# Metalation States versus Enzyme Activities of Cu, Zn-Superoxide Dismutase Probed by Electrospray Ionization Mass Spectrometry

**ARTICLE** *in* ANALYTICAL CHEMISTRY · NOVEMBER 2008

Impact Factor: 5.64 · DOI: 10.1021/ac801324b · Source: PubMed



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# Metalation States versus Enzyme Activities of Cu, Zn-Superoxide Dismutase Probed by Electrospray Ionization Mass Spectrometry

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**Cu, Zn-superoxide dismutase (SOD-1), an enzyme that catalyzes the disproportionation reaction of superoxide to produce oxygen and hydrogen peroxide, thereby protecting cells from oxidative stress, is a homodimer that coordinates one copper and one zinc ion per monomer. $Cu^{2+}$ and $Zn^{2+}$ ions play important roles in enzyme activity and structural stability, respectively. In addition, dimer formation is also essential for fulfilling the function of SOD-1. We here report on the reconstitution and enzyme activities of several metalation states of SOD-1 ($Cu_4$-, $Cu_3Zn$-, and $Cu_2Zn_2$-homodimers). Each metalation state of the reconstituted SOD-1 could be unambiguously differentiated by electrospray ionization mass spectrometry, the metal ions of which had been completely replaced by 99 atom % $^{63}Cu$ and $^{68}Zn$ stable isotopes. It was found that (1) the $Cu_4$-dimer possessed 84% of the activity of the native enzyme, (2) the Cu-site resisted being coordinated with $Zn^{2+}$ ions while the Zn-site could be bound with $Cu^{2+}$ ions, and (3) the simultaneous addition of the $Cu^{2+}$ and $Zn^{2+}$ ions to generate a fully metalated form produced the multiply metalated SOD-1 ($Cu_4$-, $Cu_3Zn$-, and $Cu_2Zn_2$-dimers), which were clearly distinguishable from one another by the use of the stable isotopes, while the sequential addition of $Zn^{2+}$ followed by the $Cu^{2+}$ ion predominantly produced a $Cu_2Zn_2$-dimer comparable to the native enzyme.**

Various metal ions are widely distributed in organisms and often deeply involved in the functions of proteins. Metal ions are essential for some proteins to gain stability or to express enzymatic activity. Typical metals and their order of abundance in living organism are iron, zinc, and copper. Especially, a zinc ion is known to be coordinated to a specific site of a protein, which can be read out from the sequence as a Zn-binding motif. According to Zn-binding motifs in protein sequence databases, the number of proteins with zinc-binding potential in the human proteome is estimated to be 2800 sequences (~10% of the human proteome).[1] The coordination of metal ion(s) takes place in a quite specific manner: the number and kinds of metal ions, and, furthermore, the order of loading of metal ions into a protein, in the case of multiple metal ions being bound to a protein, are most likely to be controlled by the folding process in the physiological environment or three-dimensional structure of a protein.

Cu, Zn-superoxide dismutase (SOD-1) is one of the intensively studied metalloproteins that involves a divalent copper and a zinc ion in a molecule. SOD-1 exists in various organisms as a 32-kDa homodimer protein and catalyzes dismutase reactions, which eliminate toxic radical anion oxygen.[2] While copper plays the main role of the activity, the zinc ion contributes to the extraordinary high stability of SOD-1. $Cu^{2+}$ is coordinated to four His residues placed in the same plane, and $Zn^{2+}$ is coordinated to three His and one Asp residue placed at the vertex of a tetrahedral structure. One among three His residues forms the bridging imidazolate, which coordinates to both the $Zn^{2+}$ and $Cu^{2+}$ ions and functions in maintaining the pH independence of the catalytic property of SOD-1.[2−4] This unique chelation between the divalent metal ions and the protein has been exhaustively studied and was found to be crucial for the catalytic activity of SOD-1 with pH over the range 5−9.5.[5,6] In this regard, Cu-apo SOD, the zinc-deficient form of the enzyme, has been one of the research targets, which was shown, in turn, to have a pH-dependent catalytic property in a range of pH <8 [7−9] and to display 80% activity of the native enzyme at pH 6.0.[7] It is worth mentioning that Cu-apo SOD at pH >8 suffered appreciable migration of the $Cu^{2+}$ ion from the native copper site of one subunit of the zinc-free protein to the empty zinc site of another subunit,[7] which resulted in loss of the enzyme activity. In spite of the interesting behavior of Cu-apo SOD, it could be hardly produced in vivo (see below).

Strange et al. reported on the variable metalation of human SOD-1 and showed the structural stability of Zn,Zn-SOD-1 which

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10.1021/ac801324b CCC: $40.75 
Published on Web 10/01/2008

was comparable to that of Cu,Zn-SOD-1, in spite of the significant change in the structure at the copper site compared with the Cu, Zn-form; a $Zn^{2+}$ ion placed at the copper site could easily be changeable with a $Cu^{2+}$ ion in vitro.[10] Thus, they speculated that the $Zn^{2+}$ ion was likely to first occupy both the Zn and Cu binding sites in vivo, which contains a much lower level of $Cu^{2+}$ ions than $Zn^{2+}$, and the $Zn^{2+}$ ion at the copper site, then, might be replaced by $Cu^{2+}$ with the aid of a specific metal-binding chaperone for SOD-1.[11,12]

The structural aspects of SOD-1 have been recently highlighted because familial amyotrophic lateral sclerosis (ALS), sporadic ALS, or both are possibly caused by the aggregation of SOD-1, which could be triggered by the misfolding of SOD-1 resulting from gain-of-function mutations.[13−16] It is suggested that some mutations, typically A4V at a dimer interface, cause a small perturbation of the interface, leading to dissociation of a dimer to a monomer.[17−19] In conjunction with some unfavorable events, such as oxidation of His residues near the metal-binding sites,[20] an exposed disulfide bond,[21−23] and mis-metalation during a folding process,[24,25] the dissociation could give rise to the oligomerization or aggregation, eventually leading to expression of toxicity.[26] In view of these observations, the variability of the metal loading in SOD-1 and the relevance to enzyme activity is of considerable interest.

While many analytical methods, such as gel permeation chromatography,[22] NMR,[21] X-ray,[10] ESR,[7,27] visible absorption spectroscopy,[7,27] circular dichroism,[17] and thermal analysis,[17,22,26] have been applied to the investigation of metalation states and the oligomerization of a protein relative to its folding, they could not provide straightforward evidence on various metalation states or oligomerization in solution. However, electrospray ionization (ESI)-MS has been a powerful method not only for protein identification but also for detection of noncovalent complexes, such as those formed by protein−protein, protein−ligand, or protein−metal ion interactions.[28−30] In addition, it has been successfully applied to the monitoring of the folding state or refolding process of a protein upon observation of the charge-state distribution of ions that is highly sensitive to the changes of protein conformations.[31] It, thus, makes it possible to distinguish between native and denatured forms, monomer/dimer/oligomer forms, metal ion-bound and unbound forms, etc. This unique capability of ESI-MS for the above analyses prompted us to investigate the variability of the metal loading in SOD-1 and the relevance to enzyme activity.

We report here on various metalated states of SOD-1 ($Cu_4$-, $Cu_3Zn$-, and $Cu_2Zn_2$-homodimers) in relation to their enzyme activities, the preparation of which was conducted by mixing an apo dimer of SOD-1, which does not have any metal ions in the molecule, with various concentrations of $Zn^{2+}$ or $Cu^{2+}$ ions. Of note, we demonstrated that the ordinary ESI-MS technique could be successfully applied not only for distinguishing between a monomer and a dimer form but also for probing the various metalation states of the reconstituted SOD-1 when $^{63}Cu$ and $^{68}Zn$ stable isotopes, instead of the natural ones, are used as the metal ions coordinated in the protein.

## MATERIALS AND METHODS

**Materials.** Bovine Cu,Zn SOD (SOD-1) (MP Biomedicals, Solon, OH) was applied to all experiments without any further purification. Reagent grade ammonium acetate and acetic acid were obtained from Nacalai Tesque (Kyoto, Japan), and $CuCl_2$ and $ZnCl_2$ were obtained from Sigma (St. Louis, MO). A dialysis membrane (10-kDa MW-cut) was purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). All reagents for dismutase activity measurements were purchased from Wako Pure Chemical Industries (Osaka, Japan). $^{68}ZnO$ and $^{63}CuCl_2$ were obtained from Taiyo Nippon Sanso (Tokyo, Japan). $^{63}CuCl_2$ was directly applied to the experiments. $^{68}ZnO$ (10 mg) was dissolved in 50 $\mu$L of 5.7 M hydrochloric acid, and then the solution was heated until white powder was obtained.

**Sample Preparations.** SOD-1 was dissolved in distilled water and the concentration of the protein was determined to be 50 pmol (dimer)/$\mu$L (stock solution) by amino acid analysis. The stock solution was diluted to 1 or 2 pmol/$\mu$L in 10 mM ammonium acetate (pH 6.3) and subjected to MS measurement. For preparation of the apo-dimer of SOD-1, 150 $\mu$L of the stock solution was dialyzed against a 20 mM ammonium acetate buffer (pH 3.1) containing 5 mM EDTA for 2 h by using an in-house dialysis system, which has eight independent membrane (10-kDa MW-cut)-seated rooms. Then, the dialysis buffer was changed to a 20 mM ammonium acetate buffer (pH 3.1) to remove EDTA. After 2 h, the dialysis buffer was replaced with a 20 mM ammonium acetate buffer (pH 6.8) and allowed to stand for 1 h, giving the apo-dimer solution. It should be noted that since the preparation of the apo-dimer in our study was not in accordance with the previous methods,[32,33] the apo-dimer might be associated with some EDTA molecules.

**Metal Ion Loading and Enzyme Activity Assay.** Metal dichlorides were dissolved in distilled water and then diluted to 100 pmol/$\mu$L in a 10 mM ammonium acetate buffer (pH 6.3). For the experiments in Figures 2−5, the metal ion solution was further diluted in a 10 mM ammonium acetate buffer (pH 6.3) to prepare


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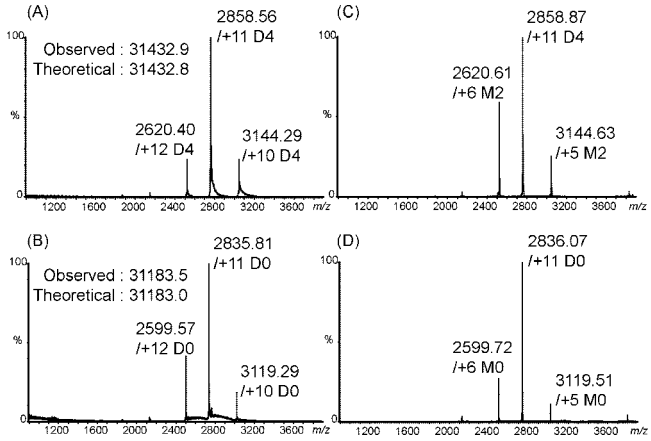


**Figure 1.** ESI-MS spectra of native SOD-1 (D4) (A) and apo-dimer (D0) (B), and ESI-MS/MS spectra from +11-charged ions in (A) (C) and (B) (D). The dimeric proteins (1 pmol/$\mu$L) were dissolved in a 10 mM ammonium acetate buffer (pH 6.3).

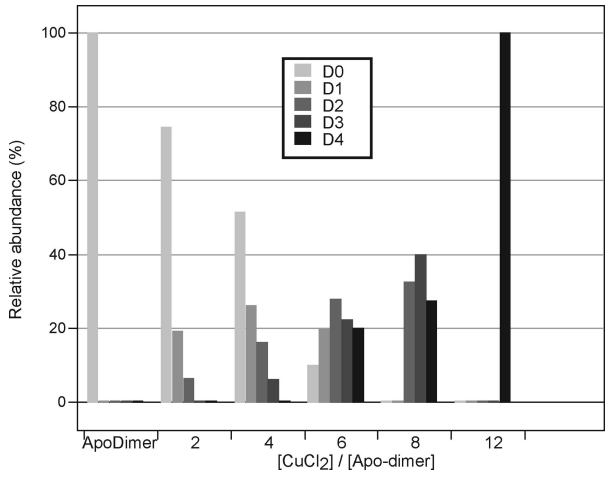


**Figure 2.** Metalation states (Cu-form) as a function of the amount of $Cu^{2+}$. The apo-dimer (D0) (1 pmol/$\mu$L) was incubated with various concentrations of $CuCl_2$ (2, 4, 6, 8, 12 pmol/$\mu$L) and subjected to ESI-MS. The relative intensities of multiply charged ion peaks observed for each metalation state of the dimer were separately summed up, and the resultant values of each form were normalized against the total (100%) to give the relative abundance of each form.

concentrations of 2.0−12 pmol/$\mu$L. The apo-dimer (D0) solution (1 $\mu$L, 50 pmol/$\mu$L) was added to the metal ion solution (49 $\mu$L, $Zn^{2+}$ or $Cu^{2+}$/D0, 2.0−12) and incubated at 37 °C for 1 h. For the experiments in Figure 6, the above apo-dimer solution (1 $\mu$L) was added to the metal ion solution (49 $\mu$L) containing $Zn^{2+}$ (4 pmol/$\mu$L → $Zn^{2+}$/D0, 3.9) and $Cu^{2+}$ ions (8 pmol/$\mu$L → $Cu^{2+}$/D0, 7.8) and incubated at 37 °C for 1 h (protocol 1) or added to the $Zn^{2+}$ ion solution (43 $\mu$L, 4.6 pmol/$\mu$L → $Zn^{2+}$/D0, 3.9), incubated at 37 °C for 1 h, thereafter mixed with the $Cu^{2+}$ ion solution (4 $\mu$L, 100 pmol/$\mu$L → $Cu^{2+}$/D0, 8.0) and incubated at 37 °C for an additional 1 h (protocol 2). An aliquot (5 $\mu$L) of the resulting mixture was used for measurements of the dismutase activity, which were carried out as described by McCord and Fridovich,[34] using the xanthine−xanthine oxidase system; the values of the activity were normalized against authentic bovine SOD-1 and obtained as the average of three experiments. Another

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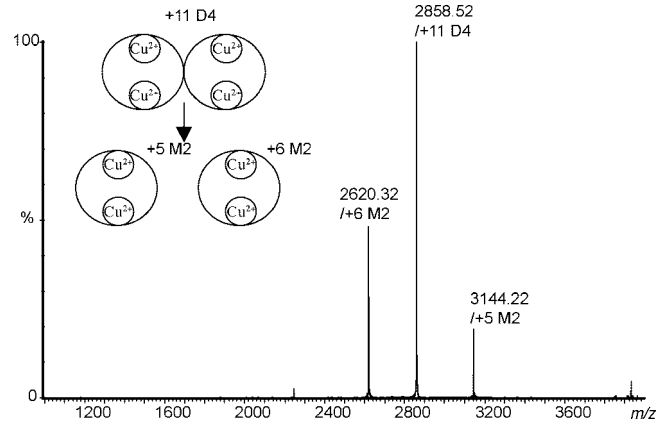


**Figure 3.** ESI-MS/MS spectrum from +11-charged ion of $Cu_4$-dimer at *m*/*z* 2858.28 (D4 in Figure 2). The collision cell was potentiated at 40 V.

5 $\mu$L of the mixture from the same tube was subjected to ESI-MS measurement.

**Mass Spectrometry.** All nano ESI-MS experiments were performed on a Q-TOF II mass spectrometer (Micromass, Manchester, UK) in a positive ion mode. To minimize artificial dimer dissociation, a capillary voltage and a cone voltage were optimized at 1.5 kV and 35 V, respectively. A nitrogen counter gas temperature was adjusted to 60 °C to avoid dimer dissociation. For MS experiments, Ar gas was introduced into a collision cell, which was potentiated at 10 V; for MS/MS, the cell voltage was elevated to 40 V. A mass range was set from an *m*/*z* at 500−4000. The sample solution was introduced into a glass capillary (Proxeon, Odense, Denmark). The inherent stability of the TOF analyzer allowed for mass accuracy of ~50 ppm using an external multiple-point calibration with NaI. All data were acquired and analyzed with MassLynx software (Micromass). Calculation of the theoretical molecular masses of various metalated SOD-1 and simulative analyses of observed peaks were carried out by Isotopica (http://coco.protein.osaka-u.ac.jp/Isotopica/), a software aid for calculating and analyzing complex isotopic envelopes, which allows automatic peak fittings of the theoretical isotopic distributions of multiple components to a raw mass spectrum.[35] Before being analyzed by Isotopica, mass spectra obtained here were subjected to adequate smoothing in the MassLynx. All the theoretical and observed masses were given as average values. To calculate molecular masses of various metalated states of a protein, protons were displaced by metals so that the net electric charge of a molecule was zero. For instance, the molecular masses of fully metalated monomer and dimer were calculated as [apo-monomer $+Zn^{2+}+Cu^{2+}-4H^+$] and [apo-dimer $+2Zn^{2+}+2Cu^{2+}-8H^+$], respectively, which were designated as M2 and D4 in this paper.

## RESULTS AND DISCUSSION

**Electrospray Ionization Mass Spectrometry of the Metalated and Apo-Forms of SOD-1.** Since the acidity, viscosity, and additives of sample solution significantly affect electrospray ionization, we first tested whether the present measurement conditions for ESI-MS (see Materials and Methods) were sufficient

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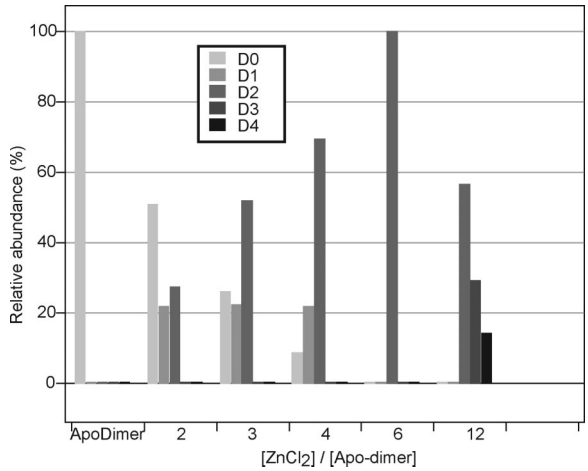


**Figure 4.** Metalation states (Zn-form) as a function of the amount of $Zn^{2+}$. The apo-dimer (D0) (1 pmol/$\mu$L) was incubated with various concentrations of $ZnCl_2$ (2, 3, 4, 6, 12 pmol/$\mu$L) and subjected to ESI-MS. The relative intensities of multiply charged ion peaks observed for each metalation state of the dimer were separately summed up, and the resultant values of each form were normalized against the total (100%) to give the relative abundance of each form.

enough to detect SOD-1 as a native form, which is a homodimer and contains a $Zn^{2+}$ and $Cu^{2+}$ ions per mole of subunits. Of note, the two metal ions, $Cu^{2+}$ or $Zn^{2+}$, loaded into the monomer of SOD-1, could hardly be differentiated from each other by the ordinary MS since the mass of copper (63.55) is close to that of zinc (65.40). Figure 1A shows the ESI-MS spectrum of SOD-1 dissolved in 10 mM ammonium acetate (pH 6.3), which was directly used for the measurement of enzyme activity in this study. As a result, the molecular ions were observed in a range of $m/z$ 2600−3200 with the charge state of +12 to +10; the molecular mass was calculated to be 31 432.9 Da, which corresponded to the homodimer with a $Cu^{2+}$ and $Zn^{2+}$ ions per mole of subunits (D4). When SOD-1 was treated with 5 mM EDTA in 10 mM ammonium acetate buffer at pH 3.1 (see Materials and Methods), the molecular ion was observed at the lower $m/z$ with the charge state of +15 to +9 (data not shown), based on which the molecular mass was calculated to be 15 591.5 Da, which corresponded to a nonmetalated monomeric form. In general, the number of charges is most probably reflected by the folding state or quarternary structure of a protein; a denatured (unfolded or extended) form carries more sites to be protonated, and thus, it is highly charged so as to be observed at a relatively lower $m/z$, while a native (folded) form has less protonation sites to be observed at a higher $m/z$. Thus, the less number of charges (+12 to +10) observed for the homodimer (D4) in Figure 1A indicated that it possessed a highly ordered structure.

When the above nonmetalated monomeric form, which gave the charge states of +15 to +9, was subjected to dialysis against a 10 mM ammonium acetate buffer (pH 6.3), it changed into the molecular species with the charge state similar to that observed for D4 (Figure 1B), implying that this molecular species regained the folding state or quaternary structure quite similar to D4; based on the +10 to +12 charged ions, the molecular mass was calculated to be 31 183.5 Da, which was distinct from D4 (calcd 31 432.8 Da) and coincided with that of a nonmetalated apo-dimer (D0) (calcd 31 183.0 Da).

Interestingly, collision-induced dissociation (CID), in the MS/MS experiments, from the ions with an odd number of charge (+11), i.e., $[D4 + 11H^+]^{+11}$ in Figure 1A and $[D0 + 11H^+]^{+11}$ in Figure 1B, gave the specific product ions, $[M2 + 6H^+]^{6+}$ and $[M2 + 5H^+]^{5+}$ (Figure 1C), and $[M0 + 6H^+]^{6+}$ and $[M0 + 5H^+]^{5+}$ (Figure 1D), respectively. The results suggested that 11 extra protons associated with D0 and D4, and the 4 metal ions in D4, were evenly distributed in the subunits (5 or 6 protons and 2 metal ions (most probably, $Cu^{2+}$ and $Zn^{2+}$) per mole of subunits); the spectral profiles were similar to each other, implying that there is little difference in conformation between D0 and D4 irrespective of coordination of metal ions and that the apo-dimer (D0) possessed sufficient stability to be observed during ESI mass measurement without any breakdown.

**Metalation States versus Enzyme Activity.** *Cu-Form.* Since the mass spectrometer used in this study could apparently differentiate among various metalation states of SOD or between a monomer and dimer form, we examined various metalation states of SOD loaded with $Cu^{2+}/Zn^{2+}$ or both ions versus the enzyme activities.

It was previously reported that bovine SOD could be reconstituted with titration of the metal ions ($Zn^{2+}$ and $Cu^{2+}$), and the resultant $Cu^{2+}{}_2$-dimeric SOD ($Cu_2$-dimer) showed approximately half the enzyme activity of an equivalent amount of the native enzyme and the $Cu_4$- and $Cu_2Zn_2$-dimers gained full activity, where two extra $Cu^{2+}$ ions of the $Cu_4$-dimer occupied the zinc-binding sites.[36] In the present study, the apo-dimer (1 pmol/uL) was titrated with 2, 4, 6, 8, and 12 equivalent amounts of the $Cu^{2+}$ ion to a dimer and an aliquot of each resultant mixture was measured by ESI-MS. It is worth mentioning that the metal dichlorides ($CuCl_2$, $ZnCl_2$), with up to 20 $\mu$M, used as the addititives in the titration experiments, did not affect the ionization efficiency of the dimer proteins, irrespective of coordination of metal ions (data not shown). As a result, a dimer that coordinated with one to four $Cu^{2+}$ ions was observed with increasing the amount of $Cu^{2+}$ (Figure 2). It had been difficult to obtain a solution containing only the $Cu_2$-dimer. A fully metalated $Cu_4$-dimer could be solely obtained with 12 equivalent amounts of $Cu^{2+}$ to a dimer, and its enzyme activity was measured (see Materials and Methods), which was normalized against the native enzyme (bovine SOD-1). The $Cu_4$-dimer displayed slightly weaker activity than bovine SOD-1 with ~84% of the activity of the native enzyme, although it was reported to have the activity compatible with Cu,Zn-SOD,[36] but its activity was comparable to the Cu-apo SOD (80 ± 5% as active as Cu,Zn-SOD).[7] It should be noted that the $Cu^{2+}$ ions of the $Cu_4$-dimer (Figure 3) and $Cu_2$-dimer (data not shown) turned out to be evenly coordinated to the constituent monomer, based on the MS/MS experiments that were carried out similarly to that in Figure 1. Furthermore, the successive incorporation of $Cu^{2+}$, up to $Cu^{2+}{}_4$, into the apo-dimer, with increasing the amounts of $Cu^{2+}$, i.e., 0.5, 1, 1.5, 2, and 3-fold excess amounts of the $Cu^{2+}$ ion to each of the four metal-binding sites, suggested the specific loading of the $Cu^{2+}$ ion into these four metal-binding sites, although the loading order of the $Cu^{2+}$ ions could not be specified with this experiment. Of note, the use of the $Cu^{2+}$ ion with more than the stoichiometric amount could be attributed to the

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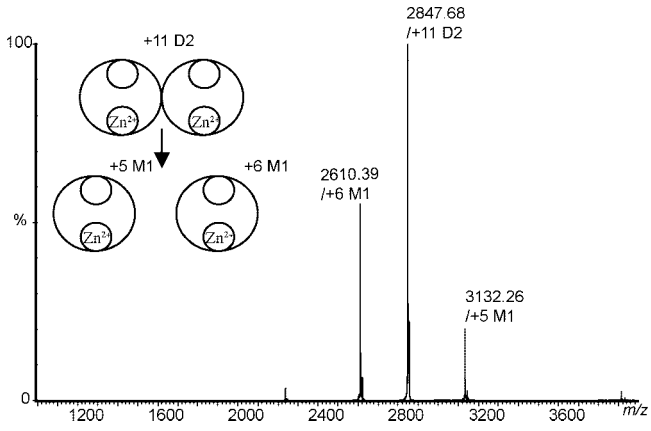


**Figure 5.** ESI-MS/MS spectrum from +11-charged ion of $Zn_2$-dimer at *m/z* 2847.41 (D2 in Figure 4). The collision cell was potentiated at 40 V.

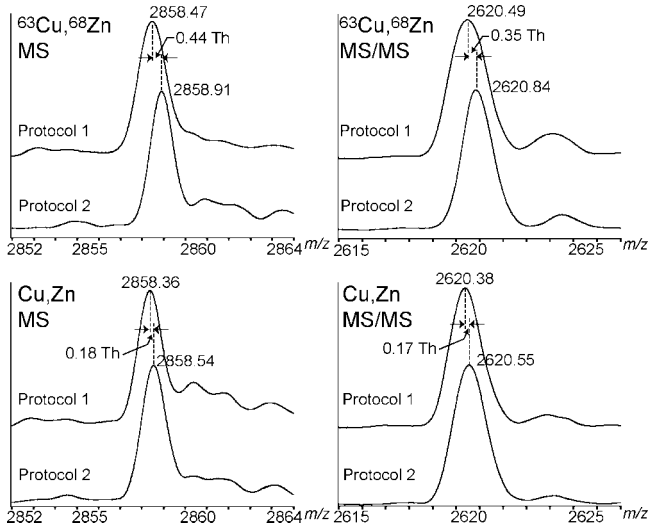


**Figure 6.** ESI-MS spectra (+11-charged ions) of the reconstituted SOD-1 (left panels) and ESI-MS/MS spectra (+6-charged ions) from the +11-charged ions in the left panel (right panels). SOD-1 was reconstituted by simultaneous loading of $Cu^{2+}$ and $Zn^{2+}$ ions (protocol 1) or by sequential loading of Zn → Cu (protocol 2) into the apo-dimer (see Materials and Methods). $^{63}Cu^{2+}$ and $^{68}Zn^{2+}$ ions, instead of their natural isotopes, were used (upper panels).

likelihood that the apo-dimer prepared in this study was associated with some EDTA molecules (see Materials and Methods).

*Zn-Form.* Next, we examined the incorporation of zinc ions into the apo-dimer under the same conditions as the above. The two $Zn^{2+}$ ions could easily be loaded into the apo-dimer, most probably, at the two Zn-sites of the dimer, with 6-fold excess amounts of the $Zn^{2+}$ ion to a dimer (Figure 4). However, only a small amount of the $Zn_4$-dimer was obtained even with the 12-fold excess amounts of the $Zn^{2+}$ ion, while the $Zn_2$-dimer was predominantly observed (Figure 4). The way of coordinating the $Zn^{2+}$ ion into the apo-dimer is likely to be distinct from that of the $Cu^{2+}$ ion (Figure 2). The $Zn_2$-dimer (+11-charged ion) split into a homogeneous $Zn_1$-monomer upon CID (Figure 5), which was mainly observed as +5- and +6-charged ions as in the case of the $Cu_4$-dimer (Figure 3), implying that the $Zn^{2+}$ ions were evenly loaded in the constituent monomer, most probably, at the Zn sites. None of the Zn forms displayed enzyme activity, as reported in a previous study.[2] Recently, Strange et al. reported on the crystal structure of the $Zn_4$-dimer and proposed that it might be the preferred metalation state for the nascent protein produced in the cell due to the fact that the concentration of the $Zn^{2+}$ ion is much higher than that of the $Cu^{2+}$ ion in a cell;[10] it then could interact with the copper chaperone for SOD, which aids in the transfer of the $Cu^{2+}$ ion to the protein, to form a mature enzyme, Cu,Zn-SOD. The present result, however, clearly showed that the $Zn^{2+}$ ion over 2 equiv to a dimer displayed some resistance to be loaded into the protein, which was distinct from the case of incorporation of the $Cu^{2+}$ ion. The higher concentration of the $Zn^{2+}$ ion over 1 mM might eventually be adequate to form a $Zn_4$-dimer, two $Zn^{2+}$ ions of which occupy the Cu sites. It is noteworthy, however, that the intracellular zinc ion is thought to be available in the picomolar to micromolar range,[37] which is comparable to the concentrations of $Zn^{2+}$ ion (2−12 $\mu$M) used in the present study; it has been difficult to examine the higher concentration of $Zn^{2+}$ ion for observation of a $Zn_4$-dimer since the concentration of the added metal ions higher than 20 $\mu$M starts to interfere with the electrospray ionization.

*Cu,Zn-Form.* Finally, we examined the incorporation of both $Cu^{2+}$ and $Zn^{2+}$ ions into the apo-dimer of SOD-1. The following two preparations were tested: (1) $Cu^{2+}$ and $Zn^{2+}$ ions were added at the same time to the apo-dimer to generate a fully metalated form (D4) (protocol 1: SOD [Zn + Cu]); (2) the $Zn^{2+}$ ion was first incubated with the apo-dimer and then with the $Cu^{2+}$ ion (protocol 2: SOD [Zn→Cu]). Both protocols were likely to predominantly yield fully metalated dimers, the observed molecular masses (protocol 1, 31 430.9 Da; protocol 2, 31 432.9 Da) of which were close enough to be identical (the lower left panel in Figure 6), taking into account the mass accuracy achieved with the instrument used in this study (see Materials and Methods). However, the enzyme activities of the products were apparently distinct from each other; i.e., SOD [Zn + Cu] displayed 78 ± 10% of the activity of the native SOD, while SOD [Zn→Cu] had 93 ± 5% of the activity (Table 1). Because the masses of naturally occurring isotopes of Cu and Zn, which distribute to $^{63}$Cu (69.2%) and $^{65}$Cu (30.8%), and $^{64}$Zn (48.6%), $^{66}$Zn (27.9%), $^{67}$Zn (4.1%), $^{68}$Zn (18.8%), and $^{70}$Zn (0.6%), are too close to be distinguishable with the present MS technique when associated with a high molecular weight compound such as a protein, the lower enzyme activity of SOD [Zn + Cu] could be attributable to the presence of non-native metalated forms, such as $Cu_4$-, $Cu_3Zn$-, $CuZn_3$, or $Zn_4$-forms. In order to determine the exact metal ion coordination in the above products, the stable isotopes of copper ($^{63}$Cu) and zinc ($^{68}$Zn) (99 atom % for each), instead of the natural ones, were loaded into the apo-dimer, and the products were examined by ESI-MS (the upper left panel in Figure 6). The molecular mass of SOD [Zn → Cu] was calculated to be 31 436.7 Da (the lower trace of the upper left panel in Figure 6), which agreed well with a theoretical mass (31 436.6 Da) for the $^{63}Cu_2{}^{68}Zn_2$-dimer. In addition, the MS/MS of the +11-charged ion split the dimer into a monomer mainly as +6- and +5-charged ions, as seen in Figure 1C. The close charge splitting of the dimer (+11) into the dissociated monomer (+5 and +6) demonstrated that the protons associated with the dimer are evenly distributed on each constituent monomer. As reported

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**Table 1. Metalation States versus Dismutase Activities of Cu, Zn-SOD-1**

| | relative ratio of metalated forms of SOD-1 (%)[a] | | | | | enzyme activity (%)[b] |
|---|---|---|---|---|---|---|
| measurement | MS | | | MS/MS | | |
| charge state | +11 (dimer) | | | +6 (monomer) | | |
| metalated form | $^{63}Cu_4$ | $^{63}Cu_3{}^{68}Zn_1$ | $^{63}Cu_2{}^{68}Zn_2$ | $^{63}Cu_2$ | $^{63}Cu_1{}^{68}Zn_1$ | |
| SOD[Zn + Cu][c] | 40.9 | 17.6 | 41.5 | 53.6 | 46.4 | 78 ± 10 |
| SOD[Zn → Cu][d] | 7.0 | 0.1 | 92.9 | 9.9 | 90.1 | 93 ± 5.0 |

[a] Obtained from Figure 7. [b] The data, normalized against SOD-1, were the average of three independent experiments. [c] Protocol 1: SOD-1 was reconstituted by simultaneous loading of $^{63}Cu$ and $^{68}Zn$. [d] Protocol 2: SOD-1 was reconstituted by sequential loading of $^{68}Zn$ → $^{63}Cu$.

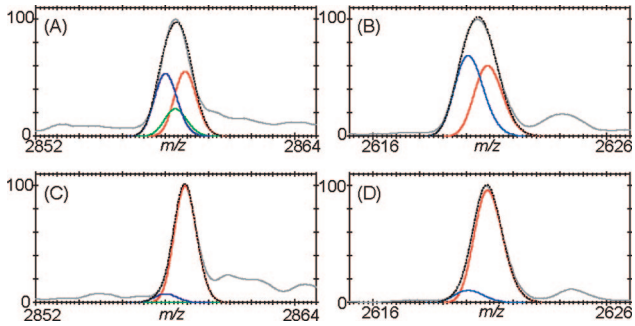


**Figure 7.** Deconvolution of the raw spectra obtained in Figure 6 into the respective isotope species, i.e., $^{63}Cu_2{}^{68}Zn_2$ (red), $^{63}Cu_3{}^{68}Zn$ (green), and $^{63}Cu_4$-dimer (blue) (A, C); $^{63}Cu^{68}Zn$ (red) and $^{63}Cu_2$-monomer (blue) (B, D). The simulative fitting of the observed peaks to the theoretical ones (dashed line) was carried out by Isotopica, which concomitantly output the relative abundances of the above components (see Table 1 and Results and Discussion).

previously,[38] the symmetric distribution of protons in a homodimer was indicative of little conformational difference between the constituent monomers. Thus, the monomers were unlikely to happen to interact with each other in a nonspecific manner during the ionization process. In addition, the observed molecular mass (15 719.1 Da) of the monomer (the lower trace of the upper right panel in Figure 6), agreed well with the theoretical mass of $^{63}Cu^{68}Zn$-monomer (15 718.3 Da), which was distinct from that of $^{63}Cu_2$- (15 713.3 Da) or $^{68}Zn_2$-monomer (15 723.3 Da). Moreover, the ion peak of this monomer could be deconvoluted into a $^{63}Cu^{68}Zn$-monomer (90.1%) and a $^{63}Cu_2$-monomer (9.9%) by simulative analysis of the peak with Isotopica[35] (Figure 7D, Table 1), based on the two most possible components of the $^{63}Cu_2$- and $^{63}Cu^{68}Zn$-monomers (the $Zn_2$-monomer was not included in this calculation, the population of which was found to be very small from the result in Figure 4). Based on these two components ($^{63}Cu_2$- and $^{63}Cu^{68}Zn$-) of the monomer, three possible dimers ($^{63}Cu_4$-, $^{63}Cu_3{}^{68}Zn$-, and $^{63}Cu_2{}^{68}Zn_2$-dimers) could be generated. The relative ratio of each dimer was calculated similarly to the above as 7.0, 0.1, and 92.9%, respectively (Figure 7C, Table 1); this composition well accounted for the overall enzyme activity of 93 ± 5.0% for SOD [Zn → Cu] (Table 1). On the other hand, SOD [Zn + Cu] gave slightly broad peaks for each charge state of ions in comparison with those observed for SOD [Zn → Cu]; typically, the centroid of the peak at +11-charged ion (the upper trace of the upper left panel in Figure 6) was evidently at a lower $m/z$ by 0.44 Th than that obtained for SOD [Zn → Cu]. In addition, the +6-charged ion peak of the monomer in the upper trace of the upper right panel in Figure 6, which was produced upon CID in the MS/MS experiment from the +11-charged ion, was observed at 0.35 Th less than that of SOD [Zn → Cu] and could be deconvoluted into those of the $^{63}Cu_2$-monomer (53.6%) and CuZn-monomer (46.4%) by simulative analysis (Figure 7B, Table 1). The result suggested that the Zn-site could be bound with both the $Cu^{2+}$ and $Zn^{2+}$ ions, while the Cu-site had a preference for $Cu^{2+}$ over the $Zn^{2+}$ ion. Moreover, based on the fact that the majority of the metalated dimers were distributed evenly into the $Cu_4$- (40.9%) and $Cu_2Zn_2$-dimers (41.5%) (Table 1), when either of the two Zn-sites was occupied with Cu or Zn, another Zn-site had a propensity to be coordinated with the same metal ion as the one in the other site. The rest of the metalated form, the $Cu_3Zn$-dimer (17.6%), might be produced by shuffling between the constituent monomers of the $Cu_4$- and $Cu_2Zn_2$-dimers. The presence of the $Cu_4$-dimer in SOD [Zn + Cu], which has ~84% of the potency of the native enzyme, might in part imply the overall deficiency of the enzyme activity (78 ± 10% of the native one) observed for SOD [Zn + Cu] in spite of the specific activity of the $Cu_3Zn$-dimer being unclear.

## CONCLUSIONS

The structure of SOD-1 in solution, i.e., native or denatured, monomeric or dimeric, and the metal ion coordination, could be probed by ESI-MS, and direct correlation with the dismutase activity has been achievable since the enzyme activity could be readily measured using the solution that had been subjected to mass measurement. The results gave insights into the functional relevance of the metal ion coordination. The native form of SOD, a homodimer associated with the $Cu^{2+}$ and $Zn^{2+}$ ions per monomer, could be observed with ESI-MS; the fact that the multiply charged ion (+11) of the dimer bore symmetric charge partitioning into +5 and +6 of the monomer upon CID suggested the structural resemblance of the constituent monomers. In the experiments on the metal loading of either $Cu^{2+}$ or $Zn^{2+}$ into the apo-dimer, $Cu^{2+}$ could occupy both the Zn- and Cu-sites; a fully metalated $Cu_4$-dimer expressed 84% of the enzyme activity of the native one. The $Zn^{2+}$ ion could hardly be loaded into the Cu-site, which predominantly forms a $Zn_2$-dimer. The successive incorporation of $Cu^{2+}$, up to $Cu^{2+}{}_4$, into the apo-dimer (Figure 2) suggested the specific loading of the $Cu^{2+}$ ion into the four metal-binding sites, although the loadaing order of $Cu^{2+}$ ions could not be specified with this experiment. Furthermore, the sequential addition of $Zn^{2+}$ followed by the $Cu^{2+}$ ion produced a $Cu_2Zn_2$-

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dimer comparable to a native enzyme; meanwhile, the simultaneous addition of $Cu^{2+}$ and $Zn^{2+}$ ions could produce an active enzyme but with apparently less activity than the native one (Table 1). The deficiency of activity (78 ± 10%) could be accounted for by the fact that the $Cu_4$-dimer, which possessed 84% of the potency of the native enzyme, turned out to constitute 40.9% of this preparation (Table 1).

ESI-MS has the great advantage of monitoring not only the folding process of a protein from the denatured form to the native one and vice versa, including a noncovalent oligomer, but also the metal ion coordination in the case of a metalloprotein. In addition, due to the fact that ESI mass measurements are feasible under physiological conditions, with respect to sample concentration (1 $\mu$M SOD was applied for the measurements in this study), pH, and the tolerance for some additives, we can rationalize the discussion on the relationship between the metal ion coordination of a protein and the function. The method can also be applicable for screening the metal ion(s) loaded into a protein; especially, the $Zn^{2+}$ ion, the second most abundant metal ion essential for an organism, is known to be incorporated into Zn-binding motifs, the proteins of which are estimated to reach ~10% of the human proteome.

## ACKNOWLEDGMENT

This study was supported by a Grant-in-Aid for Creative Scientific Research (Grant 15GS0320 to T.T.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a project on "Research on Proteomics" from the Ministry of Health, Labor and Welfare of Japan.

Received for review June 28, 2008. Accepted August 26, 2008.

AC801324B