, contains a single zinc binding site where the metal is coordinated to four cysteine residues of subunit Vb [12]. This site, however, is absent in the bacterial cytochrome oxidases as they do not have subunits homologous to the nuclear encoded proteins of eukaryotes. The pool of $Zn^{2+}$ that is not tightly bound to macromolecules has been termed 'chelatable zinc' [13] and has been receiving growing attention over the last decade [14]. Elevated chelatable zinc concentrations have been associated with, e.g. cardiac ischemia and cardiac inflammation [15,16] and were shown to be toxic to neurons [17,18]. The latter was attributed to alterations of mitochondrial function.

Early studies on intact mitochondria revealed that $Zn^{2+}$ inhibits respiration on substrates that enter the respiratory chain downstream from complex I [19,20]. This inhibition was later attributed to $Zn^{2+}$ binding to the cytochrome $bc_1$ complex [21,22]. Indeed, two $Zn^{2+}$ binding sites per cytochrome $bc_1$ monomer were identified by X-ray crystallography [23] and located close to the stigmatellin binding site that is thought to be equivalent to center $Q_o$. Recently, it was shown that zinc also inhibits the mitochondrial α-ketoglutarate dehydrogenase complex upstream from complex I [14].

Here we show that zinc binding to the cytoplasmic side of *P. denitrificans* COX causes the proton pump to slip, i.e. it leads to selective uncoupling of proton translocation from electron transfer. The electrogenic phases slowed down in

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*Abbreviations:* CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; COV, cytochrome *c* oxidase containing vesicle; COX, cytochrome *c* oxidase; RCR, respiratory control ratio


PII: S0014-5793(01)02719-3

the presence of $Zn^{2+}$ correspond to those that have been attributed to proton uptake from the cytoplasmic side or to proton pumping. We conclude that $Zn^{2+}$ binding occurs close to or within the two proton transfer pathways of the enzyme. These findings are in good agreement with a recent report by Aagaard and Brzezinski [24] showing that there is a decrease in the rates of the P→F and F→O transitions upon $Zn^{2+}$ binding to the solubilized *Rhodobacter sphaeroides* COX.

## 2. Materials and methods

### 2.1. Protein purification and reconstitution

*P. denitrificans* ATCC 13543 cells were grown, cytoplasmic membranes were isolated, and cytochrome *c* oxidase was purified by immunoaffinity chromatography as described [25]. Horse-heart cytochrome *c* was purchased from Biomol (Hamburg, Germany) and used without further purification.

Proteoliposomes for proton pumping measurements were prepared by the cholate dialysis method as described in [3] using asolectin (phosphatidyl choline type II S, Sigma) which was further purified as described in [26], at a concentration of 40 mg/ml. Lipids were dried under vacuum and resuspended in 100 mM HEPES–KOH, pH 7.3, 10 mM KCl, 2% (w/v) cholate. The suspension was stirred on ice for 1–2 h under argon and sonicated to clarity with a Branson sonifier at 30% duty cycle (power setting 3). COX was added to a concentration of 4 μM and the mixture was dialyzed against the resuspension buffer without cholate (2×2 h, 10 kDa cut-off). Subsequently, asolectin vesicles were dialyzed against two changes of 10 mM HEPES–KOH, 50 mM KCl, 50 mM sucrose (12 h each) and two changes of 55 mM KCl, 55 mM sucrose, pH 7.3 (12 h and 4 h). For the incorporation of $Zn^{2+}$, the appropriate amount of $ZnCl_2$ was added prior to sonication, and was also present during the first three dialysis steps. The COX concentration after dialysis was determined from the reduced minus oxidized optical difference spectrum with $\varepsilon_{607-630\,nm} = 23$ $mM^{-1}$ $cm^{-1}$. It was found to be 2.5–3 μM. For electrometric measurements COX was reconstituted into *Escherichia coli* lipid (acetone/ether preparation; Avanti Biochemicals) liposomes at a lipid/protein ratio of 20 (w/w) as described [27], using 2% (w/v) sodium cholate as detergent in 50 mM HEPES–KOH (pH 7.4). The proteoliposomes were loaded with $Zn^{2+}$ by adding the desired amount of $ZnCl_2$ followed by a repeated freeze-thaw-sonicate procedure. External $Zn^{2+}$ was removed by gel filtration, using 0.65 μm filter units (Millipore) and Sephadex G50 medium (Pharmacia).

The respiratory control ratio (RCR) was determined as the ratio of the rates of cytochrome *c* oxidation in the coupled and uncoupled states, respectively, essentially as described by Müller et al. [28]. Reduced cytochrome *c* was used at a concentration of 40 μM in 10 mM HEPES–KOH, 50 mM KCl, 50 mM sucrose, pH 7.3, the rate of its oxidation was measured by following the change in absorbance at 550 nm after addition of 1.2 nM reconstituted cytochrome *c* oxidase. Uncoupling was achieved by addition of 5 μM valinomycin and 10 μM carbonyl cyanide *m*-chlorophenylhydrazine (CCCP).

### 2.2. Determination of $[Zn^{2+}]$ and trapped volume

The concentration of $ZnCl_2$ within the vesicles was determined by atomic absorption spectroscopy (Perkin-Elmer AAS 4100, furnace). The internal volume of the vesicles was estimated by entrapping bromocresol purple (Aldrich) within the liposomes. The dye was added at a concentration of 100 μM prior to sonication and was also present during the first three dialysis steps. The last three dialysis steps were against 10 mM HEPES–KOH, pH 7.5, 50 mM KCl, 50 mM sucrose. Relative bromocresol purple concentrations were determined by measuring the absorbance at 590–480 nm (pH 8 minus pH 6). The difference extinction coefficient (590–480 nm, pH 8−pH 6) was determined as 44 000 $M^{-1}$ $cm^{-1}$.

### 2.3. Proton pumping experiments and electrometric measurements

Proton pumping was measured as the acidification of the external medium upon a pulse of reduced cytochrome *c* using a stopped-flow spectrophotometer and phenol red as the pH-sensitive dye [3,29].

Time-resolved potential measurements on the black lipid membrane were performed under anaerobic conditions using [Ru(2,2′-bipyridyl)$_3$]$Cl_2$ as electron donor as previously described [9,30]. Briefly, 40 μl of proteoliposomes containing *P. denitrificans* COX (4 μM) and either 0 or 100 μM internal $Zn^{2+}$ were added to 1.3 ml of buffer (50 mM HEPES–KOH, pH 7.4) containing 300 μM potassium ferricyanide. After the vesicles had attached to the black lipid membrane, 80 μM Ru II (2,2′-bipyridyl)3$Cl_2$ (Rubpy) and 300 μM EDTA were added and electron injection was induced with a short laser pulse (10 ns, 451 nm, ≈100 mJ/$cm^2$). The oxoferryl state F was generated by addition of a 100-fold excess (10 μM) of hydrogen peroxide.

### 2.4. Crystallization

Wild-type *P. denitrificans* COX was purified and crystallized as a co-complex with an antibody Fv fragment as described by Ostermeier et al. [31]. 1 mM $ZnCl_2$ was present during crystallization.

## 3. Results and discussion

Fig. 1 shows the experimental traces of the proton pumping measurements for $Zn^{2+}$ concentrations inside the vesicles between 0 and 200 μM. The *H/e* stoichiometry was determined from the ratio of the amplitudes of the absorbance changes in the coupled (trace a) and the decoupled state (trace b). Stoichiometries varied slightly for different cytochrome *c* oxidase containing vesicle (COV) preparations and were determined to be between 0.8 and 1 in the absence of $Zn^{2+}$ inside the vesicles. In the presence of the metal, a gradual decrease in the *H/e* ratio with increasing $[Zn^{2+}]_i$ was observed, with half-inhibition of proton pumping occurring at a concentration of ≈75 μM (Table 1). The rate of cytochrome *c* oxidation, however, was only slightly affected by the presence of $Zn^{2+}$, remaining >70% at $Zn^{2+}$ concentrations below 300 μM (see Table 1). No decoupling of proton pumping from catalytic turnover was observed when $Zn^{2+}$ ions were present *outside* the vesicles, even at a concentration of 500 μM (not shown).

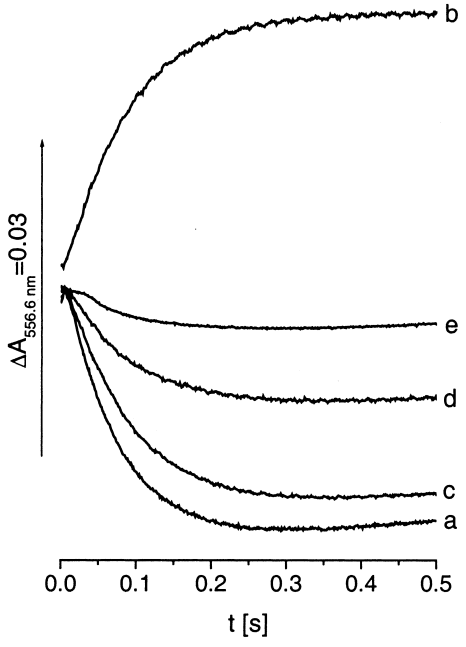


Fig. 1. Proton pumping assay using a stopped-flow spectrophotometer. pH changes were monitored by following the change in absorbance of phenol red at the isosbestic point of horse-heart cytochrome *c*. COVs were diluted to [COX] = 0.5 μM in 55 mM KCl, 55 mM sucrose, pH 7.3, 55 μM phenol red, 5 μM valinomycin and mixed with 16 μM ferrocytochrome *c* in 55 mM KCl, 55 mM sucrose, pH 7.3 (all concentrations after mixing). a, COVs without $Zn^{2+}$; b, as a, in the presence of 5 μM CCCP; c–e, COVs with internal $Zn^{2+}$ concentrations of 15, 75 and 200 μM, respectively.

Table 1
Proton pumping stoichiometries, relative RCRs and turnover rates for COVs with different internal $Zn^{2+}$ concentrations

| $[Zn^{2+}]_i$ (μM) | *H/e* (rel.) | RCR (rel.) | Rate ($s^{-1}$) |
|---|---|---|---|
| 0 | 1[a] | 4.5[b] | 166[c] |
| 15 | 0.9 | 6.3 | 147 |
| 30 | 0.9 | 5.9 | 164 |
| 50 | 0.6 | 7.2 | 139 |
| 75 | 0.5 | 8.3 | 135 |
| 100 | 0.4 | 8.6 | 156 |
| 300 | 0.2 | 12.2 | 120 |
| 500 | 0.1 | 13.5 | 90 |
| 1000 | n.d. | 18.5 | 51 |

The turnover rate was determined under decoupled conditions (5 μM valinomycin, 10 μM CCCP). The error for all values is ≈10%.
[a]Depending on the COV preparation, *H/e* ratios varied between 0.8 and 1.1. Values shown are relative stoichiometries ($\{H/e\}_{+Zn}/\{H/e\}_{-Zn}$) for COVs from the same batch, i.e. if the measured value for $\{H/e\}_{+Zn}$ was 0.45 and the $\{H/e\}_{-Zn}$ was 0.9 for COVs from the same batch, than $\{H/e\}_{rel.} = 0.5$.
[b]Varied between 2.8 and 5.8 among different COV batches. As for *H/e*, the RCRs are relative values for vesicles from the same batch.
[c]Between 160 and 170 $s^{-1}$ for different COV preparations.

Determination of $[Zn^{2+}]_i$ by atomic absorption spectroscopy showed that the absolute amount of zinc present after dialysis was about five times lower than that added initially. This is in good agreement with the result of the measurement of the average internal volume of the vesicles that was found to be 20% of the overall volume. Hence the trapped volume corresponds to about 4–6 l/mol phospholipid which is in the typical range for large unilamellar vesicles obtained by detergent dialysis [32]. Thus, with $[Zn^{2+}]_{1/2} = 75$ μM and a COX concentration after dialysis of 2.5–3 μM, a five- to six-fold excess of $Zn^{2+}$ over COX was sufficient for half-inhibition of proton pumping.

The decrease in the *H/e* ratio with increasing $[Zn^{2+}]_i$ was paralleled by an increase in the respiratory control ratio which was found to rise by a factor of 3 between 0 and 500 μM $Zn^{2+}$. This result implicates that the permeability of the proteoliposome membrane for $H^+$ is lowered in the presence of internal $Zn^{2+}$. It has been proposed that a large part of the proton leakage through the vesicular membrane occurs via the incorporated protein, because 10 times higher RCRs were obtained when a COX from a different source was reconstituted, under otherwise identical conditions [3]. Thus, the increase in the RCR observed here is probably due to $Zn^{2+}$ inhibiting proton leakage through the enzyme by binding close to or within the proton-conducting pathways of COX.

Selective uncoupling of proton translocation from cytochrome *c* oxidation has been reported for *E. coli* ubiquinol oxidase [33,34] and *P. denitrificans* COX [35] with mutations close to the entrance of the D-pathway. In the case of the *E. coli* enzyme, replacement of Asp-124 (*P. denitrificans* numbering) by asparagine led to an enzyme that retained 45% of its catalytic activity but was unable to pump protons. A similar result was obtained with an Asn-199-Asp mutant of the *P. denitrificans* enzyme whereas a Asn-131-Asp mutant showed a so-called ideally decoupled phenotype with full enzymatic activity but no proton pumping. Mutation of the central residue of the K-pathway, Lys-354, to methionine yielded an enzyme with very low activity [36] and a binuclear center that is difficult to reduce [37]. However, flow-flash experiments with the fully reduced COX [7] and measurement of proton pumping coupled to the peroxidase half cycle of the enzyme [38] showed that during the oxidative part of the catalytic reaction the Lys-354-Met mutant behaves similar to the wild-type. Thus, the selective inhibition of proton pumping indicates that $Zn^{2+}$ binding somehow impedes proton transfer along the D-pathway, but an additional effect on the proton conductivity of the K-pathway cannot be ruled out.

To distinguish between the two pathways, we have investigated the electrogenic events associated with the uptake of the first and fourth electrons in the catalytic cycle, i.e. the O→E and F→O transitions. It has been reported that uptake of the first electron by the oxidized enzyme is coupled to proton uptake via the K-pathway [9] whereas the F→O transition was shown to be linked to proton uptake and proton translocation via the D-pathway [8]. Our results are summarized in Fig. 2 and Table 2. Two electrogenic phases are observed when an electron is injected into oxidized COX. They have time constants of $\tau_1 = 22$ μs and $\tau_2 = 176$ μs and have been associated with electron transfer from $Cu_A$ to heme *a* and proton uptake from the cytoplasmic side via the K-pathway, respectively [9]. In the presence of $Zn^{2+}$ inside the proteoliposomes at a concentration of 100 μM, the time constant of the first phase was slightly larger (34 μs) whereas the second phase was significantly slower, with $\tau_2 = 1.4$ ms. A similar

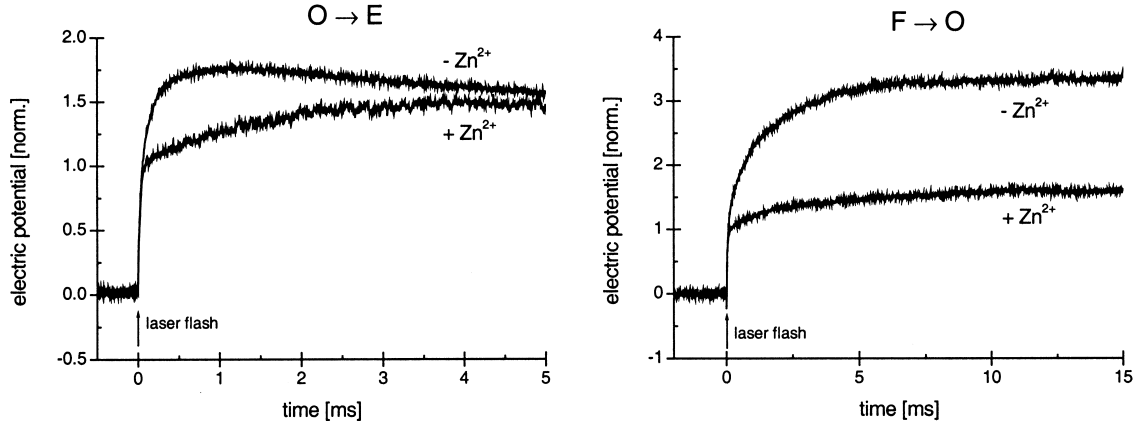


Fig. 2. Changes in electric potential observed upon one-electron reduction of the oxidized enzyme (O→E, left) and the oxoferryl state (F→O, right). The $Zn^{2+}$ concentration inside the vesicles was either 0 or 100 μM. Conditions were as described in [9] and Section 2. The traces in each panel have been normalized to the amplitude of the fast phase to aid visual comparison of the kinetics.

Table 2
Time constants ($\tau$) and relative amplitudes ($\alpha$) of electric potential generation

| Reaction | COVs ($-Zn^{2+}$) | COVs ($[Zn]_i = 100$ μM) |
|---|---|---|
| O→E | $\tau_1 = 21.7 \pm 5.5$ μs | $\tau_1 = 33.7 \pm 6.3$ μs |
| | $\alpha_1 = 0.57 \pm 0.10$ | $\alpha_1 = 0.70 \pm 0.03$ |
| | $\tau_2 = 176 \pm 50$ μs | $\tau_2 = 1.43 \pm 0.59$ ms |
| | $\alpha_2 = 0.43 \pm 0.10$ | $\alpha_2 = 0.30 \pm 0.03$ |
| F→O | $\tau_1 = 25.6 \pm 7.7$ μs | $\tau_1 = 33.3 \pm 3.0$ μs |
| | $\alpha_1 = 0.30 \pm 0.08$ | $\alpha_1 = 0.60 \pm 0.01$ |
| | $\tau_2 = 269 \pm 105$ μs | $\tau_2 = 1.91 \pm 0.47$ ms |
| | $\alpha_2 = 0.23 \pm 0.06$ | $\alpha_2 = 0.26 \pm 0.03$ |
| | $\tau_3 = 1.5 \pm 0.5$ ms | $\tau_3 = 13.4 \pm 0.9$ ms |
| | $\alpha_3 = 0.47 \pm 0.09$ | $\alpha_3 = 0.14 \pm 0.03$ |

increase in the time constants of the phases linked to proton transfer was observed for the F→O transition: In the absence of $Zn^{2+}$, three phases with time constants of $\tau_1 = 26$ μs, $\tau_2 = 270$ μs and $\tau_3 = 1.5$ ms were observed, in good agreement with the results reported by Konstantinov et al. [8]. The three phases have been attributed to electron transfer from $Cu_A$ to heme *a* (fast phase), proton uptake via the D-pathway (intermediate phase) and proton pumping, again via the D-pathway (slow phase). When $Zn^{2+}$ was incorporated into the proteoliposomes, the first phase was only slightly affected ($\tau_1 = 33$ μs) whereas the other two phases were slowed down dramatically to $\tau_2 = 1.9$ ms and $\tau_3 = 13$ ms. In addition, the relative amplitudes of the phases attributed to proton transfer were significantly smaller in the presence of $Zn^{2+}$. Thus, it can be concluded that $Zn^{2+}$ ions inside the vesicles lead to inhibition of proton transfer along both the D- and the K-pathway.

Recently, Aagaard and Brzezinski described a decrease in the rates of the P→F and F→O transitions upon $Zn^{2+}$ binding, as measured with the flow-flash technique [24]. In the presence of 100 μM $Zn^{2+}$ the overall transition rates dropped to 6200 (P→F) and 360 $s^{-1}$ (F→O) compared to 10 000 and 820 $s^{-1}$ in the absence of $Zn^{2+}$, respectively. Our results on the F→O transition, namely a decrease in rate from 3700 to 520 and from 670 to 75 $s^{-1}$ for the two phases associated with proton transfer, qualitatively agree with the data from [24].

However, a direct comparison of the actual numbers is difficult because (1) the results described in [24] were obtained with the solubilized enzyme whereas we have used proteoliposomes, (2) $Zn^{2+}$ ions were present at the given concentration in the surrounding medium in [24] whereas here they were trapped within the proteoliposomes with access only to the cytoplasmic side of the enzyme, (3) Aagaard and Brzesinski report overall transition rates determined spectrophotometrically whereas we have measured the different phases of charge translocation, and (4) the enzymes were from different sources (*R. sphaeroides* vs. *P. denitrificans*).

A similar retardation of proton transfer in the presence of $Zn^{2+}$ ions has been reported for the mammalian cytochrome $bc_1$ complex [22] and the bacterial photosynthetic reaction center [39]. In the latter case, binding of $Zn^{2+}$ or $Cd^{2+}$ led to a marked decrease in the rate of the second electron transfer from $Q_A$ to $Q_B$. This electron transfer is coupled to proton transfer from the cytoplasmic surface to $Q_B$. Normally, the proton transfer is much faster than the electron transfer, but in the presence of $Zn^{2+}$ or $Cd^{2+}$ it becomes the rate-limiting process. The metal binding site was identified by X-ray crystallography as being close to the cytoplasmic surface of the protein, with $Zn^{2+}$ or $Cd^{2+}$ being coordinated to an aspartic acid and two histidine residues [40]. For the identification of the metal binding site in COX, we have crystallized the enzyme in the presence of $Zn^{2+}$. Unfortunately, suitable crystals could only be obtained at pH 5.5, and even only a slight increase in pH resulted in destruction of the crystals. As a result, in the crystal structure, determined at 3 Å resolution, no bound $Zn^{2+}$ was found. We attribute this to histidines being doubly protonated at this acidic pH and therefore unable to serve as metal ligands. Thus, it can only be speculated about the location of the binding site(s). The simplest explanation of the inhibitory effect of $Zn^{2+}$ on proton transfer is that it binds to groups – possibly histidines and acidic amino acid side chains – that normally serve as transient proton donors or acceptors. Close to the entrance of the D-pathway, three residues, Asn-27, His-28 and Asp-124 are arranged in a way (geometry, distance) that could allow for specific binding of a metal ion, thereby blocking proton uptake via Asp-124. Alternatively, $Zn^{2+}$ might not directly coordinate to amino acid residues participating in proton transfer, but could block proton transfer by disturbing the arrangement of water molecules in the pathways or by electrostatic repulsion of incoming protons.

## 4. Conclusion

Our results show that binding of $Zn^{2+}$ to the cytoplasmic side of bacterial COX reduces the proton conductivity of the two proton transfer pathways, resulting in uncoupling of proton translocation from electron transfer and an elevated respiratory control ratio. Electrometric measurements indicate that both the D- and the K-pathway are affected by $Zn^{2+}$ binding, but the exact location of the metal site(s) remains to be identified.

*Acknowledgements:* We are greatly indebted to Hartmut Michel, Klaus Fendler and Ernst Bamberg for their generous support and valuable discussions. We thank the Lebensmittelchemisches Labor Hemmrich in Lauda, Germany, for help with atomic absorption spectroscopy. Roy Lancaster and Wolfgang Grabarse are gratefully acknowledged for their help with X-ray data acquisition and processing. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 472).

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