Biochemistry **2000,** *39,* 8125−8132

Copper(2+) Binding to the Surface Residue Cysteine 111 of His46Arg Human Copper−Zinc Superoxide Dismutase, a Familial Amyotrophic Lateral Sclerosis Mutant[†]

Hongbin Liu,[‡] Haining Zhu,[‡] Daryl K. Eggers,[‡] Aram M. Nersissian,[‡] Kym F. Faull,[§] Joy J. Goto,[‡,||] Jingyuan Ai,[⊥]
Joann Sanders-Loehr,[⊥] Edith Butler Gralla,[‡] and Joan Selverstone Valentine*[,‡]

Department of Chemistry and Biochemistry, Pasarow Mass Spectrometry Laboratory, Department of Chemistry & Biochemistry and Psychiatry & Biobehavioral Science, and The Neuropsychiatric Institute, University of California, Los Angeles, Los Angeles, California 90095-1569, and Department of Biochemistry and Molecular Biology, Oregon Graduate Institute of Science and Technology, Beaverton, Oregon 97006-8921

Received April 14, 2000

ABSTRACT: Mutations in copper−zinc superoxide dismutase (CuZnSOD) cause 25% of familial amyotrophic lateral sclerosis (FALS) cases. This paper examines one such mutant, H46R, which has no superoxide dismutase activity yet presumably retains the gain-of-function activity that leads to disease. We demonstrate that Cu^{2+} does not bind to the copper-specific catalytic site of H46R CuZnSOD and that Cu^{2+} competes with other metals for the zinc binding site. Most importantly, Cu^{2+} was found to bind strongly to a surface residue near the dimer interface of H46R CuZnSOD. Cysteine was identified as the new binding site on the basis of multiple criteria including UV−vis spectroscopy, RR spectroscopy, and chemical derivatization. Cysteine 111 was pinpointed as the position of the reactive ligand by tryptic digestion of the modified protein and by mutational analysis. This solvent-exposed residue may play a role in the toxicity of this and other FALS CuZnSOD mutations. Furthermore, we propose that the two cysteine 111 residues, found on opposing subunits of the same dimeric enzyme, may provide a docking location for initial metal insertion during biosynthesis of wild-type CuZnSOD *in vivo*.

A connection between familial amyotrophic lateral sclerosis (FALS)[1] and copper−zinc superoxide dismutase (CuZnSOD), SOD1, was established in 1993 with the reports of a variety of different mutations in the *sod1* gene in FALS patients (*1*, *2*). Nearly 25% of FALS patients (2.5% of total ALS patients) carry a mutant form of this gene, typically a missense autosomal dominant point mutation. ALS is a progressive neurodegenerative disease resulting in slow loss of motor neurons in the motor cortex, brain stem, and spinal cord (*3*). The etiology of ALS is incompletely understood, and there is no known cure. To date, more than 70 disease-causing mutations have been identified in CuZnSOD, including two early termination mutations.

CuZnSOD (*4*−*6*) is a 32 kDa homodimer containing one copper ion and one zinc ion in each subunit. The copper ion, which alternates between Cu^{2+} and Cu^{+}, is the catalytic metal, while the zinc ion, Zn^{2+}, is believed to play a structural role. This enzyme is thermally stable, resistant to denaturants, and specific for the superoxide substrate (*7*).

It has been further established that the mutations cause a gain rather than a loss of function, but the nature of the gain of function is as yet uncertain (*8*). Three hypotheses have been advanced: (1) enhancement of a deleterious catalytic peroxidative activity (*9*−*11*), possibly associated with undermetalation or mismetalation of the mutant enzymes (*4*, *12*−*16*); (2) enhancement of a deleterious catalytic activity resulting in tyrosine nitration (*17*), again possibly associated with alterations in metal binding properties of the mutant enzymes (*18*−*20*); and (3) protein aggregation (*21*−*25*). The first and second of these hypotheses are based on the presumed cellular toxicity of redox-active copper centers: copper is essential for cell survival; however, copper-mediated redox chemistry (e.g., the Fenton reaction that produces hydroxide radical or copper-catalyzed oxidation of thiols by dioxygen to give disulfides) could be toxic.

[†] This work was supported by grants from the National Institute of General Medical Sciences, GM28222 (J.S.V.) and GM8865 (J.S.L.), and the ALS Association. Financial support from the W. M. Keck Foundation for instrument acquisition (Pasarow Mass Spectrometry Laboratory, to P.I. and K.F.F.) is acknowledged.

* To whom correspondence should be addressed: fax (310) 206-7197; e-mail jsv@chem.ucla.edu.

[‡] Department of Chemistry and Biochemistry, UCLA.

[§] Pasarow Mass Spectrometry Laboratory, Department of Chemistry & Biochemistry and Psychiatry & Biobehavioral Science and The Neuropsychiatric Institute, UCLA.

[||] Current address: Department of Neurology, Massachusetts General Hospital, Building 149, 13th St., Charlestown, MA 02129-2060.

[⊥] Department of Biochemistry and Molecular Biology, Oregon Graduate Institute of Science and Technology.

[1] Abbreviations: ALS, amyotrophic lateral sclerosis; FALS, familial amyotrophic lateral sclerosis; CuZnSOD, copper−zinc superoxide dismutase; hCuZnSOD, human copper−zinc superoxide dismutase; H46R, His46Arg mutant human copper−zinc superoxide dismutase; DMPO, 5,5′-dimethyl-1-pyrroline *N*-oxide; EPR, electron paramagnetic resonance; UV−vis, ultraviolet−visible; RR, resonance Raman; 4VP, 4-vinylpyridine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); LCMS, liquid chromatography−mass spectrometry; ICP-MS, inductively coupling plasma mass spectrometry; DTT, dithiothreitol; CCS, copper chaperone for copper−zinc superoxide dismutase; hCCS, human CCS. Nomenclature of CuZnSOD derivatives: $M_2M'_2SOD$, M- and M′-substituted SOD with M in the copper site and M′ in the zinc site (an E represents an empty site).

10.1021/bi000846f CCC: $19.00

Most FALS CuZnSOD mutants do not bind copper or zinc ions as strongly as wild-type protein (*18*) (H. Liu, J. Goto, and J. S. Valentine, unpublished results). In addition, the ability to bind copper and zinc ions to their correct metal-specific sites has been compromised in many of the mutants (*16*). Changes in the catalytic properties of copper in the mutant CuZnSOD enzymes could also result from zinc deficiency or mismetalation (*16*, *19*). Alternatively, given the fact that CuZnSOD is abundant in cells, the effect of altered copper or zinc binding could significantly disrupt metal ion homeostasis and expose other cellular components to abnormally high levels of free or available copper or zinc ions.

Data from CuZnSOD-associated FALS patients and from transgenic mice expressing FALS mutant CuZnSODs have provided the following evidence that the disease is associated with elevated levels of oxidative stress: (1) the appearance of excess oxidative damage in tissues of ALS patients (*26*), (2) the ability of vitamin E, an antioxidant, to slow disease development in transgenic mice expressing G93A mutant CuZnSOD (*27*), (3) the observation of enhanced spin trapping of reactive radicals in G93A transgenic mice (*28*), and (4) direct observation of enhanced oxidative damage to proteins, including CuZnSOD, in G93A mice (*29*). The observation that the copper chelator penicillamine delays onset and extends survival in the mouse model (*30*) implicates copper ions as the catalyst of the deleterious oxidation reactions that appear to be involved in CuZnSOD-associated FALS.

How can CuZnSOD enzymes be both antioxidants and prooxidants at the same time? This seeming paradox is actually chemically straightforward: antioxidant activities, e.g., SOD and catalase reactions, and prooxidant activities, e.g., peroxidase and peroxynitrite formation reactions, utilize similar redox mechanisms. Wild-type CuZnSOD appears to achieve its high specificity for superoxide not by modulating its reactivity but rather by limiting access of potential substrates other than superoxide. Thus, structural perturbations in the FALS CuZnSOD mutants may permit access of alternative substrates to the copper center, leading to less selectivity for superoxide and more diverse catalytic redox reactions.

The FALS mutations H46R (*31*) and H48Q (*32*) are of interest since they occur in the copper site and reduce SOD activity relative to other FALS mutants and the wild-type enzyme (*33*), thereby providing a model for further studies on the involvement of copper in the gain-of-function mechanism. Previous studies showed that both mutants are structurally stable (*33, 34*) and that the EPR spectra of H46R display an axial shape geometry for the Cu^{2+} site, which is different from that of the wild-type protein (*35*). A recent paper also reported superoxide-dependent peroxidase activity in H48Q (*36*). Analysis of blood from H46R FALS patients showed a ratio of mutant to normal CuZnSOD of about 1:7 (*37*).

In the study reported here, an extensive investigation of the metal binding characteristics of the human FALS mutant H46R CuZnSOD revealed a previously unidentified metal binding site with cysteine 111 acting as a ligand. Cysteine 111 is located at the subunit interface. Previous studies of human CuZnSOD have used a thermostable and fully active mutant in which cysteine 111 and cysteine 6 were replaced with serine (*38*). However, our results suggest that cysteine 111 could influence in vivo metalation and other properties of this metalloenzyme and thus could play a role in causing FALS.

EXPERIMENTAL PROCEDURES

Mutagenesis and Protein Purification. Mutant genes were constructed by PCR (*39*) on YEP351-hSOD plasmid carrying human *sod1* under the control of the yeast *SOD1* promoter. The sequences of the outside primers were 5′-CACTCCATGGCGACGAAGGCCGTGTGCGT (*Nco*I) and 5′-CCGGTCGACAGGGAATGTTTATTGGGCGAT (*Sal*I); inside primers for H46R were 5′-CTGCATGGATTCCGTGTTCATGAG and 5′-ACTCATGAACACGGAATCCATGCAG; inside primers for C111S were 5′-CAGGAGACCATTCCATCATTGGCCGCA and 5′-GCGGCCAATGATGGAATGGTCTCCTGA. The H46R/C111S double mutant was made using the H46R gene as the template. PCR products were ligated into *Nco*I and *Sal*I sites of YEP351-hSOD, which placed the new gene under the control of the yeast *SOD1* promoter. The mutant proteins were expressed in the *Saccharomyces cerevisiae* SOD1-EG118 strain and purified according to published procedures (*9*).

Metal Titration Protocols. Apoproteins were prepared using earlier published procedures (*12*, *40*), and their concentrations were determined using an extinction coefficient of 10 800 $M^{-1}\cdot cm^{-1}$ at 280 nm for the dimer protein. Cu^{2+}, Co^{2+}, Zn^{2+}, and Ag^{+} were added as aqueous $CuSO_4$, $CoSO_4$, $ZnSO_4$, and $AgNO_3$ (Baker, Fisher, Sigma, and Mallinckrodt, respectively). Apoprotein solutions, usually 200 μL at a dimer concentration of 0.2−0.3 mM, were titrated with 20 mM solutions of the appropriate metal ion. All titrations were carried out in 100 mM acetate buffer at pH 5.5, if not otherwise indicated.

Sodium hydroxide titrations of remetalated protein solutions were monitored with an Ingold microelectrode (Wilmington, MA). Reduction conditions in Figure 1 were achieved by addition of crystalline sodium dithionite.

Elemental Analysis by ICP-MS. Metal contents of CuZnSOD preparations were determined using a PE/Sciex Elan 5000A ICP-MS. About 10 nmol of protein (usually between 40 and 100 μL) was first washed twice with double-distilled water using a Centricon filter (Amicon, MA) and then diluted in 5 mL of 0.1% metal-free HNO_3 (optima grade, Fisher). Operating conditions for ICP-MS were as follows: cross-flow pneumatic nebulizer in a room temperature spray chamber; argon gas flows (in L/min) of plasma 14, auxiliary 1, and nebulizer 0.9; RF power 1000 W; Ni cones.

Raman Spectroscopy. Apo H46R (0.4 mM in 100 mM acetate, pH 5.5) was titrated with 2 equiv of Zn^{2+} (per dimer) and then incubated at 4 °C overnight. A total of 8−10 equiv of Cu^{2+} was then added, and the protein solution was concentrated to 1.0 mM in a Microcon 10 concentrator. Samples were illuminated with 413 nm excitation and 90° scattering geometry at room temperature in a moving capillary. Spectra were collected using a custom McPherson 2061/207 spectrograph (0.67 m, 2400 groove grating) with a Princeton Instruments liquid N_2-cooled (LN-1100PB) CCD detector. Rayleigh scattering was attenuated with a Kaiser Optical holographic super-notch filter. The excitation source was provided by a Coherent Innova 302 Kr laser. Frequencies (accurate to 1 cm^{-1}) were calibrated relative to 2-(acetyloxy)-benzoic acid.

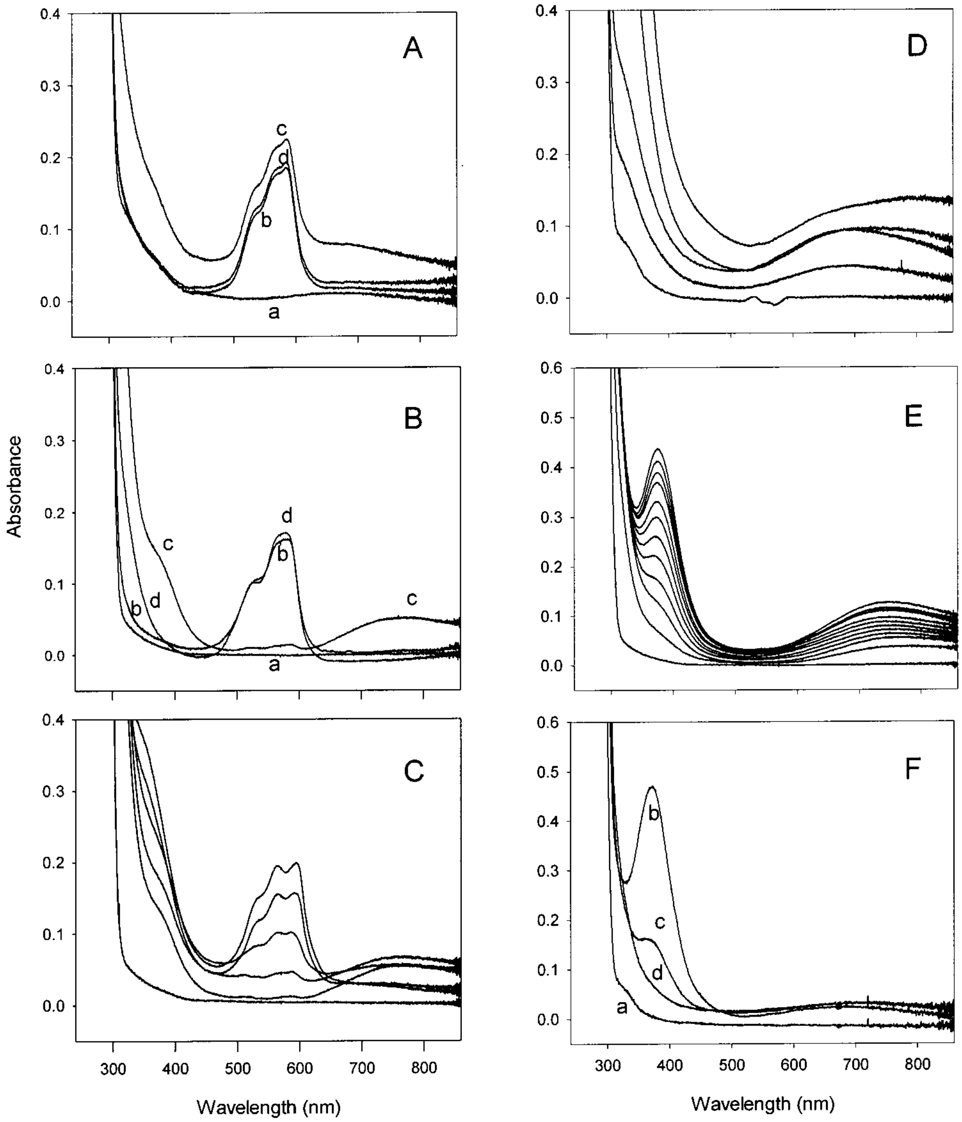

FIGURE 1: UV−visible spectra of metal titrations of CuZnSOD. Absorption spectra for Cu^{2+} and Co^{2+} derivatives of hWT SOD (A), hH46R SOD (B), and Cu_2Co_2-hH46R SOD at different pH values (C), Cu^{2+} titration of hWT SOD (D) and hH46R SOD (E), and Ag^+Cu^{2+} titration of hH46R SOD (F). Individual spectra of panels A and B: (a) apoprotein, pH 5.5; (b) 2 Co^{2+} per dimer, pH 5.5; (c) 2 Cu^{2+} plus 2 Co^{2+} per dimer, pH 5.5; (d) after addition of several small crystals of sodium dithionite to the sample shown in spectrum c. Panel C (from bottom to top at 500−600 nm): apoprotein and Cu_2Co_2 derivatives at pH 5.5, 6.0, 6.8, 7.4, and 7.8. Panel D (from the bottom spectrum upward): apoprotein and 1, 2, 3, and 4 Cu^{2+} per dimer. Panel E (from the bottom): apoprotein and 1−11 Cu^{2+} per dimer. Individual spectra of panel F: (a) 2 Zn^{2+} per dimer; (b) 2 Cu^{2+} and 2 Zn^{2+} per dimer; (c) 2 Ag^+, 2 Cu^{2+}, and 2 Zn^{2+} per dimer; (d) 2.5 Ag^+, 2 Cu^{2+}, and 2 Zn^{2+} per dimer.

Cysteine Modification and Mass Spectrometry. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) and 4-vinylpyridine (4VP) were used at 100- or 1000-fold molar excess over protein sulfhydryls in 50 mM Tris, pH 8.0, at room temperature for 3 h. Molecular weights were determined by electrospray mass spectrometry using a PE Sciex API III triple quadrupole mass spectrometer (Thornhill, Canada) fitted with an ion spray source. Purified protein samples or HPLC fractions were analyzed by direct injection (10−20 μL/injection) into a stream of water/acetonitrile/formic acid (50/50/0.1 v/v) entering the ion source (10 μL/min). Alternatively, modified proteins or trypsin digestion mixtures were injected onto a reverse-phase HPLC column that was directly interfaced with the mass spectrometer (LCMS). Normal spectra were obtained by scanning between m/z 600 and m/z 2400 with 0.3 Da step size and a scan speed of about 6 s at an orifice voltage of 90. Positive ion tandem spectra (MS/MS) of Q1 preselected parent ions were obtained after collisionally induced dissociation (collision gas, 10% nitrogen in argon) by scanning Q3 from m/z 50 to m/z 2000.

RESULTS

Cobalt and Copper Titrations. Because Zn^{2+} cannot be observed by UV−vis spectrosopy, due to completely filled d-orbitals, cobaltous ion was used as a zinc replacement to investigate metal binding to Zn sites in SOD (*41*). When Co^{2+} was added to wild-type apo human CuZnSOD at pH 5.5, a peak appeared between 500 and 600 nm, characteristic of Co^{2+} in a distorted tetrahedral zinc binding site (Figure 1A, curve b). The peak absorbance stopped rising immediately after the addition of 2 equiv of Co^{2+} per dimer (equivalents mentioned heretofore and hereafter are with reference to the dimeric proteins). This result indicates strong and saturable binding at the zinc site. When Cu^{2+} was subsequently added to this solution of E_2Co_2SOD, a broad peak around 680 nm appeared, indicating Cu^{2+} occupation of the native copper sites (Figure 1A, curve c).

When H46R was titrated in a similar fashion at pH 5.5, the addition of Co^{2+} caused formation of a peak similar to that found in wild type, again with an end point of 2 equiv of Co^{2+} (Figure 1B, curve b). However, subsequent addition of Cu^{2+} to the solution resulted in disappearance of the characteristic cobalt peak and appearance of a new peak around 750 nm, presumably resulting from the d−d transition of Cu^{2+} newly bound to the protein. The decrease in the Co^{2+} absorbance occurred immediately upon addition of each aliquot of Cu^{2+}, and the amount of the decrease in each case was correlated with the amount of Cu^{2+} added to the solution. Increasing the pH of this solution from pH 5.5 to pH 7.8 by addition of NaOH resulted in the disappearance of the 750 nm peak assigned to Cu^{2+} in concert with the reappearance of a band characteristic of tetrahedral Co^{2+} (Figure 1C). To test the hypothesis that Cu^{2+} was displacing Co^{2+} from E_2-Co_2 H46R to form an E_2Cu_2 derivative, the copper-containing solution at pH 5.5 was loaded onto a gel filtration column to separate unbound metal from the protein. No Co^{2+} peak was observed in the reduced eluted protein, indicating that Co^{2+} was removed by gel filtration and did not remain bound to H46R in the presence of Cu^{2+} (also see below).

Dithionite reduction of wild-type protein resulted in the disappearance of the 680 nm peak, due to the absence of d−d transition bands in d^{10} Cu^{+} ion, while the characteristic tetrahedral Co^{2+} band was maintained (Figure 1A, curve d). Interestingly, addition of dithionite to the similarly prepared H46R at pH 5.5 gave a similar final spectrum characteristic of Co^{2+} bound in the tetrahedral configuration characteristic of the zinc site (Figure 1B, curve d).

Cu^{2+} Titrations. The Cu^{2+} binding properties of wild-type hCuZnSOD apoprotein at pH 5.5 are virtually identical to those of the bovine apoprotein (*40*). Two equivalents of Cu^{2+} per dimer bind first to the copper site (Figure 1D), giving a characteristic d−d transition band at 670 nm. Additional Cu^{2+} then binds to the zinc site, giving an additional overlapping absorption band with a maximum at 810 nm. Both sites bind Cu^{2+} strongly; i.e., the titration curves have well-defined end points.

By contrast, when Cu^{2+} was added to the mutant hH46R CuZnSOD apoprotein, the spectra were dramatically different. Titration with up to 2 equiv per dimer of Cu^{2+} at pH 5.5 produced an absorption band at 750 nm (Figure 1E). Addition of >2 equiv of Cu^{2+} resulted in the appearance of a new peak at 370 nm. The absorbance of this peak increased as more Cu^{2+} was added, suggestive of a relatively weak binding site that required a large excess of Cu^{2+} to reach saturation.

Zn^{2+} and Ag^{+} Titrations. To investigate further the origin of the new 370 nm absorption peak that resulted from Cu^{2+} binding to H46R, titrations with Zn^{2+} and Ag^{+} were employed (Figure 1F). Two equivalents of Zn^{2+} was first added to the hH46R apoprotein at pH 5.5 (Figure 1F, curve a). Subsequent titration with Cu^{2+} resulted in maxima at 370 and 680 nm (Figure 1F, curve b). Further addition of 2 equiv of Ag^{+} resulted in a decrease in the 370 nm peak (Figure 1F, curve c). After 2.5 equiv of Ag^{+}, the peak had almost completely disappeared (Figure 1F, curve d).

The new 370 nm absorption band has a similar maximum and extinction coefficient to certain sulfur-to-Cu^{2+} charge-transfer transitions (*42*) and, thus, suggests the presence of a thiolate−Cu^{2+} moiety. Such an assignment was supported by the disappearance of this band upon addition of Ag^{+}, an ion which binds strongly to thiolates and is likely to displace Cu^{2+} from such a site. The above results thus imply that a thiol, presumably from a cysteine residue, is capable of binding Cu^{2+} in a long-lived complex in H46R.

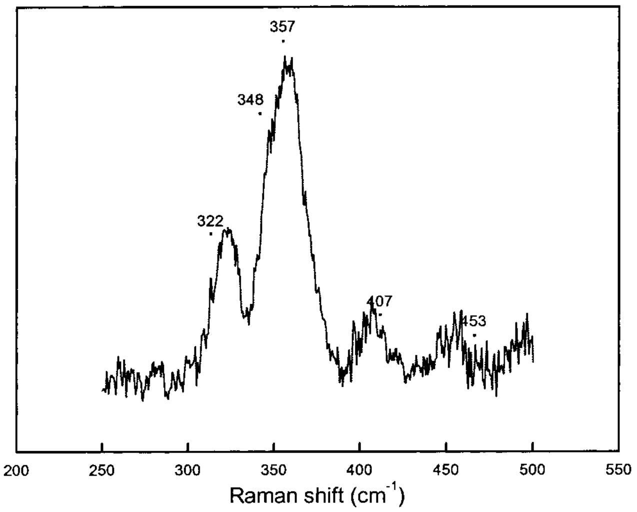

FIGURE 2: Resonance Raman spectrum of hH46R SOD at pH 5.5. The spectrum was obtained using 413 nm excitation (20 mW), a spectral resolution of 4 cm^{-1}, and 30 min of data collection. The Raman spectrum of the apoprotein was subtracted to remove protein and glass contributions.

Raman Spectroscopy. The resonance Raman spectrum of human H46R CuZnSOD showed fundamentals at 322, 348, 357, and 407 cm^{-1} that most likely originate from Cu−S (Cys) stretching (Figure 2). In addition, there are higher frequency vibrations at 751 cm^{-1} (the S−C stretch of cysteine) and at 675 and 698 cm^{-1}, which can be assigned as combination bands of $322 + 357$ (sum 679) and $348 + 357$ (sum 705), respectively. The same RR spectrum is observed after reconstitution either with Cu^{2+} only or with Zn^{2+} plus Cu^{2+} as described in the preceding section. Thus the appearance of this RR spectrum is correlated with the appearance of the 370 nm band.

The RR spectrum of H46R CuZnSOD is similar to that of other copper−cysteinate proteins with tetragonal type II copper sites, as in the His−ligand mutants of azurin and SOD (*43*). All are characterized by the appearance of two to four RR fundamentals in the 295−365 nm region. However, the Cu−S combination bands in the RR spectrum of H46R SOD have not been detected for the single thiolate tetragonal sites of azurin and SOD. They could be indicative of coordination of two thiolates to a single Cu^{2+} in H46R SOD.

Chemical and Posttranslational Modification of Cysteine 111. There are four cysteine residues in each human SOD subunit, but cysteine 111 appeared to be the most likely candidate ligand for Cu^{2+} binding because it is the most solvent-exposed cysteine in hCuZnSOD (*2*). One approach to studying the properties of this surface cysteine was to use chemical modification reagents, and the reactions of the enzyme with 4VP and DTNB, two reagents specific for cysteine modification, were investigated.

LCMS of 4VP reactions with apo hH46R showed residual unreacted protein and a single modification product with a molecular mass of 15 969 Da, corresponding to incorporation

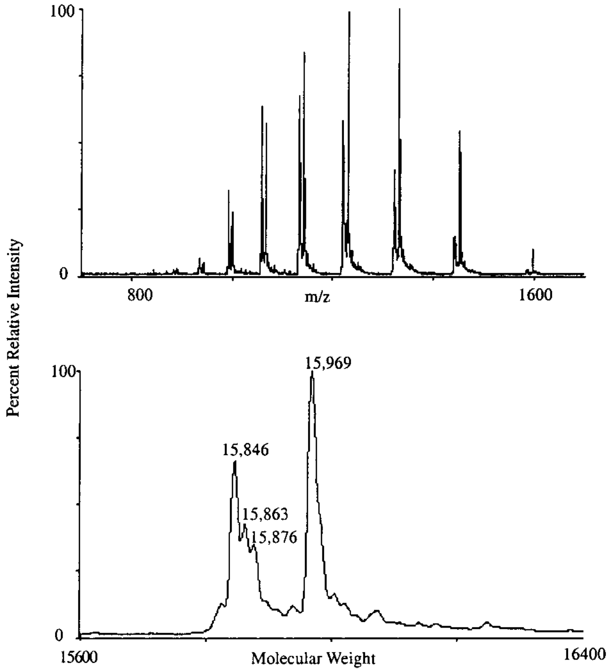

FIGURE 3: Electrospray mass spectrum (top) and reconstructed molecular weight profile (bottom) of human H46R CuZnSOD following covalent modification with 4-vinylpyridine. The calculated mass of hH46R is 15 864, and the expected mass upon modification is 15 969.

of one 4VP moiety per subunit (observed mass increase 105 Da, theoretical mass increase 105.1 Da/modification). Similar results showing a single modification per subunit were obtained with DTNB (data not shown). However, the yield of the modified product in the case of either reagent never exceeded approximately 50% irrespective of the molar excess of reagent (Figure 3). Reconstructed molecular mass profiles of H46R CuZnSOD following 4VP reaction show the two major peaks with approximately equal distribution representing the unmodified SOD monomer and the one-4VP modified SOD monomer (Figure 3, bottom panel). This result suggests only one-half of the SOD subunits react with the modification reagent. When hWT SOD was subjected to the same experiments, 50% modification was also obtained.

Cysteine 111 is near the dimer/dimer interface, and the distance between the two cysteine side chains is about 10 Å, as estimated from the crystal structure of the wild-type protein (*2*). The 50% yield suggests that modification of the cysteine 111 side chain on one subunit of the dimer impedes access of the modifying reagent to the cysteine 111 on the other subunit because of their close proximity.

It is interesting to note here some apparently related mass spectrometry results that have been observed for recombinant human WT and many of the mutant human CuZnSODs. Very frequently, the purified samples have been found to contain 30−50% of a species 118 Da heavier (cysteine disulfide residue mass is 118.1 Da) than the predicted subunit mass, with the percentage varying from batch to batch. When the purified proteins were treated with DTT, the 118 Da adduct peak disappeared from the mass spectra. We assign this adduct species as SOD with a cysteine attached.

To determine the location of the adducted 118 Da mass, human wild-type protein that had been purified without DTT treatment was subjected to trypsin digestion and then analyzed by LCMS. From the digestion map, a fragment with the measured mass of 2576 was tentatively identified as the cysteine-adducted D92−R115 tryptic peptide (DGVADVSIEDSVISLSGDHCIIGR, predicted mass of 2458 Da, $\Delta = 118$). The 2576 Da fragment was further studied by MS/MS, which unambiguously confirmed that the extra cysteine was on the peptide containing Cys111 (data not shown).

The results described above suggest that as-isolated H46R may be modified naturally or artificially by an additional cysteine at cysteine 111 in the absence of DTT. We therefore titrated both DTT-treated and untreated hH46R apoprotein with Cu^{2+} at pH 5.5. As predicted, the untreated H46R had a much lower absorbance at 370 nm as compared with the treated protein.

Cu^{2+} Titration of the C111S/H46R Double Mutant. Another piece of direct evidence for binding of Cu^{2+} to cysteine 111 in H46R was gleaned from mutagenesis studies. The C111S/H46R double mutant gene was made and expressed in yeast, and the protein was purified (see Experimental Procedures). Pure protein was first characterized by mass spectrometry. The results showed the presence of a molecule of mass 15 849 Da, corresponding to the predicted double mutant (calculated mass 15 850 Da), with no detectable level of a Cys-adducted species. The double mutant was then demetalated and titrated with Cu^{2+}, resulting in the appearance of a peak at 750 nm, similar to that seen in Figure 1E. No peak was observed at 370 nm, even after the addition of 6 equiv of Cu^{2+} (data not shown). Thus, removing cysteine 111 totally eliminated the appearance of the ligand-to-Cu^{2+} charge-transfer band, strongly supporting our hypothesis that cysteine 111 is the ligand that produced the unusual band in H46R.

DISCUSSION

Metal Binding of H46R. Previous studies have shown that most FALS mutant CuZnSOD proteins display abnormal metal binding abilities (*13*, *16*); however, there is no detailed study of either of the metal binding site mutants, H46R and H48Q. Unlike other FALS mutants, which retain most of the enzymatic activity of WT, these two mutations result in complete loss of SOD activity and represent the extreme case of functional perturbation in the enzyme.

Histidine 46 is a copper ligand in wild-type CuZnSOD, and it has been reported that Cu^{2+} binding is impaired when histidine is replaced by arginine in H46R (*35*). Our results confirm that the native copper site is indeed highly affected by this mutation, and in the case of Cu^{2+} or Co^{2+} binding, we see evidence for only one *strong* metal binding site (per subunit), for which Cu^{2+} and Co^{2+}compete. When this site binds Co^{2+}, the resulting spectrum is very similar to that of wild-type CuZnSOD in which Co^{2+} is bound to the zinc site (Figure 1). When this site binds Cu^{2+}, the resulting spectrum has an absorption maximum at 750 nm, considerably red shifted from the characteristic peak of Cu^{2+} in the copper site. We conclude, therefore, that Cu^{2+} and Co^{2+} are competing for binding to the zinc site and that the modified copper site in H46R has little affinity for either of these ions. From the shape and extinction coefficient value of the absorption attributed to Co^{2+}, we conclude that the zinc site of H46R has a relatively wild-type-like tetrahedral geometry when Co^{2+} is bound.

Addition of Cu^{2+} to E_2Co_2 H46R at pH 5.5 results in displacement of Co^{2+}, but the Co^{2+} apparently rebinds when the pH is raised, presumably due to an increased affinity of the site for Co^{2+} relative to Cu^{2+} at the higher pH (where the Cu^{2+} is also less soluble) (Figure 1C). Reduction of Cu^{2+} to Cu^{+} at pH 5.5 also caused rebinding of Co^{2+} (Figure 1B, curve d).

The New Cu^{2+} Binding Site. A distinctive new 370 nm band was observed in the UV−vis spectra of human H46R CuZnSOD (Figure 1E). A similar band has been observed in a mutant form of yeast CuZnSOD, H46C (*42*), and in this case the peak was assigned to a cysteine-to-copper charge-transfer band due to the new cysteine in the copper site. However, there is no cysteine in or near the zinc or copper sites in human H46R CuZnSOD.

There are two "free" cysteines in each CuZnSOD subunit, one of which might bind to Cu^{2+} and give the 370 nm band. To test our hypothesis, we used Ag^{+} to compete for copper binding to the possible site. If cysteine is indeed one of the ligands in the new site, a "softer" metal ion such as Ag^{+} should be able to replace Cu^{2+} from the site, resulting in a decrease of the 370 nm charge-transfer band. As shown in Figure 1F, the 370 nm charge-transfer band disappeared after addition of 2.5 equiv of Ag^{+}, indicating that Ag^{+} replaces Cu^{2+} at this new site.

A comparison of the resonance Raman of H46R CuZnSOD with other copper−cysteinate proteins also suggests that H46R contains a tetragonal copper site (Figure 2). The RR spectrum of H46R has its most intense feature at 357 cm^{-1}, which is within the 320−365 cm^{-1} range of the Cu−S(Cys) stretch in tetragonal type 2 copper−cysteinate proteins. The same pattern of RR fundamentals is also seen in tetragonal Cu sites with two cysteinate ligands, such as Cu-substituted liver alcohol dehydrogenase (*44*). However, multiple thiolate coordination may be distinguished by the appearance of a low-energy S−C(Cys) stretch near 750 cm^{-1} and a series of combination bands between 650 and 800 cm^{-1}. Both of these features are observed in the RR spectra of Cu-substituted LADH (*45*) and H46R SOD but not in the type 2 tetragonal Cu−cysteinate proteins, which are devoid of combination bands and have their S−C(Cys) stretch near 765 cm^{-1}. Thus, it is likely that the extra copper ion in H46R SOD is coordinated to two cysteinate ligands.

All of above experiments establish that a cysteine in the protein becomes a ligand for copper ions in H46R. There are four cysteines in each subunit of CuZnSOD at positions 6, 57, 111, and 146, with 57 and 146 forming an internal disulfide bond. From the published human CuZnSOD structure, cysteine 6 is buried in the protein and cysteine 111 is on the surface, near the dimer interface. Next to cysteine 111 are histidine 110 and aspartate 109. Since all of these residues are near the subunit interface and near to each other, some combination of the six residues from the two subunits could form one or more metal ion binding sites (Figure 4).

To obtain more information about the accessibility of the cysteine residues of the protein, two thiol-specific reagents, 4VP and DTNB, were used to modify reactive cysteines (Figure 3). However, to our surprise, after long reaction times and large excesses of the modification reagents, only about 50% protein modification was observed, possibly due to steric blockage generated by the first modified cysteine. As shown in Figure 4, cysteine 111 from one subunit is in close spatial proximity to cysteine 111 on the other subunit. The RR spectrum suggests that these two residues may coordinate a single copper ion in H46R.

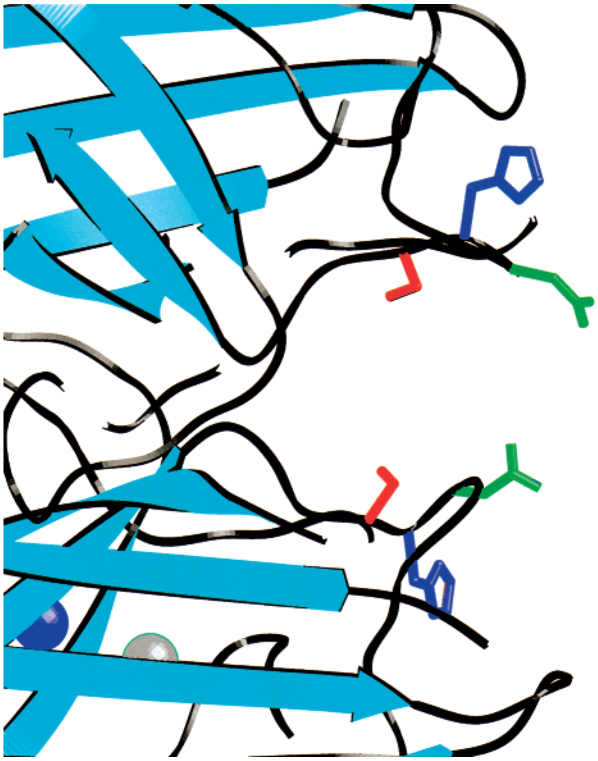

FIGURE 4: Partial structure of the human CuZnSOD dimer near cysteine 111, drawn with Setor. Cys111 residues are shown in red, His110 residues are in blue, and Asp109 residues are in green.

Posttranslationally modified CuZnSOD has been observed frequently in the isolated protein with two common adducts, cysteine and glutathione (*37*, *46*, *47*). It is not known whether these adducts are made naturally *in vivo* or artificially following cell lysis. This study has shown that it is cysteine 111 and not cysteine 6 that is modified. Similar to chemical modification, the percentage of the posttranslationally modified form of SOD is rarely more than 50% as determined by mass spectrometry. These half-modified H46R CuZnSODs are ideal for testing whether cysteine 111 is a ligand for binding copper. H46R SOD with posttranslationally modified cysteine 111 was found to have a much lower absorbance at 370 nm compared with that of the unmodified form (data not shown). To further our investigation, H46R/C111S CuZnSOD was made. The absence of the 370 nm peak in the Cu^{2+} titration of the double mutant is firm evidence that cysteine 111 is a ligand in the new site.

Possible Significance of the New Copper Binding Site for FALS. The observation of Cu^{2+} binding to cysteine 111 in the human FALS CuZnSOD mutant H46R raises the possibility that such binding may be occurring more transiently in the other FALS mutants. The only reason the site was identified in H46R was because of the occurrence of the unusual and stable 370 nm band upon addition of Cu^{2+}. Either Cu^{+} or Zn^{2+} may be binding to some combination of cysteine 111, histidine 110, and aspartate 109 near the subunit interface of the other FALS CuZnSOD mutants, but no spectroscopic property would be perturbed to signal their presence. The binding of copper ions at this solvent-exposed site could conceivably promote deleterious reactions resulting in the oxidative damage observed in ALS patients and in transgenic mice.

Possible Significance of the New Copper Binding Site for Metal Ion Insertion into CuZnSOD. Insertion of copper ions into CuZnSOD under normal conditions in vivo requires the presence of the copper chaperone for SOD (CCS) (*48*). A copper-transfer mechanism for CCS has been proposed in which copper ions are bound to solvent-exposed sulfur ligands on CCS prior to transfer to CuZnSOD (*49*, *50*). It is interesting to speculate that cysteine 111 may play a role as an intermediate ligand, one which accepts the copper ion from hCCS through sulfur ligand exchange and then transfers it to the final destination in the active site of the enzyme. In this scenario, transient metal binding at cysteine 111 is a normal and necessary event for wild-type CuZnSOD. It is possible that the species responsible for the 370 nm cysteine sulfur-to-Cu^{2+} charge-transfer band is detectable and long-lived in H46R because the affinity of the native copper site for Cu^{2+} is markedly decreased.

The transient appearance of a similar band in the human wild-type CuZnSOD was actually reported in 1975 (*51*). A 370 nm band was observed in the wild-type SOD immediately after the addition of Cu^{2+} and then it quickly disappeared. This phenomenon is most likely explained by copper being transferred through the surface cysteine 111 to the copper site.

To investigate further the importance of cysteine 111 for copper insertion, we carried out sequence alignment of CuZnSOD from 60 different organisms; only chicken CuZnSOD has cysteine 111. However, no additional primate CuZnSOD sequences are available. On the other hand, histidine 110 and aspartate 109 are conserved in 15 organisms. Examination of Figure 4 reveals that, even without cysteine 111, histidine 110 and aspartate 109 may form one or more binding sites across the subunit interface and thus remain possible candidates for copper transfer. It is important to note here that the structure of the apoprotein of CuZnSOD has not yet been reported, and therefore the orientations and proximity of these potential ligands across the subunit interface may differ from those deduced from examination of the crystal structure of the fully metalated protein.

In conclusion, we have found that H46R has markedly abnormal metal binding properties relative to wild type; it binds neither Cu^{2+} nor Co^{2+} at its native copper site but forms a new copper binding site at its surface, near the subunit interface, which could catalyze the deleterious reactions in this and other FALS mutants. Moreover, these results lead to the hypothesis that metal ion binding sites near cysteine 111 on the surface of wild-type CuZnSOD may be involved in the mechanism of metal ion insertion into the apoprotein *in vivo*.

REFERENCES

1. Rosen, D. R., Siddique, T., Patterson, D., Figlewicz, D. A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J. P., Deng, H.-X., Rahmani, Z., Krizus, A., McKenna-Yasek, D., Cayabyab, A., Gaston, S. M., Berger, R., Tanzi, R. E., Halperin, J. J., Herzfeldt, B., van den Bergh, R., Hung, W.-Y., Bird, T., Deng, G., Mulder, D. W., Smyth, C., Laing, N. G., Soriano, E., Pericak-Vance, M. A., Haines, J., Rouleau, G. A., Gusella, J. S., Horvitz, H. R., and Brown, R. H., Jr. (1993) *Nature 362*, 59−62.
2. Deng, H. X., Hentati, A., Tainer, J. A., Iqbal, Z., Cayabyab, A., Hung, W. Y., Getzoff, E. D., Hu, P., Herzfeldt, B., Roos, R. P., Warner, C., Deng, G., Soriano, E., Smyth, C., Parge, H. E., Ahmed, A., Roses, A. D., Hallewell, R. A., Pericak-vance, M. A., and Siddique, T. (1993) *Science 261*, 1047−1051.
3. Adams, R. D., Victor, M., and Ropper, A. H. (1998) *Principles of Neurology*, 6th ed., McGraw-Hill Health Professions Division, New York.
4. Lyons, T. J., Gralla, E. B., and Valentine, J. S. (1999) *Met. Ions Biol. Syst. 36*, 125−177.
5. Bertini, I., Mangani, S., and Viezzoli, M. (1998) *Adv. Inorg. Chem. 45*, 127−250.
6. Cabelli, D. E., Riley, D., Rodriguez, J. A., Valentine, J. S., and Zhu, H. (2000) in *Biomimetic Oxidations Catalyzed by Transition Metal Complexes* (Meunier, B., Ed.) pp 461−508, Imperial College Press, London.
7. Forman, H. J., and Fridovich, I. (1973) *J. Biol. Chem. 248*, 2645−2649.
8. Morrison, B. M., and Morrison, J. H. (1999) *Brain Res. Rev. 29*, 121−135.
9. Wiedau-Pazos, M., Goto, J. J., Rabizadeh, S., Gralla, E. B., Roe, J. A., Lee, M. K., Valentine, J. S., and Bredesen, D. E. (1996) *Science 271*, 515−518.
10. Yim, M. B., Kang, J. H., Yim, H. S., Kwak, H. S., Chock, P. B., and Stadtman, E. R. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 5709−5714.
11. Yim, H. S., Kang, J. H., Chock, P. B., Stadtman, E. R., and Yim, M. B. (1997) *J. Biol. Chem. 272*, 8861−8863.
12. Nishida, C. R., Gralla, E. B., and Valentine, J. S. (1994) *Proc. Natl. Acad. Sci U.S.A. 91*, 9906−9910.
13. Lyons, T. J., Liu, H., Goto, J. J., Nersissian, A., Roe, J. A., Graden, J. A., Cafe, C., Ellerby, L. M., Bredesen, D. E., Gralla, E. B., and Valentine, J. S. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 12240−12244.
14. Lyons, T. J., Nersissian, A., Huang, H., Yeom, H., Nishida, C. R., Graden, J. A., Gralla, E. B., and Valentine, J. S. (2000) *J. Biol. Inorg. Chem. 5*, 189−203.
15. Goto, J. J., Gralla, E. B., Valentine, J. S., and Cabelli, D. E. (1998) *J. Biol. Chem. 273*, 30104−30109.
16. Goto, J. J., Zhu, H., Sanchez, R. J., Nersissian, A., Gralla, E. B., Valentine, J. S., and Cabelli, D. E. (2000) *J. Biol. Chem. 275*, 1007−1014.
17. Beckman, J. S., Carson, M., Smith, C. D., and Koppenol, W. H. (1993) *Nature 364*, 584.
18. Crow, J. P., Sampson, J. B., Zhuang, Y., Thompson, J. A., and Beckman, J. S. (1997) *J. Neurochem. 69*, 1936−1944.
19. Estevez, A. G., Crow, J. P., Sampson, J. B., Reiter, C., Zhuang, Y., Richardson, G. J., Tarpey, M. M., Barbeito, L., and Beckman, J. S. (1999) *Science 286*, 2498−2500.
20. Facchinetti, F., Sasaki, M., Cutting, F. B., Zhai, P., MacDonald, J. E., Reif, D., Beal, M. F., Huang, P. L., Dawson, T. M., Gurney, M. E., and Dawson, V. L. (1999) *Neuroscience 90*, 1483−1492.
21. Durham, H. D., Roy, J., Dong, L., and Figlewicz, D. A. (1997) *J. Neuropathol. Exp. Neurol. 56*, 523−530.
22. Tu, P. H., Gurney, M. E., Julien, J. P., Lee, V. M., and Trojanowski, J. Q. (1997) *Lab. Invest. 76*, 441−456.
23. Bruijn, L. I., and Cleveland, D. W. (1996) *Neuropathol. Appl. Neurobiol. 22*, 373−387.
24. Brown, R. H., Jr. (1998) *Nat. Med. 4*, 1362−1364.
25. Kaytor, M. D., and Warren, S. T. (1999) *J. Biol. Chem. 274*, 37507−37510.
26. Ferrante, R. J., Browne, S. E., Shinobu, L. A., Bowling, A. C., Baik, M. J., MacGarvey, U., Kowall, N. W., Brown, R. H., Jr., and Beal, M. F. (1997) *J. Neurochem. 69*, 2064−2074.
27. Gurney, M. E., Cutting, F. B., Zhai, P., Doble, A., Taylor, C. P., Andrus, P. K., and Hall, E. D. (1996) *Ann. Neurol. 39*, 147−157.
28. Liu, R., Althaus, J. S., Ellerbrock, B. R., Becker, D. A., and Gurney, M. E. (1998) *Ann. Neurol. 44*, 763−770.
29. Andrus, P. K., Fleck, T. J., Gurney, M. E., and Hall, E. D. (1998) *J. Neurochem. 71*, 2041−2048.

30. Hottinger, A. F., Fine, E. G., Gurney, M. E., Zurn, A. D., and Aebischer, P. (1997) *Eur. J. Neurosci. 9*, 1548−1551.
31. Aoki, M., Ogasawara, M., Matsubara, Y., Narisawa, K., Nakamura, S., Itoyama, Y., and Abe, K. (1994) *J. Neurol. Sci. 126*, 77−83.
32. Enayat, Z. E., Orrell, R. W., Claus, A., Ludolph, A., Bachus, R., Brockmuller, J., Ray-Chaudhuri, K., Radunovic, A., Shaw, C., Wilkinson, J., King, A., Swash, M., Leigh, P. N., de Belleroche, J., and Powell, J. (1995) *Hum. Mol. Genet. 4*, 1239−1240.
33. Ratovitski, T., Corson, L. B., Strain, J., Wong, P., Cleveland, D. W., Culotta, V. C., and Borchelt, D. R. (1999) *Hum. Mol. Genet. 8*, 1451−1460.
34. Ogawa, Y., Kosaka, H., Nakanishi, T., Shimizu, A., Ohoi, N., Shouji, H., Yanagihara, T., and Sakoda, S. (1997) *Biochem. Biophys. Res. Commun. 241*, 251−257.
35. Carri, M. T., Battistoni, A., Polizio, F., Desideri, A., and Rotilio, G. (1994) *FEBS Lett. 356*, 314−316.
36. Liochev, S. I., Chen, L. L., Hallewell, R. A., and Fridovich, I. (1997) *Arch. Biochem. Biophys. 346*, 263−268.
37. Nakanishi, T., Kishikawa, M., Miyazaki, A., Shimizu, A., Ogawa, Y., Sakoda, S., Ohi, T., and Shoji, H. (1998) *J. Neurosci. Methods 81*, 41−44.
38. Hallewell, R. A., Imlay, K. C., Lee, P., Fong, N. M., Gallegos, C., Getzoff, E. D., Tainer, J. A., Cabelli, D. E., Tekamp-Olson, P., Mullenbach, G. T., and Cousens, L. S. (1991) *Biochem. Biophys. Res. Commun. 181*, 474−480.
39. Higuchi, R. (1990) in *PCR Protocols* (Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., Eds.) pp 177−183, Academic Press, New York.
40. Valentine, J. S., and Pantoliano, M. W. (1981) in *Copper Proteins* (Spiro, T. G., Ed.) pp 292−358, John Wiley and Sons, Inc., New York.
41. Maret, W., and Vallee, B. L. (1993) *Methods Enzymol. 226*, 52−71.
42. Lu, Y., Roe, J. A., Bender, C. J., Peisach, J., Banci, L., Bertini, I., Gralla, E. B., and Valentine, J. S. (1996) *Inorg. Chem. 35*, 1692−1700.
43. Andrew, C. R., Yeom, H., Valentine, J. S., Karlsson, B. G., Bonander, N., Vanpouderoyen, G., Canters, G. W., Loehr, T. M., and Sandersloehr, J. (1994) *J. Am. Chem. Soc. 116*, 11489−11498.
44. Maret, W., Shiemke, A. K., Wheeler, W. D., Loehr, T. M., and Sanders-Loehr, J. (1986) *J. Am. Chem. Soc. 108*, 6351−6359.
45. Maret, W., Zeppezauer, M., Sanders-Loehr, J., and Loehr, T. M. (1983) *Biochemistry 22*, 3202−3206.
46. Marklund, S. L., Andersen, P. M., Forsgren, L., Nilsson, P., Ohlsson, P. I., Wikander, G., and Oberg, A. (1997) *J. Neurochem. 69*, 675−681.
47. Schininà, M. E., Carlini, P., Polticelli, F., Zappacosta, F., Bossa, F., and Calabrese, L. (1996) *Eur. J. Biochem. 237*, 433−439.
48. Harrison, M. D., Jones, C. E., Solioz, M., and Dameron, C. T. (2000) *Trends Biochem. Sci. 25*, 29−32.
49. Schmidt, P. J., Rae, T. D., Pufahl, R. A., Hamma, T., Strain, J., O'Halloran, T. V., and Culotta, V. C. (1999) *J. Biol. Chem. 274*, 23719−23725.
50. Zhu, H., Shipp, E., Sanchez, R. J., Liba, A., Stine, J. E., Hart, P. J., Gralla, E. B., Nersissian, A. M., and Valentine, J. S. (2000) *Biochemistry 39*, 5413−5421.
51. Calabrese, L., Federici, G., Bannister, W. H., Bannister, J. V., Rotilio, G., and Finazzi-Agrò, A. (1975) *Eur. J. Biochem. 56*, 305−309.

BI000846F