Relationship between the Ligand Structure of Copper and the Stability of Superoxide Dismutase

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The ligand structure of chelated copper in recombinant human $\text{Cu}_2\text{Zn}_2\text{SOD}$ (r-hSOD) and its metal-substituted SODs was examined. In r-hSOD the configuration of the copper binding site was axially symmetrical and that of metal-substituted SODs had a tendency to change towards a tetrahedral type compared with r-hSOD except for $\text{Cu}_2(\text{VO})_2\text{SOD}$. $\text{Cu}_2(\text{VO})_2\text{SOD}$ was not tetrahedral and in this case the ligand atom of copper was inclined to convert from nitrogen towards oxygen. In $\text{Cu}_2\text{E}_2\text{SOD}$, $\text{Ag}_2\text{Cu}_2\text{SOD}$, and $\text{Cu}_2(\text{VO})_2\text{SOD}$ the specific activities were lower but in $\text{Cu}_2\text{Cu}_2\text{SOD}$ and $\text{Cu}_2\text{Co}_2\text{SOD}$ they were nearly identical with that of r-hSOD. These results suggested the ligand structure of chelated copper was not related to the specific activity of SOD. However, these SODs were less stable than r-hSOD against heat treatment and denaturing reagent. Further they were less stable against attack by an inactivator (hydrogen peroxide) except for $\text{Cu}_2(\text{VO})_2\text{SOD}$. In this case the decreased stability of these SODs was associated with the change of the ligand structure of copper from that of r-hSOD. These results suggested that the presence of zinc contributed highly to the stable formation of the ligand structure of copper and the enzymic stability.

Superoxide dismutase (SOD) catalyzes the dismutation of superoxide anion radicals and has been found in a variety of organisms.^{1,2)} SOD is a metalloprotein classified according to the metal ion, including Fe, Mn, or Cu, which is necessary for SOD activity.³⁾ Bovine and human $\text{Cu}_2\text{Zn}_2\text{SOD}$ are dimeric enzymes, made of 16K identical subunits, each containing Cu(II) and Zn(II) ion.⁴⁾ Cu(II) is important in the catalytic cycle,⁵⁾ while Zn(II) stabilizes the protein structure. Study of the ligand structure of bovine $\text{Cu}_2\text{Zn}_2\text{SOD}$ (b-SOD) has been done by electron spin resonance (ESR),^{6,7)} nuclear magnetic resonance (NMR),^{8,9)} and X-ray crystallography.¹⁰⁾ The Cu(II) is coordinated to four histidines (44, 46, 61, and 118), one (his 61) of which it shares with the Zn(II) ion. The zinc is also bonded to two additional histidines (69 and 78) and aspartic acid (81).¹⁰⁾ The geometry around the Cu is a slightly distorted square plane while Zn is at

the center of a distorted tetrahedron.¹⁰⁾ The copper is also reported to have a coordinated water molecule.¹²⁾ The ESR properties of b-SOD and its various metal-substituted derivatives has been extensively investigated.¹¹⁻¹³⁾ In this report we examined the ligand structure of chelated copper in several metal-substituted SODs prepared from recombinant human $\text{Cu}_2\text{Zn}_2\text{SOD}$ (r-hSOD) and the relationship between that ligand structure and the specific activity or the stability of SOD activity was investigated.

Materials and Methods

Recombinant human $\text{Cu}_2\text{Zn}_2\text{SOD}$ (r-hSOD) was cloned and expressed in *E. coli*.¹⁴⁾ It was then highly purified by the method of McCord and Fridovich.¹⁾ Apo-SOD was prepared by the method of Weser *et al.*¹⁵⁾ The metal-substituted SODs were prepared by dialysis against buffer solutions containing metal ions as follows:

Type of SOD	Starting materials	Dialyzed buffer
Cu ₂ Cu ₂ SOD	Apo-SOD	1 mM CuSO ₄ /0.2 M acetate buffer (pH 6.0)
Cu ₂ E ₂ SOD (E = empty)	Apo-SOD	1 mM CuSO ₄ /0.1 M acetate buffer (pH 3.8)
Cu ₂ Co ₂ SOD	Cu ₂ E-SOD	1 mM CoCl ₂ /0.1 M K-phosphate buffer (pH 7.4)
Cu ₂ (VO) ₂ SOD	Cu ₂ E-SOD	1 mM VOSO ₄ /0.1 M acetate buffer (pH 5.4)
Ag ₂ Cu ₂ SOD	Apo-SOD (1st) (2nd)	1 mM AgNO ₃ /0.1 M acetate buffer (pH 3.8) 1 mM CuSO ₄ /0.1 M K-phosphate buffer (pH 7.4)

The resulting metal-substituted SOD solutions were exhaustively dialyzed against deionized distilled water. Amounts of incorporated metals were measured by X-ray fluorescence. SOD activity assays were done as described by McCord and Fridovich,¹¹ using the xanthine-xanthine oxidase system. Protein concentrations were measured by the method of Lowry *et al.*¹⁶ with bovine serum albumin (BSA) as a standard. ESR measurements were done with a JEOL FE-1XG spectrometer with a temperature controller, and analyzed with a computer system (ES-PRIT23). X-ray fluorescence was measured on a Gieger flex IKF-3064 (Rigaku Denki, Co., Japan).

Results and Discussion

ESR spectra are shown in Fig. 1 and ESR parameters are shown in Table I. ESR parameters of r-hSOD were obtained as ($g//$ 2.265, $A//$ 0.0143 cm⁻¹) and these values agreed well with those of native human and bovine Cu₂Zn₂SOD.¹⁸⁻²³ In this case the ligand structure was estimated to be an axially symmetric type as reported before. Broadened hyperfine splitting patterns of copper in Cu₂E₂SOD, Cu₂(VO)₂SOD, and Cu₂Co₂SOD indicated that the absence of zinc induced instability in the ligand structure of copper. The spectrum of Cu₂Cu₂SOD was nearly a single peak. This suggests that an interaction between two coppers occurs at the metal binding site. The configurations of copper binding site were estimated based on the model of Yokoi *et al.*²⁴ (Fig. 2).

In r-hSOD the configuration of the copper

Table I. SPECIFIC ACTIVITY AND ESR SPECTRAL PARAMETERS OF Cu₂Zn₂SOD AND METAL-SUBSTITUTED SOD

Type of SOD	Specific activity (U/mg)	g//	A/(cm ⁻¹)
Cu ₂ Zn ₂ SOD	3660	2.265	0.0143
Cu ₂ E ₂ SOD	2680	2.277	0.0138
Cu ₂ Cu ₂ SOD	3560	2.289	0.0128
Cu ₂ Co ₂ SOD	3620	2.301	0.0121
Cu ₂ (VO) ₂ SOD	1760	2.346	0.0131
Ag ₂ Cu ₂ SOD	670	2.316	0.0108

Fig. 1. ESR Spectra of Cu₂Zn₂SOD and Its Metal-substituted SODs.

A, Ag₂Cu₂SOD; B, Cu₂(VO)₂SOD; C, Cu₂Co₂SOD; D, Cu₂Cu₂SOD; E, Cu₂E₂SOD; F, Cu₂Zn₂SOD.

All samples were dissolved in 25 mM potassium phosphate buffer (pH 7.4) and measured at 173 K.

Instrument settings: microwave power, 4 milliwatts; modulation amplitude, 6.3 G; time constant, 0.1 sec; scan rate, 500 G per min.

binding site was an axially symmetric type. That of metal-substituted SOD had a tendency to change towards a tetrahedral type compared with r-hSOD except for Cu₂(VO)₂SOD. Ag₂Cu₂SOD in particular shifted to a tetrahedral type (Fig. 2, E). This suggested the copper chelated in the zinc site was typically

Fig. 2. Ligand Structure of Recombinant $\text{Cu}_2\text{Zn}_2\text{SOD}$ and Its Metal-substituted SODs Estimated from ESR Parameters.

A, $\text{Cu}_2\text{Zn}_2\text{SOD}$; B, $\text{Cu}_2\text{E}_2\text{SOD}$; C, $\text{Cu}_2\text{Cu}_2\text{SOD}$; D, $\text{Cu}_2\text{Co}_2\text{SOD}$; E, $\text{Ag}_2\text{Cu}_2\text{SOD}$; F, $\text{Cu}_2(\text{VO})_2\text{SOD}$.

tetrahedral and this result is consistent with that of the bovine SOD derivative.^{13,25)} In this SOD a little activity (18% or r-hSOD) remained. In this case the decreased activity was out of all relation to the ligand structure of copper because the copper site was occupied by Ag. In contrast, however, $\text{Cu}_2(\text{VO})_2\text{SOD}$ was not a tetrahedral type. With this SOD, the ligand atom of copper was inclined to convert from nitrogen towards oxygen (Fig. 2, F) and it may suggest that this change contributes to the low specific activity (1760 U/mg) of this SOD (Table I).

In $\text{Cu}_2\text{E}_2\text{SOD}$ the specific activity was 73% of r-hSOD. This suggests that the chelation of zinc contributes to the increase of the activity. In $\text{Cu}_2\text{Cu}_2\text{SOD}$ and $\text{Cu}_2\text{Co}_2\text{SOD}$ the specific activities were nearly identical to that of r-hSOD. These results suggested that the ligand structure of chelated copper was not related to the specific activity of SOD. Next the relationship between the ligand structure of copper and the stability of these SODs except for $\text{Ag}_2\text{Cu}_2\text{SOD}$ was examined, comparing them with r-hSOD. $\text{Ag}_2\text{Cu}_2\text{SOD}$ was excluded because copper was not chelated in the copper binding site. The stability against heat treatment (100°C, 1 min) and the denaturing re-

agent (1% SDS) are summarized in Fig. 3. The heat treatment reduced the activity of these SODs, but r-hSOD was more stable than the others (remaining activity: 73% in r-hSOD, 42% in $\text{Cu}_2\text{Cu}_2\text{SOD}$, 34% in $\text{Cu}_2\text{Co}_2\text{SOD}$, 17% in $\text{Cu}_2\text{E}_2\text{SOD}$, and 8% in $\text{Cu}_2(\text{VO})_2\text{SOD}$). R-hSOD was also more stable than the others against SDS treatment (remaining activity: 48% in r-hSOD, 4% in $\text{Cu}_2\text{Cu}_2\text{SOD}$, 34% in $\text{Cu}_2\text{Co}_2\text{SOD}$, 2% in $\text{Cu}_2\text{E}_2\text{SOD}$, and 5% in $\text{Cu}_2(\text{VO})_2\text{SOD}$). $\text{Cu}_2\text{Co}_2\text{SOD}$ was somewhat stable against SDS and this result was confirmed by activity staining after SDS-polyacrylamide gel electrophoresis.¹⁷⁾ Second, the effects of inhibitors on SOD activity were examined. Hydrogen peroxide is an inhibitor which attacks the ligand of copper in SOD. Most of the SODs were inactivated by this reagent (final conc. 3 mM), but r-hSOD was more stable (remaining activity: 40% in r-hSOD, 11% in $\text{Cu}_2\text{Cu}_2\text{SOD}$, 16% in $\text{Cu}_2\text{Co}_2\text{SOD}$, and 13% in $\text{Cu}_2\text{E}_2\text{SOD}$) (Fig. 4). Surprisingly only $\text{Cu}_2(\text{VO})_2\text{SOD}$ was resistant to the hydrogen peroxide. This may be associated with the change of the ligand atom from nitrogen to oxygen in this SOD. Sodium azide competitively inhibits the superoxide anion binding

Fig. 3. The Stability of SODs Against Heat Treatment and SDS Treatment.

(from the left): $\text{Cu}_2\text{Zn}_2\text{SOD}$, $\text{Cu}_2\text{Cu}_2\text{SOD}$, $\text{Cu}_2\text{Co}_2\text{SOD}$, $\text{Cu}_2\text{E}_2\text{SOD}$, $\text{Cu}_2(\text{VO})_2\text{SOD}$.

1, no treatment; 2, heat treatment (100°C , 1 min); 3, SDS treatment (final conc. 1% SDS, at room temperature for 30 min).

In these treatment 500 U/ml of each SOD solution was used. The value of percentage indicates the remained activity when the value of no treatment is fixed as 100%.

Fig. 4. The Stability of SODs against Inhibitors.

(from the left): $\text{Cu}_2\text{Zn}_2\text{SOD}$, $\text{Cu}_2\text{Cu}_2\text{SOD}$, $\text{Cu}_2\text{Co}_2\text{SOD}$, $\text{Cu}_2\text{E}_2\text{SOD}$, $\text{Cu}_2(\text{VO})_2\text{SOD}$.

1, no addition; 2, addition of hydrogen peroxide (final conc. 3 mM); 3, addition of sodium azide (final conc. 0.1%).

Hydrogen peroxide was added to 500 U/ml of each SOD solution and after 10 min. SOD activity was measured. Sodium azide was added to the reaction mixture containing about 10 U of each SOD and SOD activity was measured. The value of percentage indicates the remained activity when the value of no addition is fixed as 100%.

to the copper site of SOD molecule.¹¹⁾ The degree of inhibition by this reagent (final conc. 0.1%) was almost identical in all SODs (Fig. 4). This suggested the effect of sodium azide was out of all relation to the ligand structure of copper. R-hSOD was more stable than the others against the heat treatment and SDS

treatment, and further more stable against the attack by hydrogen peroxide except for $\text{Cu}_2(\text{VO})_2\text{SOD}$. In these SODs CD spectra were nearly identical and it was suggested the secondary or tertiary structure was almost identical.¹⁷⁾ These results suggested the different stability in these SODs did not derive

from the difference of secondary or tertially structure but from the difference of the ligand structure of chelated copper. In these SODs the decreased stability was associated with the change of the ligand structure of copper from that of r-hSOD. These considerations suggest that the presence of zinc contributes highly to the stable formation of the ligand structure of copper and the enzymic stability.

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