

Roles of zinc and copper in modulating the oxidative refolding of bovine copper, zinc superoxide dismutase

Hong-Tao Li, Ming Jiao, Jie Chen, and Yi Liang*

State Key Laboratory of Virology, College of Life Sciences, Wuhan University, Wuhan 430072, China

*Correspondence address. Tel/Fax: +86-27-68754902; E-mail: liangyi@whu.edu.cn

The structural integrity of the ubiquitous enzyme copper, zinc superoxide dismutase (SOD1) depends critically on the correct coordination of zinc and copper. We investigate here the roles of the stoichiometric zinc and copper ions in modulating the oxidative refolding of reduced, denatured bovine erythrocyte SOD1 at physiological pH and room temperature. Fluorescence experiment results showed that the oxidative refolding of the demetalated SOD1 (apo-SOD1) is biphasic, and the addition of stoichiometric Zn^{2+} into the refolding buffer remarkably accelerates both the fast phase and the slow phase of the oxidative refolding, compared with without Zn^{2+} . Aggregation of apo-SOD1 in the presence of stoichiometric Zn^{2+} is remarkably slower than that in the absence of Zn^{2+} . In contrast, the effects of stoichiometric Cu^{2+} on both the rates of the oxidative refolding and the aggregation of apo-SOD1 are not remarkable. Experiments of resistance to proteinase K showed that apo-SOD1 forms a conformation with low-level proteinase K resistance during refolding and stoichiometric Cu^{2+} has no obvious effect on the resistance to proteinase K. In contrast, when the refolding buffer contains stoichiometric zinc, SOD1 forms a compact conformation with high-level proteinase K resistance during refolding. Our data here demonstrated that stoichiometric zinc plays an important role in the oxidative refolding of low micromolar bovine SOD1 by accelerating the oxidative refolding, suppressing the aggregation during refolding, and helping the protein to form a compact conformation with high protease resistance activity.

Keywords copper, zinc superoxide dismutase; protein aggregation; protein refolding; protease resistance activity; refolding kinetics

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Introduction

The information required to drive a disordered polypeptide spontaneously to its native three-dimensional structure (called protein folding) under physiological conditions is

known to be encoded within its amino acid sequence [1]. Protein misfolding is now known to be associated with a wide range of serious diseases [2]. Understanding the roles of metal-ion binding and release in modulating the folding and misfolding of metalloproteins is quite relevant with respect to the complete comprehension of the stability, function, and gain of function of these proteins [3].

Copper, zinc superoxide dismutase (SOD1) isolated from bovine blood has been the subject of extensive investigation since the discovery of its catalytic function in 1969 [4]. From a comparison of the sequences between bovine and human enzymes, the homology is found to be 90% [5], and the structure of SOD1 proteins from many species are highly conserved [6]. Using bovine SOD1 as a flagship enzyme, detailed structural information about the SOD1 family has been provided over the years by a plethora of analytical techniques [7]. It is a dimer consisting of two identical 16-kDa subunits, each of which is constituted by the classical β -barrel made of eight antiparallel β -strands and connected by three loop regions. Two of these loops (called the electrostatic loop and the zinc-binding loop) together with a section of the β -barrel form the walls of a channel from the enzyme surface to the active site. The copper and zinc ions are bound at the base of the active site cavity and are bridged by the imidazole ring of His61. Cu^{2+} is coordinated to four histidines (His44, His46, His61, and His118) and a water molecule in a distorted square pyramidal geometry, whereas Zn^{2+} coordination is completed by three histidines (His61, His69, and His78) and an Asp residue (Asp81) in a distorted tetrahedral geometry [8]. In addition, bovine SOD1 monomer contains three cysteine residues. Two of these residues, Cys55 and Cys144, can form an intramolecular disulfide bond which is stable and conserved among all structurally determined SOD1 [9].

Many early investigations of the ubiquitous enzyme SOD1 focused on the metal-binding properties of the enzyme. The structural integrity of SOD1 depends critically on the correct coordination of zinc and copper [10]. Both metal ions can be removed by dialysis at pH 3.8

against a metal chelator such as ethylenediaminetetraacetic acid (EDTA) [4]. At pH 3.8, by adding Cu^{2+} , Cu,E-SOD1 (E-empty) is obtained [11]. By increasing the pH value, zinc ion and other metal ions (M), such as Co^{2+} , Ni^{2+} , and Cu^{2+} , can bind to the apoenzyme at the zinc-binding site to give E,M-SOD1 derivatives [12,13]. The addition of one Zn^{2+} per subunit of the apoprotein results in a conformational rearrangement of the metal-binding region to a state closely resembling that of the native structure [13,14]. Differential scanning calorimetric studies of the apoprotein and zinc-only derivatives suggest that zinc plays a stabilizing role [15,16]. Since the connection between amyotrophic lateral sclerosis (ALS) and SOD1 has been established [17,18], there is more and more interest in the roles of zinc and copper in modulating SOD1 refolding and aggregation related to ALS [10,19–23].

The visible inclusions in SOD1-linked ALS contain neurofilament proteins, ubiquitin, and a variety of other components in addition to SOD1, but it is unknown whether copper and zinc are present in the inclusions or are involved in their formation [6]. Defective metal binding or decreased affinity for zinc [24] and copper [25,26] is a feature of many SOD1 mutants and has been suggested to play a role in the pathogenic mechanism of ALS. In particular, SOD1 aggregation appears to arise from metal-deficient SOD1 [27]. Loss of these cofactors not only promotes SOD1 aggregation *in vitro* but also seems to be a key prerequisite for pathogenic misfolding in ALS [10]. It has been reported that the acid-induced unfolding of wild-type apo-SOD1 and apo-SOD1 mutants is much faster than that of the holo forms of the enzyme [28]. In contrast, moderate supplementation of zinc ions delays the death in G93A-mutant SOD1 mice by 11 days compared with mice on a zinc-deficient diet [29]. It has been suggested that ALS mutations result in destabilization affecting the zinc affinity of the enzyme and that this, in turn, may result in a protein that is less stable and more prone to aggregation and denaturation than wild-type SOD1 [7]. However, the molecular mechanism by which SOD1 causes neural damage in ALS as well as the roles of zinc and copper in the pathogenesis of ALS is not well understood so far. Indeed, understanding the role of zinc in ALS is important for the study of mutations that cause ALS [18].

In the current study, our goal was to carry out an in-depth biophysical study of bovine SOD1 in different states of metalation and in the metal-free apo state to compare and contrast the difference in the refolding courses of this protein in these different states at physiological pH and room temperature. Our data here demonstrate that stoichiometric zinc plays an important role in the oxidative refolding of low micromolar bovine SOD1 by accelerating the oxidative refolding, suppressing the aggregation during refolding and helping the protein to form

a compact conformation with high protease resistance activity.

Materials and Methods

Materials

Lyophilized proteinase K, 8-anilino-1-naphthalenesulfonic acid (ANS), and reduced and oxidized glutathione (GSH/GSSG) were obtained from Sigma (Sigma-Aldrich, St Louis, USA). Dithiothreitol (DTT) was a Biomol product (Biomol Co., Hamburg, Germany). Ultra pure guanidine hydrochloride was purchased from Promega (Madison, USA). 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) was obtained from Alfa Aesar (Ward Hill, USA). All other chemicals used were made in China and of analytical grade. The reagents were prepared in 50 mM Tris-HCl buffer (pH 7.4) for reduction/denaturation and renaturation of SOD1, and 50 mM Tris-HCl buffer at pH 8.2 was used for SOD1 activity assay. The buffers used in this study were treated with ion exchange resins to remove trace amounts of divalent cations present as contaminants in the solutions.

Protein preparation

Bovine erythrocyte SOD1 was isolated from bovine blood according to the method of McCord and Fridovich [4]. Material eluted from the final DEAE-52 cellulose column was dialyzed against deionized water, lyophilized, and stored as a powder at -20°C . Bovine erythrocyte demetallated SOD1 (apo-SOD1) was obtained by dialysis the holoenzyme against 100 mM sodium acetate buffer at pH 3.8 in the presence of 10 mM EDTA, and EDTA was then removed by dialysis against the sodium acetate buffer [4,7]. Metal content was determined by atomic absorption using an AAnalyst-800 atomic absorption spectrometer (PerkinElmer Life Sciences, Shelton, USA) to confirm that the samples were indeed in the apo state, containing less than 5% of residual metal ions. The concentrations of apo-SOD1 were determined at 258 nm using molar extinction coefficients of $2920 \text{ M}^{-1} \text{ cm}^{-1}$ [30], and the remetallated SOD1 concentrations were quantified using the Bio-Rad protein assay (Bio-Rad, Hercules, USA) using apo-SOD1 as a standard.

Remetallated SOD1 samples except E,Zn-SOD1 were prepared following the procedures reported [26]. Cu,E-SOD1 (only containing copper ions in copper-binding site) was prepared by the addition of copper chloride into the apo-SOD1 in 50 mM sodium acetate buffer and by incubating overnight with a certain molar ratio of copper ions to SOD1 dimer. E,Zn-SOD1 (only containing zinc ions in zinc-binding site) was prepared by the addition of zinc chloride into the apo-SOD1 in 50 mM sodium acetate buffer and by incubating overnight with a certain molar ratio of zinc ions to SOD1 dimer. The relative

activity of SOD1 was determined at 25°C using a pyrogallol autoxidation inhibition assay [31].

SOD1 denaturation and refolding

SOD1 was fully reduced and denatured in 50 mM Tris-HCl buffer (pH 7.4) containing 6.8 M guanidine hydrochloride and 40 mM DTT at 4°C for 4 h. Oxidative refolding of the reduced, denatured SOD1 was initiated by a rapid 50-fold dilution of the concentrated solution into the refolding buffer at 25°C, effected by vigorous agitation with a Vortex mixer for 3–5 s, giving reduced, denatured SOD1 a final concentration of 6.5 µM. The refolding buffer (pH 7.4) consisted of 50 mM Tris-HCl, 1.0/0.20 mM GSH/GSSG, and different concentrations of zinc and copper ions. Stock solutions of GSH/GSSG in 50 mM Tris-HCl buffer at pH 7.4 were made immediately prior to use.

Estimation of free thiol groups in SOD1

To confirm the formation of disulfide bonds upon refolding, the presence of free thiol groups in SOD1 during refolding was monitored by reacting DTNB with the protein using a method described previously [32]. A 3-mM stock of DTNB was prepared in DMSO. The final concentrations of SOD1 and DTNB were 6.5 µM and 1 mM, respectively. The reaction mixture of SOD1 and DTNB was incubated at room temperature for 15 min, and the concentration of the released chromogenic product was determined by measuring the absorbance at 412 nm, using a molar extinction coefficient of 13,600 M⁻¹ cm⁻¹ [32]. When measuring the time dependence of the formation of disulfide bonds, aliquots of 1.0 ml refolding sample were taken and the folding reactions were quenched by the addition of 40 µl of 2.5 M HCl, to give a pH value of 2.

Fluorescence spectroscopy

Refolding samples of reduced, denatured SOD1 was transferred to a 1-cm thermostated quartz fluorescence cuvette quickly, and the time-course fluorescence curves were recorded immediately on an LS-55 luminescence spectrometer (PerkinElmer Life Sciences) using a kinetic scan method at 25°C. The excitation wavelength at 280 nm was used for the intrinsic fluorescence spectroscopic experiments, and the excitation and emission slits were pre-determined by control experiment in order to make the intensity of fluorescence spectroscopy in a measurable region. The intrinsic fluorescence curves were recorded at 310 nm during SOD1 refolding. For ANS-binding experiments, ANS (final concentration of 300 µM) was added into the protein samples. The fluorescence emission spectra were then recorded between 450 and 600 nm at 25°C with excitation at 380 nm. The excitation and emission slits were both 10 nm and the scan speed was 100 nm/min.

Assays in the absence of the protein were performed in order to correct for unbound ANS emission fluorescence intensities.

Refolding kinetics analyses by fluorescence spectroscopy

The refolding kinetic analyses of SOD1 by fluorescence spectroscopy were performed as described previously [33]. The kinetic data of monomolecular refolding determined from fluorescence measurements can be described by the following double exponential model [Equation (1)]:

$$F(t) - F(\infty) = F_1 \exp(-k_1 t) + F_2 \exp(-k_2 t) \quad (1)$$

where $F(t)$ is the intrinsic fluorescence at time t , $F(\infty)$ is the fluorescence at the end of the reaction, k_1 and k_2 are the apparent rate constants of the first phase and the second phase respectively, and F_1 and F_2 are the corresponding fluorescence intensities of the first phase and the second phase, respectively. The time-dependent profiles of fluorescence were modeled by Equation (1), and their corresponding rate constants were determined from the change of the intrinsic fluorescence during apo-SOD1 refolding using OriginLab Origin 7.0 software. The dead time needed to mix the solutions, fill the cuvette, and start the scan was 1 min and was taken into account for data processing.

Circular dichroism measurement

Circular dichroism (CD) spectra were obtained by using a Jasco J-810 spectropolarimeter (Jasco Corporation, Tokyo, Japan) with a thermostated cell holder. Quartz cell with a 1-mm light-path was used for measurements in the far-UV region. Spectra were recorded from 200 to 250 nm for far-UV CD. The final concentration of bovine SOD1 was kept at 15 µM. The averaged spectra of several scans were corrected relative to the buffer blank. Measurements were made at 25°C.

Measurement of SOD1 aggregation

Aggregation of SOD1 during refolding was followed immediately after the initiation of the oxidative refolding by monitoring the 90° light scattering of the refolding sample using an LS-55 luminescence spectrometer at 25°C. An excitation wavelength of 450 nm was used with an emission wavelength of 455 nm (emission and excitation slit widths were 2.5 and 2.5 nm, respectively) in order to measure light scattering [34]. In all experiments, the blanks were subtracted to correct for scattering and absorbance of buffer components (including zinc and copper ions).

Proteinase K digestion

Oxidative refolding of reduced, denatured SOD1 was initiated by a rapid 50-fold dilution of the concentrated

solution into the refolding buffer at 25°C, giving reduced, denatured SOD1 a final concentration of 6.5 μM . Aliquots were taken from the refolding sample at various time intervals and digested with 1.0 or 0.010 mg/ml proteinase K for 10 min on ice [21]. Phenylmethylsulfonyl fluoride (5 mM) was then added to halt digestion. Samples were boiled for 5 min after the addition of Laemmli sample buffer, and then separated by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Band intensities in the gel were determined by Glyko BandScan gel analyzing software (Glyko, Novato, USA). Under optimal conditions, the density of the bands is proportional to the amount of the refolded, proteinase K-resistant protein.

Results

Kinetics of apo-SOD1 refolding

Bovine erythrocyte SOD1 contains a single solvent-exposed tyrosine residue (Tyr108) per subunit, which is completely conserved, and no tryptophan residues [35,36]. When 280 nm is selected as the excitation wavelength, only tyrosine residues (Tyr108) contribute to the intrinsic fluorescence of the protein. When reduced, denatured apo-SOD1 was diluted 50 folds into a 50 mM Tris–HCl buffer (pH 7.4) in the presence of GSH/GSSG at 25°C, the enzyme structure was restored to some extent. The conformational change of refolded SOD1 was studied by intrinsic fluorescence spectroscopy, and a rapid decrease in the intrinsic fluorescence intensity of tyrosine residues of SOD1 together with a constant maximum emission wavelength of 310 nm took place upon increasing the refolding time. As shown in **Fig. 1(A)**, the refolding kinetics of reduced, denatured apo-SOD1 monitored by fluorescence decrease in tyrosine at 310 nm were found to be biphasic during the first

60 min, because a semi-logarithmic plot of the fluorescence intensity versus time gave a curve which was resolved into two straight lines [the inset in **Fig. 1(A)**]. The corresponding rate constants were determined from the decay of the fluorescence during SOD1 refolding by global fitting the fluorescence intensity versus time to Equation (1), and these kinetic parameters are summarized in **Table 1**. For apo-SOD1, the fast and slow phase apparent rate constants were determined to be $k_1 = (27.2 \pm 0.1) \times 10^{-2} \text{ min}^{-1}$ and $k_2 = (3.31 \pm 0.04) \times 10^{-2} \text{ min}^{-1}$, respectively. The fast component ($27.2 \times 10^{-2} \text{ min}^{-1}$) of the kinetic profiles suggested that burial of tyrosine residues occurred rapidly after initiation of folding. The slow component of the kinetic profiles ($3.31 \times 10^{-2} \text{ min}^{-1}$) indicated that structural rearrangements during folding took place with significant changes in the environment of the tyrosine residues, accompanied by the formation of a tightly packed structure.

To obtain qualitative information about the existence of exposed hydrophobic patches on the intermediate, the kinetics of refolding were measured by allowing the protein to refold in the presence of ANS. This hydrophobic molecule binds to clusters of apolar groups in proteins when they are hydrated [37,38]. If the folding intermediate contains solvent-exposed apolar clusters, ANS will bind to these sites [38]. The folding of the intermediate to the native state is accompanied by dissociation of ANS, which can be monitored by measuring its extrinsic fluorescence. The time course of decrease in ANS fluorescence during apo-SOD1 refolding is shown in **Fig. 1(B)**. Global fitting the data to Equation (1) yields k_1 of $29.6 \times 10^{-2} \text{ min}^{-1}$ and k_2 of $3.40 \times 10^{-2} \text{ min}^{-1}$ (**Table 1**) comparable with the folding rate 27.2×10^{-2} and $3.31 \times 10^{-2} \text{ min}^{-1}$ measured by the intrinsic fluorescence spectroscopy.

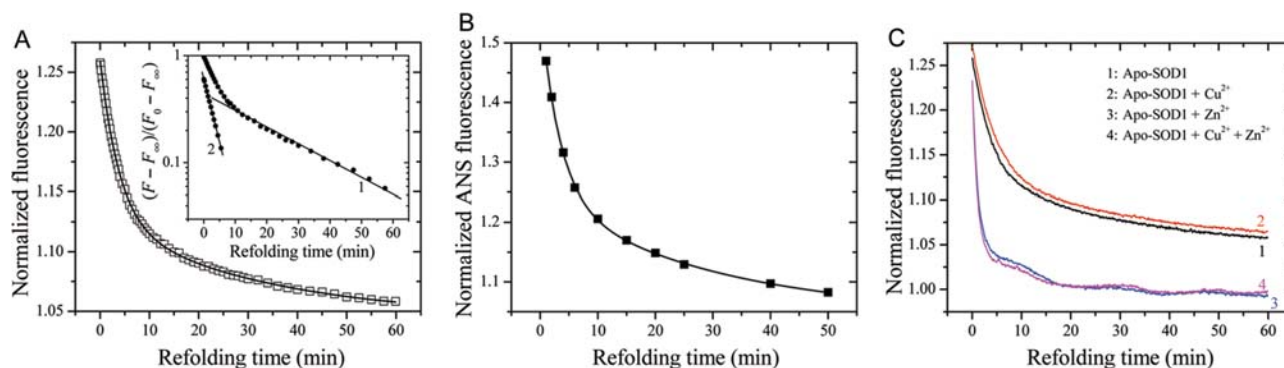


Figure 1 Kinetic analyses of the oxidative refolding of reduced, denatured apo-SOD1 by fluorescence spectroscopy Data of the intrinsic fluorescence intensity at 310 nm (A) and ANS fluorescence intensity at 512 nm (B) were selected for kinetic analyses. The inset in (A) shows semi-logarithmic plots: 1, the experimental data; 2, points obtained by subtracting the contribution of the slow phase from the original curve. The kinetic parameters were obtained by global fitting the intrinsic fluorescence intensity at 310 nm (A, open square) or ANS fluorescence intensity at 512 nm (B, filled square) versus time to Equation (1). (C) Refolding kinetics of apo-SOD1 in 50 mM Tris–HCl buffer containing or not containing $\text{Zn}^{2+}/\text{Cu}^{2+}$ at pH 7.4 in the presence of GSH/GSSG. Trace 1, in the absence of $\text{Zn}^{2+}/\text{Cu}^{2+}$. Trace 2, in the presence of Cu^{2+} at a $\text{Cu}^{2+}/\text{SOD1}$ dimer molar ratio of 2:1. Trace 3, in the presence of Zn^{2+} at a $\text{Zn}^{2+}/\text{SOD1}$ dimer molar ratio of 2:1. Trace 4, in the presence of both Zn^{2+} and Cu^{2+} at molar ratios to SOD1 dimer of 2:1. Time-course intrinsic fluorescence curves were recorded at 310 nm. All fluorescence intensities were normalized against those of 100% refolded apo-SOD1 (the refolding time was 8 h). The concentration of SOD1 was 6.5 μM . Measurements were made at 25°C.

Table 1 Kinetic parameters for the oxidative refolding of reduced, denatured bovine erythrocyte SOD1 measured by fluorescence spectroscopy

Refolding system	k_1 ($\times 10^{-2} \text{ min}^{-1}$)	k_2 ($\times 10^{-2} \text{ min}^{-1}$)
Apo-SOD1	27.2 ± 0.1	3.31 ± 0.04
Apo-SOD1 ^a	29.6 ± 2.6	3.40 ± 0.99
Apo-SOD1 + Cu ²⁺	22.1 ± 0.1	2.61 ± 0.05
Apo-SOD1 + Zn ²⁺	109.9 ± 1.2	8.72 ± 0.10
Apo-SOD1 + Cu ²⁺ + Zn ²⁺	110.6 ± 1.1	8.87 ± 0.13

The refolding buffer contained all kinds of stoichiometric Zn²⁺/Cu²⁺. The corresponding rate constants were determined from the decay of the fluorescence during SOD1 refolding by global fitting the intrinsic fluorescence intensity at 310 nm or ANS fluorescence intensity at 512 nm (^a) versus time to Equation (1), using OriginLab Origin 7.0 software. Measurements were made at 25°C. Errors shown are standard errors of the mean.

Disulfide bonds, required for stability and function of proteins, are one of the few post-translational covalent modifications that occur during protein folding [39]. Bovine SOD1 monomer contains three cysteine residues, of which two (Cys55 and Cys144) can form an intramolecular disulfide bond [9]. To provide evidence that the disulfide bridge forms upon apo-SOD1 refolding, we measured the number of free thiol groups in refolded apo-SOD1. As shown in **Fig. 2(A)**, the number of free thiol groups per dimer was observed to decrease from six of the fully reduced, denatured SOD1 to two of the refolded apo-SOD1, indicating that one disulfide bond formed in each subunit of apo-SOD1 during refolding and that metal ions did not significantly affect the oxidation state of SOD1. Kinetics of disulfide bond formation of

apo-SOD1 during refolding are shown in **Fig. 2(B)**. Fitting the data to Equation (1) yields k_1 of $23.7 \times 10^{-2} \text{ min}^{-1}$ and k_2 of $3.0 \times 10^{-2} \text{ min}^{-1}$ comparable with the folding rate 27.2×10^{-2} and $3.31 \times 10^{-2} \text{ min}^{-1}$ measured by the intrinsic fluorescence spectroscopy.

Proteolysis of a protein substrate can occur only if the polypeptide chain can bind and adapt to a specific stereochemistry of the protease's active site [40]. This method to probe refolding should yield specific information about the accessibility and hence the extent of folding of particular regions of the polypeptide chain in the course of refolding [41]. To monitor the kinetics of apo-SOD1 folding, refolding products were digested with proteinase K at various time points following initiation of protein refolding. **Figure 3** shows SDS-PAGE of refolding of apo-SOD1 by the proteinase K-pulse method [**Fig. 3(A,B)**] and its densitometric evaluation [**Fig. 3(C)**]. The time-dependent increase in optical density of the bands revealed an increase in protease resistance activity during refolding of apo-SOD1. As shown in **Fig. 3(A,C)**, at a low concentration of proteinase K (0.010 mg/ml), refolded apo-SOD1 had a relative low protease resistance activity at the early stage of refolding and ~80% apo-SOD1 was digested by proteinase K at 2 min, but after 20 min, the refolded apo-SOD1 was completely resistant to the digestion. No proteolytic fragments of apo-SOD1 from low-concentration proteinase K digestion were resolved by SDS-PAGE [**Fig. 3(A)**], suggesting that folding of apo-SOD1, as expected for a single-domain protein, did not proceed through any stable protease-resistant intermediates. As shown in **Fig. 3(B,C)**, at a high concentration of proteinase K (1.0 mg/ml), no proteinase-resistant band but only the band of proteinase K itself was observed, indicating that apo-SOD1 alone could not refold to a compact conformation which would be resistant to high

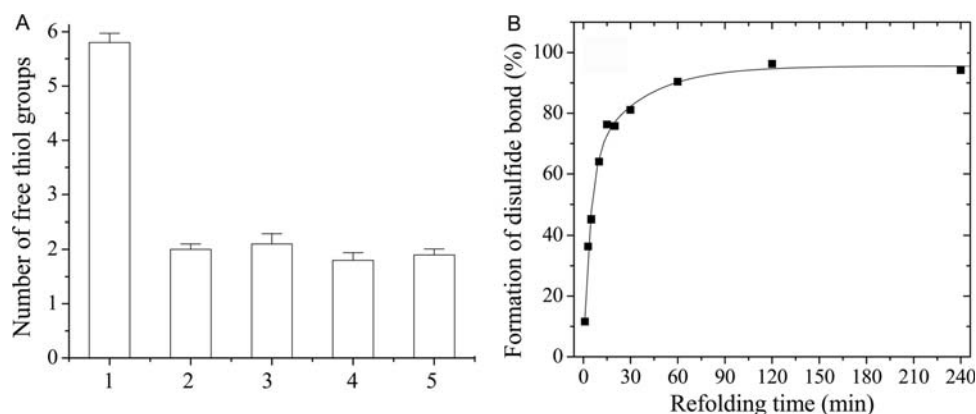


Figure 2 Formation of disulfide bonds in SOD1 during refolding (A) The number of free thiol groups per dimer in refolded SOD1 after 6 h refolding. 1, fully reduced, denatured apo-SOD1; 2, refolded apo-SOD1; 3, refolded apo-SOD1 in the presence of Cu²⁺ at a Cu²⁺/SOD1 dimer molar ratio of 2:1; 4, refolded apo-SOD1 in the presence of Zn²⁺ at a Zn²⁺/SOD1 dimer molar ratio of 2:1; and 5, refolded apo-SOD1 in the presence of both Zn²⁺ and Cu²⁺ at molar ratios to SOD1 dimer of 2:1. The data were expressed as the mean $\pm$ SD ($n = 3$). (B) Kinetics of disulfide bond formation of apo-SOD1 during refolding. All kinetic data were normalized against and expressed as a percentage of 6.5 μM solution of native SOD1. The kinetic parameters were obtained by fitting the kinetic data (filled square) versus time to Equation (1). Measurements were made at 25°C.

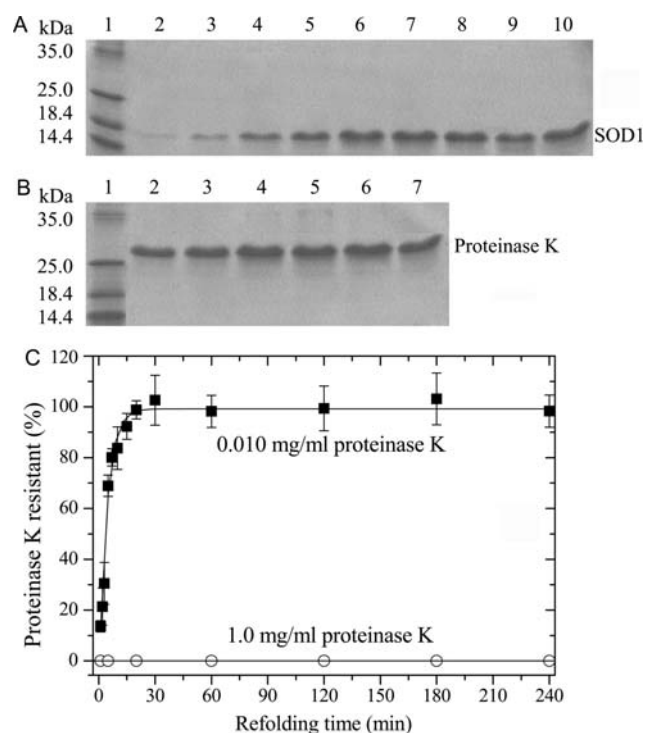


Figure 3 Kinetics of acquisition of resistance to proteinase K during apo-SOD1 refolding (A) SDS-PAGE of resistance to 0.010 mg/ml proteinase K during apo-SOD1 refolding. Lane 1, protein molecular weight marker: lactate dehydrogenase (35.0 kDa), restriction endonuclease Bsp98 I (25.0 kDa), β -lactoglobulin (18.4 kDa), and lysozyme (14.4 kDa). Lanes 2, 3, 4, 5, 6, 7, 8, and 9 represent the resistance to proteinase K during apo-SOD1 refolding at refolding time of 1, 2, 3, 5, 7, 10, 15, and 20 min, respectively. Lane 10 represents native apo-SOD1 at the same concentration. (B) SDS-PAGE of resistance to 1.0 mg/ml proteinase K. Lane 1, molecular weight marker. Lanes 2, 3, 4, 5, 6, and 7 represent the resistance to proteinase K during apo-SOD1 refolding at refolding time of 1, 5, 20, 60, 120, and 300 min, respectively. (C) Kinetics of acquisition of resistance to 0.010 mg/ml (filled square) and 1.0 mg/ml (open circle) proteinase K. The normalized data from multiple experiments were expressed as the mean $\pm$ SD ($n = 2-4$) and fitted using Equation (1). The concentrations of GSH and GSSG were 1.0 and 0.20 mM, respectively. The concentration of SOD1 was 6.5 μ M. Measurements were made at 25°C.

concentrations of proteinase K. In other words, apo-SOD1 formed a conformation with low-level, but not with high-level, proteinase K resistance during refolding.

Effects of zinc and copper on kinetics of SOD1 refolding

Wild-type SOD1 is a homodimeric metalloenzyme that binds a single atom each of copper and zinc per monomer [21]. To examine the requirement for these metals for SOD1 refolding, copper and/or zinc ions were added into the refolding buffer containing GSH/GSSG. The corresponding kinetic parameters were determined from the decay of the fluorescence during SOD1 refolding by fitting the fluorescence intensity versus time to Equation (1). As shown in **Fig. 1(C)**, the intrinsic fluorescence of the protein decreased more quickly

when the refolding buffer contains stoichiometric Zn^{2+} than those without Zn^{2+} . **Table 1** summarizes the kinetic parameters obtained for the oxidative refolding of reduced, denatured SOD1 in the absence and presence of $\text{Zn}^{2+}/\text{Cu}^{2+}$. As shown in **Table 1**, the apparent rate constants for the fast phase of the oxidative refolding of SOD1 in the presence of stoichiometric Zn^{2+} were about 1.1 min^{-1} , whereas those in the absence of Zn^{2+} were $0.22-0.27 \text{ min}^{-1}$. Furthermore, the apparent rate constants for the slow phase of the oxidative refolding of SOD1 in the presence of stoichiometric Zn^{2+} were $8.7-8.9 \times 10^{-2} \text{ min}^{-1}$, whereas those in the absence of Zn^{2+} were $2.6-3.3 \times 10^{-2} \text{ min}^{-1}$. The above results indicated that the addition of stoichiometric Zn^{2+} into the refolding buffer remarkably accelerated both the fast phase and the slow phase of the oxidative refolding, compared with no Zn^{2+} . In contrast, the effects of stoichiometric Cu^{2+} on the rates of the oxidative refolding of apo-SOD1 were not remarkable [**Fig. 1(C)** and **Table 1**].

The roles of the stoichiometric zinc and copper ions in modulating the oxidative refolding of reduced and denatured SOD1 were further investigated by the proteinase K digestion method. As shown in **Fig. 4(A,C)**, at a high concentration of proteinase K (1.0 mg/ml), refolded apo-SOD1 in the presence of Zn^{2+} at a $\text{Zn}^{2+}/\text{SOD1}$ dimer molar ratio of 2:1 had a relative low protease resistance activity at the early stage of refolding and $\sim 80\%$ apo-SOD1 was digested by proteinase K at 5 min. With the increase in refolding time, however, refolded apo-SOD1 incubated with stoichiometric Zn^{2+} became progressively resistant to the digestion of 1.0 mg/ml proteinase K and was mostly resistant ($\sim 25\%$ apo-SOD1 was digested by proteinase K) to the degradation after 60 min. In other words, the addition of stoichiometric zinc ions gained high protease resistance activity of SOD1 to a high concentration of proteinase K (1.0 mg/ml). In contrast, the addition of stoichiometric copper ions [**Fig. 4(B,C)**] had no obvious effect on the acquisition of protease resistance to high concentrations of proteinase K. Our data here indicate that zinc is essential for SOD1 to refold to a native-like hyperstable conformation (see below) with high protease resistance activity. Taken together, these results indicated that apo-SOD1 formed a conformation with low-level proteinase K resistance during refolding and stoichiometric Cu^{2+} had no obvious effect on the resistance to proteinase K. In contrast, when the refolding buffer contained stoichiometric Zn^{2+} , SOD1 formed a compact conformation with high-level proteinase K resistance during refolding.

Structural characteristics of refolded apo-SOD1 in the presence of zinc

The refolded apo-SOD1 in the presence of stoichiometric zinc ions at 25°C was characterized by fluorescence spectroscopy and CD. As shown in **Fig. 5(A)**, native SOD1 had

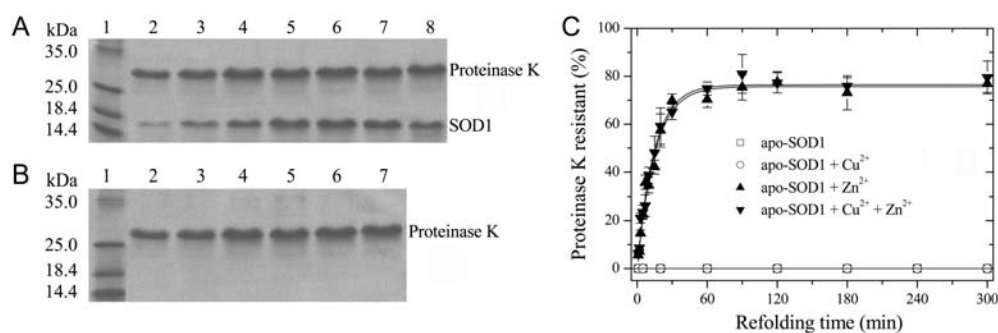


Figure 4 Kinetics of acquisition of resistance to proteinase K during apo-SOD1 refolding in the presence of Zn²⁺/Cu²⁺ (A) SDS-PAGE of resistance to 1.0 mg/ml proteinase K during apo-SOD1 refolding in the presence of Zn²⁺ at a Zn²⁺/SOD1 dimer molar ratio of 2:1. Lane 1, protein molecular weight marker. Lanes 2, 3, 4, 5, 6, 7, and 8 represent the resistance to proteinase K during apo-SOD1 refolding in the presence of Zn²⁺ at refolding time of 1, 2, 3, 5, 7, 10, and 15 min, respectively. (B) SDS-PAGE of resistance to 1.0 mg/ml proteinase K in the presence of Cu²⁺ at a Cu²⁺/SOD1 dimer molar ratio of 2:1. Lane 1, molecular weight marker. Lanes 2, 3, 4, 5, 6, and 7 represent the resistance to proteinase K during apo-SOD1 refolding in the presence of Cu²⁺ at refolding time of 1, 5, 20, 60, 120, and 300 min, respectively. (C) Kinetics of acquisition of resistance to 1.0 mg/ml proteinase K in the absence of Zn²⁺/Cu²⁺ (open square), in the presence of Cu²⁺ (open circle), in the presence of Zn²⁺ (filled triangle), and in the presence of both Zn²⁺ and Cu²⁺ at molar ratios to SOD1 dimer of 2:1 (filled inverse triangle). All proteinase K resistance values were normalized against and expressed as a percentage of 6.5 μ M solution of native SOD1. The normalized data from multiple experiments were expressed as the mean $\pm$ SD ($n = 2-4$) and fitted using Equation (1). The concentration of SOD1 was 6.5 μ M. Measurements were made at 25°C.

an intrinsic fluorescence emission maximum of about 310 nm when excited at 280 nm. The intrinsic fluorescence emission maximum of the refolded apo-SOD1 in the presence of stoichiometric zinc ions was similar to that of native SOD1. Far-UV CD spectroscopy was used to compare the secondary structural changes in the different samples. As shown in **Fig. 5(B)**, no obvious secondary structural change was observed for the refolded apo-SOD1 in the presence of stoichiometric zinc ions, compared with native SOD1. The above results indicated that a native-like hyperstable conformation was obtained by the addition of zinc ions.

Effects of zinc and copper on SOD1 activity and refolding yield

To examine the requirement for these metals for SOD1 activity, relative activity of copper-reconstituted SOD1 as a

function of copper content was investigated. As shown in **Fig. 6(A)**, a gradual increase in relative activity of SOD1 took place upon increasing the molar ratio of Cu²⁺/SOD1 dimer, reaching about 85% at the molar ratio of 2:1 and about 90% at the molar ratio of 3:1 or greater. The presence of Zn²⁺ enhanced the relative activity of SOD1 to some extent, reaching about 90% at the molar ratio of 2:1 and about 95% at a Cu²⁺/SOD1 dimer molar ratio of 3:1 or greater. These results indicated that copper played a dominant role in SOD1 activity and zinc only made a small contribution to SOD1 activity. When the Cu²⁺-binding sites were fully saturated, it is possible that the extra Cu²⁺ could bind to one of the Zn²⁺ sites to replace Zn²⁺. That may be the reason why the relative activity of SOD1 at a Cu²⁺/SOD1 dimer molar ratio of 3:1 or greater is slightly higher than that in the presence of stoichiometric Cu²⁺.

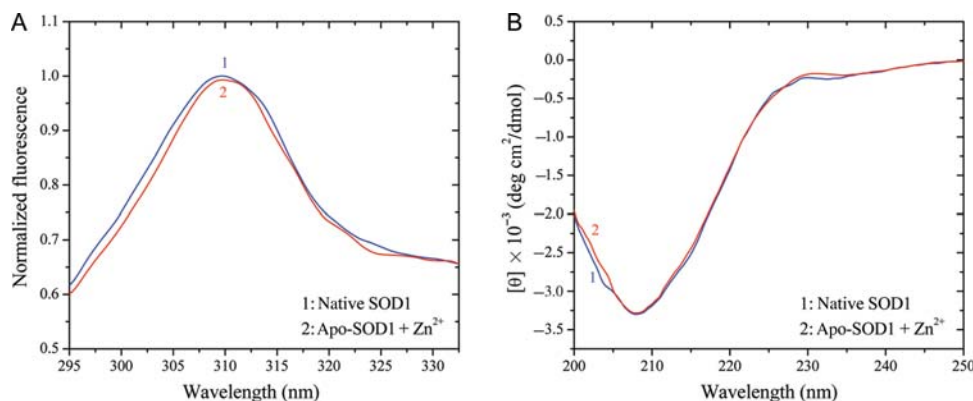


Figure 5 Structural characteristics of refolded apo-SOD1 in the presence of zinc ions detected by intrinsic fluorescence emission and far-UV CD. Curves 1 and 2 represent the spectra of native SOD1 and refolded apo-SOD1 in the presence of stoichiometric zinc ions, respectively. The refolding time was 6 h. (A) Intrinsic fluorescence emission spectra of native SOD1 and refolded apo-SOD1. The concentration of SOD1 was 6.5 μ M. All fluorescence intensities were normalized against those of native SOD1. (B) Far-UV CD spectra of native SOD1 and refolded apo-SOD1. The concentration of SOD1 was 15 μ M. Measurements were made at 25°C.

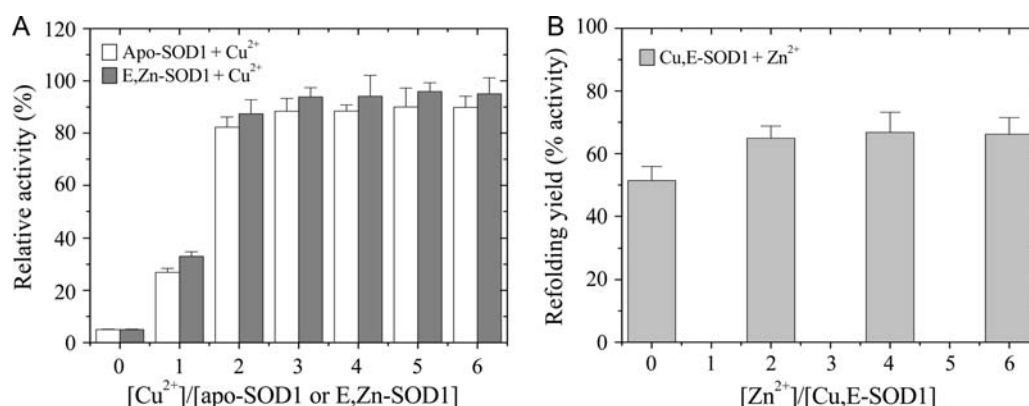


Figure 6 Effects of zinc and copper on the relative activity of SOD1 and the reactivation of reduced, denatured SOD1 (A) Relative activity of copper-reconstituted superoxide dismutase as a function of copper content. Apo-SOD1, E,Zn-SOD1, and Cu,E-SOD1 were achieved by the methods as described in ‘Materials and Methods’. The activity was measured after incubating copper chloride with apo-SOD1 or E,Zn-SOD1 in 100 mM sodium acetate buffer for 12 h. The relative activities of apo-SOD1 (white bar) and E,Zn-SOD1 (gray bar) incubated with copper chloride. (B) Refolding yield of Cu,E-SOD1 as a function of zinc content. The refolding time was 6 h. All enzymatic activities were normalized against and expressed as a percentage of native Cu,Zn-SOD. Measurements were made at 25°C. The data were expressed as the mean $\pm$ SD ($n = 3$).

To confirm that native SOD1 is obtained in the kinetic experiments, we measured the refolding yield of Cu,E-SOD1 as a function of zinc content and the refolding yield of E,Zn-SOD1 as a function of copper content. As shown in **Fig. 6(B)**, the refolding yield of Cu,E-SOD1 after refolding for 6 h in the presence of Zn²⁺ at a Zn²⁺/SOD1 dimer molar ratio of 2:1 or greater increased about 15%, compared with that in the absence of Zn²⁺ (~52%). We further used different metal-loading orders to compare the roles of these two ions [**Figs. 6(B)** and 7]. As shown in **Fig. 7**, the refolding yield of E,Zn-SOD1 after refolding for 6 h in the presence of Cu²⁺ at a Cu²⁺/SOD1 dimer molar ratio of 2:1 or greater increased about 60%, compared with that in the absence of Cu²⁺ (~4%). These results indicated that copper made a major contribution to the reactivation of reduced, denatured SOD1, and that zinc made a minor contribution to the

recovery of SOD1 activity by suppressing the aggregation during refolding (see below). Meanwhile, since it has been reported that the binding of Zn²⁺ could facilitate the following Cu²⁺ loading *in vivo* [42,43], the increased 15% activity by adding zinc could be resulted from a higher affinity of SOD1 to Cu²⁺ besides preventing aggregation.

Effects of zinc and copper on SOD1 aggregation during refolding

For *in vitro* refolding of SOD1, correct folding competes kinetically with SOD1 aggregation. Effects of zinc and copper on apo-SOD1 aggregation during refolding were monitored by light scattering. As shown in **Fig. 8(A)**, the 90° light scattering value of apo-SOD1 increased more quickly in refolding buffer without Zn²⁺ ($k = 0.795 \text{ min}^{-1}$) than that in buffer containing stoichiometric Zn²⁺ ($k = 0.467 \text{ min}^{-1}$) and that in the presence of Zn²⁺ at a Zn²⁺/SOD1 dimer molar ratio of 3:1 or greater (average $k = 0.453 \text{ min}^{-1}$). As shown in **Fig. 8**, aggregation of apo-SOD1 in the presence of stoichiometric Zn²⁺ was remarkably slower than that in the absence of Zn²⁺ or that in the presence of Cu²⁺ at the same molar ratio of metal ions to apo-SOD1 ($k = 0.791 \text{ min}^{-1}$). In contrast, the effects of stoichiometric Cu²⁺ on the aggregation of apo-SOD1 were not remarkable (**Fig. 8**).

Discussion

Understanding the dynamical roles of zinc and copper in SOD1 folding and aggregation [44] is quite relevant with respect to the complete comprehension of the mechanism of SOD1 folding and aggregation. SOD1 was first isolated in 1938 and determined to be a copper protein of unknown function [45]. It was not until 1970, a year after the SOD1 function was discovered, that zinc was proven to be a

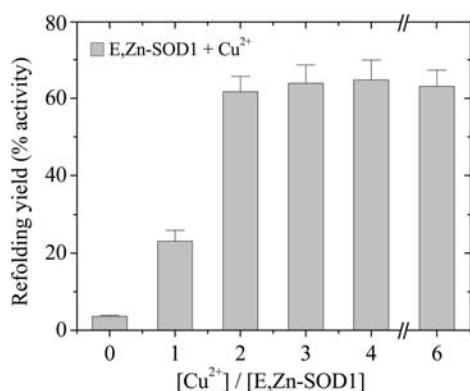


Figure 7 Refolding yield of E,Zn-SOD1 as a function of copper content E,Zn-SOD1 was achieved by the methods as described in ‘Materials and Methods’. The refolding time was 6 h. All enzymatic activities were normalized against and expressed as a percentage of native SOD1. Measurements were made at 25°C. The data were expressed as the mean $\pm$ SD ($n = 3$).

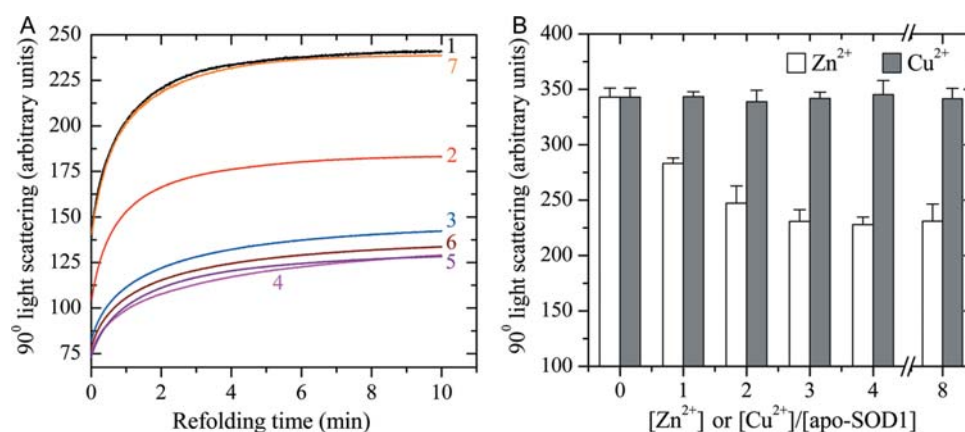


Figure 8 Effects of zinc and copper on apo-SOD1 aggregation during refolding monitored by light scattering (A) Time-course SOD1 aggregation at different Zn^{2+} /SOD1 dimer molar ratios. For curves 1, 2, 3, 4, 5 and 6, Zn^{2+} /SOD1 dimer molar ratios were 0, 1:1, 2:1, 3:1, 4:1, and 8:1, respectively. For curve 7, Cu^{2+} /SOD1 dimer molar ratio was 2:1. (B) Effects of zinc and copper on the extent of apo-SOD1 aggregation during refolding. Here, the extent of aggregation was the 90° light scattering value recorded after 12 h of refolding. The concentration of SOD1 was 6.5 μM . Measurements were made at 25°C. The data were expressed as the mean $\pm$ SD ($n = 3$).

cofactor of the enzyme [46]. Although the activity of the holoenzyme is relatively constant over a wide pH range, the activity of the zinc-free enzyme is markedly pH dependent and falls off rapidly above pH 7 [47]. Banci *et al.* [42] have found that E,Zn-SOD1 is very similar to the holo form but different from the apo form, and the binding of zinc has dramatic structuring effects: it organizes all the β -strands with the full length as found in the native state and induces the complete tertiary structure and the optimal conformation of the electrostatic loop VII. Once zinc binds, the protein undertakes the final folding, leaving only the copper-binding site disordered and somewhat open [42]. In the present study, we found that at physiological pH and room temperature, the rate constants for the fast phase of the oxidative refolding of SOD1 in the presence of stoichiometric Zn^{2+} were about 1.1 min^{-1} , whereas those in the absence of Zn^{2+} were $0.22 - 0.27 \text{ min}^{-1}$, and that the rate constants for the slow phase of the oxidative refolding in the presence of stoichiometric Zn^{2+} were $8.7 - 8.9 \times 10^{-2} \text{ min}^{-1}$, whereas those in the absence of Zn^{2+} were $2.6 - 3.3 \times 10^{-2} \text{ min}^{-1}$. Clearly, the addition of stoichiometric Zn^{2+} into the refolding buffer remarkably accelerates both the fast phase and the slow phase of the oxidative refolding, compared with no Zn^{2+} , suggesting that binding of zinc ions is of fundamental importance to define the tertiary interactions of SOD1 during refolding. We also found that aggregation of apo-SOD1 in the presence of stoichiometric Zn^{2+} was remarkably slower than that in the absence of Zn^{2+} . Our data here showed that the presence of Zn^{2+} during refolding decreased the amount of material that scatters light, and this is consistent with other reports in the literature that suggests zinc binding protects the protein against aggregation [20,22,27,29,48]. Richardson and Richardson [48] have indicated that the interactions between edges of β -strands of SOD1 are crucial

to regulate aggregation propensity of the protein. It is the structural changes in the zinc-binding loop and in the electrostatic loop induced by zinc that reduce non-native interactions between the two β -strands of SOD1 and hence decrease the edge-to-edge aggregation of SOD1.

So far, the role of stoichiometric zinc in SOD1 is to provide a positive charge and a structural stability to the holoenzyme, to provide the correct structure for the interaction with the copper chaperone for SOD1 (CCS) and to be involved in the catalytic mechanism through the histidine-histidinato 63 link [15,16,42,43]. Potter *et al.* [49] have shown that the binding of the two zinc ions to the SOD1 dimer is thermodynamically asymmetric and that the binding of the first zinc ion to apo-SOD1 exerts far more influence on the structure than the binding of the other three metal ions. Mulligan *et al.* [19] have specifically described how zinc and copper are involved in SOD1 unfolding. Kayatekin *et al.* [20] have observed a remarkable increase in the rate of folding of SOD1 with the disulfide bond intact in the presence of stoichiometric zinc, demonstrating a significant role for a pre-organized zinc-binding loop in the transition state ensemble for the monomer folding reaction in this β -barrel protein. Recent findings have pointed to a critical role for the native zinc site in controlling SOD1 misfolding and have shown that even subtle changes of zinc-loading sequence can render the wild-type protein the same structural properties as ALS-provoking mutations [10]. First, the present study demonstrated that stoichiometric zinc played a significant role in the oxidative refolding of low micromolar bovine SOD1 by accelerating the oxidative refolding and suppressing the aggregation during refolding. There is relatively little data in the literature on refolding from the apo, reduced state of SOD1, which represents the most

immature form of SOD1 in cells [27]. It has been reported that familial ALS mutations impair either zinc binding, disulfide bond formation, or both, leading to accumulation of such an aggregation-prone, immature form of human SOD1 [50]. It has also been suggested that such mutations may cause aggregation/disease by increasing the unfolding of folded SOD1 rather than impairing folding [19,51]. Therefore, this paper provides evidence for the conclusion that stoichiometric zinc plays an important role in modulating SOD1 folding *in vivo*. Second, we found that Zn^{2+} loading rendered the protein stable against digest, whereas Cu^{2+} loading had no obvious effect. This is a complement to previous NMR data [42,43] that shed new light on how metalation controls, not only the dynamics, but also the solvent accessibility of the active-site loops of SOD1. Therefore, this paper presents findings concerning the influence of Zn^{2+} loading upon the inherent stability and protease resistance activity of SOD1 and provides support for the hypothesis that the role of stoichiometric zinc in SOD1 is mainly structural. Previous studies [42,43,52] sustain this hypothesis. Zinc binding to apo-SOD1 orders loops IV (the zinc-binding loop) and VII (the electrostatic loop) and also pre-organizes the copper-binding site [42,43,52]. In this paper, by using fluorescence technique and proteinase K digestion, we showed that the addition of stoichiometric zinc to such an immature form of SOD1 increased the folding rate of the protein and consequently made it more stable with respect to proteinase K digestion and aggregation.

The present study demonstrated that stoichiometric Cu^{2+} had no obvious effects on refolding and aggregation of apo-SOD1, but made a major contribution to the recovery of SOD1 activity. Even the presence of a higher concentration of Cu^{2+} (325 μM), in which the molar ratio of Cu^{2+} to SOD1 dimer is 50:1, had no obvious effect on the rates of the oxidative refolding and exerted a small enhancing effect on the aggregation of apo-SOD1 (data not shown). Because of its inherent toxicity, free copper in cells is maintained at vanishingly low concentrations [53]; this metal could be delivered to immature apo-SOD1 molecules by carriers such as CCS [21]. The extension of the study of bovine apo-SOD1 refolding in the absence of CCS to human apo-SOD1 mutant refolding in the presence of CCS should lead to a better understanding of how SOD1 folds in its intracellular environments.

Human SOD1 monomer contains four cysteine residues, of which two, Cys6 and Cys111, exist as free cysteine residues [21]. A pseudo-wild-type form of human SOD1, in which the free cysteine residues have been mutated to prevent aberrant intermolecular disulfide bonds and to increase the reversibility of folding, has been extensively used for the study of apo-SOD1 refolding [19,20,22,51,54]. Bovine SOD1 monomer contains three

cysteine residues but only Cys6 exists with free hydrosulfide group [9]. Cys111 plays a critical role in the intermolecular disulfide formation and aggregation process of human SOD1 mutant [55]. As a result, the absence of Cys111 in bovine SOD1 makes it unable to form a non-physiological intermolecular disulfide bond between Cys6 and Cys111 during refolding, and aggregation of bovine apo-SOD1 is less serious than that of human apo-SOD1. Our additional experiments demonstrated that aggregation of bovine apo-SOD1 in the presence of GSH and GSSG had no obvious effects on the tyrosine and ANS fluorescence data as well as the protease sites of the protein (data not shown).

The predominant model in the literature for folding kinetics of SOD1 (with disulfide bonds intact) for the protein in the apo state [20,51,54], in the presence of Zn^{2+} [20,52], and in the presence of Zn^{2+} and Cu^{2+} [52] is that rate-limiting monomer formation is followed by very rapid dimer association that is too fast to measure. The observed folding kinetics are single exponential for apo and in the presence of Zn^{2+} (corresponding to monomer folding), and double exponential in the presence of Zn^{2+} and Cu^{2+} (likely due to parallel pathways for monomer folding with different metal ligation) [20,52,54,56,57]. In the present study, the observed oxidative folding kinetics of denatured, reduced SOD1 were double exponential for apo, in the presence of Zn^{2+} , and in the presence of Zn^{2+} and Cu^{2+} . An interpretation is that the two observed folding phases are for parallel (monomer) folding reactions with concomitant formation of disulfide bonds. In order to resolve the nature of the two phases, we conducted folding experiments as a function of SOD1 concentration and found that the fast and slow phase apparent rate constants were independent of SOD1 concentration (data not shown), indicating that our interpretation is reasonable.

In conclusion, three techniques, tyrosine fluorescence, ANS binding, and proteinase K digestion, were used to monitor the progress of reduced, denatured apo-SOD1 folding at physiological pH and room temperature. The results obtained using all three techniques suggest that stoichiometric zinc significantly increases the folding rate of such an immature form of SOD1, whereas stoichiometric copper has no noticeable effects. Stoichiometric zinc also has a significant stabilizing effect on the protein, rendering it resistant to digestion by high concentrations of proteinase K. Finally, stoichiometric zinc is also shown to decrease the rate and extent of SOD1 aggregation during refolding. Our data here demonstrate that stoichiometric zinc plays an important role in the oxidative refolding of low micromolar bovine SOD1 by accelerating the oxidative refolding, suppressing the aggregation during refolding and helping the protein to form a compact conformation with high protease resistance activity.

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