[Chem. Pharm. Bull. 36(6)2103—2108(1988)]

Studies of Copper-Binding Behavior of Copper-Free and Apo-superoxide Dismutase by High-Performance Liquid Chromatography

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(Received September 28, 1987)

Various enzyme species differing in copper content were formed when various amounts of Cu^{2+} were added to copper-free superoxide dismutase (E_2Zn_2SOD) and the apo-enzyme (E_2E_2SOD), and were separated by high-performance liquid chromatography. The three peaks, I, II and III, arising from the former were assigned as Cu_2Zn_2SOD, $CuEZn_2SOD$ and E_2Zn_2SOD, respectively, based on the copper content and specific activity. The three peaks, A, B and C, from the latter were assigned as $Cu_2Cu_2SOD + Cu_2CuESOD$, $CuEE_2SOD + Cu_2E_2SOD$, and E_2E_2SOD, respectively.

Keywords——superoxide dismutase; metal binding; HPLC; apo-superoxide dismutase

Introduction

Superoxide dismutase in bovine erythrocytes (Cu_2Zn_2SOD[1]; susperoxide: superoxide oxidoreductase E.C. 1.15.1.1) has two identical subunits, each of which contains one copper (II) ion and one zinc (II) ion.[2] It is important to know how the copper and zinc ions bind to the native binding sites in apo-superoxide dismutase after completion of protein synthesis of apo-superoxide dismutase on a ribosome *in vivo*. To solve this problem, we first measured the binding constants of copper (II) and zinc (II) ions to apo-superoxide dismutase (metal-free enzyme; E_2E_2SOD) by the equilibrium dialysis method.[3-5] At pH 6.25, the stepwise apparent binding constants of copper ions to the native copper and zinc sites in E_2E_2SOD were $10^{13.9}\,M^{-1}$ (native copper site), $10^{13.4}\,M^{-1}$ (native copper site), $10^{11.1}\,M^{-1}$ (native zinc site), and $10^{10.6}\,M^{-1}$ (native zinc site). The stepwise apparent binding constants of zinc ions to the native copper and zinc sites in E_2E_2SOD were $10^{10.9}\,M^{-1}$ (native zinc site), $10^{11.1}\,M^{-1}$ (native zinc site), $10^{7.8}\,M^{-1}$ (native copper site) and $10^{6.5}\,M^{-1}$ (native copper site.)[3-5] When these stepwise apparent binding constants of copper and zinc ions in E_2E_2SOD are simply compared with one another, native copper sites in the enzyme have 10^6 times larger binding constant for copper ions than for zinc ions at pH 6.25. On the other hand, native zinc sites have almost the same binding constants for both zinc and copper ions. Therefore, the thermodynamic selectivity for copper and zinc ions is high in the native copper sites but low in the native zinc sites.

Fee and coworkers[6-8] have indicated that the direct addition of metal ions (copper and zinc ions) to apo-superoxide dismutase does not result in an adequate reconstitution of the apoenzyme (dialysis of E_2E_2SOD against a diluted metal solution containing a 10-fold excess of metals ($Cu^{2+}:Zn^{2+}=1:1$) led to about 70% yield of native protein).

Jewett *et al.*[9] showed that, upon the simultaneous addition of copper and zinc ions or the addition of only copper ions to apo-superoxide dismutase (E_2E_2SOD), the electrophoretic pattern in polyacrylamide gel showed only three peaks. On the basis of the theoretical

probability of metal binding to apo-superoxide dismutase, the presence of five species containing different numbers of metal ions (0, 1, 2, 3, and 4 metal ions in apo-superoxide dismutase) is expected.

High-performance liquid chromatography (HPLC) is a useful technique to separate proteins for both analytical and preparative purposes because it can provide high resolution without denaturation,[10-12] compared with the electrophoretic method in polyacrylamide gel. In this work, we searched for suitable conditions of HPLC to separate many species containing various numbers of metal ions. Each peak was fractionated and characterized according to metal contents (flameless atomic absorption spectroscopy) and enzyme activities.

Experimental

Chemicals and Reagents——Bovine superoxide dismutase (Cu_2Zn_2SOD) was purified from bovine erythrocytes by the method of McCord and Fridvich.[13] The apo-bovine superoxide dismutase (E_2E_2SOD) (E=empty) was prepared by dialysis against ethylenediaminetetraacetic acid (EDTA) (10^{-3} M) at pH 3.8 (0.05 M acetate buffer) and then 0.1 M sodium perchrolate solution (0.05 M potassium phosphate buffer, pH 7.4) to remove excess EDTA.

The copper-free bovine superoxide dismutase (E_2Zn_2SOD) was prepared by the method of Calabrese *et al.*[14] The dialysis of Cu_2Zn_2SOD against 0.05 M KCN after ferrocyanide reduction was performed in 0.1 M potassium phosphate buffer, pH 6.0, for 8—10 h.

Apparatus and Conditions for HPLC——The copper and zinc contents were measured with Shimadzu AA-630-12 flame and AA-670G flameless atomic absorption spectrophotometers. The enzyme activity was measured by the method of McCord and Fridvich.[13]

A high-performance liquid chromatograph (Shimadzu LC-3A) was equipped with a TSK-GEL DEAE-2SW column (4.6 i.d. × 250 mm) connected to its precolumn (4.6 i.d. × 50 mm) and ultraviolet (UV) detector (Shimadzu SPD-2A; at 260 nm). The elution system (flow rate, 0.5 ml/min) was a linear gradient from 0.006 M K_2HPO_4–HCl buffer (pH 7.0) containing 10^{-4} M ethylenediaminediacetic acid (EDDA) to 0.05 M K_2HPO_4–HCl buffer (pH 7.0) containing 10^{-4} M EDDA (gradient rate: 0.5%/min in the copper binding reaction for copper-free superoxide dismutase (E_2Zn_2SOD): 2%/min in the copper-binding reaction of apo-superoxide dismutase (E_2E_2SOD)). The metal contamination of E_2E_2SOD and E_2Zn_2SOD was prevented by the addition of EDDA (10^{-4} M) to the buffer solution. The enzyme activity was measured immediately after fractionation and then the fractionated solution was concentrated by the use of a collodion bag and dialyzed against metal-free distilled water to measure the metal contents of the enzyme.

Enzyme Activity and Protein Concentration——The enzyme activity was measured at pH 7.8 in 0.05 M potassium phosphate buffer with 0.1 mM EDTA (McCord and Fridvich[13]). The Shimadzu UV-200 spectrophotometer was thermostated at 25±0.5 °C with a SPR-5 Shimadzu temperature controller.

The concentration of protein was determined by the method of Lowry with bovine serum albumin as a standard.

Preparation of Sample——Sample preparation was typically carried out by the following procedure. About 300 μl of 2×10^{-4} M E_2Zn_2SOD (or E_2E_2SOD) in 0.05 M Tris–HCl buffer at pH 7.0 (or pH 8.0) was mixed with the same buffer solution (300 μl) containing various copper concentrations, and 20 μl of was injected into the HPLC column.

Results and Discussion

Copper Ion Binding to Apo-superoxide Dismutase (E_2E_2SOD)

The HPLC of apo-superoxide dismutase (E_2E_2SOD) containing various concentrations of copper ion was run with a linear gradient (2%/min) from 0.006 to 0.05 M K_2HPO_4–HCl buffer containing 10^{-4} M EDDA. The HPLC chromatogram of E_2E_2SOD containing copper ions at an $[Cu^{2+}]/[E_2E_2SOD]$ ratio of 2.0 has three main peaks (A, B, and C) as shown in (b) of Fig. 1. The retention times of copper-saturated superoxide dismutase (Cu_2Cu_2SOD) and apo-superoxide dismutase (E_2E_2SOD) are similar to those of peaks A and C, respectively. When copper ions were added to E_2E_2SOD at an $[Cu^{2+}]/[E_2E_2SOD]$ ratio of 2.0, peaks A and B were not sharp in (b) of Fig. 1. However, peak A became extremely sharp on the addition of a large excess of copper ions (4 times larger than the apo enzyme concentration) ((a) in Fig. 1). In order to characterize peaks A, B, and C, these peaks were fractionated at a $[Cu^{2+}]/[E_2E_2SOD]$ ratio of 2.0. The copper contents and specific enzyme activity of each peak were determined. Table I shows these results.

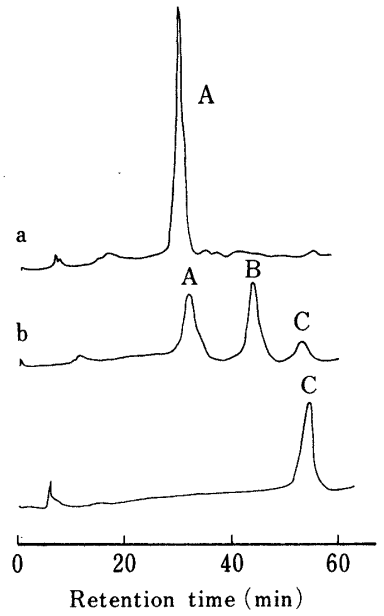

Fig. 1. Chromatogram Illustrating the Separation of Various Species in Copper Binding to Apo-superoxide Dismutase (E_2E_2SOD), as Observed at 260 nm

Cu^{2+} to E_2E_2SOD molar ratio: 3.5 (a), 2.0 (b), 0 (c).

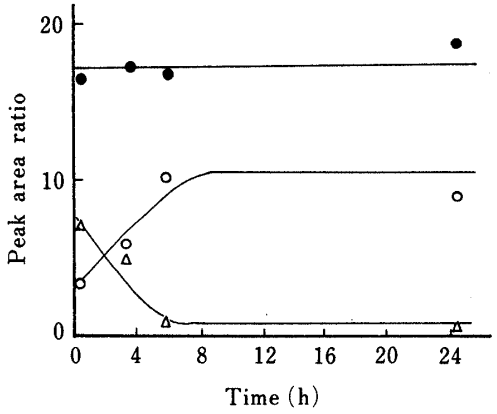

Fig. 2. Time Course of Each Peak at a Cu^{2+}-to-E_2E_2SOD Molar Ratio of 1.5

○, peak A; ●, peak B; △, peak C.

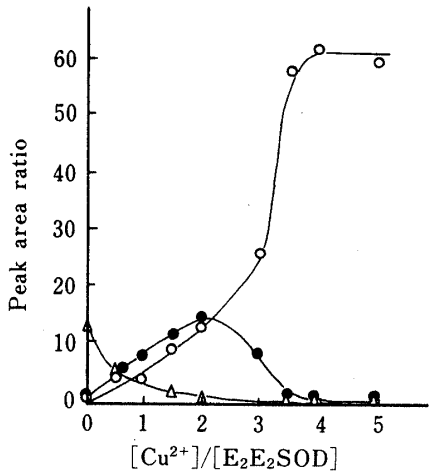

Fig. 3. Change of Each Peak as a Function of the Added Copper Ion

○, peak A; ●, peak B; △, peak C.

TABLE I. Metal Contents and Enzyme Specific Activity of Each Peak in Copper Binding to Apo-superoxide Dismutase (E_2E_2SOD)

	Peak A	Peak B	Peak C
Concentration of protein	1.11×10^{-6} M	2.77×10^{-6} M	1.11×10^{-6} M
Concentration of copper	3.76×10^{-6} M	3.64×10^{-6} M	0.33×10^{-6} M
$[Cu^{2+}]$/[enzyme]	3.39	1.31	0.3
Specific enzyme activity (unit/mg protein)	3340	860	0

In superoxide dismutase, the metal binding and dissociation rates (the half-time of metal binding for the enzyme is a few hours and that of the dissociation from the enzyme is a few days) are very slow. Therefore, the time dependence of the content of each peak of E_2E_2SOD containing copper ion at an $[Cu^{2+}]/[E_2E_2SOD]$ ratio of 1.5 was measured (Fig. 2). Each peak reached equilibrium after 8 h.

Figure 3 also shows the distribution of the three main peaks (A, B, and C) as a function of added copper ions. Peak C content continuously decreased, while peak A content increased with increasing copper concentration. Peak B content as a function of added copper ions increased with increasing concentration of copper ions at low copper concentrations ($[Cu^{2+}]/[E_2E_2SOD]$ ratio <2.0), then it reached maximum at an $[Cu^{2+}]/[E_2E_2SOD]$ ratio of 2.0 and decreased with increasing concentration of copper ions in higher copper concentrations ($[Cu^{2+}]/[E_2E_2SOD]$ ratio >2.0). This behavior indicates that peak B is an intermediate and copper-deficient species.

Table I shows that peak A can be assigned to an enzyme containing 3—4 copper ions (Cu_2Cu_2SOD and $Cu_2CuESOD$) on the basis of its copper content (3.39/enzyme) and high specific activiy (3340 units/mg protein) (the specific activity was almost the same as that of the native enzyme). In spite of the different kinds of metal ion, the retention times of native superoxide dismutase (Cu_2Zn_2SOD) and the cobalt-substituted enzyme (Co_2Zn_2SOD) were essentially identical with that of the copper enzyme (Cu_2Cu_2SOD) (data not shown). Peak A is broadened at low copper concentration and this behavior suggests that peak A contains more than one species (Cu_2Cu_2 SOD, and $Cu_2CuESOD$). The copper content of peak B was 1.3 copper ions per enzyme and the specific activity was very low (about 1/4 of that of the native enzyme). These results indicate that $CuEE_2SOD$ and Cu_2E_2SOD may be present in this peak, and the main peak is $CuEE_2SOD$. Peak C is assigned as apo-superoxide dismutase (E_2E_2SOD) from th low metal content and low specific enzyme activity. Excess copper ions were added to a part of the pooled peak C. Its specific enzyme activity was restored by the addition of copper ions (3100 unit/mg protein).

The contents of peaks A, B, and C changed as a function of copper concentration added to E_2E_2SOD, but no other peak could be found. These results suggest that some peaks overlapped each other, because the presence of five peaks (Cu_2Cu_2SOD, $Cu_2CuESOD$, Cu_2E_2SOD, $CuEE_2SOD$, and E_2E_2SOD) is theoretically expected. Jewett *et al.*[9] also showed that three peaks appeared in polyacrylamide electrophoresis of E_2E_2SOD containing unsaturated copper ion, though the peaks were not assigned. The number of peaks in polyacrylamide electrophoresis is the same as that obtained by HPLC, so that the electrophoretic peaks should correspond to those of HPLC.

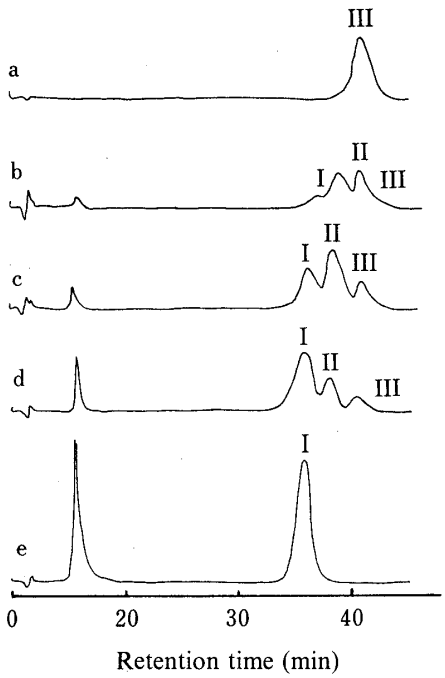

Fig. 4. Chromatograms Illustrating the Separation of Various Species in Copper Binding to Copper-Free Superoxide Dismutase (E_2Zn_2-SOD), as Observed at 260 nm

Cu^{2+} to E_2Zn_2SOD molar ratio: 0 (a), 0.25 (b), 0.50 (c), 1.0 (d), 2.0 (e). All samples were injected into the HPLC column after incubation for 6 h at 4 °C.

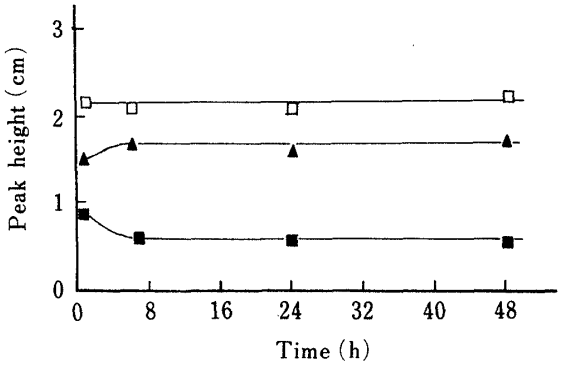

Fig. 5. Time Course of Each Peak at a Cu^{2+}-to-E_2Zn_2SOD Molar Ratio of 0.50

▲, peak I; □, peak II; ■, peak III.

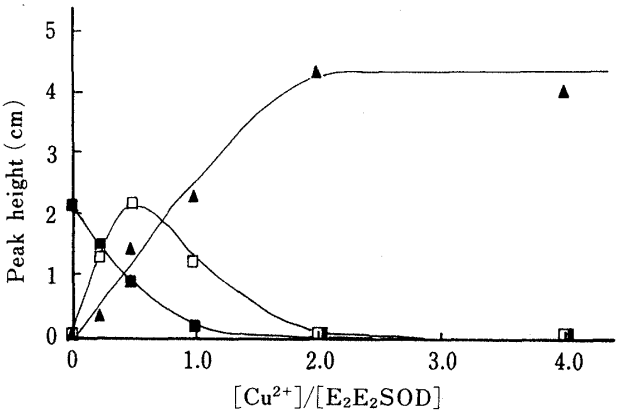

Fig. 6. Change of Each Peak as a Function of the Added Copper Ion

▲, peak I; □, peak II; ■, peak III.

TABLE II. Metal Contents and Enzyme Specific Activity of Each Peak in Copper Binding to Copper-Free Superoxide Dismutase (E_2Zn_2SOD)

	Peak I	Peak II	Peak III	Native enzyme
Concentration of protein	8.6×10^{-6} M	3.98×10^{-5} M	7.16×10^{-6} M	4.84×10^{-4} M
Concentration of copper	1.35×10^{-5} M	2.91×10^{-5} M	6.3×10^{-7} M	8.7×10^{-4} M
$[Cu^{2+}]$/[enzyme]	1.57	0.73	0.09	1.80
Concentration of zinc	1.68×10^{-5} M	7.89×10^{-5} M	1.45×10^{-5} M	8.78×10^{-4} M
$[Zn^{2+}]$/[enzyme]	1.95	1.98	2.03	1.81
Enzyme specific activity (units/mg protein)	2600	1144	0	3120

Copper Binding to Copper-Free Superoxide Dismutase (E_2Zn_2SOD)

HPLC chromatograms of E_2Zn_2SOD containing various concentration of copper ion were obtained under the same conditions as used for E_2E_2SOD containing copper ion except for the gradient rate of 0.5%/min (the three peaks (I, II, and III) of E_2Zn_2SOD containing copper ion overlapped with each other at the gradient rate of 2%/min).

Figure 4 shows an HPLC chromatogram of E_2Zn_2SOD with different $[Cu^{+2}]$/$[E_2Zn_2SOD]$ ratios from 0.25 to 2.0 (1 h after mixing), and reveals three peaks (I, II, and III). The retention times of native superoxide dismutase (Cu_2Zn_2SOD) and copper-free superoxide dismutase (E_2Zn_2SOD) were consistent with those of peaks I and III, respectively. In superoxide dismutase, the metal binding and dissociation rates are very slow. Figure 5 shows the time dependence of each peak under the conditions of E_2Zn_2SOD with 0.5 copper ion added per enzyme molecule. Each peak reached equilibrium after 6 h at 4 °C. Figure 6 shows the distribution of the three peaks (I, II, and III) as a function of added copper ions.

Peaks I, II, and III were collected in the case of E_2Zn_2SOD containing copper ion at a $[Cu^{2+}]/[E_2Zn_2SOD]$ ratio of 0.5. The metal contents and specific activities were determined to characterize these peaks (Table II). From the metal contents, enzyme activities and retention times (Table II and Fig. 4), peaks I, II and III are easily assigned to Cu_2Zn_2SOD, $CuEZn_2SOD$ and E_2Zn_2SOD, respectively. In Fig. 6, the peak II content as a function of added copper ions increased with increasing concentration of copper ions at low copper concentration, then reached the maximum at a $[Cu^{2+}]/[E_2Zn_2SOD]$ ratio of 0.5 and thereafter decreased with increasing concentration of copper ions at higher copper concentrations. On the addition of various concentration of copper ions to E_2Zn_2SOD, possible

species are E_2Zn_2SOD, $CuEZn_2SOD$, and Cu_2Zn_2SOD. Therefore, the results in Table II are consistent with expectation, and also with the result obtained by electrophoresis.[9)]

References and Notes

1) Cu_2Zn_2SOD is native superoxide dismutase which has two copper ions in the copper-binding sites and two zinc ions in the zinc-binding sites. E_2E_2SOD represents the enzyme which has no metal ions (E indicates empty). $ECuZn_2SOD$ is the enzyme which has one copper ion in the copper sites and two zinc ions in the zinc sites.
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