# The Effects of Imidazolato-bridge and Zinc on a Model Complex for the Active Site of Cu-Zn SOD

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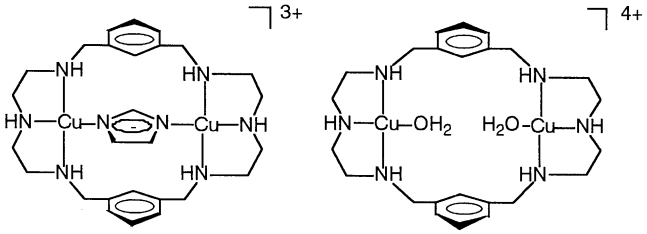
The SOD-like activity of model dicopper complexes was described. The role of imidazolato-bridged zinc was revealed by biochemical and electrochemical methods.

Copper-zinc superoxide dismutase (Cu-Zn SOD) catalyzes the disproportionation of toxic $O_2^-$ to $O_2$ and $H_2O_2$, associated with copper redox processes, as follows:[1]

$$Cu^{II} + O_2^- \rightarrow Cu^{I} + O_2$$
$$Cu^{I} + O_2^- + 2H^+ \rightarrow Cu^{II} + H_2O_2$$

Its active site has an imidazolato-bridged dimetallic center which consists of an uneven square-planar copper and tetrahedral zinc complexes. The role of the imidazolato-bridged zinc has been proposed to be the rapid dissociation of the product peroxide from the copper coordination sphere.[2] The SOD-like activity was reported for the model complexes which have an imidazolato-bridged Cu-Cu or Cu-Zn dinuclear structure.[3] But the role of imidazolato-bridged zinc was not revealed. In this paper, the model complex, which retains an imidazolato-bridged dimetallic center, was designed to investigate the effects of zinc and imidazolato-bridge on the activity of Cu-Zn SOD. Macrocyclic hexaamine was used as a dinucleating ligand which can mimic the active site of Cu-Zn SOD. The SOD-like activity was determined by using xantine, xantine oxidase, and nitro blue tetrazolium.[4]

The dicopper complexes, $[Cu_2Lim]^{3+}$ **1** and $[Cu_2L(H_2O)_2]^{4+}$ **2**, were prepared from the aqueous solutions of domestic hexaaza macrocyclic ligand L[5, 6] and $Cu^{2+}$ with or without imidazolate anion, im.[7]




**1** **2**

On the other hand, the complex formation constants of L with zinc in $H_2O$ were obtained by the simulations of the pH titration curves (Figure 1) using the program BEST[8], as shown in the table of reference 9. The overall complex formation constants were 14.48 for $[ZnL]^{2+}$, and 14.88 for $[Zn_2L]^{4+}$ : those for copper(II) are 13.79 and 23.47 respectively.[6] This means that both $[ZnL]^{2+}$ and $[Zn_2L]^{4+}$ coexist in aqueous solution and $[Cu_2L]^{4+}$ is the most stable species.

The SOD-like activities of **1** and **2** were described by $IC_{50}$[4], as shown in Table 1, with the literature data of model complexes and native enzyme : the lower value of $IC_{50}$ means the higher activity.[3] The observed values of $IC_{50}$ for **1** and **2** were less than the literature data for another model complexes : the SOD-like activities of the present model-complexes were higher. When zinc was added to aqueous solution of **1** or **2**, the SOD-like activity was higher. The difference in $IC_{50}$ between **1** and **2** means that the imidazolato-bridge also enhanced the SOD-like activity. Here, the activities of the model complexes for the present and previous works were not higher than native bovine erythrocyte SOD[3d].

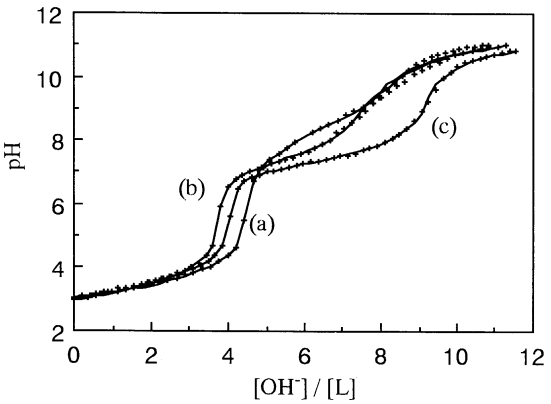



**Figure 1.** The experimental (+) and calculated ( solid line ) pH titration curves for the aqueous solutions of L and $Zn(NO_3)_2$ at 25 °C and $I$ = 0.10 M $KNO_3$, [Zn]/[L] = 0 (a), 1 (b), and 2 (c).

The cyclic voltammograms of **1** and **2** showed the effects of the imidazolato-bridge and zinc on the copper redox process as shown in Figure 2. The effect of the imidazolato-bridge was observed as the negative shift of anodic potential from (i') to (i) between solid lines of (a) and (b). The two copper(I)s of **1** and **2** were oxidized to copper(II) at -330 (i) and -240 (i') mV/SCE, respectively. This potential shift was caused by a bridging ligand at the equatorial position for dicopper(I) **1** because the bridging imidazolato increased the molecular strain of the dicopper(I)

**Table 1.** The SOD-like activities described by $IC_{50}$ for copper(II) complexes

| Complex | $IC_{50}$ / μM |
|---|---|
| **1** | 0.27 |
| **1** + Zn(II) [a] | 0.18 |
| **2** | 0.39 |
| **2** + Zn(II)[a] | 0.27 |
| $[CuZn(TMXT)(\mu\text{-im})]^{3+}$ [b] | 0.50 |
| $[Cu_2(bpzbiap)Cl_3]$[c] | 0.26 |
| $[Cu_2(pip)_2(\mu\text{-im})]^{3+}$ [d] | 0.50 |
| native Cu-Zn SOD[d] | 0.04 |

[a] [Zn]/[Cu] = 1. [b] reference 3 a) and 3 b). [c] reference 3 c ). [d] reference 3 d).

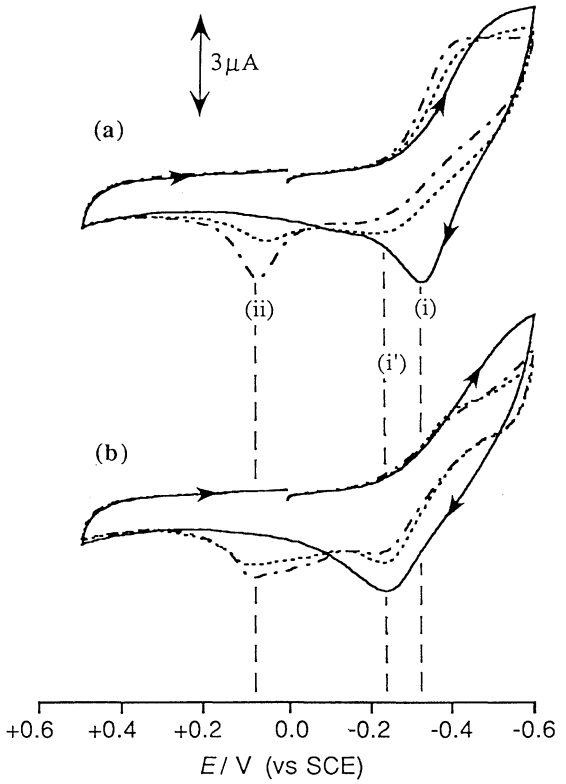



**Figure 2.** Cyclic voltammograms of dicopper complexes (a) **1** and (b) **2** recorded on a glassy carbon electrode at 50 mV/s ( pH = 7.5 in 0.10 M $KNO_3$ ) : [Cu] = 1.0 mM ; [Zn] = 0 (solid line), 1.0 mM (dot line), 2.0 mM(dot-dash line).

complex and acted on coordinated copper(I) ions as base. The copper redox process was also affected by zinc. However, the UV-vis spectra showed no substantial effect of zinc on dicopper complexes in aqueous solution.[10] One of the remarkable effects of zinc on the copper redox process was the appearance of the anodic waves (ii) at *ca.* 70 mV/SCE. As zinc concentration increased, the anodic current of (ii) increased and that of (i') decreased for both **1** and **2**. Since the new anodic waves lay around the redox potential of $Cu^{+}(aq)$ / $Cu^{2+}(aq)$[11], the exchange between Cu(I) in dicopper(I) complex and $Zn^{2+}(aq)$ creates $Cu^{+}(aq)$ during the potential sweep. Another zinc effect was the positive redox potential shift *ca.* 100 mV in the case of **1**. Since the dicopper(I) **1**, thus, is unstable and zinc has a variety of fashions in coordination around itself[12, 13], the conversion from $[Cu(I)_2Lim]^{+}$ to $[Cu(I)Zn(II)Lim]^{2+}$ can be expected from the view of their stability.

Thus, **1** was a more functional SOD model complex in comparison with **2**. When the substrate of $O_2^{-}$ or the reaction product of peroxide coordinates to copper at an axial position, they dissociate easily from the copper coordination sphere because of the much weaker interaction between the copper(II) and its axial ligands, compared with its equatorial ligands.[2] Since the equatorial coordination sites of **1** were occupied by bridging ligand, the produced peroxide coordinated to the axial position. To our knowledge, the effects of imidazolato-bridge and zinc on the SOD-like activity of model complexes were revealed for the first time from the experimental evidence.

## References and Notes


1 a) J. A. Tainer, E. D. Getzoff, J. S. Richardson, and D. C. Richardson, *Nature (London)*, **306**, 284 (1983). b) J. A. Tainer, E. D. Getzoff, K. M. Beem, J. S. Richardson, and D. C. Richardson, *J. Mol. Biol.*, **160**, 181 (1982). c) I. Freidovich, *Annu. Rev. Biochem.*, **44**, 147 (1975).

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4 C. Beauchamp and I. Fridovich, *Anal. Biochem.*, **44**, 276 (1971). Superoxide anions were generated by the xantine - xantine oxidase system and detected spectrophotometrically by the reduction of nitro blue tetrazolium (NBT) to blue formazane at 560 nm. Reactions were carried out in NBT (100 μM) and xantine (50 μM) in a phosphate buffer (10 mM) at pH 7.8 and 25.0 ± 0.1 °C . An appropriate amount of xantine oxidase (final concentration of 0.06 μM) was added into the aqueous solution mentioned above to cause a change of absorbance ( $\Delta A_{560}$ ) by 0.024 unit $min^{-1}$ which corresponds to a production rate 1.2 mM· $min^{-1}$ superoxide radical. The copper concentration which showed the 50 % inhibition of NBT reduction ($\Delta A_{560}$ = 0.012 ) is called as $IC_{50}$. The NBT reduction rate was determined within 300 s to avoid problems coming from the natural inactivation of the enzymic system. The formation of uric acid was monitored spectrophotometrically at 293 nm, if the examined complex gives rise to the generation of superoxide anions by directly interacting with the enzymic system.

5 An aqueous solution (30 ml) of L·6HBr (1.0 g), which was prepared by a literature method[6], was passed through an anion exchange column (Amberlite IRA-400) and colorless solid of L·$5H_2O$ was obtained (Yield 46%. Found: C, 57.44; H, 9.71; N, 16.50%. Calcd for $C_{24}H_{48}N_6O_5$ : C, 57.57; H, 9.66; N, 16.78%).

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7 The blue cubic crystal of **1**·$(ClO_4)_3$·$2H_2O$ was obtained by the reaction between $Cu(ClO_4)_2$·$6H_2O$, L, and imidazole (molar ratio 2:1:1) in aqueous solution (Found: C, 34.49; H, 4.99; N, 11.68%. Calcd for $C_{27}H_{45}N_8O_{14}Cl_3Cu_2$ : C, 34.53; H, 4.82; N, 11.93%). The blue powder of **2**·$(ClO_4)_4$·$2H_2O$ was obtained by the reaction between $Cu(ClO_4)_2$$6H_2O$ and L (molar ratio 2:1) in aqueous solution (Found: C, 29.94; H, 4.31; N, 8.73%. Calcd for $C_{24}H_{42}N_6O_{18}Cl_4Cu_2$ : C, 29.67; H, 4.36; N, 8.65%).

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9 The stepwise complex formation constants (log*K*) of L. Titrations were carried out under $N_2$ atmosphere at 25.0 ± 0.1 °C and $I$ = 0.10 M $KNO_3$. The test solutions were acidified with $HNO_3$ and titrated with NaOH. The protonation constants of L were previously determined.[6b]

| Equilibrium | log*K* |
|---|---|
| [ZnL] / [Zn][L] | 14.48 |
| [ZnHL] / [H][ZnL] | 5.24 |
| [ZnL(OH)][H] / [ZnL] | -7.20 |
| [$Zn_2$L] / [Zn][ZnL] | 0.40 |
| [$Zn_2$L(OH)][H] / [$Zn_2$L] | -7.90 |
| [$Zn_2$L$(OH)_2$][H] / [$Zn_2$L(OH)] | -8.11 |

10 UV-vis spectra of dicopper(II) complexes for the presence and the absence of zinc showed no difference between their broad d-d band of Cu(II) : for **1** $\varepsilon_{633}$ = 270 $M^{-1}$·$cm^{-1}$ and for **2** $\varepsilon_{644}$ = 250 in aqueous solution at pH = 7.5. The values of the above data were reproduced also in phosphate buffer solution at pH = 7.8.

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