Silver-Binding Properties of Bovine Cuprozinc Superoxide Dismutase and the Overall Stability of Selected Metal-Ion Derivatives

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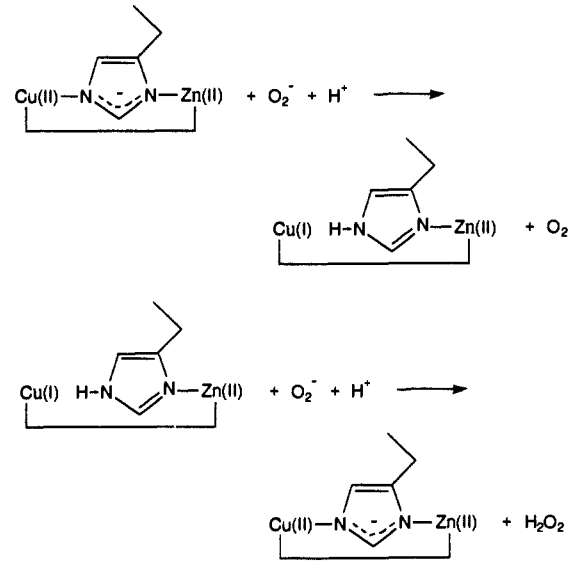
Abstract: We have determined the silver-binding constants for the copper-depleted (E, Zn-SOD), zinc-depleted (Cu, E-SOD), and the metal-free (apo-SOD) derivatives of Cu, Zn-superoxide dismutase (SOD). The constants were obtained over a large pH range by complexometric titration of protein-bound Ag^+. The courses of these titrations were followed by the detection of silver ion activity with an ion selective electrode (ISE). We have observed that the apoprotein dimer tightly binds up to 4 equiv of Ag^+ in two classes of binding sites. By contrast, E, Zn-SOD, in which two zinc ions remain bound at the zinc-binding sites, and Cu, E-SOD, in which two copper ions remain bound at the copper sites, exhibited large affinities for only 2 equiv of Ag^+, which were bound at only one class of site. The values of the silver-binding constants of all the derivatives reached maxima at pH > 7.5 with the copper-depleted derivative exhibiting the largest affinity for Ag^+ measured in this study, i.e., log K = 12.95 at pH 10.1. This final result indicates that occupancy of the zinc site enhanced the binding of Ag^+ at the copper site. Interestingly, at the same pH of 10.1, the value of the silver-binding constant of Cu, E-SOD was very near to that constant which characterizes the binding of the first 2 equiv of silver ion to the apoprotein, i.e., log K = 10.3 and 10.2, respectively. In addition, we present evidence that supports the conclusion that the thermodynamically preferred configuration of the metal-ion derivative of SOD which contains two ions of Ag^+ and two of Cu^{2+} has the silver ion bound at the native copper binding site with the copper ion bound at the zinc site. Finally, we define the overall solution stability constant, $S_{M,N}$, which quantitates the stability of the protein metal-ion derivative, in which the metal ion M occupies the copper-binding site and the metal-ion N occupies the zinc-binding site of SOD.

Bovine cuprozinc superoxide dismutase (Cu_2Zn_2SOD)[1] is a water soluble dimeric protein with M_r = 31 200 which contains one Zn^{2+} and one Cu^{2+} in each of its two identical subunits.[2,3] The cupric ion is coordinated by the imidazoyl nitrogens of four histidine residues located at the active site and by a water molecule. One of the imidazoles bound to the copper ion is deprotonated and coordinated to the zinc ion as well and serves as a bridge between the two ions. It is generally believed on the basis of spectroscopic and proton uptake studies[2,4] that the imidazolate bridge is broken and protonated on the copper side when the copper ion is reduced from 2+ to 1+, with the imidazole remaining as a zinc ligand in the reduced protein. In addition to the bridging imidazolate ligand, the zinc ion is coordinated by two more histidyl imidazoles and by the carboxylate group of an aspartyl residue. The X-ray crystal structure has been determined at 2 Å resolution and indicates that the copper binding site has a distorted square-pyramidal symmetry, whereas the zinc site is close to tetrahedral.[5]

Cu_2Zn_2SOD is one of the most thoroughly characterized non-heme metalloproteins. The protein has served as a unique "ligand" in many spectroscopic and bioinorganic investigations of bridged metal-ion systems, especially of those derivatives in which the native metal ions have been replaced by others. These derivatives include Co_2Co_2SOD, Cu_2Co_2SOD, Cu_2Ni_2SOD, Ni_2Co_2SOD, and Cu_2Cu_2SOD, all of which are believed to contain intact imidazolate bridges, and $Cu^I_2Cu^{II}_2SOD$, $Cu^I_2Zn_2SOD$, Ag_2Cu_2SOD, Ag_2Co_2SOD, $Cu^I_2Co_2SOD$, Ag_2Ni_2SOD, Cu^I_2-Ni_2SOD, and Ag_2Zn_2SOD, which do not appear to maintain the bridge.[6-10] Many of these metal ions, which have been substituted for the native metals, have served as structural probes of the metal-binding sites. Coordination changes at the active site are known to arise from several sources including the strain induced at the binding sites by the introduction of the non-native metal ion itself as well as the effects of anion binding to the metal and changes in pH.[11-13] Nonetheless, the information gained by these

Scheme I



(1) Abbreviations: Cu_2Zn_2SOD represents the native form of cuprozinc superoxide dismutase as isolated from bovine liver. In general, X_2Y_2SOD signifies those derivatives of the native protein in which the metal ions X and Y have been substituted for the native Cu^{2+} and Zn^{2+}, respectively (X and Y may be the same: E = empty). On occasion, the $XYSOD_{sub}$ is used to represent one of the identical subunits. All metal ions are assumed to be 2+ unless noted otherwise, except for silver which has an oxidation state of 1+.

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metal-ion probes has helped to clarify the structure and the mechanism by which SOD catalyzes the disproportionation of superoxide anion to dioxygen and hydrogen peroxide.

The current understanding of the disproportionation mechanism catalyzed by Cu_2Zn_2SOD is illustrated in Scheme I.[14] In the first step, an O_2^- molecule reduces the cupric center to cuprous and is itself oxidized and released as dioxygen. The reduction of the copper ion is accompanied by the breaking of the imidazolate bridge and an uptake of a proton.[15] Zinc EXAFS data on both the native and reduced forms of the protein suggest that in the reduced form the Cu^+ may be coordinated to three imidazoles and Zn^{2+} is coordinated by the remaining imidazoles, including the bridging imidazole and the aspartate residue.[16] The second step is the oxidation of the reduced copper center by superoxide which converts O_2^- into hydrogen peroxide and the metal-binding region into its oxidized or native configuration with the bridge reformed. There has been no X-ray crystal study of the reduced form and consequently the details of the structure of the metal-binding region of reduced SOD are unknown.

Since Ag^+ and Cu^+ both have the same electron configurations (d^{10}) and similar coordination preferences, silver ion may be considered a reasonable substitute and mimic for Cu^+ in the reduced form of the protein as well as a stable probe of the structure of the metal-binding region of the reduced native protein. Interestingly, though many metal ions preferentially bind to SOD at the zinc site, Ag^+ is the only nonnative metal ion which will bind preferentially at the copper site, even in the presence of stoichiometric amounts of Cu^{2+}, which binds instead to the zinc site.[9] The spectroscopic characteristics of Ag_2Cu_2SOD (UV–vis, ESR) are very similar to those of $Cu^{I}_2Cu^{II}_2SOD$, where the copper in the copper site has been reduced to 1+ oxidation state, affirming the use of Ag^+ as a stable model for the reduced form of copper in the protein. In addition, comparisons of Ag_2Co_2SOD with $Cu^{I}_2Co_2SOD$ by NMR and optical spectroscopy and the NMR comparisons of Ag_2Ni_2SOD with $Cu^{I}_2Ni_2SOD$ indicate that the Ag^+ derivatives are nearly spectroscopically identical with the cuprous ones.[6]

We have undertaken the study of the interaction between the apoprotein and Ag^+ by determining the apparent binding constants for silver derivatives of SOD (i.e., Ag_2E_2SOD, Ag_2Ag_2SOD, Ag_2Cu_2SOD, and Ag_2Zn_2SOD) as functions of pH and competition with the native metal ions. We have chosen to do so for three reasons: (1) in order to further the thermodynamics of metal binding by this "unique" binuclear biological ligand; (2) to study the effects of the occupancy of the zinc site on the metal-binding affinity of the copper site, i.e., cooperativity; and (3) to gauge the importance of both the protein itself and the occupancy of the zinc site on the increased reduction potential of Cu^{2+} in SOD (relative to aqueous Cu^{2+}); i.e., in what ways does SOD stabilize the reduced state of the protein? The final point is certainly to be considered important in the understanding of the mechanism of SOD, which may involve cycling between the cupric and cuprous states.

Experimental Section

Materials. HEPES buffer (*N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid) was obtained from Sigma and used as received. The metal ion sources were commercially obtained standard solutions: 100 mM $Cu(NO_3)_2$ from Orion; 1000 ppm $Zn(NO_3)_2$ from Ricca; and 1000 ppm $AgNO_3$ from Fisher. All other reagents were AR grade and used without purification. Triply deionized water (Millipore) was used exclusively throughout the study. The concentrations of the NaI and NaCN stock solutions were determined by titration against the commercially obtained silver standard as monitored by a Ag_2S-silver selective electrode (vide infra). The methods of Asplund[17] were followed for the titration of NaCN solutions. Typically, NaI solutions were ca. 5 mM, and those of NaCN were ca. 10 mM.

Protein Manipulation and Derivatives. Bovine liver Cu_2Zn_2SOD was obtained from Diagnostic Data, Inc. (Mountain View, CA) as a lyophilized powder. Protein concentrations of native SOD and the apoprotein were determined spectrophotometrically, with ϵ_{258nm} = 10 300 and 2920 M^{-1} cm^{-1}, respectively. The protein concentration of metal-substituted derivatives was determined by the Lowry method.[18] Metal content of protein derivatives were determined by atomic absorption measured by a Perkin-Elmer Model 303 atomic absorption spectrometer. The preparation of apo-SOD and Cu_2E_2SOD has been described previously.[8] The method of Beem et al.[9] was followed to prepare Ag_2Cu_2SOD. Before use, apoprotein solutions were filtered through 0.22 micron sieves.

NMR of the Silver Derivatives. The ^1H NMR spectra of the metal-substituted derivatives of SOD were measured with a Bruker AM 500 MHz spectrometer, by using a 1-3-3-1 hard pulse sequence.[19] Typically, the concentration of a protein sample was 0.5 mM, and 1000 acquisitions were sufficient to produce good spectra.

The metal-substituted derivatives were prepared by direct infusion of metal ions into the apoprotein solution over a period of several minutes under constant stirring. The reduced native derivative containing Cu^+ was produced by the reduction of bound Cu^{2+} by direct addition of small quantities of solid sodium dithionite until the color of the protein solution (blue-green) had faded completely to the eye.

Measurement of Free Silver Ion Activity. Ion selective electrode (ISE) measurements were performed at room temperature (18–22 °C) by an Orion 901 digital ion analyzer equipped with a Model 605 electrode switch. Although we realized that an error in the calculation of the silver-binding constants would arise from data measured under mildly uncontrolled temperature conditions (i.e., a 4° range), we judged that the error in the magnitude of the constants would be slight. For instance, if we were to assume that the enthalpy ($|\Delta H°|$) of binding Ag^+ to the protein was 10 Kcal/mol (cf. $AgEDTA^{3-}$ for which $\Delta H°$ = −5.3 Kcal/mol, ref 20), the change in the magnitude of the binding constant over that 4° range would only be ca. 0.1 log unit (ref 21, p 7.). Silver activity was determined with an Orion (94-15A) Ag_2S-ISE referenced against a single junction electrode (Orion Model 900100) filled with a 0.5 M solution of KCl, which had been saturated with Ag^+. The surface of the silver ISE was polished with Orion polishing strips, placed in 20 mM H_2SO_4 for 5 min, and, then, thoroughly rinsed with triply deionized water prior to electrode use.

Electrode readings (in mV) were correlated with free silver concentration by the use of a series of silver standards as calibration. These standards included Ag^+-saturated solutions of NaI, KSCN, and NaCl as well as Ag^+ in 100 mM $NaNO_3$ solutions. The ionic strength of these standards was adjusted with $NaNO_3$ to approximately 100 mM. The electrode readings were then calibrated over a large range of silver concentrations, as calculated with the known solubility products of AgI, AgSCN, and AgCl, from pAg = 3 (1 mM Ag^+ in 100 mM $NaNO_3$) to pAg = 15 (100 mM solution of NaI saturated with Ag^+).[22] The response over this range was Nernstian. Both preparation and calibration procedures employed here are modification of the protocols of Avdeef and Bucher.[23]

Direct Titration of Ag^+ by Apo-SOD in the Presence of Excess Ag^+. In order to determine the number of silver-binding sites on apo-SOD, titrations of Ag^+ by apo-SOD were carried out in which the total concentration of protein dimer added never exceeded one-fourth of the total concentration of Ag^+. Four μmol of Ag^+ were added to 25 mL of the appropriate buffer. The concentration of unbound Ag^+ was monitored by the ISE. Aliquots of known amounts of apo-SOD were added stepwise until the concentration of protein dimer was one-quarter that of the total silver ion concentration. It was observed that maintaining a concentration of Ag^+ in excess to that of the protein allowed for rapid equilibration of the ISE. By this method we saturated the silver-binding sites with Ag^+.

The quantities dR and R_o were calculated at each point along the titration. R_o is the simple ratio of the total concentration of Ag^+ in the solution to the concentration of added protein. dR is the number of equivalents of Ag^+ bound by the protein, as the difference of total added Ag^+ and that amount remaining unbound, divided by the concentration

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of the protein in the solution. A plot of dR vs R_o may be extrapolated to large R_o to obtain the value of dR under saturating conditions, i.e., the number of silver-binding sites per dimer.

Complexometric Titration of Protein-Bound Silver Ion. Direct titration of the protein with added silver ion proved difficult, as the electrode required a long period of time to equilibrate when very small amounts of silver (total silver concentration less than 10^{-6} M) had been added to the protein solution. In addition the protein itself appeared to slow the response of the electrode considerably. Consequently, we decided to employ complexometric titrations to quantify the binding of Ag^+ to apo-SOD by monitoring the removal of protein-bound Ag^+ by a suitable complexometric reagent.

A typical silver ion–complexometric titration was carried out in the following manner. Approximately 2 μmol of $AgNO_3$ was added to 25 mL of buffer at the appropriate pH, and the silver activity was measured. A total of 0.5 μmol of apo-SOD was added in aliquots, and the solution was allowed to reach equilibrium. We define equilibrium as that point in time when the rate change in the electrode reading has fallen below 0.5 mV/h. At this point, a suitable complexometric reagent was added to the solution, by aliquots, in order to remove Ag^+ from the protein. The pAg was then measured, and R, the metal content of the protein (vide infra), was calculated by assuming that the addition of the complexometric reagent quantitatively and stoichiometrically removed silver from the protein sites. Alternatively, R was determined graphically by comparison with the results of a control titration, which was identical in all aspects with the titration of protein-bound silver, except that no protein was added to the solution.

At pHs above 7.5, we chose NaCN as the complexometric reagent for titrations. Cyanide is a very strong coordinator of Ag^+ and has a stoichiometry illustrated in eq 1

$$Ag^+ + 2CN^- \overset{\beta_2}{\rightleftharpoons} Ag(CN)_2^- \quad (1)$$

where $\log \beta_2 = 20.4$. Generally, one would expect that cyanide would quantitatively coordinate and remove protein-bound Ag^+ if the affinity of the protein for Ag^+ were significantly less than the affinity of cyanide. Equation 2 illustrates this process

$$(Ag^+)P + 2CN^- \overset{K_e}{\rightleftharpoons} P + Ag(CN)_2^- \quad (2)$$

where P represents some protein metal-binding site, and K_e is the constant quantifying the equilibrium. However, cyanide has a rather high pK_a (9.0) so that at pHs significantly lower than 9.0, protons compete more and more effectively with silver for cyanide. For this reason, we chose NaI as the complexometric reagent for silver titrations at pHs below or equal to 7.5. Iodide forms a highly insoluble salt with Ag^+ ($-\log K_{sp} = 16$) and would be expected to remove protein-bound silver in the manner illustrated in eq 3.

$$(Ag^+)P + I^- \overset{K_e}{\rightleftharpoons} P + AgI_{(s)} \quad (3)$$

Binding Model and Data Analysis. On the basis of our experimental results (vide infra), we chose a silver-binding model for apo-SOD which contains two sets of two identical sites per dimer of protein. Each subunit contains one of each type of binding site. The Ag^+ is assumed to bind sequentially, or stepwise, to the two sites on each subunit. Each subunit is considered to be an independent binding domain. This model is depicted in eq 4 and 5, where the binding of silver is considered to occur in two steps

$$Ag^+ + EESOD_{sub} \overset{K_1}{\rightleftharpoons} AgESOD_{sub} \quad (4)$$

$$Ag^+ + AgESOD_{sub} \overset{K_2}{\rightleftharpoons} AgAgSOD_{sub} \quad (5)$$

$$SOD_{sub} = \text{subunit of SOD}$$

which are described by the equilibrium constants K_1 and K_2. Inherent in this model are two points: (1) the second site is dependent on occupancy of the first site and does not "exist" until the first site is filled; and (2) interactions between sites on different subunits are considered negligible. This model is a simplified form of that used by Hirose et al. in their copper-binding study of SOD.[24] Their model differs from ours in that they assumed that interactions exist between subunits when metal ions are being bound to the protein. In contrast to our binding model of two sequential and dependent sites, Hirose et al. assumed that all four sites per dimer bind copper sequentially and dependently and that four different binding constants were required to describe the system fully.

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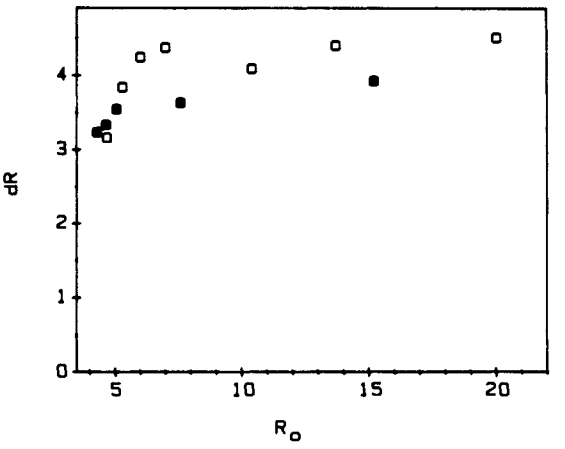

Figure 1. Saturation binding of Ag^+ to apo-SOD: open symbols, 100 mM HEPES, pH 7; solid symbols, 100 mM acetate, pH 5.5.

The derivation of mathematical models for evaluating protein metal binding, such as the ones described above, has been given elsewhere,[24,25] and thus we present only the final form of a function which describes the equivalents of Ag^+ bound per subunit (R_{sub}) as a function of aqueous silver-ion concentration ($A = [Ag^+]_{aq.}$) for a protein subunit that binds 2 equiv of silver in a sequential and dependent fashion as eq 6. We chose

$$R_{sub} = \frac{K_1A + 2K_1K_2A^2}{1 + K_1A + K_1K_2A^2} \quad (6)$$

a more convenient form of eq 6, which reflects the binding of 4 equiv to a dimer, as the final mathematical expression of our binding model. The function is given in eq 7,

$$R = \frac{2K_1A + 4K_1K_2A^2}{1 + K_1A + K_1K_2A^2} \quad (7)$$

where R is the number of equivalents of metal ion bound per dimer of protein, and A is as described above. In the limit of saturation, i.e., A becomes very large, and R approaches the expected value of 4: when $A = 1/K_1$ and $1/K_2$, i.e., points corresponding to half-filled sites, $R = 1$ and 3, respectively.

For silver binding to the derivatives Cu_2E_2SOD and E_2Zn_2SOD, we also chose a mathematical model which assumes no intersubunit interactions in binding (eq 8), where R and A are defined as above, and K_1

$$R = \frac{2K_1A}{1 + K_1A} \quad (8)$$

is the single constant necessary for quantifying the binding of Ag^+ to two independent but identical sites on the protein dimer.

We fitted the data to our binding models and determined binding constants by minimizing the squared error between the observed values of R (R_{obs}) and the ones calculated (R_{calc}) by either eq 7 or 8. This squared error is defined by eq 9. The initial value of U was calculated

$$U = \sum_{i=1}^{N} (R_{calc_i} - R_{obs_i})^2 \quad (9)$$

with estimates of the binding constants. The fitting program then incrementally changes the value of each binding constant in sequence until U has been minimized. The increment of search used in the variation of the binding constants was 0.05 log unit.

Results

Stoichiometry of Silver Binding. Figure 1 depicts the results of Ag^+-saturation of apo-SOD binding sites at pHs 5.5 and 7.5. R_o represents the ratio of total concentration of added silver ion over total protein concentration. dR is the ratio of the concentration of protein-bound silver ion over apo-SOD dimer concentration. Under saturating conditions (i.e., $R_o \gg$ dR), the value of dR is approximately 4 at both pH 5.5 and 7.5. This result indicates that a dimer of apo-SOD binds up to 4 equiv of Ag^+ tightly in four well-behaved and specific sites at these pHs.

Proton NMR of the Silver Derivatives and of the Reduced Form of the Native Protein. Traces A and B of Figure 2 depict the

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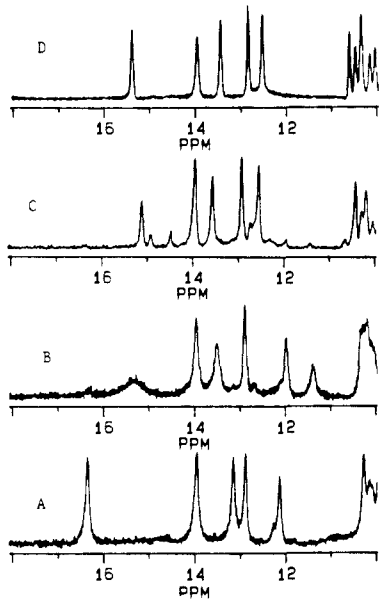

Figure 2. ^1H NMR of metal-ion derivatives of SOD in 50 mM phosphate buffer, pH 6.5: (A) Ag_2E_2SOD, (B) Ag_2Ag_2SOD, (C) Ag_2Zn_2SOD, and (D) reduced native enzyme, $Cu^I_2Zn_2SOD$.

NMR of the Ag_2E_2SOD and Ag_2Ag_2SOD, respectively. The resonances displayed in these spectra are those of the N–H protons of histidyl imidazoles. Both spectra differ significantly from each other as well as the previously published spectrum of the apoprotein.[26] These differences suggest that (1) silver ions associate closely with the protein at specific sites, (2) these sites number at least four, (3) these sites either lie close to or involve histidyl imidazoles directly, and (4) there are at least two classes of structurally different binding sites.

In addition, we measured the NMR spectra of Ag_2Zn_2SOD and compared them with the previously published spectra of $Cu^I_2Zn_2SOD$ (Figure 2 (parts C and D)). The spectra are nearly identical. There are, however, two small resonances which appear in the spectra of Ag_2Zn_2SOD between 14.5 and 15 ppm which do not correspond with resonances in the spectra of $Cu^I_2Zn_2SOD$. We attribute these additional resonances to traces present in the sample of a metal ion derivative which contains a zinc ion in both the zinc site and the copper site.[26] We have also carried out NOE studies on the silver-zinc derivative and found the results to be very similar to those Redfield and co-workers had observed for the reduced native protein.[27]

The close similarity between the spectra of $Cu^I_2Zn_2SOD$ and Ag_2Zn_2SOD is surprising since the atomic radii of Cu^+ and Ag^+ differ significantly (0.96 vs 1.26 A). Nonetheless, the NMR data indicate that Ag^+ serves as a reasonable model for Cu^+ in the reduced form of the native protein.

Complexometric Titrations of Ag_4SOD. Panel A of Figure 3 depicts the results of two complexometric titrations of silver bound to apo-SOD. The one measured at pH 4.5 was carried out by titrating the protein-bound silver ion with I^-. At pH 10.1, Ag^+ was removed by complexation with CN^-. The titration carried out at pH 4.5 is representative of the titrations measured below pH 8, where the silver activity decreased monotonically with the addition of NaI and where noticeable breaks occurred upon the addition of 2 and 4 equiv of I^-. This binding behavior indicates that there are two types of silver binding sites on apo-SOD, in support of the NMR results, and that they have very different affinities for the silver ion at this pH.

By contrast, the data from the pH 10.1 cyanide titration of Ag_4SOD do not show a clear break at $R = 2$. Four equiv of silver, which were bound by the protein, were removed by 8 equiv of

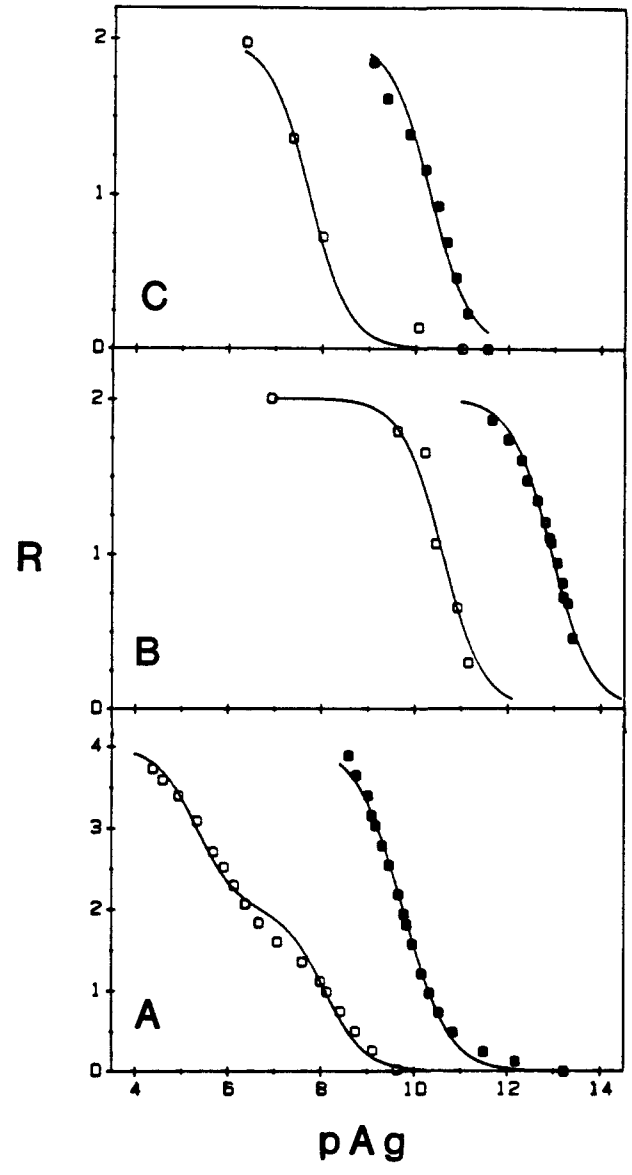

Figure 3. Equivalents of Ag^+ bound per dimer of protein (R) as a function of pAg. Ag^+ binding by (A) the apo-SOD, (B) E_2Zn_2SOD, and (C) Cu_2E_2SOD. Measurements were made at pH 4.5 in 100 mM acetate buffer (open symbols) or at pH 10.1 in 100 mM borate buffer (solid symbols).

cyanide, and a break occurred at $R = 4$. Such behavior suggests that at high pH the two types of metal binding sites have very similar affinities for Ag^+.

We chose to analyze the binding of the 4 equiv of silver ion to apo-SOD in terms of two apparent binding constants, reflecting differences in affinity between the two types of binding sites. Both the NMR spectra and the silver titrations at lower pH justify the choice of two constants, rather than the more general treatment of four constants taken by Hirose and co-workers.[24] We also felt that our electrochemical technique was not sensitive enough to yield results which would meaningfully quantify any subunit–subunit interactions in the process of silver binding. We do recognize that subunit interactions have been implicated in several physical and metal-binding studies of SOD. For example, Rigo et al.[29] noted cooperative effects in the kinetics of the reconstitution of cupric ion into the protein, that is, the rate of the incorporation of Cu^{2+} decreased as the metal content of the protein increased, indicating that the copper sites were interacting through the

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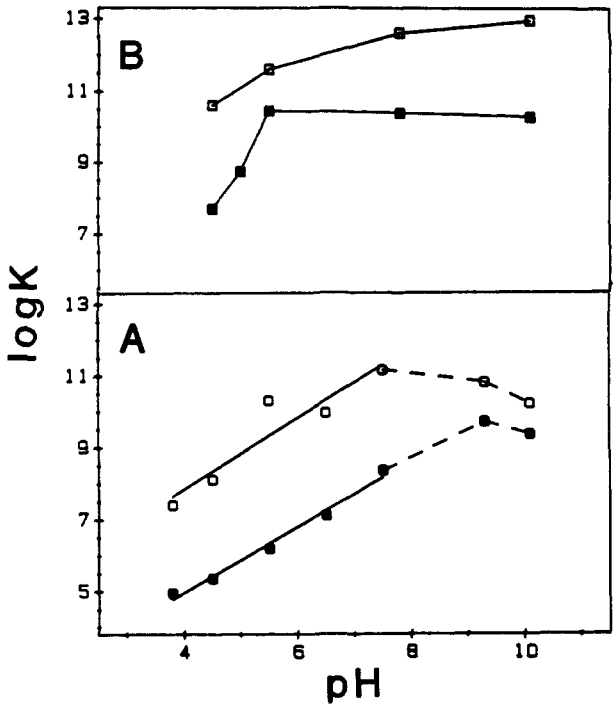

Figure 4. The pH dependence of the apparent silver-binding constants of SOD derivatives: (A) log K_1 (open symbols) and log K_2 (solid symbols) of apo-SOD (Solid lines are linear least-squares fits to the data.) and (B) log K_1 of E_2Zn_2SOD (open symbols) and of Cu_2E_2SOD (solid symbols).

subunit interface. In addition, Roe et al.[32] observed apparent cooperative effects in a differential scanning calorimetric study of Zn^{2+} binding by SOD.

The curves drawn through the titration data in Figure 3A are theoretical fits of the data. The method involved in producing these fits is described in the Experimental Section. The binding constants obtained for the titrations in Figure 3A as well as those obtained from fits to titrations which were carried out at pHs 4.5, 5.5, 6.5, and 7.5 with NaI, and at pH 9.3 with NaCN, are listed in Table I. In order to estimate the precision of our calculated values of the binding constants, a nonlinear least-squares error analysis was carried out.[28] The results indicated that the worse imprecision in the magnitude of the binding constants was less than ±0.12 log unit. We take this value as the imprecision in our measurement, since it is larger than our search increment of 0.05 log unit and greater than the estimated error involved in temperature variation.

Panel A of Figure 4 illustrates the pH dependence of the silver-apo-SOD apparent binding constants. The lowest values for the two binding constants occur at pH 3.8. From pH 3.8 to 7.5, the values of the constants appear to increase linearly with pH. From linear least-squares analysis of the first five points on each curve, the slope obtained is very close to unity, i.e., 0.99 for site 1 and 0.92 for site type 2. The magnitude of the apparent binding constant for site 1 was observed to decrease slightly at pHs 9.3 and 10.1 from a maximum at pH 7.5. By contrast, the constant for site 2 continues to increase with pH and approaches the magnitude of the first constant at the highest pH at which they were determined.

Complexometric Titration of Ag_2Zn_2SOD and Ag_2Cu_2SOD. In addition to the titrations of all-silver derivatives, we also carried out titrations of silver ion bound to two mixed-metal derivatives of SOD, Ag_2Zn_2SOD, and Ag_2Cu_2SOD. We were able to remove silver selectively from these derivatives since the affinities of I^- and CN^- for Ag^+ are much higher than the affinities for either Cu^{2+} or Zn^{2+}.[20] Figure 3B depicts the results of two titrations of the silver bound to Ag_2Zn_2SOD. Iodide was the titer at pH 4.5, and cyanide was used to remove the silver ion at pH 10.1. Again the activity of Ag^+ dropped stepwise with the addition of aliquots of the complexometric reagents. In contrast with the silver binding curves of Ag_2Ag_2SOD at pHs at or below 7.5, the curves formed by the low pH titration of Ag_2Zn_2SOD show no break at the midpoint of the titration. Since there is no break we felt confident in analyzing these curves in terms of a single apparent binding constant, which characterizes the binding of Ag^+ to E_2Zn_2SOD, and in assuming that interactions between sites on different subunits were negligible. The solid curves drawn through the data in Figure 3B are theoretical fits to a model (vide supra) which defines the binding of silver to two identical binding sites (i.e., one on each subunit). This choice was made on the basis that our titrations indicated no break at $R = 1$ and that data fit the single constant model reasonably well (vide supra). The binding constants calculated for these titrations, and for those determined by iodide complexation at pH 5.5 and by cyanide at pH 7.8, are listed in Table I.

Table I. Apparent Binding Constants

	Ag^+ binding to			
pH	E_2E_2SOD log K_1	Ag_2E_2SOD log K_2	E_2Zn_2SOD log K_1	Cu_2E_2SOD[b] log K_1
3.8	7.4	4.95		
4.5	8.1	5.35	10.6	7.7
5.0				8.75
5.5	10.3	6.2	11.6	10.45
6.25[a]	10.05	7.0	12.1	10.45
6.5	9.95	7.1		
7.5	11.15	8.35		
7.8			12.6	10.4
9.3	10.8	9.7		
10.1	10.2	9.35	12.95	10.3

[a] Interpolated. [b] Final product is Ag_2Cu_2SOD; i.e., Ag^+ binds to the copper site and Cu^{2+} migrates and binds to the zinc site. For evidence see Discussion.

The binding of Ag^+ by Cu_2E_2SOD was also investigated by complexometric titration in a manner identical with the titration of Ag_2Zn_2SOD. The results of two such titrations are depicted in Figure 3C. The titration of Ag_2Cu_2SOD at pH 4.5 was carried out against I^- and at pH 10.1 by CN^-. The qualitative results are very similar to those of the Ag_2Zn_2SOD derivative, and two identical binding sites were again assumed for fitting, for the same reasons stated above. The binding constants calculated for Ag_2Cu_2SOD, determined from titrations by iodide at pHs 4.5, 5.0, and 5.5, and by cyanide at pHs 7.8 and 10.1, are also listed in Table I. We again estimate that the imprecision in our determination of the binding constants of Ag^+ to Cu_2E_2SOD and E_2Zn_2SOD as ±0.12 log unit (vide supra).

The pH dependences of silver binding to the derivatives, E_2Zn_2SOD and Cu_2E_2SOD, are depicted in Figure 4B. The silver-binding behavior of the zinc derivative differs from that of the copper derivative, especially at low pH. The value of the binding constant of the zinc derivative increased 10-fold over the pH range of 4.5 to 5.5. This behavior is very similar to the behavior of Ag^+ binding to the apoprotein. In contrast, the constant which characterizes silver binding to Cu_2E_2SOD increased 1000-fold (3 log units) over the same pH range. Above pH 5.5, the affinity of Cu_2E_2SOD for Ag^+ became independent of pH up to pH 10.1. The affinity of E_2Zn_2SOD for Ag^+, on the other hand, continued to increase above pH 5.5. However, the steepness of increase declined significantly above pH 7.8.

The abrupt change in the silver-binding constant of Ag_2Cu_2SOD was further investigated by titrating a solution containing Ag_2Cu_2SOD ($R_{Ag} = 1.8$; 0.02 mM) with 20 mM H_2SO_4 from pH 5.6 to 3.0. There was a dramatic change in the pAg between 5.5 and 4.5. The free silver concentration increased by two magnitudes (pAg changed from 8.0 to 6.0). When the pH was returned to 5.5, the free silver-ion concentration dropped appropriately in-

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(31) Calabrese, L.; Cocco, D.; Morpurgo, L.; Mondovi, B.; Rotilio, G. *Eur. J. Biochem.* **1976**, *64*, 465–470.
(32) Roe, J. A.; Butler, A.; Scholler, D. M.; Valentine, J. S.; Marky, L.; Breslauer, K. J. *Biochemistry* **1988**, *27*, 950–958.

dicating that the procedure was reversible. In another experiment we dialyzed Ag_2Cu_2SOD against a pH 3.5 buffer. The UV–vis and ESR of the dialyzed sample exhibited the same spectral characteristics as Cu_2E_2SOD at this pH. We conclude then that the stability of the silver–copper derivative is dramatically pH sensitive between the pHs of 4.5 and 5.5 and that at low pH the silver ion is released from the protein and the copper ion migrates back to copper site.

The Order of Metal-Ion Addition and the Formation of the Silver–Copper Derivative of SOD. A change in the order of metal-ion addition to the apoprotein in order to form Ag_2Cu_2SOD did not alter the results of the complexometric titration of Ag_2Cu_2SOD (vide supra). Yet some question remained with us (and in the literature) as to whether Ag_2Cu_2SOD, with the silver ion occupying the native copper site, have Ag^+ and Cu^{2+} bound in their thermodynamically preferred sites or whether this derivative, first reported by Beem et al.,[9] actually has the metal ions kinetically "trapped" in this configuration. To determine if Ag_2Cu_2SOD is the thermodynamically preferred configuration, we attempted to prepare the identical derivative by reversing the order of metal-ion addition used by Beem and co-workers. In the method of Beem et al. to prepare Ag_2Cu_2SOD, 2 equiv of Ag^+ were infused first into a sample of apo-SOD at pH 3.8 and dialyzed against a pH 7.8 phosphate buffer overnight. Into this protein solution were infused 2 equiv of Cu^{2+} to yield Ag_2Cu_2SOD. Since it is known that some metal incorporation reactions involving SOD require a great deal of time to reach equilibrium,[29,30] it was unclear to us whether the configuration of the metal binding sites had reached equilibrium or whether the initial binding of Ag^+ to the apoprotein "forced" the Cu^{2+} into the zinc site to form a metastable derivative, namely Ag_2Cu_2SOD, which, given enough time, would rearrange to Cu_2Ag_2SOD. Consequently, we adopted the opposite procedure by infusing 2 equiv of Ag^+ into a preparation of Cu_2E_2SOD at pH 5.5 in acetate buffer. Almost complete formation of Ag_2Cu_2SOD was observed after 20 min as judged by the ESR and the visible spectra. Specifically, the visible absorbance maximum shifted upon the addition of silver from about 680 to 780 nm. This latter value is characteristic of Cu^{2+} bound to the zinc site.[8] In addition the ESR of the final derivative is identical with that measured previously for Ag_2Cu_2SOD.[9] We also observed that the addition of 2 equiv of Ag^+ per subunit to an identical solution of native protein had no effect on the spectra of that derivative.

Excess Cyanide and Thiocyanate Remove Ag^+ from Ag_2Cu_2SOD. When 2 mL of a 0.32 mM solution of Ag_2Cu_2SOD (20 mM acetate; pH 5.5) was dialyzed overnight against 4 L of 0.1 mM KCN (20 mM acetate, pH 5.5) followed by two changes of acetate buffer, the optical and ESR spectroscopic characteristics of Ag_2Cu_2SOD were replaced by those of the Cu_2E_2SOD derivative, which has unoccupied zinc sites. Atomic absorption analysis of the dialyzed sample revealed that 92% of the original copper remained, whereas 92.7% of the bound silver ion had been removed by the dialysis procedure. Unlike a similar cyanide experiment carried out by Beem et al.,[9] where they added 2 equiv directly to a solution of Ag_2Cu_2SOD, we did not observe any spectroscopic evidence that some added cyanide remained bound to the copper of Cu_2E_2SOD. This absence of cyanide may be explained as the consequence of the two cyanide-free dialyses which essentially removed all the cyanide from the sample. The addition of a small excess of Co^{2+} to the solution after dialysis against buffer yields a species whose UV–vis spectral parameters were identical with that of Cu_2Co_2SOD.[31] This final result supports the conclusion that the Cu^{2+} has migrated back to the copper site after the removal of Ag^+ and is consistent with the observation that Ag^+ can be quantitatively removed from Ag_2Cu_2SOD during the course of a complexometric titration at high pH.

Similar results as above (i.e., the removal of Ag^+) were obtained when 0.7 mmol of Ag_2Cu_2SOD was dialyzed first against a 500 mM KSCN in a 50 mM phosphate buffer, pH 7.8, and then against two changes of buffer alone. Specifically, atomic absorption indicated that at least 98% of the silver had been removed by KSCN and that the species which remained contained 100% (±2%) of the bound copper. The ESR and UV–vis revealed that the Ag_2Cu_2SOD spectral parameters were replaced by those of Cu_2E_2SOD. In addition, UV–vis and ESR showed that in the presence of the 500 mM KSCN the thiocyanate adduct of Cu_2E_2SOD, where the thiocyanate anion is bound to the copper and from which Ag^+ has been removed. These results are identical with those obtained by Strothkamp and Lippard[7] in all but one detail. These authors reported that after the large excess of thiocyanate was dialyzed away against buffer they obtained an SOD derivative which was free of silver but retained the cupric ion in the zinc site. They based this conclusion on the ESR of this species, which was found to have spectral parameters very close to that of Ag_2Cu_2SOD, namely a visible maximum at 780 nm and A of 110 G. Unfortunately, in our hands, we were not able to observe this species, which Strothkamp and Lippard had reported, and are at a loss to explain the contradictory results.

Discussion

The Stoichiometry and Identity of the Silver-Binding Sites on Apo-SOD. Taken together, the titration data depicted in Figure 1 and the NMR of the silver derivatives of SOD (Figure 2) demonstrate that (1) 4 equiv of Ag^+ are specifically bound by a dimer of SOD, (2) there exist two classes of binding sites, and (3) there are two sites of each classes. These conclusions are further supported by the iodide titration of protein-bound Ag^+ as shown in Figure 3A. It seems likely then that these sites are related to or identical with the native copper and zinc-binding sites. The evidence for this final conclusion is 2-fold: comparison of the spectroscopic properties of the cuprous and silver SOD derivatives and the competition between Ag^+ and Zn^{2+} or Cu^{2+} for the binding sites on SOD. Specifically, the spectroscopic similarities between $(Ag^+)_2M_2$ and $(Cu^+)_2M_2$ derivatives strongly suggest that Ag^+ and Cu^+ occupy the same location, i.e., the copper site, on SOD, while M binds to the zinc site for M = Zn^{2+}, Ni^{2+}, or Co^{2+}.[6-10] In addition the ESR and the electronic spectral parameters of Ag_2Cu_2SOD, which show that the cupric ion has been displaced from its native environment to a more tetrahedral one (presumably the zinc site), support the conclusion that one of the silver binding sites is the copper binding site.

We have also observed in iodide titrations of Ag_2Zn_2SOD and Ag_2Cu_2SOD that the apoprotein will bind tightly only 2 equiv of Ag^+ in the presence of 2 previously added equiv of either Zn^{2+} or Cu^{2+}, which are certainly bound to the zinc site. In addition, it has been shown that Ag^+ does not bind to the native protein, Cu_2Zn_2SOD.[9]

In light of the above experimental evidence, we conclude that two classes of binding sites are related closely to the native metal binding sites. Since Ag^+ is able to displace Cu^{2+} from the copper site when the zinc site in unoccupied and that Ag^+ does not displace either Zn^{2+} or Cu^{2+} from the zinc site, we propose that the first or stronger class of silver-binding site is related to the copper site and may have coordination symmetry similar to the cuprous ion binding site and that the second and weaker class of Ag^+ binding sites is located either at or close to the zinc sites. Therefore, the binding of 2 equiv of Ag^+ by an apoprotein subunit can be considered to be stepwise and dependent, since the native sites lie so close to each other (6.4 Å; ref 5), as proposed above.

Bound Zn^{2+} Enhances SOD Binding of Ag^+. Figure 4 depict the apparent binding constants of Ag^+ to the apo-SOD, E_2Zn_2SOD, and Cu_2E_2SOD as functions of pH. The lower binding affinity of Ag^+ for Cu_2E_2SOD as compared to that for E_2Zn_2SOD presumably stems from the competition between Ag^+ and Cu^{2+} for the copper site, whereas Zn^{2+} does not compete effectively due to its relatively low affinity for the copper site. A more interesting comparison may be made between the affinity of the silver ion for the copper site in the apoprotein and the affinity of Ag^+ for the empty copper sites of E_2Zn_2SOD. It is apparent that the presence of the zinc ion in E_2Zn_2SOD enhances Ag^+ binding to the copper site.

The molecular origins of this enhancement are not clear. Similar observations have been made in other studies. Hirose et al.[24] have suggested that the presence of Cu^{2+} in the copper site

greatly increases the affinity of the zinc sites for additional equivalents of Cu^{2+} at pHs > 7.5. They proposed that the zinc site becomes "organized" by the nearby occupied copper site to yield a new conformation of ligands which binds copper more effectively. In fact, they suggest that, at high pHs, the zinc site, when the copper site is occupied by Cu^{2+}, has a greater affinity for Cu^{2+} than the copper site itself. Valentine et al.[33] have demonstrated another manifestation of this enhancement of metal binding in their pH study of Cu_2E_2SOD, where they observed that at pHs > 8 one-half of the bound copper migrated to the zinc sites to form imidazolate-bridged Cu^{2+}–im^-–Cu^{2+} subunits. The remaining half of the subunits presumably were metal-depleted. In a similar manner the binding of the zinc ion to the zinc site may lead to a reorganization of the ligands of the copper-binding site, thereby increasing affinity for Ag^+. EXAFS results have indicated that the configuration of the ligands around the zinc ion in the derivatives, E_2Zn_2SOD and $Cu^I_2Zn_2SOD$, are very similar.[16] This observation suggests that upon reduction the bond between the bridging imidazole and the copper becomes broken, and the imidazole remains attached to the zinc ion. Thus it is possible that by "tugging" the bridging imidazole away from the copper binding site Zn^{2+} may produce a less "cluttered" site which binds reduced copper and Ag^+ more tightly.

Role of Zn^{2+} in SOD. We have observed that the occupancy of the zinc site by zinc ion greatly enhances the binding of Ag^+ to the copper site. Interestingly, Hirose et al. in their study of copper and zinc binding by apo-SOD[34] report that the effect of Zn^{2+} on Cu^{2+}-binding by SOD is negligible at pH 6.25, i.e., zinc ion, when occupying the zinc sites, neither enhances nor decreases the affinity of the copper site for Cu^{2+}.

These observations on the effect (or noneffect) of Zn^{2+} on metal-ion binding at the copper site suggest that a role for the zinc ion in SOD is the stabilization of the cuprous or reduced state of the protein over the cupric or oxidized state. Alternatively, the Lewis acid nature of Zn^{2+} acting through the imidazolate bridge may be destabilizing the cupric ion, allowing it to be more easily reduced. The silver-binding studies support this conclusion: Zn^{2+} enhances the stability of the protein/Ag^+ complex, which serves as a model for the SOD/Cu^+ complex, whereas SOD-bound Zn^{2+} does not stabilize the cupric complex. We suggest that this increased stabilization of the cuprous state may be a source of the increased reduction potential of the Cu(II)/Cu(I) couple of the protein-bound copper as compared to the aqueous value, 0.42 and 0.153 V, respectively.[4] This stabilizing role of Zn^{2+} is consistent with a differential scanning calorimetric study of SOD[32] in which it was shown that the reduced form of the protein was more resistant to thermal denaturation than was the native or oxidized form.

The pH Dependence of Silver Binding. The pH dependence of the binding of Ag^+ to apo-SOD is shown by Figure 4A. The logarithms of the apparent binding constants increase linearly with pH, having slopes of approximately 1.0, up to pH 7.5. These slopes suggest that a proton is released from the protein upon binding of each Ag^+. In addition, the pH at which the log K's reach plateaus can be interpreted as evidence for the presence of one ligand at each binding site which has a pK_a of about 7.5. Imidazolium moieties may serve as such ligands. By reasoning similar to ours, Hirose et al.[24] concluded that the pH dependence of the binding of Cu^{2+} to apo-SOD reflected the deprotonation of two imidazoliums at the copper site and the deprotonation of one imidazolium and one imidazole (to yield the imidazolate bridge) at the zinc site.

By comparison with the conclusions of Hirose et al. concerning the pH dependence of Cu^{2+} by SOD, we propose that the binding of Ag^+ to the copper site involves at least one less histidine as a ligand and that the imidazolate bridge does not form upon the introduction of 4 equiv of Ag^+ to the apoprotein. The reasoning

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Table II. Metal-Binding Competition for the Copper Site in E_2Zn_2SOD: Apparent Constants at pH 6.25

M, N	log $K_{M,N}$[a]	M, N	log $K_{M,N}$[a]
Cu, E	13.8[b]	Cu, Zn	6.65[c]
Ag, E	12.1	Ag, Zn	4.95
Zn, E	7.15[c]	Cu, Ag	1.7

[a] Refer to eq 14 for calculation of this quantity. [b] Calculated from the average of the two values reported by Hirose et al.[24] for the apparent binding constant of Cu^{2+} binding to the two copper sites of apo-SOD and $K_{Cu,Zn}$. [c] Calculated as an average of the two values given by Hirose et al.[34]

Table III. Relative Solution Stabilities of Selected Metal-Ion Derivatives of apo-SOD in Order of Stability at pH 6.25

M, N (in complex M_2N_2SOD)	$S_{M,N}$[a]	M, N (in complex M_2N_2SOD)	$S_{M,N}$[a]
Cu, Zn	24.9	Zn, Zn	18.15
Cu, Cu	24.5	(Zn, Cu)[b]	18.0
Ag, Zn	23.1	Ag, Ag	17.05
Ag, Cu	21.3	(Zn, Ag)[b]	14.15
(Cu, Ag)[b]	20.65		

[a] Refer to eq 19 for the calculation of this quantity. [b] These species have not been observed. Their calculated stabilities are theoretical.

follows: (1) The pH dependence of the log K's of silver to either site of apo-SOD is linear with a slope of one at pHs below 7.5, suggesting that only one imidazolium proton is displaced from either site, as compared to the proposed two protons released upon copper binding to the copper site and the one released from the binding of copper to the zinc site. (2) The pH dependence of silver binding at the zinc site is diminished above pH 7.5, implying that no specific proton, such as the bridging ligand's imidazole proton, is released. However, we realize that there may be additional complications in the pH dependence of the binding constants, such as distant (from the metal binding sites) structural changes or distant proton release, which may induce structural changes closer to the binding sites.

The similarity of the binding of Ag^+ to the copper site of the apoprotein with the binding of Ag^+ to E_2Zn_2SOD (Figure 4) strongly suggests that there is at least one imidazole involved in the binding of Ag^+ to E_2Zn_2SOD. On the other hand, the abrupt increase of the silver binding constant to Cu_2E_2SOD between pH 4.5 and 5.5 as well as its pH insensitivity at pHs above 5.5 is not so easily explained (Figure 4B). Equations 10, 11, and 12 depict a possible mechanism for the pH independence of the binding constant above 5.5. We break the binding of silver to a subunit

$$H^+ + CuESOD_{sub} \rightleftarrows \{ECuSOD_{sub}\} \quad (10)$$

$$Ag^+ + \{ECuSOD_{sub}\} \rightleftarrows AgCuSOD_{sub} + H^+ \quad (11)$$

$$\text{sum:} \quad Ag^+ + CuESOD_{sub} \overset{K_1}{\rightleftharpoons} AgCuSOD_{sub} \quad (12)$$

of the copper derivative into two steps. In the first step (eq 10), Cu^{2+} migrates from the copper site to the zinc site, giving the hypothetical species $\{ECuSOD_{sub}\}$. We propose that this migration is associated with protonation of an imidazole, which had served as ligand for the binding of copper at the copper site. This uptake of a single proton is in accord with the results reported by Hirose et al.[24] which suggested that two imidazolium protons are released from the copper site when Cu^{2+} is bound, and only one is released by copper binding at the zinc site. Thus if a copper ion migrates from the copper site to the zinc site, there would be a net gain of one proton at pHs below the pK_a of the moiety.

Step 2 (eq 11) represents the binding of a silver ion to the copper site of the hypothetical species $\{ECuSOD_{sub}\}$, resulting in the loss of a proton. This description of the silver-ion binding is consistent with the pH dependence of Ag^+ binding to the copper site of apo-SOD or E_2Zn_2SOD. Consequently, if the copper in the zinc site does not alter the overall proton stoichiometry at the copper site, we would expect a proton loss upon Ag^+ binding to the hypothetical species at pHs below the imidazolium proton pK_a. The overall binding expression (eq 12) does not explicitly contain

protons. The absence of a net gain or loss of a proton in eq 12 is consistent with the pH-independent behavior exhibited in the binding of Ag^+ to Cu_2E_2SOD between pHs 5.5 and 7.8.

Abrupt changes in the metal-binding properties of SOD have been observed before at low pH. In two cases,[24,30] a dramatic decrease in metal-binding affinity of the zinc site for either Cu^{2+} or Zn^{2+} at pHs below 5.5 has been described. These results are consistent with a decrease in the apparent silver-binding constant of Cu_2E_2SOD, since at lower pHs Cu^{2+} will compete more effectively with Ag^+ for the copper site. This increased competition for the copper site is reflected in the decrease in the value of the binding constant.

One possible mechanism which might trigger this low pH structural change is the protonation of the aspartate ligand at the zinc site. The pK_a of this group commonly lies in the appropriate pH range of 3.5–5.5, where the SOD structural changes occur. At pHs below the pK_a of the aspartate residue, the affinity of the zinc site for Zn^{2+} or Cu^{2+} should be significantly lower than at higher pHs due to the competition between the metal ions and protons for the ligand. In addition, the aspartate ligand (asp81) is located at the end of the zinc-binding loop, a 21 residue sequence of amino acids that contains *all* the zinc ligands. We suggest that the protonation of the aspartate residue at the zinc site not only lowers the affinity of this site for Zn^{2+} (or Cu^{2+}) but also leads to drastic change in the structure of the region by releasing constraints imposed on the zinc-binding loop.

Competition between Cu^{2+}, Zn^{2+}, and Ag^+ for the Copper-Binding Sites of E_2Zn_2SOD. It is now possible to quantify more complicated equilibria with the apparent constants determined in this study and in the works of Hirose and co-workers.[24,34] For example, we can now describe the competition between copper, silver, and zinc ions for the copper site of SOD to which 2 equiv of Zn^{2+} are bound (E_2Zn_2SOD). At this point we must define $K_{M,N}$, the constant which quantitates the competition between metal ions M and N for the copper site, as

$$M + NZnSOD_{sub} \overset{K_{M,N}}{\rightleftharpoons} MZnSOD_{sub} + N \qquad (13)$$

where

$$K_{M,N} = \frac{[N][MZnSOD_{sub}]}{[M][NZnSOD_{sub}]} \qquad (14)$$

If $K_{M,N}$ is very large, then metal ion M will easily displace N from the copper. If N = E, $K_{M,N}$ is defined as the apparent binding constant of metal ion M binding to the empty copper site, when the zinc site is occupied by Zn^{2+}. The constant $K_{M,N}$ can be calculated either by eq 15

$$K_{M,N} = K_{M,E}/K_{N,E} \qquad (15)$$

or eq 16

$$K_{M,N} = K_{M,D}/K_{N,D} \qquad (16)$$

The constants $K_{M,D}$ and $K_{N,D}$, which appear in eq 16, are defined in the same manner as $K_{M,N}$, as the constants which quantify the competition between the metal ion, M or N, and the metal ion, D, which is different than M or N.

Table II lists the results of our calculations for $K_{M,N}$ at pH 6.25. Several points must be addressed concerning the preparation of Table II. (1) Hirose et al.[24,34] carried out their measurements at 4 °C, whereas our experiments were conducted at room temperature. It is difficult to gauge the effect of ca. 20 °C difference in temperature on the magnitude of the binding constants, since no temperature study of metal-ion binding to SOD has been made. However, if we were to assume that the enthalpy of binding ($|\Delta H°|$) was as great as 10 Kcal/mol, a reasonable value for the binding of "soft" ligands to Cu^{2+}, the change in magnitude of the binding constants over 20 °C may be as large as 0.54 log units (ref 21, p 7). Consequently, the values listed in Table II should be considered qualitative in nature. (2) Hirose and co-workers also reported two constants for the binding of Cu^{2+} to each class of sites (i.e., the zinc sites and the copper sites) in order to measure the subunit–subunit interactions. As discussed above, we have chosen not to use these additional constants. Consequently, we averaged their pairs of constants to give only a single value for the binding of copper to each class of sites. This procedure is reasonable, since on the basis of our silver-binding results the average of pairs of silver-binding constants from the four-step binding sequence approximately equals the values directly obtained from the single class/single constant scheme. (3) The constants listed for the silver binding at pH 6.25 were obtained by interpolation of the data presented in Figure 4. Since the data appear smooth at this pH region, we felt the interpolations were justified.

The results listed in Table II clearly indicate that Cu^{2+} will displace stoichiometric amounts of either Ag^+ or Zn^{2+} from the copper site, when Zn^{2+} occupies the Zn^{2+} site, and that Ag^+ should displace Zn^{2+}. These results are consistent with the experimental result that the addition of stoichiometric amounts of Ag^+ into a solution containing the native protein at pH 7.8 does not displace the copper ion.[9]

Relative Stabilities of the Metal-Ion Derivatives, M_2N_2SOD. At this point, we introduce the concept of an overall stability constant for specific metal-ion derivatives, which contain bound metal at both the zinc and copper sites, as a measure of the relative configurational stability of these derivatives. Consider the binding equilibrium (eq 17) where some metal-ion M binds to the apoprotein at the copper site

$$M + EESOD_{sub} \overset{K_M}{\rightleftharpoons} MESOD_{sub} \qquad (17)$$

and the equilibrium presented in eq 18 where a second metal ion N (N may be the same as M) binds to the zinc site.

$$N + MESOD_{sub} \overset{K_N}{\rightleftharpoons} MNSOD_{sub} \qquad (18)$$

The overall stability constant $S_{M,N}$ is now defined in eq 19

$$S_{M,N} = \log K_M + \log K_N \qquad (19)$$

Values of $S_{M,N}$ have been calculated for several real and hypothetical derivatives of SOD at pH 6.25 and are listed in Table III. The same reservations exist for these calculated results as were discussed above concerning the calculations of $K_{M,N}$. Specifically, these values have been calculated on the basis of data measured at two temperatures; consequently, as discussed above, the values should be considered qualitative.

The overall stability constant allows us to predict the configuration of the metal-ion derivatives of SOD, that is, the location of each metal ion bound to the protein. For example, consider the binding of 2 equiv of Ag^+ and 2 equiv of Cu^{2+} to a dimer of apo-SOD. A comparison of the $S_{M,N}$ values should indicate whether the copper ion will occupy the copper or the zinc site. The $S_{M,N}$ value for the configuration Ag_2Cu_2SOD is greater than that for Cu_2Ag_2SOD. Consequently, we would not expect the formation of appreciable amounts of Cu_2Ag_2SOD when equilibrium is reached. It may seem odd at first that Cu^{2+} would migrate from the copper site in the presence of Ag^+, when its affinity for the copper site is so strong in general. However, it is the sum of the affinities of the metal-binding sites that determines the final configuration. Though some stability is lost when Cu^{2+} migrates to the zinc site, it is more than compensated by the stability contributed by the binding of Ag^+ at the copper site. Ag^+ prefers the copper site over the zinc site more than does Cu^{2+}.

The ordering of the $S_{M,N}$ values are consistent with experimental evidence other than the results of the titrations carried out in this study and the work of Hirose and co-workers.[24,34] Any systematic error in the calculation of the overall stability constant due to the 20 °C temperature difference between the experimental conditions employed by us and those used by Hirose et al. should not significantly change the order, since the errors are contributed to the values of $S_{M,N}$ equally.

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