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Inhibition of membrane-bound cytochrome *c* oxidase by zinc ions: High-affinity Zn^{2+}-binding site at the P-side of the membrane

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Abstract **In the presence of the uncoupler, external zinc ions inhibit rapidly turnover of cytochrome *c* oxidase reconstituted in phospholipid vesicles or bound to the membrane of intact mitochondria. The effect is promoted by electron leaks into the oxidase during preincubation with Zn^{2+}. Inhibition of liposome-bound bovine cytochrome oxidase by external Zn^{2+} titrates with a K_i of 1 ± 0.3 μM. Presumably, the Zn^{2+}-binding group at the positively charged side is not reactive in the oxidized enzyme, but becomes accessible to the cation in some partially reduced state(s) of the oxidase; reduction of Cu_B is tentatively proposed to be responsible for the effect.**
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1. Introduction

Cytochrome *c* oxidase (COX)** is a key enzyme of aerobic respiration which reduces molecular oxygen to water and conserves the free energy of this highly exergonic reaction in a form of transmembrane difference of proton electrochemical potential, $\Delta\mu H^+$ [1–4]. Elucidation of molecular mechanism of the energy-coupled oxygen reduction by COX is one of the most important issues in membrane bioenergetics. Inhibitor analysis is a classical tool in the studies of enzymatic mechanisms. Studies of bacterial oxidases have been greatly promoted by availability of mutant forms of the enzyme with impaired activity [4–7]. At the same time, the list of the inhibitors of the mammalian enzyme is rather limited, and their action is confined essentially to the sites of oxidase interaction with oxygen (KCN, CO, etc.) and with cytochrome *c* (poly-L-lysine and other alkaline polypeptides). It is therefore instrumental that zinc ions have been added recently to the list of the inhibitors of COX.

Zn^{2+} has been shown to inhibit enzymatic activity of the mitochondrial and bacterial COX [8–15]. The inhibitory effect is likely to be associated with blocking proton transfer pathways in COX. There are several Zn^{2+}-binding sites in COX located at the opposing parts of the protein exposed to the P- and N-aqueous phases separated by the membrane. Experiments with the isolated enzyme reveal inhibition of turnover by micromolar Zn^{2+} assigned to binding of the cation at the orifices of the input proton channels at the negatively charged side (N-side) of the membrane [9–11,14–16]. Similar action is exerted by Ni^{2+} and Cd^{2+} [7,15,16]. Binding of the inhibitory cations at or near the mouths of the D and K input proton channels has been directly confirmed by crystal structure of the enzyme [15,16].

The data on the inhibition of cytochrome oxidase by Zn^{2+} ions added from the outer (P-) side of the membrane are rather controversial. Phospholipid membranes are impermeable for zinc dication, therefore the binding sites at the mouths of the D- or K-channels are not accessible to Zn^{2+} from the outer (P-) aqueous phase. Inhibition of liposome-reconstituted COX by external Zn^{2+} was described originally by Nicholls and Singh [8]. More recently, Mills et al. [11] reported that Zn^{2+} added to vesicle-reconstituted cytochrome oxidase from *Rhodobacter sphaeroides* inhibited cytochrome oxidase turnover with K_i of 5 μM, but only if there was membrane potential across the vesicle membranes (see also [16]). In the de-energized membrane, or if the transmembrane difference of proton electrochemical potential was represented by its ΔpH component, very high (mM) concentrations of Zn^{2+} were required to observe the inhibition. At the same time, inhibition of membrane-bound COX by micromolar concentrations of Zn ions in the absence of energization was described by Kuznetsova et al. [13]. Inhibitory effect of 25–250 μM Zn^{2+} on proton release coupled to "$\mathbf{P}_R$"-to-**F** transition of liposome-reconstituted oxidase was reported in [14].

In this work we show that turnover of liposome-reconstituted cytochrome oxidase, as well as of the enzyme in the membrane of whole mitochondria, is readily inhibited by micromolar concentrations of external Zn^{2+} in the absence of membrane potential. The inhibition requires the presence of weak reductants providing for slow electron flux into COX during preincubation with zinc. K_i of ~1 μM has been determined for the effect of Zn^{2+} on the liposome-reconstituted oxidase in the presence of tris-bipyridyl complex of ruthenium(II) (RuBpy)+aniline. Cytochrome oxidase proteoliposomes supplemented with RuBpy+aniline have been widely used for time-resolved measurements of flash-induced membrane potential generation by COX [17–21], and external

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Abbreviations: COX, cytochrome *c* oxidase; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; EDTA, ethylenediaminetetraacetic acid; RuBpy, tris-bipyridyl complex of ruthenium(II); P- and N-sides, positively and negatively charged sides of the membrane, respectively

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Zn^{2+} was reported to hinder electrogenic intraprotein H^+-transfer coupled to the oxoferryl → oxidized transition of the enzyme [13]. Therefore, quantitative characterization of Zn^{2+} binding with COX under these conditions was one of the specific objectives of this work.

2. Methods

2.1. Biochemicals

Cytochrome *c* type III from horse heart, egg lecithin type X-E, Nagarse, $ZnCl_2$ (reagent grade), RuBpy and aniline were from Sigma-Aldrich. Carbonyl cyanide *m*-chlorophenyl hydrazone and rotenone were from Sigma. pH-buffers were purchased from Amresco. Most of the other common biochemicals of high purity were purchased from Sigma-Aldrich.

2.2. Preparations

COX was isolated from bovine heart essentially as described in [22] (a modified Fowler-type preparation) according to a protocol kindly provided by Dr. A. Musatov (Department of Biochemistry, UTHSC, San Antonio). The enzyme was reconstituted in egg lecithin vesicles by cholate dialysis method [23]. Mitochondria were isolated from rat liver or rat skeletal muscle by a procedure involving treatment with nagarse [24] which renders their outer membrane permeable for added cytochrome *c*. His-tagged cytochrome oxidase from *R. sphaeroides* was purified from the bacterial membranes on NTA-agarose (Quiagen) as described in [25].

2.3. Titrations with Zn^{2+}

Zinc ions bind to phospholipids and proteins as well as plastic (e.g. [14]). Therefore, 5 mM citrate was used in this work as a Zn^{2+} buffer to ensure accurate Zn^{2+} titrations in the micromolar range [13]. Concentration of free Zn^{2+} at given experimental conditions (pH 8, $I \sim 0.1$ M and 25 °C) was calculated from the known concentration of added $ZnCl_2$ with the Internet-available free program WinMAXC 2.05 (see [13] for more details).

2.4. COX activity assay

Liposome-reconstituted COX or mitochondria were preincubated in a spectrophotometric cell for 4–5 min in a medium with or without Zn^{2+} (see figure legends). Ferrous cytochrome c^{2+} was added (6 μM if not indicated otherwise) and its oxidation was monitored spectrophotometrically at 550 nm vs. 535 nm reference in a dual-wavelength mode of SLM-Aminco 2000 spectrophotometer. The kinetic traces were imported into Origin 7 program (Microcal) for finding the pseudo-first order rate constants for cytochrome c^{2+} oxidation traces and fitting the data.

All the experiments were performed under weakly alkaline conditions (pH 8) for two reasons. First, some of the typical Zn^{2+}-reactive protein groups in COX, like histidyl residues, have p*K* values around 7 and bind cation in the deprotonated form only [11]. Second, we would like to avoid spontaneous transition of the oxidized COX to the so-called slow form which becomes noticeable at pH < 7.5.

3. Results

As shown in Fig. 1A, preincubation of the fully oxidized uncoupled cytochrome oxidase vesicles with 100 μM Zn^{2+} does not inhibit COX activity. This observation agrees with the data of Mills et al. [11] and Kuznetsova et al. [13].

However, if the preincubation is carried out in the presence of 40 μM RuBpy and 10 mM aniline, 100 μM Zn^{2+} brings about significant inhibition of cytochrome *c* oxidation (Fig. 1B). The inhibition is released by excess ethylenediaminetetraacetic acid added after (Fig. 1B) or before (not shown) onset of the reaction. RuBpy/aniline system has been widely used for flash-induced rapid reduction of COX in the time-resolved measurements of membrane potential generation by COX [17–21]. Control experiments show that upon prolonged anaerobic incubation of COX with RuBpy/aniline at deem light, there occurs very slow electron flux into COX. The flux is too slow to be monitored with an oxygen electrode under steady-state aerobic conditions, but can be detected as partial reduction of the hemes upon prolonged anaerobic incubation (2-3% reduction of the hemes after 2 h, 5–7% after 4 h, data not included). Low concentrations of ferrocyanide could substitute for RuBpy/aniline during preincubation of COX with zinc (data not shown, [13]).

Similar pattern of the inhibitory effect of external Zn^{2+} on the COX activity was observed with the whole mitochondria from rat liver, heart, kidney or skeletal muscle. Some typical results are shown in Fig. 2.

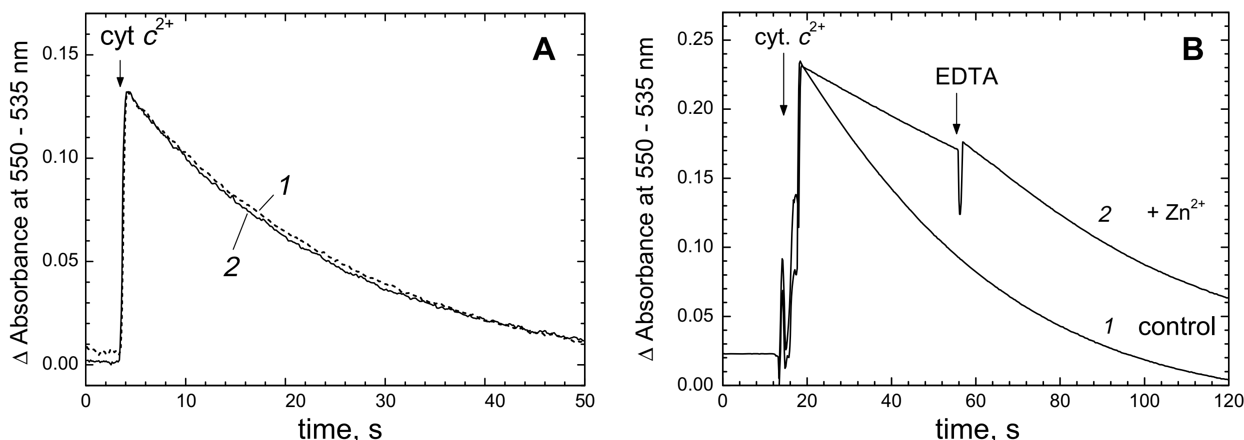

Fig. 1. Effect of external Zn^{2+} on the rate of ferrocytochrome *c* oxidation by liposome-reconstituted cytochrome oxidase. Liposome-reconstituted COX (final enzyme concentration, 4 nM) in the aerobic reaction medium containing 50 mM Tris–HCl pH 8, 50 mM KCl, 100 μM EDTA and 1 μM the uncoupler CCCP. Ferrocytochrome *c* is added to initiate the reaction. Where indicated, the mixture was preincubated with Zn^{2+} for 4-5 min before addition of cytochrome c^{2+}. **(A)** Fully oxidized enzyme. Trace *1*, control without Zn^{2+}. Trace *2*, with 200 μM Zn^{2+} added (100 μM excess over EDTA). (B) The same experiment as in (A), but in the presence of of 10 mM aniline and 40 μM RuBpy. Addition of EDTA, 300 μM. Ferrocytochrome *c* addition was 6 μM in (A) and 12 μM in (B).

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