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Inhibitory Effects of Exogenous Cu^{2+} and Zn^{2+} on the Cytochrome c Oxidase Activity

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Abstract: Exogenous Cu^{2+} or Zn^{2+} at micromolar concentration had a strong inhibitory effect on detergent-solubilized cyto chrome *c* oxidase. A similar effect was observed when Cu^{2+} was added to vesicular cytochrome *c* oxidase, although the extent of inhibition was significantly larger for the uncoupled state than for the coupled state. Interestingly, the inhibition by Zn^{2+} was almost negligible for both the coupled and uncoupled states. These results suggest that the binding sites for Cu^{2+} ions are exposed to the extravesicular side, whereas those for Zn^{2+} are exposed to the matrix side. The EPR spectra of bound Cu^{2+} ions at 77 K indicate that each of the first two Cu^{2+} ions is ligated by three or four histidine residues, as evidenced by distinct ^{14}N superhyperfine splitting. These Cu^{2+} ions can not be removed readily by EDTA and inhibit the enzyme activity by as much as 80%.

Key words: cytochrome c oxidase, electron paramagnetic resonance, exogenous metal, inhibition.

In mitochondria, reducing electrons of NADH undergo successive oxidation by redox proteins in the respiratory chain. Cytochrome *c* oxidase (CcO), the terminal enzyme in the chain, accepts electrons from cytochrome *c* and reduces molecular oxygen to water. As an integral membrane protein, CcO pumps protons across the inner mitochondrial membrane by using the free energy released from the reduction of oxygen. According to the chemiosmotic hypothesis, ATP synthase couples this proton electrochemical potential gradient to the synthesis of ATP (Babcock & Wikström, 1992; Trumpower and Gennis, 1994).

Due to the difficulties shared by other membrane proteins, a three dimensional structure has not been determined by X-ray diffraction studies for any one of the terminal oxidases. Extensive biochemical and spectroscopic studies, however, envisage the enzyme as a homodimer containing two *a*-type hemes and at least two copper ions as the minimal catalytic unit for the reduction of oxygen (Wikström *et al.*, 1981). The stoichiometry of 2 : 2 Fe : Cu was generally accepted by the notion that the four electrons required for the reduction of oxygen to water may be distributed over the four metal centers. All the experimental data confirm that there are two Fe ions per monomeric CcO. The exact number of copper ions, however, has been a matter of controversy for quite some time (Azzi and Müller, 1990; Palmer, 1993). Recently, there has been growing evidence (Bombelka *et al.*, 1986; Yewey and Caughey, 1988; Pan *et al.*, 1991; Lappalainen *et al.*, 1993; Vonwachenfeld *et al.*, 1994) for the existence of additional copper ions, although the stoichiometry varies from one laboratory to another. Zinc and magnesium ions are also believed to be intrinsic components of the enzyme. The biological functions, if any, of these newly found metal ions are yet to be defined.

In addition to these endogenous metal ions, CcO has binding sites for exogenously added metal ions such as Cu^{2+}, Zn^{2+}, Mg^{2+}, and Ca^{2+}. By carefully analyzing the molar ratio of metal ions bound to CcO, Yewey and Caughey (1988) and Pan *et al.* (1991) found that CcO binds several exogenous Cu^{2+} and Zn^{2+} ions. Binding appears to be tight as some (Yewey and Caughey, 1988) or all (Pan *et al.*, 1991) of bound metal ions can be dissociated from the enzyme upon prolonged dialysis against a buffer containing EDTA. Identity of the binding sites and the consequences of such binding are largely unknown.

In the present study, we have examined the effects of exogenous metal ions on the activity of both micellar (detergent-solubilized) and vesicular CcO. Only Cu^{2+} and Zn^{2+} were found to decrease the activity of CcO significantly although their binding sites are different from each other. Electron paramagnetic resonance (EPR) spectra of the added copper ions provided structural information of the binding sites.

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Materials and Methods

CcO was isolated from a bovine heart according to the Yonetani method (1960). Enzyme concentration was determined spectrophotometrically by using extinction coefficient $\varepsilon_{605-630}$ (red-ox)=27 $mM^{-1}\cdot cm^{-1}$.

Ferrocytochrome *c* was prepared by reducing cytochrome *c* (Sigma, type VI) with ascorbate in the presence of an electron mediator, TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine). Excess reductants were removed by gel filtration on a Sephadex G-15 column. All the manipulations were carried out under anaerobic conditions to achieve a high reduction level (typically well over 95%) of ferrocytochrome *c*. Extinction coefficient $\varepsilon_{550}=27.7\ mM^{-1}\cdot cm^{-1}$ was used for the determination of ferrocytochrome *c* concentration.

The enzyme was reconstituted in liposomes by a cholate-dialysis method (Casey *et al.*, 1982). 250 mg of asolectin (Sigma, type IVs) was stirred anaerobically in 5 ml of 75 mM K-Hepes (pH 7.4) containing 25 mM cholate. The cloudy suspension was sonicated under nitrogen with occasional cooling on ice until it became translucent. CcO was added to a final concentration of 5 μM and dialyzed for 4 h against 1 l of 75 mM K-Hepes, for 4 h against 1 l, 12 h against 2 l, and finally 4 h against 1 l of buffer containing 10 mM K-Hepes, 41 mM KCl, and 38 mM sucrose. Typically, the respiratory control ratio (RCR) was greater than 8. More than ~90% of the enzyme was incorporated with right-side out orientation as measured by the method of Casey *et al.* (1982).

Enzyme activity of CcO was determined by measuring the rate of ferrocytochrome *c* oxidation. The desired amount (typically 10 nM final concentration) of micellar or vesicular CcO was added to an assay buffer and the reaction was initiated by adding ferrocytochrome *c*. Oxidation of ferrocytochrome *c* was followed by measuring the absorption at 550 nm. The reaction is of the first order in cytochrome *c* and the rate constant can be calculated accordingly.

X-band EPR spectra of copper ions were recorded at 77 K on a Bruker ER-200D spectrometer operating at 9.37 GHz with 100 KHz field modulation. A cylindrical quartz tube (4 mm i.d.) containing a CcO sample was immersed in a liquid nitrogen dewar that was fit in the microwave cavity.

Results

Catalytic activities of detergent-solubilized CcO were measured spectrophotometrically in the presence of various metal ions (Fig. 1). Among the four metal ions testec, only Cu^{2+} and Zn^{2+} were very effective in lowering the activity of CcO. Inhibition by Ca^{2+} or Mg^{2+} was marginal. When 10 nM CcO was reacted with 38 μM ferrocytochrome *c*, the CcO activity decreased by as much as ~70% when 3 μM of Cu^{2+} or Zn^{2+} ions were included in the reaction mixture. The degree of inhibition by Cu^{2+} was (coincidentally) the same as that by Zn^{2+}, and in both cases it leveled off at higher concentration of metal ions indicating that there is a limited number of binding sites that affect the enzyme activity.

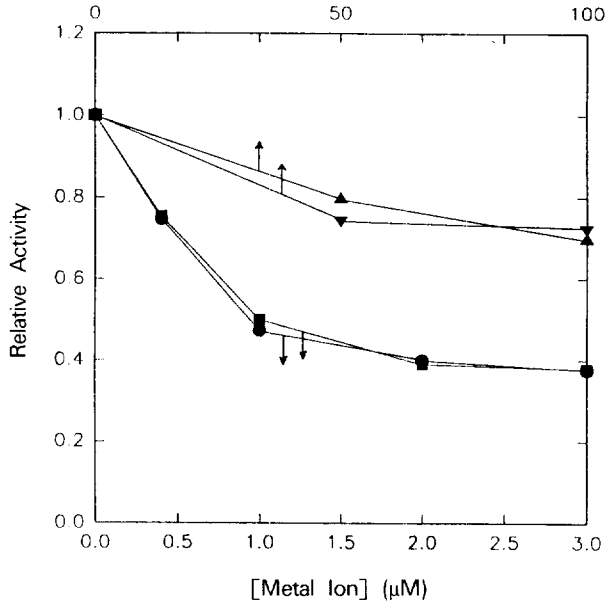

Fig. 1. Effects of metal ions on the activity of detergent-solubilized CcO. To 3 ml of 20 mM K-Hepes (pH 7.4) containing 100 mM KCl, 0.5% Tween 80, and given concentration of metal ion was added CcO to a final concentration of 10 nM. The reaction was initiated by adding 38 μM ferrocytochrome *c* and the absorption at 550 nm was followed to measure the rate of ferrocytochrome *c* oxidation. Cu^{2+} (●); Zn^{2+} (■); Mg^{2+} (▲); and Ca^{2+} (▼).

Measurements were repeated with vesicular CcO and the results are shown in Fig. 2. The enzyme was reconstituted into asolectin vesicles with a high respiratory control ratio (RCR) of 8 or larger. Inclusion of 3 μM of Cu^{2+} ions in the reaction mixture decreased the enzyme activity of uncoupled CcO vesicles to ~15% of that of untreated CcO, which is even lower than in the case of detergent-solubilized CcO. The coupled state, however, was affected by Cu^{2+} ions to a much less extent. This discriminatory effect of Cu^{2+} on the coupled and uncoupled states of vesicular CcO resulted in a dramatic change in the RCR values from 8.2 to 2.5. In contrast to the effective inhibition by Cu^{2+} of both detergent-solubilized and vesicular CcO, Zn^{2+} affected the detergent-solubilized CcO, but not the vesicular CcO, suggesting that the binding sites for Cu^{2+} lie in the extravesicular side whereas those for Zn^{2+} lie in the matrix side.

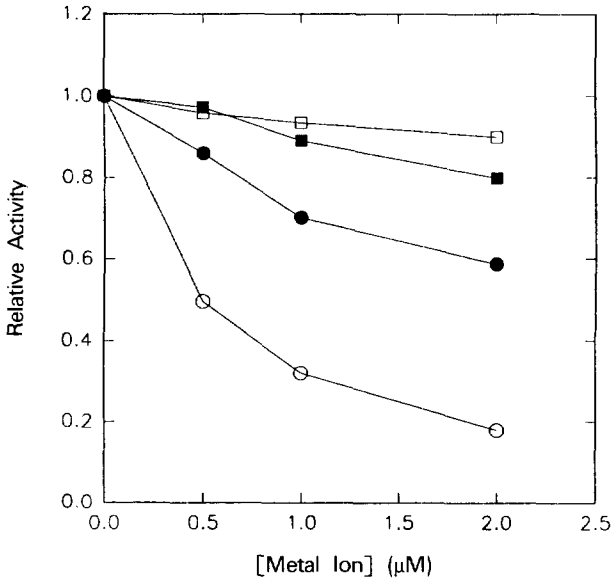

Fig. 2. Effects of Cu^{2+} and Zn^{2+} on the activity of CcO vesicles. Experimental conditions were the same as those in Fig. 1 except the final dialysis buffer (see Materials and Methods) was used instead. For uncoupled state, 0.2 μM valinomycin and 0.6 μM CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) were included in the reaction mixture. Cu^{2+}, coupled (●); Cu^{2+}, uncoupled (○); Zn^{2+}, coupled (○); and Zn^{2+}, uncoupled (□).

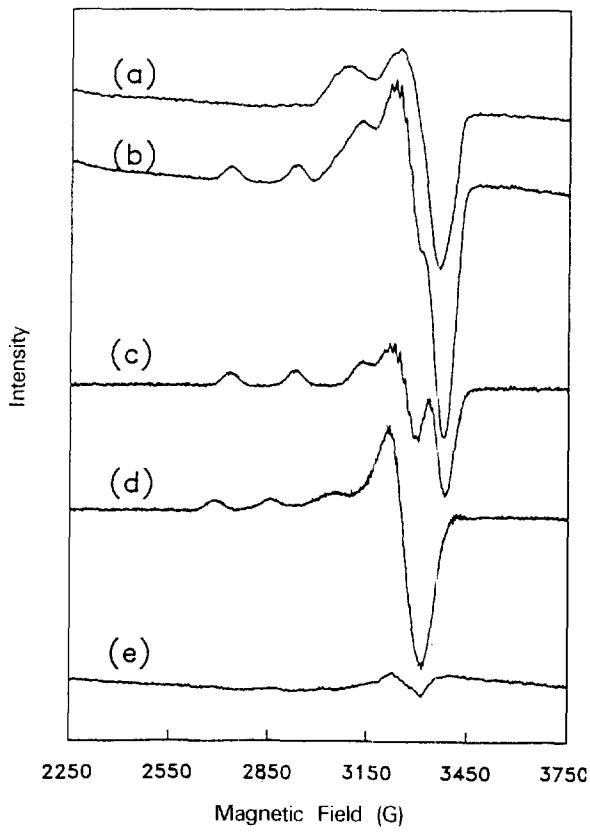

Fig. 3. EPR spectra of exogenous Cu^{2+}. (a) resting enzyme (200 μM); (b) resting enzyme with 400 M Cu^{2+}; (c) spectrum (b) − spectrum (a); (d) spectrum of the enzyme with 6-fold Cu^{2+} minus that with 4-fold Cu^{2+}; (e) 400 μM Cu^{2+} in the absence of the enzyme. A sample in a cylindrical quartz tube (4 mm i.d.) was frozen at 77 K and immersed in a cold finger cooled by liquid nitrogen. EPR spectra were recorded on a Bruker X-band spectrometer operating at 9.37 GHz (64 mW power) with 100 KHz field modulation.

In order to obtain structural information about the copper-binding sites, we measured EPR spectra of copper ions at various Cu : CcO ratios. In the absence of exogenous Cu^{2+}, the EPR spectrum (Fig. 3a) exhibits characteristics of the CuA center present in the enzyme. Upon raising the Cu : CcO ratio to 2, a new set of EPR lines emerge (Fig. 3b). Subtracting the intrinsic Cu_A signal (Fig. 3a), one obtains a pure spectrum of added copper as shown in Fig. 3c. The spectrum shows a rhombic distortion as evidenced by the three *g*-values, and the g_z component is split into four lines due to interaction with the nuclear spin of Cu^{2+}. It also shows a well-resolved superhyperfine structure that can be attributed to either three or four nitrogen ligands depending on the structural model used in the spectral simulation. Similar spectrum was obtained for a CcO sample denatured by urea (Greenaway *et al.*, 1977) or by *p*-hydroxymercuribenzoate (Gelles and Chan, 1985). At a higher ratio of Cu : CcO, there appear yet another set of EPR lines that suggest binding of non-nitrogenous ligands to copper (Fig. 3d). If the sample with exogenous copper ions was reduced by ascorbate and cytochrome *c*, an EPR spectrum identical to that in Fig. 3c was obtained (data not shown). Since Cu_A is readily reduced by the reductants, it can be concluded that the exogenous copper ions bind to CcO but do not participate in the electron transfer pathway.

If EDTA is added to a sample of native CcO, no spectral change is observed indicating that the enzyme as isolated does not contain a significant amount of exogenous copper (data not shown). If Cu^{2+} is added to an enzyme solution which was preincubated with EDTA, one obtains a spectrum shown in Fig. 4c (after subtraction of the Cu_A spectrum), which is different from the spectrum of Cu^{2+}-EDTA complex alone (Fig. 4d). The implication is that the Cu^{2+}-EDTA complex somehow binds to the enzyme and its spectral characteristics change. Fig. 4b represents the spectrum that can be obtained when the order of additions is reversed, i.e. 2 equivalents of Cu^{2+} were added prior to EDTA. Spectral simulation demonstrates that the spectrum in Fig. 4b is a composite of those in Fig. 4c (40%) and Fig. 4a (60%). This suggests that the first two Cu^{2+} ions bind to the enzyme very tightly so that they can not be completely removed by immediate addition of EDTA.

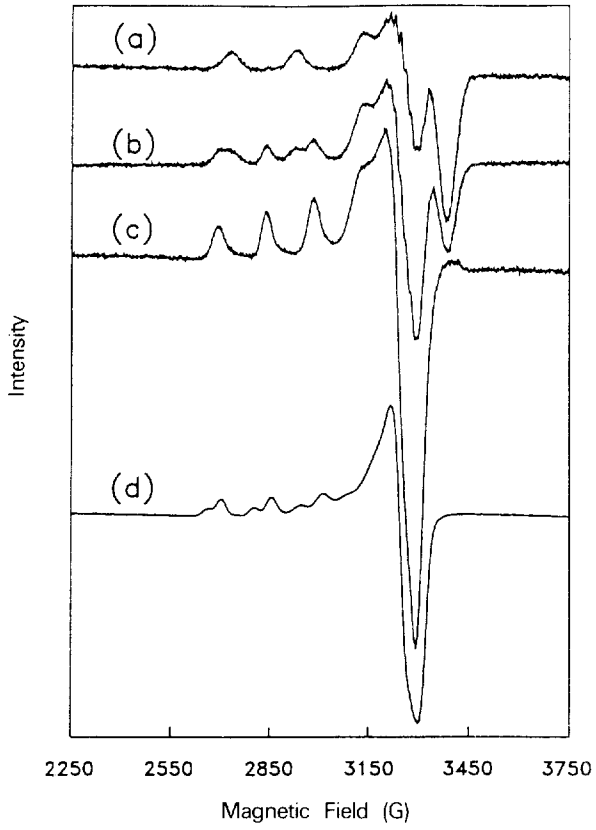

Fig. 4. Effects of EDTA on the EPR spectrum of exogenous Cu^{2+}. (a) resting enzyme (200 μM) with 2-fold excess Cu^{2+}; (b) Excess EDTA was added to (a); (c) Cu^{2+} (400 μM) was added to 200 μM CcO which was preincubated with excess EDTA; (d) 200 μM Cu^{2+} complexed by EDTA in the absence of CcO. The spectrum of CuA was subtracted from (a), (b), and (c). Spectral conditions same as in Fig. 3.

Discussion

The redox active metal centers of CcO include two *a*-type hemes and two copper ions. The enzyme contains two Zn^{2+}, two Mg^{2+}, and one or two more Cu^{2+} per dimer whose biological functions are yet to be characterized. Whether or not these extra Cu^{2+} ions are adventitious is not free from dispute (Palmer, 1993). In addition to these endogenous metal ions, CcO binds various metal ions when exogenously added. According to Yewey and Caughey (1988), CcO binds 11 Cu^{2+} and 9 Zn^{2+} ions per dimer. Dialysis against EDTA leaves 3 Cu^{2+} and 2 Zn^{2+} ions tightly bound to the enzyme. Pan *et al.* (1991) found that the enzyme binds 11 Cu^{2+} and 12 Zn^{2+} ions all of which can be dissociated upon prolonged dialysis against EDTA. These results suggest that CcO has very tight binding sites for Cu^{2+} and Zn^{2+} which can not be removed easily by EDTA dialysis. Among the four metal ions examined in this work, only Cu^{2+} and Zn^{2+} had a strong influence on the activity of CcO. Both Cu^{2+} and Zn^{2+} decreased the enzyme activity by up to ~70%. It is clear that the effect is not due to binding of metal ions to cytochrome *c*, since the concentration of the metal ions was much lower than that of cytochrome *c*. These findings are in contrast to those obtained by the above authors who performed the activity assays presumably in the presence of EDTA (*vide infra*). Absorption spectrum of CcO, however, was hardly affected by these metal ions (data not shown) suggesting that the structural changes induced by the metal binding were not large enough to alter the structure of hemes. Addition of EDTA or other chelators restores the enzyme activity to some extent. That the enhancement of enzyme activity by EDTA is due to removal of these metal ions is not clear because polyanions, including EDTA, are known to affect the enzyme activity via unknown mechanisms (Malatesta *et al.*, 1987; Reimann *et al.*, 1988).

Cu^{2+} and Zn^{2+} decrease the enzyme activity of CcO with exactly same efficiency (see Fig. 1). This is simply coincidental because the two metal ions affect the vesicular CcO differently. From the fact that Cu^{2+} acts on both micellar and vesicular CcO to lower the activity but Zn^{2+} does on only micellar CcO, it can be concluded that Cu^{2+} binds from the extravesicular side whereas Zn^{2+} from the matrix side. Further evidence for the different binding sites comes from the observation that one obtains the same EPR spectrum of added Cu^{2+} regardless of the presence of Zn^{2+}.

As the binding sites are different, their mechanism of action must accordingly be different from each other. Cu^{2+} may well bind to the negatively charged amino acids localized around subunit II, which is involved in the charge interaction with the positively charged region of cytochrome *c* (Bisson *et al.*, 1978). In addition to this weakening of the CcO-cytochrome *c* interaction, Cu^{2+} binding appears to interfere with the electron transfer from cytochrome *a* to a_3, as evidenced by the elevation of steady-state reduction level of cytochrome a in the presence of Cu^{2+} (data not shown). Zn^{2+}, on the other hand, binds to the water exposed peptides extruded into the matrix. Inhibition by Zn^{2+} binding probably involves conformational changes rather than blocking the charge interaction. Differential effects of Cu^{2+} on the coupled and uncoupled state of the vesicular enzyme suggest that the Cu^{2+} binding may induce conformational changes as well.

Analyses of the EPR spectra (Figs. 3 and 4) show that the enzyme binds several Cu^{2+} ions. An EPR titration curve (data not shown) indicates that binding of the first two Cu^{2+} ions is very strong and EDTA can not immediately displace Cu^{2+} ions from the enzyme. Each binding site is provided by three or four histidines as evidenced by the EPR superhyperfine structure. The

enzyme binds a few more Cu^{2+} ions at sites composed of non-nitrogenous ligands.

It is not straightforward, however, to tell if the first two Cu^{2+} ions are responsible for the inhibitory effects shown in Figs. 1 and 2. Analyses of a series of spectra like Fig. 3a reveal that the intensity grows until the ratio Cu^{2+} : CcO becomes ~2. So we assume for the moment that the number of Cu^{2+} ions bound to the nitogenous ligands is 2 and write the reaction as

$$CcO + 2\ Cu^{2+} \rightleftarrows CcO \cdot Cu_2$$

where $CcO \cdot Cu_2$ denotes the enzyme with two bound Cu^{2+} ions. The dissociation constant K_d is defined as

$$K_d = \frac{[CcO][Cu^{2+}]^2}{[CcO \cdot Cu_2]}$$

From the data in Figs. 1 and 2, we estimate K_d to be $\sim 2.5 \times 10^{-11}$ M because the activity drops by half when $[Cu^{2+}] \approx 0.5$ μM. This implies that the Cu^{2+} binding is quantitative in the EPR samples. Therefore the analysis is self-consistent and we conclude that it is indeed the first two Cu^{2+} ions bound to histidines that inhibit the enzyme.

In order to estimate the effect of Cu^{2+} binding on the individual electron transfer steps, we have meaured steady-state reduction levels of cytochrome *c* (absorption at 550 nm) and cytochrome *a* (absorption at 605 nm). To a reaction mixture of 1 μM CcO, 4 μM cytochrome *c*, and 100 μM TMPD, was added 20 mM ascorbate to initiate the reaction and the steady-state absorption spectrum was obtained. The reduction levels of both cytochromes *c* and a were higher in the presence of 10 μM Cu^{2+} than in its absence (data not shown). Effect of 10 μM Zn^{2+} was similar but a little weaker. This indicates that binding of these metal ions retards the electron transfer from cytochrome *a* to a_3 and/or cytochrome *c* to *a*.

In conclusion, CcO has tight binding sites for Cu^{2+} and Zn^{2+} ions both of which strongly modulate the enzyme activity. Although equally potent inhibitors, Cu^{2+} and Zn^{2+} bind to different parts of the enzyme. EPR studies of the bound Cu^{2+} indicate that the first two Cu^{2+} ions bind tightly to the sites formed by histidyl nitogen atoms and account for the inhibitory effect observed in the oxidation of ferrocytochrome *c*. Biochemical consequences of the binding of Cu^{2+} and Zn^{2+}, including the inhomogeneity in the CcO preparation, are currently under investigation.

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