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Binding of a Single Zinc Ion to One Subunit of Copper−Zinc Superoxide Dismutase Apoprotein Substantially Influences the Structure and Stability of the Entire Homodimeric Protein

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Abstract: The thermodynamics of zinc binding to metal-free (apo) human and bovine copper−zinc superoxide dismutases (SOD1) were measured using isothermal titration calorimetry. The apparent thermodynamics of zinc binding to the apoproteins were favorable ($K_a > 10^8$ M^{-1}), with an observed stoichiometry of one zinc per homodimer. The change in heat capacity for the one-zinc binding event was large and negative (~ -650 cal mol^{-1} K^{-1}), suggestive of significant structural changes to the protein upon zinc binding. We further characterized the one-zinc derivative by circular dichroism and determined that this derivative had nearly the same secondary structure as the two-zinc derivative and that both are structurally distinct from the metal-free protein. In addition, we monitored the effect of zinc binding on hydrogen−deuterium exchange and accessibility of histidyl residues to modification by diethyl pyrocarbonate and observed that more than 50% protection was afforded by the binding of one zinc in both assays. Differential scanning calorimetry on the human SOD1 zinc derivatives also showed increased thermostability of the protein due to zinc binding. Further, the melting transitions observed for the one-zinc derivative closely resembled those of the two-zinc derivative. Finally, we observed that the quaternary structure of the protein is stabilized upon binding of one and two zinc ions in analytical ultracentrifugation experiments. Combined, these results suggest communication between the two monomers of SOD1 such that the binding of one zinc ion per homodimer has a more profound effect on the homodimeric protein structure than the binding of subsequent metal ions. The relevance of these findings to amyotrophic lateral sclerosis is discussed.

1. Introduction

Copper−zinc superoxide dismutase (CuZnSOD1 or SOD1) is a metalloenzyme that catalyzes the disproportionation of superoxide to give dioxygen and hydrogen peroxide.[1] The enzyme contains two identical subunits of ∼150 amino acids, each one built around a β-barrel core plus several long loops of irregular structure. Each subunit contains a metal binding region that binds one copper and one zinc ion, positioned such that they share a common histidyl imidazolate ligand when the copper ion is in the 2+ oxidation state. Each metal binding region is contained entirely within its subunit and is far removed from the metal binding region of its partner subunit. Consequently, the zinc and copper ions are approximately 40 and 32 Å apart, respectively, from their counterparts across the dimer interface (Figure 1).

The metal ions have an enormous effect on the thermostability of SOD1.[2−4] The binding of metal ions also increases the affinity of the two subunits within the protein dimer and decreases the susceptibility toward proteolysis relative to that of apo SOD1.[5−7] The gain in stability of SOD1 upon metal binding is likely related to a restructuring of the apoprotein: structures of SOD1 proteins devoid of metals show differences compared to proteins that contain metal ions (see below).[8−12]

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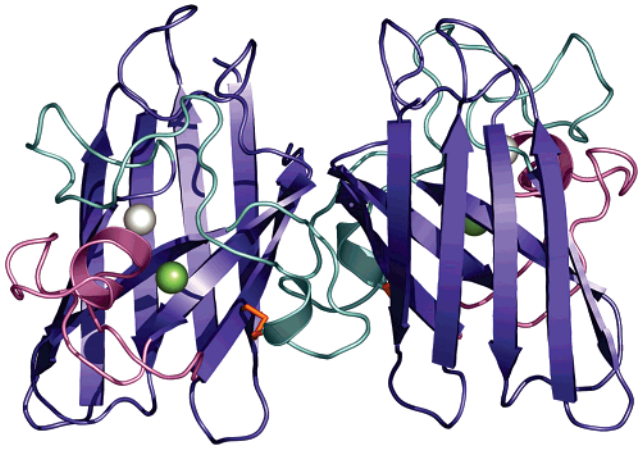

Figure 1. The hSOD1 three-dimensional fold (1HL5). The copper ions are colored green and the zinc ions, gray. The intramolecular disulfide bonds are colored orange, and the zinc loops and electrostatic loops are colored cyan and pink, respectively.

Understanding the metal binding processes of SOD1 is currently of great interest because autosomal dominant SOD1 mutations are known to cause a familial form of the neurodegenerative disease amyotrophic lateral sclerosis (ALS) by a toxic gain of function.[13−15] Over 100 different mutations have now been identified,[16] and yet the mechanism by which the mutant proteins cause the disease is unclear. One of the pathological hallmarks of the disease is the presence of protein deposits in the motor neurons of ALS patients. Protein aggregates are also found in other neurological diseases, such as Alzheimer's disease, Huntington's disease, and prion diseases, which are believed to be linked to protein destabilization and misfolding;[17] increasingly, a consensus is now being reached that mutant SOD1-mediated ALS is likely a protein misfolding disease (reviewed in refs 16 and 18).

If SOD1-linked ALS is indeed a protein folding disease, it is possible that the mutations may interfere with one of the post-translation modification events required to reach the fully active protein. To understand fully where mutant SOD1 proteins may have aberrant behavior in these steps, it is essential to characterize completely the maturation pathway of the wild-type protein. The requirement of copper for enzymatic activity, together with the spectroscopically active nature of the cupric ion, has prompted careful and thorough study of the enzyme *in vitro*.[19−21] There is also considerable information available about how copper proteins, including SOD1, are metalated *in vivo* (reviewed in refs 22 and 23). The Zn^{2+} ion, however, has a $3d^{10}$ electronic configuration, seriously limiting spectroscopic studies, and little is known about how SOD1 (or any other zinc-containing protein) obtains that metal ion *in vivo*. Furthermore, certain ALS mutant SOD1 proteins have been shown to have a decreased affinity for zinc,[24,25] and zinc-deficient SOD1 proteins have been postulated to be the toxic species in ALS (reviewed in ref 26).

Crystal structures of apo human SOD1 typically display disorder in both the zinc and the electrostatic loops, while those of fully metalated SOD1s have well-ordered loop structure.[12,27] Differences were observed between NMR solution structures of various metal derivatives of engineered monomeric hSOD1 that have suggested that zinc binding has a more profound effect on structural tightening of the apoprotein than the subsequent binding of copper. Namely, the structures of the zinc-bound monomer and the copper- and zinc-bound monomer are very similar, while the apo monomer displays significant structural perturbations relative to the other derivatives: the electrostatic and zinc loops are disordered, the two β-sheets that comprise the β-barrel have moved apart, several β-strands are shortened, and the empty zinc site is solvent exposed.[8,10,11] Thus, zinc binding led to structural rearrangements in the engineered monomer, including ordering of the large electrostatic and zinc binding loops.

In this study, we focus on the effects of zinc binding on the structure and stability of wild-type, homodimeric human and bovine SOD1. We demonstrate that zinc binding strongly affects the structure and stability of the SOD1 protein overall. Moreover, the binding of one zinc ion to the homodimer has a more profound effect on the SOD1 structure than the binding of the second zinc ion, and the binding of one zinc to the metal-free dimer stabilizes the opposite apo subunit.

2. Experimental Section

2.1. Protein Preparation. Bovine SOD1 (bSOD1) was purchased from Oxis International. Human SOD1 (hSOD1) was expressed in yeast and purified following previously published protocols.[6] Both proteins were made apo following established methods.[6] Metal content was confirmed by inductively coupled plasma mass spectrometry (ICP-MS), and apoproteins contained less than 0.1 equiv per dimer each of copper and zinc. Zinc derivatives were made by the slow, stepwise addition of a stoichiometric amount of metal from a stock solution of 10−20 mM $ZnSO_4$ (Fisher) in 100 mM acetate, pH 5.5, to the protein sample (∼5−10 mg/mL in 100 mM acetate buffer, pH 5.5) while stirring on ice. After the final addition, the protein was incubated at 4 °C overnight. The two-copper-plus-two-zinc derivative was made by the titration of 20 mM $CuSO_4$ (Fisher) into the two-zinc derivative. The d−d transition at 680 nm due to copper in the copper site of CuZnSOD was monitored

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during the titration by UV−vis. Prior to all experiments except for those with diethyl pyrocarbonate (DEPC) and circular dichroism (CD), the metalated derivatives were washed with metal-free buffer (prepared by treatment with Chelex (Biorad)) by carrying out at least three cycles of 5−10-fold concentration and dilution using 10 000 molecular weight cutoff (MWCO) microcon filters (Millipore).

2.2. Isothermal Titration Calorimetry. Metal binding thermodynamics were measured by isothermal titration calorimetry (ITC) using the VP-ITC instrument (MicroCal, Inc.). All solutions were degassed by stirring under vacuum using a ThermoVac instrument (MicroCal, Inc.). The reference cell was filled with 1.4 mL of degassed water. Protein samples were either dialyzed or washed, as described above, using 10 000 MWCO centricons (Millipore), with 100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 5.5, which had been treated with Chelex resin (Biorad) in order to remove all metals. A 100 μM stock solution of apoprotein was made, and the concentration was determined by the absorbance at 280 nm using previously determined extinction coefficients of 3000 M^{-1} cm^{-1} for bSOD1 dimers and 10 800 M^{-1} cm^{-1} for hSOD1 dimers.[28,29] In a typical ITC experiment, 100 μL of the stock was diluted into 1.9 mL of degassed buffer to give a concentration of 5 μM. This solution was degassed again before 1.4 mL was loaded into the sample cell.

Metal samples were prepared with volumetric flasks using $ZnSO_4$ (Fisher) or $CoSO_4$ (Mallinkrodt). A 100 mM stock solution was made of each metal in water before dilution into the appropriate buffer using volumetric flasks. Metal concentrations were confirmed by ICP-MS. Titrations typically used 0.2 mM metal in the titration syringe with 1−3 μL injections. The time between injections ranged typically from 5 to 10 min. Either a titration of metal into buffer or the last few injections of a titration into protein taken to completion were used to determine the heats of dilution.

The MicroCal Origin software package was used for ITC data analysis. The enthalpy due to metal dilution was subtracted from the experimental data. All data were fit to the one- or two-site model, depending on the best fit determined by the fitting program (least-squares fit).

2.3. Native Gels and Subunit Exchange Assays. Stock solutions of 50 μM apo bSOD1 and fully metalated (as purchased) bSOD1 were prepared in 100 mM acetate buffer, pH 5.5, and 0.5 mM ethylenediaminetetraacetic acid (EDTA). Five-microliter portions of each sample were mixed together, and different samples were allowed to incubate for various times at room temperature or at 37 °C. The mixed samples were run on 7.5% native protein gels (no sodium dodecyl sulfate (SDS) or reducing agents) and stained with coomassie blue.

2.4. Diethyl Pyrocarbonate Modification. The apo, one-zinc, two-zinc, and two-copper-plus-two-zinc derivatives were modified by DEPC using a previously published protocol.[20] A 98 μL aliquot of 16 μM protein in 100 mM sodium phosphate buffer, pH 6.0, was incubated with 2 μL of 0.5 M DEPC. After no further change was observed (~20 min), the final absorbance at 240 nm was measured, and the number of modified histidines was determined using the extinction coefficient of 3200 M^{-1} cm^{-1} at this wavelength.

The DEPC modification of apo hSOD1 was also monitored by electrospray ionization-mass spectrometry (ESI-MS) using a Perkin-Elmer/Sciex API III mass spectrometer equipped with an ion spray source at the UCLA Pasarow mass spectrometry laboratory. A 5000-fold excess of DEPC was added to 30 μL of 0.2 mM apo hSOD1, and 2 μL of the reaction mixture was injected into the mass spectrometer immediately and at 2- to 3-min intervals thereafter. The sample was injected into a stream of acetonitrile/water/formic acid, 50/50/0.1 by volume.

2.5. Circular Dichroism. CD spectra of the metal derivatives in 100 mM acetate buffer, pH 5.5, were recorded at room temperature on a Jasco 715 spectropolarimeter. Each spectrum represents an average of five scans, where the step resolution was 0.5 nm and the scan speed was 20 nm/min. The spectra are reported per protein subunit, where the subunit concentration was 0.6 mM.

2.6. H/D Exchange, DSC, and AUC. Hydrogen−deuterium (H/D) exchange experiments were completed using previously described protocols.[2,30] Proteins in 10 mM potassium phosphate, pH 7.4, were diluted 10-fold into deuterated buffer and equilibrated for varying times at 37 °C before ESI-MS analysis. Differential scanning calorimetry (DSC) experiments were performed following the protocols outlined previously.[2,3] Briefly, 2 mg/mL protein samples in 100 mM phosphate buffer, pH 7.0, were analyzed on a Nano II differential scanning calorimeter (Calorimetry Sciences Corp.). The analytical ultracentrifugation (AUC) experiments were carried out at the Center for Analytical Ultracentrifugation of Macromolecular Assemblies at the University of Texas Health Science Center (San Antonio, TX). Data analysis was carried out using the UltraScan software package.[31] Sample preparation and experimental apparatus were previously detailed.[6]

3. Results and Discussion

3.1. Thermodynamics of Zinc Binding to Apo SOD1 Proteins. Isothermal titration calorimetry was used to determine directly the thermodynamics of zinc binding to apo bovine and human SOD1 proteins (bSOD1 and hSOD1, respectively). The buffer system used was 100 mM MES, pH 5.5. We found MES to be ideal because it does not complex Zn^{2+},[32] thereby removing thermodynamic contributions of metal−buffer interactions. The preferential binding of zinc (or cobalt) ions to the zinc site is well characterized, and it is documented that, at pH < 6.5, Zn^{2+} binds first to the zinc site (reviewed in refs 19 and 21). Thus, the low pH was selected for the ITC experiments to prevent Zn^{2+} binding to the copper site.

It is well established that apo hSOD1 in the disulfide-oxidized form is an extremely stable dimer,[5,6,33] and ultracentrifugation experiments have demonstrated that the disulfide-oxidized apoprotein remains a dimer at the concentrations used in this study (5 μM).[6] Previous studies have shown that apo bovine and apo human SOD1 are similarly resistant to subunit dissociation with SDS or urea in both the metalated and apo forms,[34,35] and we therefore infer that the apo bovine protein is also dimeric under these conditions. As mentioned above, both the apo bSOD1 and apo hSOD1 proteins are folded, with the loops disordered, while the zinc-bound forms have well-ordered loop elements. Therefore, the basic reaction monitored here is the binding of zinc ions to the apo dimeric conformation, resulting in the distinct zinc-bound dimeric structure.

Figure 2a shows representative raw ITC and integrated heat data for $ZnSO_4$ binding to apo bSOD1 and apo hSOD1. Each titration resulted in one observable binding event. The apparent thermodynamic parameters determined for Zn^{2+} and Co^{2+} binding to apo bSOD1 and apo hSOD1 are shown in Table 1. The binding constants for metal binding to both apo bSOD1 and apo hSOD1 were too high to measure directly by ITC, even

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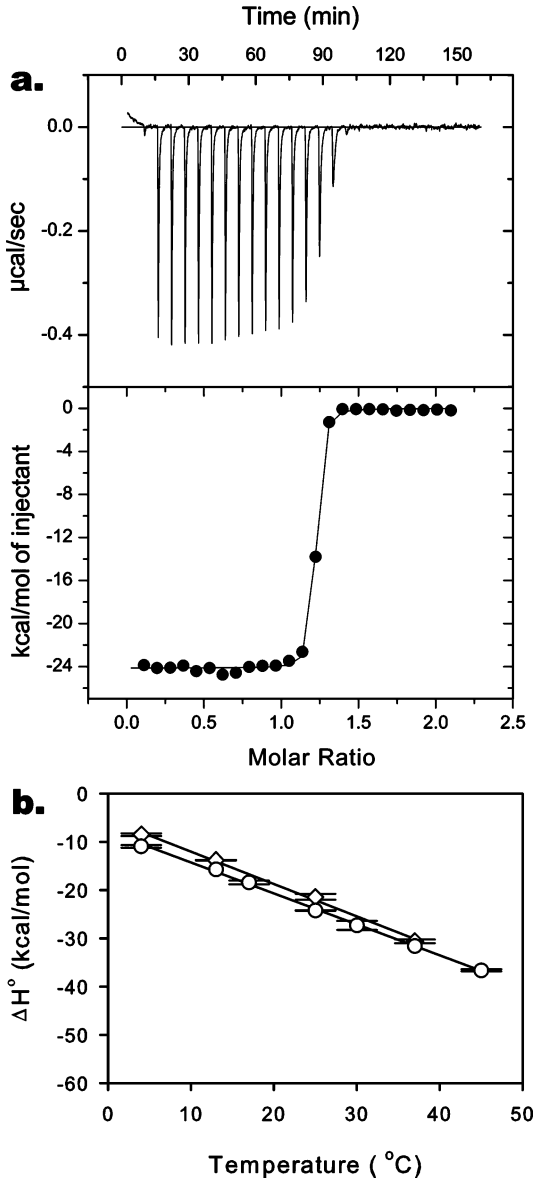

Figure 2. Zinc binding to apo SOD1 proteins is energetically favorable and thermodynamically asymmetric, as determined by ITC. (a) Representative ITC titration of $ZnSO_4$ into apo bSOD1. The top box contains the raw ITC data, and the bottom contains the integrated heats. (b) The temperature dependence on observed $\Delta H°$ due to zinc binding to apo hSOD1 (◇) and apo bSOD1 (○). Zinc binding experiments were carried out in 100 mM MES buffer, pH 5.5, at a protein dimer concentration of 5 μM. Error bars represent the standard deviations of at least two experiments. bSOD1 at 13 and 37 °C was only determined once. The change in heat capacity for zinc binding to hSOD1 was calculated to be -670 (± 30) cal mol^{-1} K^{-1} and for bSOD1, -640 (± 10) cal mol^{-1} K^{-1}.

at the low (5 μM) concentrations of protein used here; however, $\Delta H°$ and the stoichiometry of binding, n, could be obtained from the titration experiments. Despite the fact that the apoprotein dimer contains two equivalent zinc binding sites, under these conditions, both hSOD1 and bSOD1 exhibited stoichiometries of approximately one Zn^{2+} per dimer ($n = 1.2$ and 1.3, respectively). The binding was enthalpically favorable, with $\Delta H°_{bSOD1} = -24.2$ kcal mol^{-1} and $\Delta H°_{hSOD1} = -21.4$ kcal mol^{-1}.

To confirm the observed stoichiometry of approximately one metal ion per homodimer under these conditions, the ITC experiments were repeated using $CoSO_4$ in place of $ZnSO_4$. Previous studies of Co^{2+} binding to apo bSOD1, followed by visible spectroscopy and at the protein concentrations required for the spectrophotometric visualization, have established that two Co^{2+} ions bind selectively to the two zinc sites of the homodimer at pH 5.5.[21,28] The protein concentrations used in those studies were typically 2 orders of magnitude higher than those used in the ITC titrations presented here. We therefore repeated the spectrophotometric titrations using the same protein and buffer system used in the ITC experiments, but at a higher concentration of protein ($\sim$300 μM), and we confirmed that the stoichiometry was two Co^{2+} per apoprotein dimer (data not shown). Nevertheless, when we substituted Co^{2+} for Zn^{2+} in our ITC experiments, we again observed stoichiometries of approximately one Co^{2+} per homodimer. Although still strong, the energetics for cobalt binding were less favorable than those observed for zinc binding to apo bSOD1, consistent with differences in ligand-field-splitting stabilization energies between the two metals.[36,37]

Equilibrium dialysis experiments on bSOD1 have previously revealed a tight binding constant of $\sim 10^{11}$ M^{-1},[38] and a competition assay resulted in binding constants of $\sim 10^{14}$ M^{-1} for hSOD1.[24] The latter study necessitated the use of urea to loosen (but not unfold) the SOD1 structure and highlights the fact that these binding constants are condition specific (temperature, buffer, pH, etc.). Nevertheless, it is apparent that our lower limit for zinc binding to apo SOD1 determined by ITC ($K_a \geq 10^8$ M^{-1}) agrees well with previous measurements and that the binding affinities of apo bSOD1 and hSOD1 proteins for zinc ions are extremely favorable.

The apparent thermodynamic parameters obtained in our studies include contributions from deprotonation of any protonated zinc binding ligands, protonation of the MES buffer, and the breaking of the six $Zn-OH_2$ bonds of the dissolved Zn^{2+} ions.[37] Of more interest is the fact that the observed thermodynamics also include contributions from protein restructuring that occurs upon metal binding. To determine the degree of protein restructuring upon zinc binding, we determined the change in heat capacity ($\Delta C_p°$) due to zinc binding to SOD1 by measuring $\Delta H°$ over a range of temperatures (Figure 2). The change in heat capacity for hSOD1 was calculated to be -670 (± 30) cal mol^{-1} K^{-1} and for bSOD1, -640 (± 10) cal mol^{-1} K^{-1}. The stoichiometries and related errors were approximately the same as those observed at 25 °C (data not shown).

Large, negative $\Delta C_p°$ values arise from the restructuring of water at the surface of the protein as apolar residues become internalized.[39−42] Zinc binding to a 26-amino-acid zinc-finger domain, where the apo form is unfolded, produced an observed $\Delta C_p°$ of ~ -500 cal mol^{-1} K^{-1},[37] while zinc binding to the 261-amino-acid apo carbonic anhydrase, where the apo and zinc-loaded structures are almost identical,[43] produced a change in heat capacity of ~ -100 cal mol^{-1} K^{-1}.[44] Therefore, the

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Table 1. Observed Thermodynamics of Zinc Binding to 5 μM Apo SOD1 Proteins in 100 mM MES, pH 5.5, at 25 °C

SOD1	n[a]	$K_{a,obs}$ (M^{-1})	$\Delta H°_{obs}$ (kcal mol^{-1})	$\Delta G°_{calc}$ (kcal mol^{-1})	$\Delta S°_{calc}$ (cal mol^{-1} K^{-1})
bSOD1	1.3 (±0.1)[b]	(>10^8)[c]	−24.2 (±0.1)[b]	(<−10.9)[c]	(>−45)[c]
hSOD1	1.2 (±0.2)[b]	(>10^8)[c]	−21.4 (±0.6)[b]	(<−10.9)[c]	(>−35)[c]
bSOD1[d]	1.1	(1.8 ± 0.1) × 10^7[e]	−19.4	−9.9	−31.9

[a] Stoichiometry, n, refers to the number of metal ions per protein dimer. [b] Errors represent standard deviations from at least three experiments. [c] Binding constant was too high to measure directly, and therefore $\Delta G°$ and $\Delta S°$ could not be determined. [d] $CoSO_4$ was used instead of $ZnSO_4$. [e] The error is from the fit of the data using the one-site model.

observed$\Delta C_p°$ for zinc binding to these SOD1 proteins is significant relative to these other systems and suggests that large changes in SOD1 protein structure occur upon zinc binding and, more specifically, upon one zinc binding to the homodimer. The change in polar and apolar surface area that occurs upon zinc binding to SOD1[40−42] cannot be calculated at this time because the electrostatic and zinc loops in apo SOD1 are disordered in the crystal structure and, in addition, because there is no structure available for a one-zinc derivative. Therefore, we cannot, at this point, attribute the change in heat capacity we observe to defined differences in structure.

The apparent negative change in heat capacity that we observed was for a binding event for which the stoichiometry was approximately one zinc ion per protein homodimer. At first blush, the observed stoichiometry of one strong binding event per homodimer is surprising, considering there are two identical zinc binding sites in apo SOD1. However, a significant structural change is accompanied by the binding of one zinc per dimer, as evidenced by the large change in heat capacity. Therefore, we conclude that binding of one zinc ion produces large conformational changes to the homodimeric protein structure, such that the thermodynamics of zinc binding to the zinc site in the remaining apo subunit is different. In other words, although the two empty zinc sites are identical in the apo dimer, once zinc binds to one subunit, the empty zinc site in the apo subunit loses its equivalency through negative cooperativity. Thus, we conclude that the binding of Zn^{2+} to the second site is thermodynamically different and too weak or too slow for direct measurement under these conditions. Our results are not without precedent: a recent ITC study of Mn^{2+} binding to homodimeric tartrate dehydrogenase found a similar thermodynamic asymmetry in the binding of metal ions to the protein.[45]

When we carried out the ITC experiments using higher concentrations of SOD1 protein, we found that the number of zinc ions participating in the strong binding event increased (data not shown). For example, experiments using 100 μM bSOD1 protein exhibited stoichiometries of ~1.7 Zn^{2+} per homodimer, more consistent with expected results. We hypothesize that "subunit exchange" may be involved in the zinc remetalation mechanism at higher concentrations. In such a mechanism, a single zinc ion would bind tightly to an apo homodimer, producing a one-zinc dimer. This species could then exchange subunits with a second one-zinc dimer, producing a two-zinc homodimer and an apo homodimer (Figure 3b).

We tested the feasibility of subunit exchange mechanisms in SOD1 by mixing fully metalated CuZn bSOD1 with apo bSOD1 and following the results using native polyacrylamide gel electrophoresis. The apo bSOD1 protein ran as one band on the native gel (Figure 3). Fully metalated bSOD1 also ran as a single band and migrated more slowly than the apo species due to differences in size and charge. The migration of the fully metalated and apo bSOD1, each as a single band, was ideal for this study: partially metalated SOD1 proteins have previously been shown to run as multiple bands on non-denaturing gels.[28,46] Fully metalated and apo bSOD1 samples were mixed together and allowed to incubate for different time periods at either room temperature or 37 °C. EDTA was present to prevent metalation of the apo subunits via the diffusion of metal ions. Protein bands that migrated between the apo and fully metalated SOD1 species appeared after 1 h of incubation at 37 °C and grew in intensity over time (Figure 3). The appearance of these bands at 37 °C is dependent on the presence of both apo and metalated proteins: Incubated separately at 37 °C overnight, the fully metalated proteins and the apoproteins ran similarly to the non-incubated species shown in lanes 7 and 8 (data not shown). Apo bovine SOD1 melts at temperatures >50 °C;[4] therefore, the apoprotein is still folded at 37 °C. The rate of the reaction appears to be temperature dependent, since the new bands were not observed after 21 h of incubation at 25 °C. At this point, it is difficult to define the precise nature of the two new bands, but we believe it highly likely that they represent the products of subunit exchange.

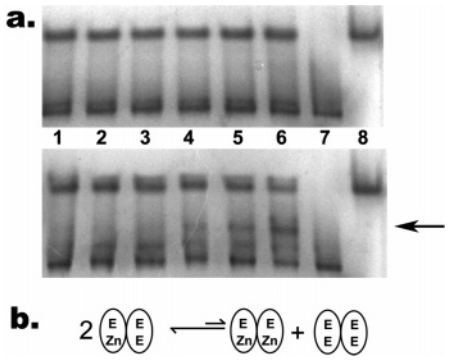

Figure 3. Evidence of subunit swapping using native gels. (a) Lanes 1−6: 1:1 mixtures of apo and fully metalated bSOD1 were incubated at room temperature (top) or at 37 °C (bottom) in 100 mM acetate buffer, pH 5.5, 0.5 mM EDTA for increasing lengths of time. Left to right, 30 min, 1 h, 2 h, 3.5 h, 5.5 h, and 21 h. Lane 7 is apo SOD1, and lane 8 is fully metalated SOD1. The arrow indicates the mixed dimer bands that appear over time in lanes 1−6. (b) Scheme representing the proposed subunit swapping equilibrium of the one-zinc derivative based on the native gel results (see text).

We are hypothesizing that, in metal titration experiments, subunit exchange occurs between two one-zinc dimers to produce one apo dimer and one two-zinc dimer (Figure 3b) and that, at higher concentrations of protein, the reaction can go forward to produce more strong zinc binding sites as the apo homodimer is regenerated. As more zinc is added, the equilib-

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rium shown in Figure 3b is pushed to the right. At low concentrations of protein, the equilibrium lies far to the left, and only one strong binding event is observed by ITC.

Previous studies have demonstrated similar formation of SOD1 heterodimers when different types of SOD1 were present.[47−50] In addition, an analogous reaction has been observed to occur in the formation of a heterodimer when CCS and SOD1 were cocrystallized. The resulting heterodimer was comprised of one SOD1 monomer and one CCS monomer, with a dimer interface similar to the interfaces in the individual homodimers of CCS and SOD1.[51]

The results presented here clearly demonstrate negative cooperativity in zinc binding to apo bSOD1 and hSOD1. Unfortunately, the failure to appreciate this phenomenon has had some adverse consequences. Recently, a kinetic study by Michel et al. on remetaled species of bSOD1 determined rate constants different from those previously reported for the dismutation of superoxide by SOD1.[52] The newly reported rate constant, 1.2×10^9 M^{-1} s^{-1}, was approximately half of the previously accepted value; their result was subsequently disputed, but no explanation was given for the discrepancy.[53] In their study, Michel et al. reported that they determined the protein concentrations using ITC titrations. According to our results, if they assumed a zinc binding stoichiometry of two per dimer in their ITC experiments, it would lead to an underestimation of their protein concentrations and their calculated rate constants would therefore be artificially low. The ITC conditions used in that study were different from ours, leading to differences in the observed thermodynamics; however, the protein concentrations were of the same order of magnitude for which we observe strong asymmetry. Michel et al. also noted that, when they added what they assumed to be excess copper, EDTA did not affect the rate of dismutation, and they proposed that copper−phosphate complexes were also catalyzing disproportionation of the superoxide in these solutions. However, we suspect that the "excess" copper was binding to copper sites in the protein structure that they had misunderstood to be already full. Given this behavior, we conclude that ITC is not a straightforward means of determining SOD1 protein concentrations and that further studies should eschew the methodology.

3.2. Biophysical Properties of the One-Zinc Derivative of SOD1. To probe further the effects of the binding of one zinc to homodimeric apo SOD1 protein, zinc derivatives were made and characterized biophysically. We used DEPC, a modification reagent specific for histidyl residues, to assess the accessibility of the various histidine residues in SOD1. Human and bovine SOD1 proteins contain a total of 16 histidine residues (eight in each subunit), of which six are in the metal binding region of each monomer. Earlier studies using DEPC modification of histidine residues in bovine SOD1 and its derivatives established that (1) all 16 histidine in the apoprotein could be modified by DEPC, (2) two histidines in the fully metalated protein were modified by DEPC, and (3) only two histidines were modified by DEPC in protein that was reconstituted with 2 equiv of zinc per dimer, despite the fact that the copper site, with its three uncoordinated histidine ligands, was empty.[54]

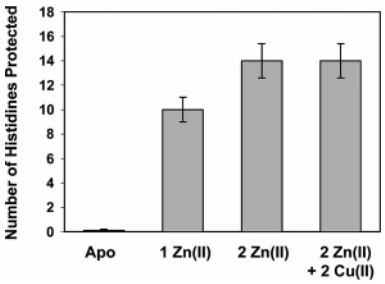

Figure 4. DEPC modification of hSOD1 demonstrates that the binding of one zinc per homodimer affects the structure of the apo subunit. Excess DEPC was added to apo or metal derivatives of hSOD1 in 100 mM sodium phosphate buffer, pH 6.0, and the reaction was monitored by changes in the absorbance at 240 nm. hSOD1 has 14 histidines per dimer that can be protected from DEPC modification (see text). The addition of one Zn^{2+} to the protein protects 10 of these 14 histidine residues in the dimer, meaning that at least three histidines were protected in the apo subunit. The addition of a second equivalent of zinc protects the other four histidine residues, while the further addition of copper has no effect.

In this study, we used human SOD1 zinc derivatives. Similar to the previous results obtained using bSOD1,[54] all 16 histidines of the apoprotein were accessible to modification by DEPC. The reaction of DEPC with apo hSOD1 was also monitored over time by electrospray mass spectrometry. The modification occurred in a stepwise fashion where the protein was modified by several DEPC molecules every 2−3 min and complete addition was observed after 8 min (data not shown). These results suggest that the modification had a denaturing effect on the SOD1 protein; i.e., more histidine residues became accessible as the protein was modified by DEPC. While eventually all 16 histidines in apo hSOD1 were modified, in the two-zinc derivative only two histidines had reacted after equilibrium was reached, indicating that the homodimer contained 14 "protectable" histidines, seven in each subunit. These results further suggest that the two-zinc species is stable toward any denaturing effect due to histidine modification. In the one-zinc derivative, 10 histidines were protected, indicating that some of the histidines in the apo subunit must be protected against histidine modification (Figure 4). This result strongly suggests that subunit−subunit communication across the dimer interface occurs upon Zn^{2+} binding. Similar DEPC protection studies using Co^{2+}, which binds in the zinc site, gave the same results (data not shown). However, in a one-copper derivative of hSOD1, in which Cu^{2+} was bound to the copper site and the zinc site was empty, only seven histidines were protected (data not shown). Therefore, the subunit−subunit communication that allows for the strong asymmetry upon metal binding appears to be linked to metal occupancy in the zinc site.

CD spectroscopy was employed to measure the differences in secondary structure between different metalated derivatives of hSOD1. The CD spectra of the apoprotein and its remetalated derivatives are shown in Figure 5. A significant change in the spectrum occurred when the first zinc equivalent was added to the apoprotein; however, only minor changes were observed upon the addition of the second zinc and the further addition of

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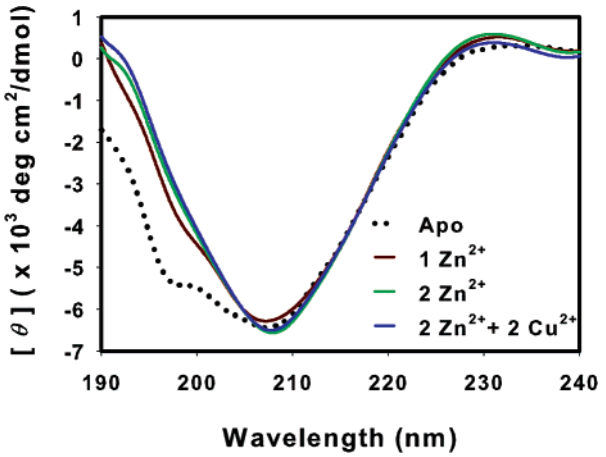

Figure 5. One-zinc hSOD1 has nearly all the same secondary structure of the fully metalated protein, as determined by room-temperature CD spectroscopy. The CD spectrum of the one-zinc derivative is very similar to those of the two-zinc and fully metalated derivatives suggesting that the binding of one zinc significantly affects the structure of the apo subunit. The spectra are reported per molar SOD1 monomer.

the copper ions. Thus, binding of one zinc ion per protein dimer had a more profound effect than the addition of any further equivalent of metal ions in obtaining the structure of the fully metalated protein.

We also used amide H/D exchange, measured by electrospray ionization mass spectrometry (ESI-MS) to study how zinc binding affects the global structure and dynamics of hSOD1 and bSOD1 proteins at 37 °C. Backbone amide hydrogen exchange rates are sensitive probes of secondary, tertiary, and quaternary structure, and it is thought that the exchange rate is dependent upon hydrogen bonding and solvent accessibility. Bovine and human apo SOD1 both exhibit approximately 25 unexchanged protons per monomer after a 60-min incubation in deuterated buffer (Figure 6). These protons most likely participate in strong hydrogen bonds or are buried and inaccessible to solvent.[30] The binding of the first Zn^{2+} ion increased the number of unexchanged protons to approximately 33 in both bovine and human SOD1, suggesting substantial structural ordering. In the case of the human protein, the binding of the second Zn^{2+} had a smaller effect upon the structure: after 60 min in D_2O, the number of unexchanged protons in the two-zinc derivatives remained approximately 33. Thus, the binding of the first zinc ion must be protecting hydrogens across the dimer interface from exchange. This effect also appears in the bovine protein, albeit to a lesser extent.

Molecular dynamic simulations have shown that a high degree of interconnectivity exists between two subunits in apo SOD1 homodimers.[55,56] However, at this point, we can only speculate on the nature of the communication across the dimer interface that leads to asymmetry in the zinc binding thermodynamics. It is understood that the electrostatic and zinc loops both go from a disordered to an ordered state upon zinc binding in both crystal and NMR structures.[10−12] Amino acids near the zinc loop participate in subunit−subunit contacts through interactions with a β-strand in the opposite monomer.[6] It is therefore conceivable that the zinc and the electrostatic loops become ordered upon

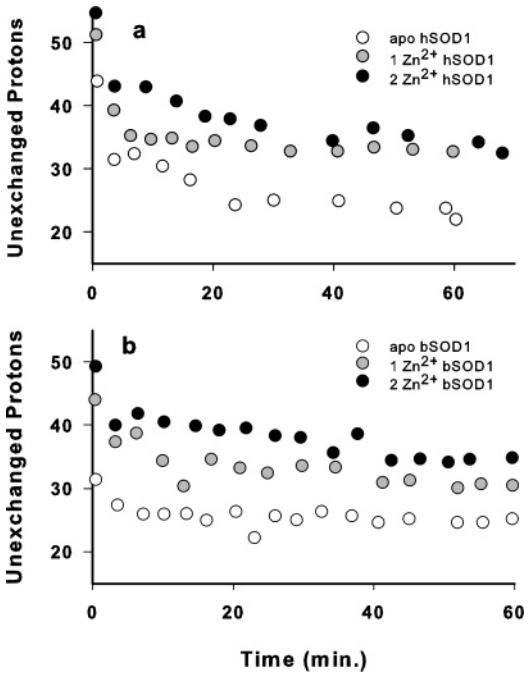

Figure 6. Effect of Zn^{2+} coordination on the global hydrogen−deuterium (H/D) exchange kinetics of bovine and human WT SOD1 measured with ESI-MS. In both human (a) and bovine (b) apo SOD1, there were approximately 25 hydrogens per monomer that remained unexchanged with solvent after a 60-min incubation in deuterated buffer. The coordination of the first Zn^{2+} increases the number of hydrogens that are protected from exchange from approximately 25 to 33 in both bovine and human SOD1. In the human protein, the binding of the second Zn^{2+} has a smaller effect relative to the first, and the two-zinc species exhibits a similar number of unexchanged protons relative to the one-zinc species. This effect is also seen, albeit to a lesser extent, in the bovine protein.

binding of one Zn^{2+}, an effect which is then translated across the dimer interface through contacts made by the zinc loop at the interface.

3.3. Stability of the Apo Subunit in One-Zinc SOD1. To quantify the stabilizing effect of binding one zinc ion to the apo subunit in the dimeric hSOD1 structure, apo and metalated derivatives of the enzyme were studied in the analytical ultracentrifuge using sedimentation velocity and equilibrium methods as described previously.[6] As discussed above, apo disulfide-oxidized hSOD1 is a stable dimer, displaying a dissociation constant of $<10^{-8}$ M,[6] and we expect the metals to enhance the dimer stability further. Thus, we used a denaturant (guanidine hydrochloride, GuHCl) to weaken interface interactions in order to compare the dimer stability of apo and metalated SOD1 proteins.

Figure 7a shows the $G(s)$ plots (van Holde and Weischet plots,[57] corrected to eliminate the contribution of diffusion on the experiment) for apo hSOD1 and the one-zinc and two-zinc derivatives in the absence of denaturant. Similar plots, generated from experiments where the same derivatives were exposed to increasing amounts of GuHCl (data not shown), were used to generate a plot of average S value versus GuHCl concentration[6] (Figure 7b). The two-zinc dimer remained intact until approximately 2 M GuHCl was added. The one-zinc derivative remained dimeric ($S_{20,w} \approx 2.6$) in 1 M GuHCl, whereas the apo SOD1[6] was already monomeric ($S_{20,w} \approx 1.4$) at this concentration. It is apparent from these results that the binding

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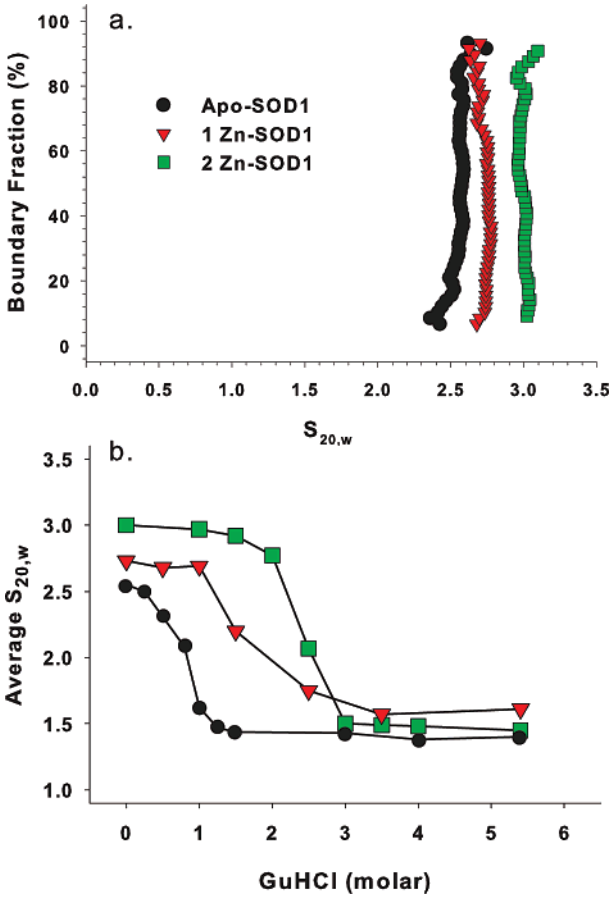

Figure 7. Analytical ultracentrifugation of zinc derivations of hSOD1 shows increased dimer stability upon zinc binding. (a) Sedimentation velocity experiments in acetate buffer, pH 5.5, 1 mM EDTA without denaturant. (b) Average $S_{20,w}$ values of SOD1 derivatives with increasing amounts of GuHCl. The one-zinc derivative stays dimeric ($S_{20,w} \approx 2.6$) at denaturant concentrations where the apo derivative[6] is monomeric ($S_{20,w} \approx 1.4$).

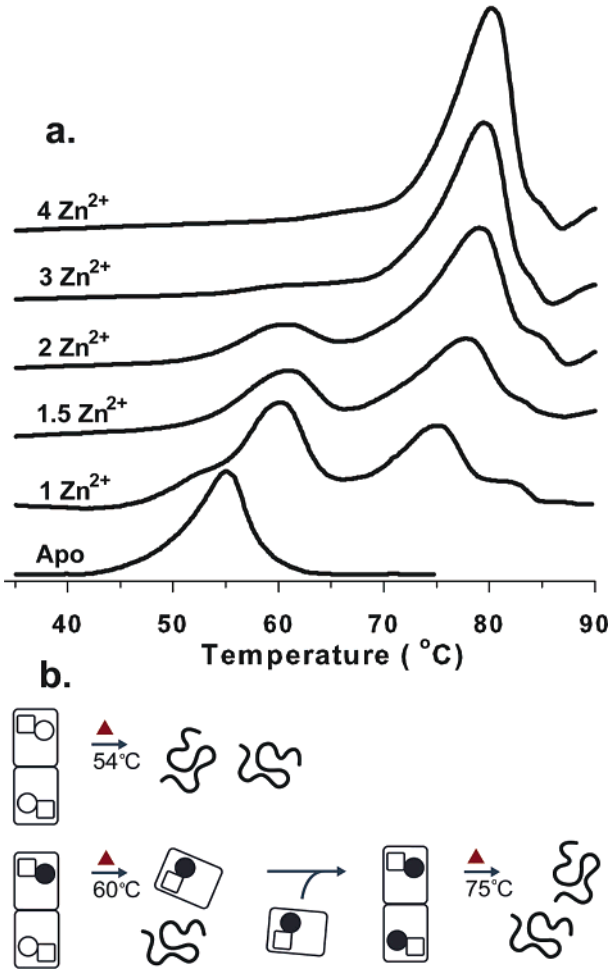

Figure 8. Thermostabilities of apo and metal SOD1 derivatives. (a) DSC scans of apo and zinc derivatives of hSOD1 in phosphate buffer, pH 7.0. Metal derivatives were made by the stoichiometric addition of $ZnSO_4$ to apo hSOD1 and allowed to equilibrate overnight. (b) The proposed scheme for the mechanism of melting of the apo and one-zinc SOD1 derivatives. Empty copper and zinc sites are represented by open squares and circles, respectively. The filled zinc site is represented as a filled circle.

of a single zinc ion to the protein stabilizes the dimer, since a higher concentration of denaturant is needed to monomerize the one-zinc protein compared to the metal-free protein.

We observed a similar result in DSC experiments on the zinc derivatives of the human SOD1 protein. Figure 8 shows a series of DSC traces arising from a zinc titration of apo SOD1. The unfolding of apo disulfide-oxidized human SOD1 produced one endotherm centered at 54.4 °C. Addition of 1.0 equiv of Zn^{2+} per dimer nearly eliminated the apoprotein peak and produced two new peaks with T_m values of 61.4 and 75.9 °C. An additional 0.5 equiv of zinc completely abolished the 54 °C transition and decreased the magnitude of the transition at 61.4 °C, while the higher melting endotherm increased in magnitude and was shifted to 76.8 °C. It should be emphasized that this DSC profile is nearly identical to that obtained using the as-isolated recombinant enzyme, which also contained 1.5 equiv of zinc.[3] Addition of 0.5 equiv more, to bring the total to 2.0 equiv of Zn^{2+} per apoprotein dimer, further decreased the magnitude of the endotherm at 61.4 °C, resulting in a DSC trace with one major transition at 78.1 °C. Addition of a third and fourth equivalent of zinc resulted in single endotherms at 78.6 and 79.3 °C, respectively.

Figure 8b depicts our proposed scheme for the mechanism of the melting of the apo and one-zinc derivatives. The endotherm at 54 °C is due to melting of apo homodimeric hSOD1. We propose that the melting of the one-zinc derivative proceeds in two steps. First, the apo subunit of the one-zinc dimer melts (at 60 °C). Two remaining one-zinc subunits then combine to form the two-zinc dimer, which melts when the temperature reaches 75 °C. Importantly, the one-zinc derivative does not exhibit a strong endotherm at 54 °C (corresponding to the melting of the metal-free apoprotein), indicating that the metalated subunit stabilizes the metal-free subunit.

3.4. Implications for SOD1-Linked ALS. We have shown in this study that SOD1 dimers can exchange subunits with other SOD1 molecules. In SOD1-linked ALS, an autosomal dominant disease, wild-type hSOD1 may also make mixed dimers with mutant SOD1 proteins. Recently, two lines of transgenic mice expressing the ALS disease-causing SOD1 mutant A4V were developed that were asymptomatic. Interestingly, when these mice were crossed with mice overexpressing human wild-type SOD1, which also do not develop the disease, the progeny mice became sick,[58] suggesting that the combination of the two proteins is necessary to cause disease. The authors hypothesized that human wild-type SOD1 is converted from a soluble form to an insoluble toxic aggregate through an interaction with the mutant protein.[58] We hypothesize that this interaction is a

subunit exchange event, a process we have shown here to occur with the wild-type bovine protein.

Here, we have shown that the binding of the two zinc ions to the SOD1 dimer is thermodynamically asymmetric. 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 has important implications *in vivo*. Due to the significant subunit-to-subunit communication that allows for a "one-zinc effect", conditions that affect zinc binding may have far-reaching effects in the protein structure. Certainly, the converse would hold as well; i.e., structural perturbations far removed from the zinc site may significantly alter zinc binding affinities to SOD1. Previous work has shown that some of the ALS mutant SOD1 proteins have decreased affinities for zinc ions relative to wild-type.[24,25] Further, amino acid substitutions, such as those that cause familial ALS (FALS), have already been shown in some cases to disrupt intersubunit coupling in molecular dynamic simulations.[59] A recent study using a fluorescence cell imaging technique demonstrated conformational changes of FALS mutants in live cells, suggesting that the structural perturbations to SOD1 *in vivo* are relevant.[60]

If intersubunit communication is disrupted, e.g., by means of an amino acid substitution, the binding of a single zinc ion may not have the same effect on the holoprotein fold. Indeed, certain FALS mutant hSOD1 proteins did not exhibit increased protection against DEPC modification in the second subunit upon zinc binding.[20] This apparent decrease in structural cooperativity between FALS-variant subunits during metalation, relative to the wild-type protein, suggests that metal-free or under-metalated FALS variant subunits will continue to be substantially more disordered and destabilized, in spite of being bound to a metalated SOD1 subunit.

The identification of significant intersubunit communication upon metal binding in this state has important implications for understanding how mutations to SOD1 cause ALS, as outlined above. It is important to note that our studies to date have addressed the behavior of zinc binding to the disulfide-oxidized apoprotein. Work is under way in our laboratory to extend these studies to zinc binding to the disulfide-reduced apoprotein, a form that may also be relevant to FALS causation. In addition, we plan to study the thermodynamics of zinc binding to FALS mutant hSOD1 proteins under the same conditions used here in order to compare directly the energetics of zinc binding to wild-type hSOD1 and disease-causing mutant proteins.

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Supporting Information Available: Complete refs 13 and 14. This material is available free of charge via the Internet at http://pubs.acs.org

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