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MEASUREMENT OF APPARENT BINDING CONSTANT BETWEEN COPPER ION AND APO-BOVINE SUPEROXIDE DISMUTASE

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SUMMARY The binding constants of copper ions to apo-bovine superoxide dismutase were measured by the method of equilibrium dialysis. The binding constant ($10^{8.9}$ M^{-1}) of copper ion to the native copper site was much larger (10^6 times) than that to the native zinc site at pH 4.0. The two native binding sites of copper ions are identical and show no interaction in the measurement of the binding constants. The binding of copper ions to the native zinc sites involved release of two protons and competition between these two protons and copper ion was governed by the pH dependence of copper binding constant to the native zinc sites.

INTRODUCTION

Bovine erythrocytes superoxide dismutase (Cu_2Zn_2SOD; superoxide:superoxide oxidoreductase, EC 1.15.1.1) has two identical subunits, each of which contains one copper(II) ion and one zinc(II) ion [1].

Cass et al. [2] have investigated the binding of zinc ions to apo-superoxide dismutase and reported that two zinc(II) ions are bound to each subunit of the apoenzyme and that the first one has a binding constant at least one order of magnitude larger than the second one at pH 6.3. Recently Pantoliano et al. [3] have reported that metal ions bound to the native zinc sites in Cu_2Zn_2SOD, Cu_2Cu_2SOD or Cu_2Co_2SOD are released in low pH but copper ion to the native copper site is strongly bound to the enzyme. This result indicates that the binding constants of various metal ions to the native zinc binding site are much lower than that of copper ion to the native copper binding site. Therefore, we have been interested in the apparent binding constants of metal ions to the native copper site or zinc site.

In this paper, the apparent binding constant of copper to each binding site in Cu_2Cu_2SOD was measured by the method of equilibrium dialysis.

MATERIALS AND METHODS

Bovine erythrocytes superoxide dismutase (Cu_2Zn_2SOD) was purchased from Miles Laboratories, Inc. and used without further purification. The copper and zinc contents were measured by a Shimadzu AA-630-12 atomic absorption spectrophotometer. The enzyme activity was measured by the method of McCord and Fridvich [4]. The apo-superoxide dismutase (E_2E_2SOD) (E=Empty) was prepared by dialysis 2,6-pyridinedicarboxylate (10^{-2} M) at pH 4.0 (0.1 M acetate buffer) and then water. The four-

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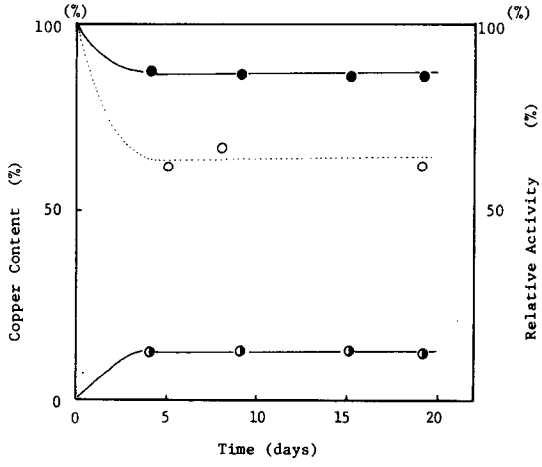

Fig. 1 Time course of equilibrium dialysis against 0.2 M acetate buffer (pH 4.7) 5.2×10^{-5} M Cu_2Cu_2SOD are dialyzed against 0.2 M acetate buffer at pH 4.7. The solid line, -●- and -◑- represent the percentage of copper content in the enzyme and ligand chambers, respectively and the dotted line and ···O··· represent the residual relative activity.

copper derivative, Cu_2Cu_2SOD was prepared by dialysis against 10^{-3} M Cu^{2+} solution at pH 6.0 (0.2 M acetate buffer) and then water. The concentration of Cu_2Cu_2SOD was determined from the copper content which was measured by an atomic absorption spectrophotometry, and its specific activity was 4500-4800 uint/mg and almost identical to that of native enzyme [5]. The two-copper ion derivative, Cu_2E_2SOD was prepared by dialysis of Cu_2Cu_2SOD against 0.1 M acetate buffer at pH 4.0 [3] and then water. The concentration of Cu_2E_2SOD was determined from the copper content, and its specific activity was 1400-1700 unit/mg and 25-35 % of the native enzyme. These values were almost the same as the literature value [5]. Equilibrium dialysis was performed by the equilibrium dialysis cell (capacity 1 ml). The equilibrium dialysis cell was assembled by using a sheet of dialysis tubing membrane. The typical procedure for this experiment was as follows. Approximately 0.8 ml of 5.0×10^{-5} M enzyme in a buffer solution of pH 4.0 was placed in compartment a, called enzyme chamber, and the same volume of various concentrations of chelating agent dissolved in the same buffer was placed in compartment b, called ligand chamber. Dialysis was allowed to proceed for about 10-15 days in cold room (at 4°). At various intervals, a certain volume of the solution was removed from both ligand and enzyme chambers. The copper content and enzyme activity were measured.

RESULTS

The Copper Content of Four-copper Superoxide Dismutase at Various pHs

Cu_2Cu_2SOD was dialyzed against various pH buffers in an equilibrium dialysis cell, and both copper content and enzyme activity were measured at regular intervals. Figure 1 shows the time course of the release of copper ion from Cu_2Cu_2SOD at pH 4.7 (0.2 M acetate buffer). The copper content and enzyme activity reached equilibrium after 3 days. The copper content and enzyme activity in equilibrium are plotted against pH as shown in Fig. 2. Figure 2 shows that copper ion bound to the native zinc site is easily released in the range $5.3 > pH > 3.2$ and this behavior was consistent with the result reported qualitatively by Pantoliano et al. [3]. The

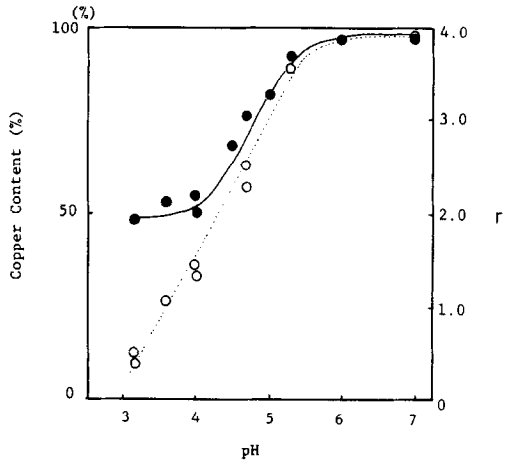

Fig. 2 The relationship between copper content of four-copper superoxide dismutase (Cu_2Cu_2SOD) and pH
Cu_2Cu_2SOD (5.2×10^{-5} M) was dialyzed against various pH buffers (pH 3.18-pH 6.0, 0.2 M acetate buffer, pH 7.0, 0.2 M Tris-HCl buffer) in the equilibrium dialysis cell. —○— Enzyme activity, —●— Copper content

copper release behavior indicates the competition between proton and copper ion against the native zinc site. On the other hand, copper ion bound to the native copper site was not release at pH 3.18. This result indicate that copper ion is strongly bound to the native copper site even at low pH. At low pH, the binding constant of copper ions to the native zinc site would be so smaller than that to the native copper site so that the binding of copper ion to the native zinc site would be have no influence on the measurement of the binding constant of copper ions to the native copper site. Therefore, the binding constant of copper ion to the native copper site was measured at pH 4.0.

Measurement of Apparent Binding Constant of Copper Ions against Native Copper Site at pH 4.0

The copper ion is strongly bound to the native copper site at low pH so that the chelating agent(2-pyridinecarboxylate) was used to remove the copper ion from Cu_2E_2SOD. Cu_2E_2SOD was dialyzed against various concentrations of 2-pyridinecarboxylate in 0.2 M acetate buffer (pH 4.0) in the equilibrium dialysis cell and the copper content of ligand and enzyme chambers were measured at regular intervals. The copper content reached equilibrium after about 3 days. The copper contents in equilibrium are plotted against the concentration of free 2-pyridinecarboxylate (L') in Fig. 3. The copper content in equilibrium decreased with an increase in concentration of 2-pyridinecarboxylate.

It may be considered that various species are distributed in the dialysis cell at equilibrium state as indicated in Chart I. The apparent binding constant of

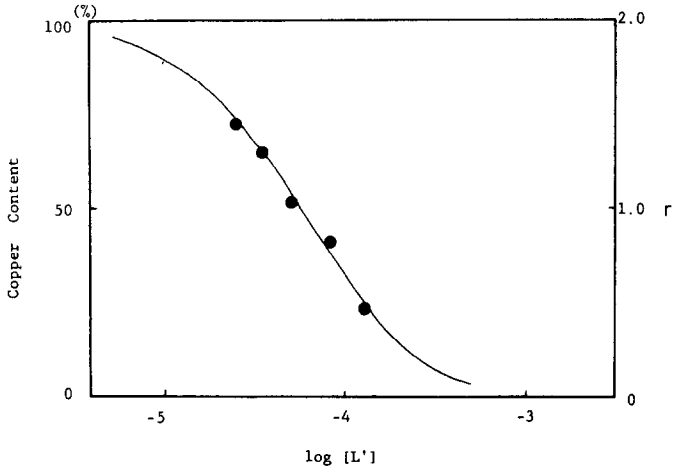

Fig. 3 The behavior of metal removal with 2-pyridinecarboxylate in 0.2 M acetate buffer (pH 4.0).
The enzyme was dialyzed in an equilibrium dialysis cell for 10-15 days at 4 °C.
● : the copper content in which Cu_2E_2SOD was dialyzed against 2-pyridinecarboxylate. The solid line represents theoretical curve as $K_1'(\text{Cu site}) = 8.0\times10^8\ M^{-1}$, R=1 and Cu_2E_2SOD (5.2×10^{-5} M).

copper ion to the native copper site was calculated by the method described in our previous paper [7] and Aasa et al. [8]. The binding of copper to apo-SOD (E_2E_2SOD) is considered to be a stepwise reaction described by the following equations at pH 4.0.

$$E_2E_2SOD + Cu^{2+} \rightleftharpoons CuE\cdot E_2SOD \qquad K_{1(\text{Cu site})} = \frac{[CuE\cdot E_2SOD]}{[Cu^{2+}][E_2E_2SOD]} \quad (1)$$

$$CuE\cdot E_2SOD + Cu^{2+} \rightleftharpoons Cu_2E_2SOD \qquad K_{2(\text{Cu site})} = \frac{[Cu_2E_2SOD]}{[Cu^{2+}][CuE\cdot E_2SOD]} \quad (2)$$

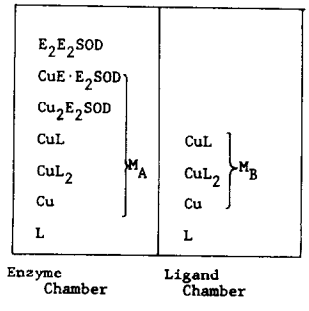

Chart 1 Distribution of various species in dialysis cells

The experimentally determined quantity r is described by the following relation[7, 8]

$$r = \frac{\text{Moles of Metal Bound}}{\text{Total Moles of Enzyme}} = \frac{[CuE\cdot E_2SOD] + 2[Cu_2E_2SOD]}{[E_2E_2SOD] + [CuE\cdot E_2SOD] + [Cu_2E_2SOD]}$$

$$= \frac{M_A - M_B}{E_0} \tag{3}$$

$$M_A = [CuE\cdot E_2SOD] + 2[Cu_2E_2SOD] + [CuL] + [CuL_2] + [Cu^{2+}] \tag{4}$$

$$M_B = [CuL] + [CuL_2] + [Cu^{2+}] \tag{5}$$

where M_A and M_B are the total metal concentration in the enzyme and ligand chambers, respectively, and E_0 is the initial concentration of the enzyme.

At given pH, apparent binding constatants K_1' and K_2' can be used, and r is represented by the following equation.

$$r = \frac{K_1'[Cu^{2+}] + 2K_1'K_2'[Cu^{2+}]^2}{1 + K_1'[Cu^{2+}] + K_1'K_2'[Cu^{2+}]^2} \tag{6}$$

The correlation between K_1 and K_2 can be given as : [8]

$$K_2 = \frac{1}{4} R\cdot K_1 \tag{7}$$

If the two binding sites are identical and show no interaction, R will be one. K_1 is given by the following equation, derived from Eqns. 6 and 7 [8].

$$K_1' = \frac{2}{R[Cu^{2+}](2-r)}\left[(r-1) \pm \sqrt{(r-1)^2 + r(2-r)R}\right] \tag{8}$$

To calculate K_1 from Eqn. 8, $[Cu^{2+}]$ must be known. It can be determined from the total metal concentration of the ligand compartment of dialysis cell [7, 8].

[L'] can be determined from the total metal concentration in the ligand chamber of dialysis cell with the aid of the known stability constants of 2-pyridinecarboxylate

$$[L'] = C_L - ([CuL] + 2[CuL_2]) \tag{9}$$

where C_L is the total concentration of the chelating agent. When the conditional stepwise stability constants of 2-pyridinecarboxylate are defined as β'_{ML_1} and β'_{ML_2} then : $\beta'_{ML_1} = \frac{[CuL]}{[Cu^{2+}][L']} = \frac{[CuL]}{[Cu^{2+}]\alpha_L[L]} = \frac{1}{\alpha_L}\beta_{ML_1}$

$$\beta'_{ML_2} = \frac{[CuL_2]}{[Cu^{2+}][L']^2} = \frac{[CuL_2]}{\alpha_L^2[Cu^{2+}][L]^2} = \frac{1}{\alpha_L^2}\beta_{ML_2} \tag{10}$$

$$\alpha_L = \frac{[L']}{[L]} = \frac{[L]+[LH]+[LH_2]}{[L]} = 1 + [H^+]K_{LH}^{H} + [H^+]^2\cdot K_{LH_2}^{H_2} \tag{11}$$

In Eqn. 10, β_{ML_1} and β_{ML_2} are stepwise stability constants, the value of which are cited from the literature[2] ($\beta_{ML_1}=5\times10^6$, $\beta_{ML_2}=2.7\times10^{12}$) [9]. α_L is calculated from Eqn. 11, because pH and pK_a values of 2-pyridinecarboxylate are known ($K_{LH}^{H}=1.7\times10^5$, $K_{LH_2}^{H_2}=1.2\times10^6$)[9]. The following expression for $[Cu^{2+}]$ can be derived from Eqn. 5 and 9-11.

$$[Cu^{2+}] = \frac{M_B}{1 + [L']\beta'_{ML_1} + [L']^2\beta'_{ML_2}} \tag{12}$$

$[Cu^{2+}]$ is derived from Eqn. 12 and when it is used in Eqn. 8, the binding constant

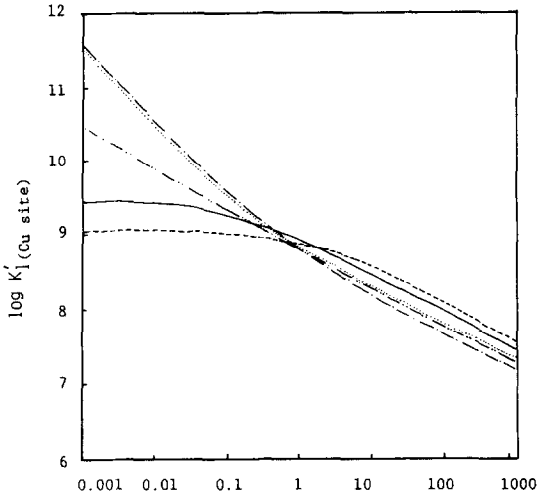

Fig. 4 The effect of adjustment of parameter R on the calculated value of $\log K'_{1(\text{Cu site})}$ for the binding of Cu^{2+} to apo-SOD. The binding data (Table I) have been used. 2-Pyridinecarboxylate concentration (C_L) : a) 5×10^{-5} M, b) 7×10^{-5} M, c) 10^{-4} M, d) 1.4×10^{-4} M, e) 2.0×10^{-4} M

between apo-SOD(E_2E_2SOD) and copper ions can be determined. But R in Eqn. 8 is unknown so that different R values are assumed and K_1 values are calculated by Eqn. 8. The correlation between R and $\log K'_{1(\text{Cu site})}$ is shown in Fig. 4. In Fig. 4, all curves cross at R=1. This result indicates that these two native binding sites are identical and show no interaction in the copper binding of protein. Therefore, $K'_{1(\text{Cu site})}$ is calculated by Eqn. 8 as R=1 and shown in Table I. Table I also

Table I

THE BINDING OF Cu^{2+} TO THE NATIVE COPPER SITE OF APO-SOD WITH 2-PYRIDINECARBOXYLATE AS COMPETING CHELATING AGENT AT pH 4.0

Various concentration of 2-pyridinecarboxylate, and Cu_2E_2SOD(5.2×10^{-5} M) were presented in both cell compartments.

Concn. of 2-pyridinecarboxylate (C_L)	Concn. of Enzyme Chamber	Concn. of Ligand Chamber	[L']	r	$\log K'_{1(\text{Cu site})}$
($\times10^{-5}$M)	($\times10^{-5}$M)	($\times10^{-5}$M)	($\times10^{-5}$M)		
5.0	8.62	1.25	2.5	1.42	8.85
7.0	8.23	1.73	3.5	1.25	8.87
10.0	7.79	2.46	5.1	1.03	8.80
14.0	6.92	2.86	8.3	0.78	8.92
20.0	6.15	3.67	12.7	0.48	8.86

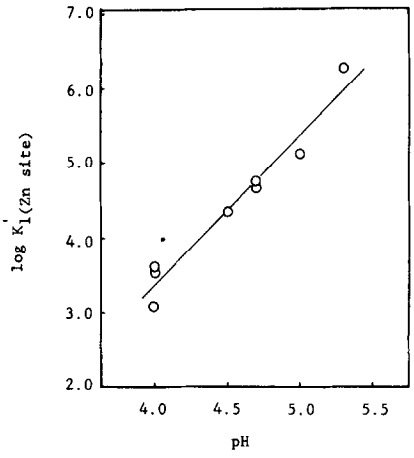

Fig. 5 The relationship between log K_1' (Zn site) for copper binding to the native zinc site and pH

includes the primary data from a set of dialysis cells. The apparent binding constant of copper ion to the native copper site was not dependent on the concentrations of the chelating agent. This fact indicates the validity of R=1.

Measurement of Apparent Binding Constant of Copper Ion against Native Zinc Site at Various pHs (4.0-5.3)

In equilibrium dialysis at low pH, copper ion in Cu_2Cu_2SOD was released from the native zinc site (in Fig. 2) and the free copper concentration were easily measured by metal concentration of none protein side of the dialysis cell.

$$[Cu^{2+}]=M_B \quad (13)$$

Therefore, the apparent binding constant of copper ions to the native zinc site was easily calculated by Eqn. 8 on the assumption of R=1, and Fig. 5 shows the relationship between the apparent binding constant of copper ion to the native zinc site and pH. The relationship between pH and logarithm of the apparent binding constant of copper ion to the native zinc site is linear as shown in Fig. 5 and its slope is about 2.0. Therefore, in the pH range 4.0-5.3, the apparent binding constant of the native zinc site varies according to the following relationship.

$$\log K'_{1(\text{Zn site})} = -4.7 + 2.0\text{pH} \quad (14)$$

DISCUSSION

At pH 4.0, the apparent binding constant (10^9 M^{-1}) of copper ion to the native copper binding site was about 10^6 times larger than that to the native zinc site. This phenomenon indicates the validity of the assumption that the binding of copper ion to the native zinc site would have no influence on the measurement of binding constant of copper ion against the native copper site.

Rigo et al. [10,11] reported the copper binding behavior to copper free-zinc superoxide dismutase (E_2Zn_2SOD) by distribution and kinetic methods. They proposed that two binding sites of the first Cu^{2+} ion were equivalent but that the occupation of the first sites lowered the activation energy of the binding site of the second Cu^{2+} ion [10]. But our data in this paper indicate that the two binding sites of copper ions are identical and show no interaction in the copper removal reaction by chelating agent from Cu_2E_2SOD.

The apparent binding constant of copper ion to the native zinc sites over a low pH range (4.0-5.3) was very small and the relationship between the logarithm of apparent binding constant ($\log K'_{1(\text{Zn site})}$) and pH was given in Eqn. 14. The slope of the straight line was about 2.0 in Fig. 5. Therefore, the reaction between the copper ion and Cu_2E_2SOD would be expressed by the following equation.

$$Cu_2E_2SOD + Cu^{2+} \rightleftharpoons Cu_2CuESOD + 2H^+$$
$$Cu_2CuESOD + Cu^{2+} \rightleftharpoons Cu_2Cu_2SOD + 2H^+ \qquad (15)$$

Pantoliano et al. reported that the metal ion in the native zinc site in Cu_2Zn_2SOD, Cu_2Cu_2SOD or Cu_2Co_2SOD is released in the range $4.5 > pH > 3.0$ and that this release of metal ions from the native zinc site ocuurs at very similar pH values [3]. They proposed that this phenomenon was not simple competition between metal ions and protons for the protein site but pH dependent conformational change [3].

Our data proved quantitatively the competition equilibrium (Eqn. 15) between copper ion and protons but it is not clear whether two protons released in the binding of copper ion to the native zinc site are released from zinc binding site or other residues by the conformation change.

REFERENCES

1. Lippard, S.J., Burger, A.R., Ugurbil,K. Valentine, J.S. and Pontaliano, M.W.(1977) Bioinorganic Chemistry, Vol. 2, pp 251-262, American Chemical Society, Washington
2. Cass, A.E.G., Hill, H.A.O., Bannister, J.V. and Bannister, W.H. (1979) Biochem. J. 177, 477-486
3. Pantoliano, M.W., McDonnell, P.J. and Valentine, J.S. (1979) J. Amer. Chem. Soc. 101, 6454-6456
4. McCord, J.M. and Fridovich, I. (1969) J. Biol. Chem., 22, 6049-6055
5. Beem, K.M., Rich, W. E. and Rajagopan, K.V. (1974) J. Biol. Chem., 249 7298-7305
6. Fee, J.A. and Briggs, G.R.(1975) Biochim. Biophys. Acta, 400, 439-450
7. Kidani, Y. Okuma, K., Hirose, J. and Noji, M.(1980) Arch. Biochem. Biophys., 200 452-460
8. Aasa, R., Malmström, B.G., Saltman, P. and Vanngard, T. (1963) Biochim. Biophys. Acta, 75, 203-222
9. Anderegg, G. (1960) Helv. Chim. Acta, 43, 414-424
10. Rigo, A., Viglino, P, Calabrese, L., Cocco, D. and Rotilio, G. (1977) Biochem. J. 161, 27-30
11. Rigo, A., Terenzi, M., Viglino, P. Calabrese, L., Cocco, D., and Rotilio, G. (1977) Biochem. J., 161, 31-35