Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 12240–12244, October 1996
Biochemistry

Mutations in copper–zinc superoxide dismutase that cause amyotrophic lateral sclerosis alter the zinc binding site and the redox behavior of the protein

THOMAS J. LYONS*, HONGBIN LIU*, JOY J. GOTO*, ARAM NERSISSIAN*, JAMES A. ROE†, JANET A. GRADEN*‡, CARLA CAFÉ§, LISA M. ELLERBY§, DALE E. BREDESEN§, EDITH BUTLER GRALLA*, AND JOAN SELVERSTONE VALENTINE*¶

*Department of Chemistry and Biochemistry, University of California, Los Angeles, CA 90095-1569; †Department of Chemistry and Biochemistry, Loyola Marymount University, Los Angeles, CA 90045; and §Program on Aging, The Burnham Institute, La Jolla, CA 92037

JoAnne Stubbe, Massachusetts Institute of Technology, Cambridge, MA, August 8, 1996 (received for review June 28, 1996)

ABSTRACT **A series of mutant human and yeast copper–zinc superoxide dismutases has been prepared, with mutations corresponding to those found in familial amyotrophic lateral sclerosis (ALS; also known as Lou Gehrig's disease). These proteins have been characterized with respect to their metal-binding characteristics and their redox reactivities. Replacement of Zn^{2+} ion in the zinc sites of several of these proteins with either Cu^{2+} or Co^{2+} gave metal-substituted derivatives with spectroscopic properties different from those of the analogous derivative of the wild-type proteins, indicating that the geometries of binding of these metal ions to the zinc site were affected by the mutations. Several of the ALS-associated mutant copper–zinc superoxide dismutases were also found to be reduced by ascorbate at significantly greater rate than the wild-type proteins. We conclude that similar alterations in the properties of the zinc binding site can be caused by mutations scattered throughout the protein structure. This finding may help to explain what is perhaps the most perplexing question in copper–zinc superoxide dismutase-associated familial ALS—i.e., how such a diverse set of mutations can result in the same gain of function that causes the disease.**

Mutations in the gene encoding copper–zinc superoxide dismutase (CuZnSOD) have been linked to the neurodegenerative disease amyotrophic lateral sclerosis (ALS; also known as Lou Gehrig's disease) and appear to be causative in approximately 20% of the cases of the inherited form of the disease (familial ALS or fALS) (1, 2). Most of the mutations are point mutations and are scattered throughout the protein. The pattern of inheritance of CuZnSOD-associated fALS is autosomal dominant; recent evidence from several laboratories supports a gain of function mechanism for this form of the disease (3–8). Although CuZnSOD-associated fALS accounts for only 2% of the total cases of ALS, understanding the mechanism by which mutations in CuZnSOD cause ALS should provide valuable clues in the effort to determine the causes of all forms of the disease.

We have constructed, expressed, and purified several ALS human CuZnSOD (hCuZnSOD) mutant proteins (4) and yeast analogs (yCuZnSOD) (9) to characterize their metal-binding characteristics and chemical reactivities. It was hoped that whatever defects the mutations confer on this protein would be manifested in the metal binding behavior. We report herein our observations of dramatically altered metal-binding and redox behavior for several of these ALS mutant CuZnSOD proteins.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

EXPERIMENTAL PROCEDURES

Materials. Wild-type yeast CuZnSOD (yWT) was purchased from Carlbiotech (Copenhagen) as a lyophilized powder (batch no. 8997368, product no. 3007). Purity was confirmed by gel electrophoresis. Reagent grade $CuSO_4$, $CoSO_4$, and $ZnSO_4$ were purchased from Baker, Fisher, and Sigma, respectively. $Na_2S_2O_4$ was purchased from Aldrich. L-Ascorbic acid was purchased from Sigma.

DNA Manipulations and Protein Expression. Mutations in yeast CuZnSOD were constructed via modified Kunkel site-directed mutagenesis on the pBSIIKS(−)-SOD0.5 plasmid (10). Mutagenic oligonucleotides were synthesized on a Pharmacia LKB Gene Assembler Plus. The mutant genes were subcloned into pET-3d plasmid between *Nco*I and *Bam*HI sites for expression in BL21 *Escherichia coli*. Expression and purification of the recombinant yWT protein and the mutants (yG85R, yL106V, yH46R, yH63E, and yH63A) were carried out according to published procedures (9).

Human CuZnSOD mutants were prepared by the overlap extension PCR technique using the human SOD gene cloned into the yeast expression plasmid, YEP351, as a template (11). Human wild-type (hWT), hH46R, hA4V, hG37R, and hG93A were expressed in EG118 *Saccharomyces cerevisiae*, which lacks endogenous CuZnSOD, and purified via published procedures (4).

Preparation of Metal-Free and Metal-Substituted Derivatives. Apoproteins were prepared by published procedures (9, 12). Protein concentration was determined by UV absorption with an extinction coefficient of 2900 $M^{-1}\cdot cm^{-1}$ at 278 nm for the yeast apoprotein subunit and 5400 $M^{-1}\cdot cm^{-1}$ at 280 nm for the human apoprotein subunit. Cu^{2+} and Co^{2+} were added to the protein as aqueous $CuSO_4$ and $CoSO_4$. Solutions of apoprotein, 200 μl, usually of a concentration between 0.3 and 0.4 mM per subunit, were titrated with 10 or 25 mM solutions of the appropriate metal sulfate salt until an end point was reached in the visible spectrum. In the case of CuCoSOD, Co^{2+} was added before copper. Solutions were allowed to stand for at least 1 h after the addition of Cu^{2+} before further studies were carried out. For the recombinant WT proteins, a period of 2 days at 4°C was necessary for the cobalt-reconstituted proteins to reach equilibrium. All titrations were carried out in 100 mM acetate buffer at pH 5.5.

Reduction. Reduction of active site Cu^{2+} to Cu^{+} in solutions of WT or mutant CuZnSOD or their metal substituted derivatives was carried out by addition of either a single crystal of solid sodium dithionite or aqueous L-ascorbic acid, as indi-

Abbreviations: ALS, amyotrophic lateral sclerosis; CuZnSOD, copper–zinc superoxide dismutase; h, human; y, yeast; WT, wild type.

‡Present address: Department of Medicine, University of Utah Health Sciences Center, Salt Lake City, UT 84112.
¶To whom reprint requests should be addressed.

cated in the text. Disappearance of the characteristic visible absorption peak due to *d–d* transitions of Cu^{2+} in Cu^{II}ZnSOD (680 nm for the WT enzymes) was used to monitor the rate of reduction in the presence of ascorbate, using 100 μM enzyme and 3 mM ascorbate.

pH Experiments. The pH of solutions was altered from the initial pH of 5.5 by the addition of 100–500 mM NaOH in 1-μl increments. The pH was measured using an Orion Research Microprocessor Ionalyzer/901 (Boston, MA) with an Ingold microelectrode (Wilmington, MA).

Spectroscopy. Visible/UV spectra from 900 nm to 240 nm were determined using a Cary 3 UV/Vis spectrometer (Varian; Sunnyvale, CA). Samples were centrifuged for 1 min before spectra were taken. EPR spectra were obtained with a Bruker ER200d-SRC X-band EPR spectrometer (Billerica, MA) equipped with a ER43MRD microwave bridge, an ER4111VT variable temperature unit, and a EIP 548 microwave frequency counter. Typical instrument parameters for 90 K spectra are as follows: field center, 3000 G; sweep width, 1600 G; frequency, 9.54 GHz; modulation amplitude, 5.0 G; power, 20.7 mW; time constant, 640 ms; receiver gain, 5.0×10^5; sweep time, 200 s.

RESULTS

Copper Binding. Metal ion substitution in WT CuZnSOD proteins is a useful tool to measure the metal binding affinities and to probe the geometries of their metal binding sites. In particular either Co^{2+} or Cu^{2+} substituted for Zn^{2+} provide excellent indicators of the nature of the native zinc site (12–14). We therefore carried out similar metal ion substitution experiments on the ALS mutant CuZnSOD proteins.

Apo yWT or hWT binds two equivalents of Cu^{2+} per subunit to give $Cu^{II}Cu^{II}$SOD at pH 5.5 (15). Numerous spectroscopic studies have led to the conclusion that Cu^{2+} binds first to the native copper sites of the apoprotein, resulting in the appearance of a broad band in the visible spectrum at 670 nm (Fig. 1*A*, spectrum b). After the native copper sites are full, Cu^{2+} then binds to the native zinc sites, resulting in the appearance of an additional broad band, partially overlapping the first, with a maximum at 810 nm (see Fig. 1*A*, spectrum c) (12). These observations are consistent with the Cu^{2+} ion adopting a distorted tetragonal geometry in the native copper sites, similar to that observed for copper in x-ray crystal structures of Cu^{II}ZnSOD (16, 17), and a distorted tetrahedral geometry when bound in the native zinc sites (18).

Our first indication of abnormal metal ion binding behavior in ALS mutants appeared upon titration of several ALS mutant SOD apoproteins with Cu^{2+} (9) . Addition of Cu^{2+} to apoproteins derived from the human proteins hG93A, hG37R, and hA4V and the yeast proteins yG85R, yL106V, and yH46R demonstrated binding of Cu^{2+} to the apoprotein with an endpoint at two equivalents per subunit. The visible spectra obtained during the addition of the first equivalent were similar to those observed for titrations of the WT apoproteins, i.e., a broad band developed with $\lambda_{max} \cong 680–700$ nm. However, upon binding of a second equivalent additional absorption was seen around 700 nm rather than at 810 nm, which is characteristic of Cu^{2+} in the zinc site of WT. Titration of the apoprotein from hG93A with Cu^{2+}, shown in Fig. 1*B*, should be compared with the results from a similar titration of the apoprotein from hWT in Fig. 1*A*. All the aforementioned mutants yielded similar abnormalities. We conclude that these ALS mutant proteins retain their ability to bind two equivalents of Cu^{2+} per subunit but that the geometry of Cu^{2+} bound to the native zinc site of the mutant proteins is different from that found in the $Cu^{II}Cu^{II}$ derivative of the WT protein.

Cobalt Binding. The ionic radii and geometric preferences of Cu^{2+} are different from those of Zn^{2+}, but these same properties for Co^{2+} and Zn^{2+} are similar, making Co^{2+} a particularly useful probe of zinc-binding sites in metalloproteins in general and of CuZnSOD in particular (14, 19). Co^{2+} binds readily to the zinc site in place of Zn^{2+} in WT

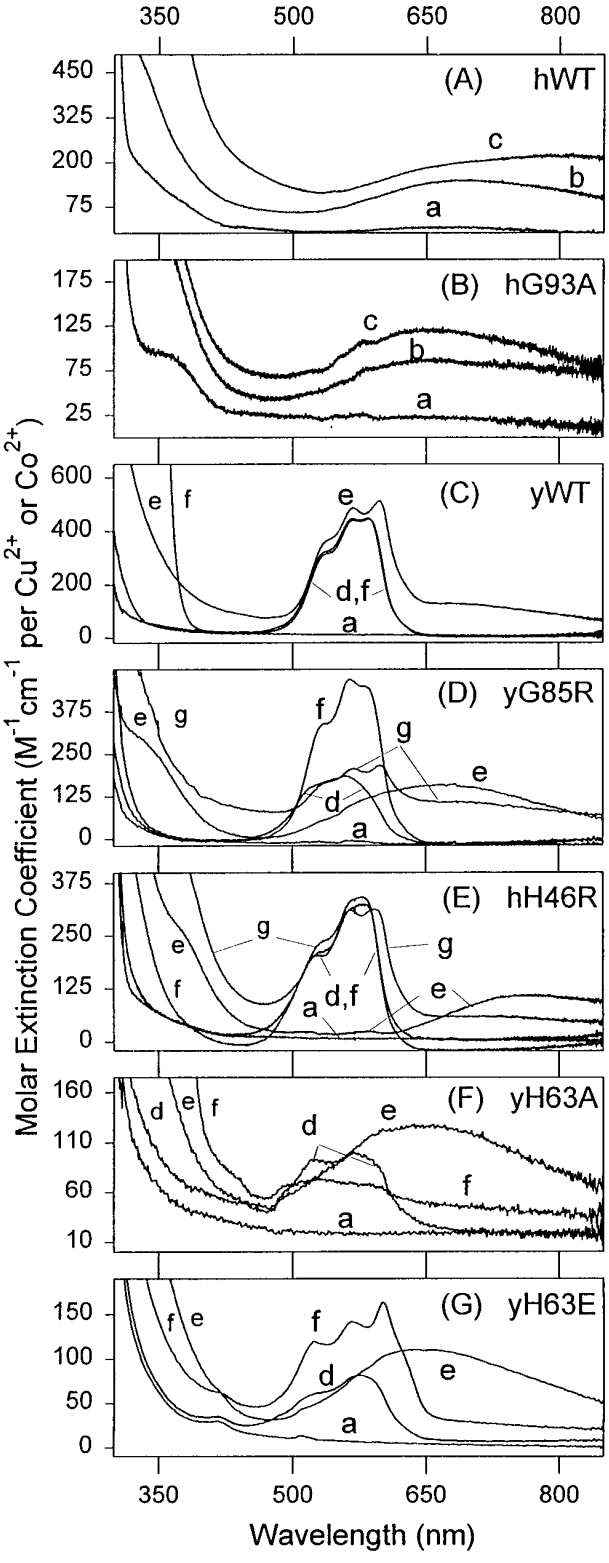

FIG. 1. UV/visible spectra. Absorption spectra for metal substituted derivatives of hWTSOD (*A*), hG93ASOD (*B*), yWTSOD (*C*), yG85RSOD (*D*), hH46RSOD (*E*), yH63ASOD (*F*), and yH63ESOD (*G*). Individual spectra: a, apoprotein, pH 5.5; b, 1 Cu^{2+} per subunit, pH 5.5; c, 2 Cu^{2+} per subunit, pH 5.5; d, 1 Co^{2+} per subunit, pH 5.5; e, 1 Cu^{2+}, 1 Co^{2+} per subunit, pH 5.5; f, 1 Cu^{+}, 1 Co^{2+} per subunit, pH 5.5; g, 1 Cu^{2+} and 1 Co^{2+} per subunit, pH 7.0.

CuZnSOD, resulting in WT CuCoSOD, with no significant change in its SOD activity (20). In addition, spectroscopic and x-ray crystallographic studies of $Cu^{II}CoSOD$ have confirmed that the imidazolate bridge linking the two metal ions in each subunit is present in WT oxidized $Cu^{II}CoSOD$ and broken in WT reduced $Cu^{I}CoSOD$, just as in WT CuZnSOD (21–25).

The nature of the visible absorption spectrum of the d^7 Co^{2+} ion is an excellent reporter of its ligand environment. The intensity of its *d–d* bands, measured by molar extinction coefficient, provides reliable information concerning the coordination number and geometry of sites to which it is bound. Thus the extinction coefficients for *d–d* bands in visible spectra of octahedral complexes of Co^{2+} are typically $\varepsilon \leq 20$ $cm^{-1}{\cdot}M^{-1}$, while those for tetrahedral complexes of the same ion are 10- to 20-fold higher; the extinction coefficients for five-coordinate Co^{2+} fall between these two extremes. Moreover, the absorption bands due to Co^{2+} frequently show considerable fine structure which changes as the identities of the individual ligands are varied (19, 26).

One equivalent per subunit of Co^{2+} was added to apo yWT to produce yWT ECoSOD (E, empty; i.e., the copper site is empty). The resulting visible spectrum shows a broad absorption band consisting of three overlapping components [535 (sh), 567, and 585 nm; Fig. 1*C*, spectrum d]. The extinction coefficient for the 585-nm absorption maximum was approximately 450 $M^{-1}{\cdot}cm^{-1}$, consistent with a tetrahedral geometry for the bound Co^{2+} ion.

Addition of one equivalent per subunit of Cu^{2+} to yWT ECoSOD resulted in the appearance of the characteristic band at 670 nm due to Cu^{II} in the copper site of $Cu^{II}CoSOD$ (Fig. 1*C*, spectrum e). This new copper band was superimposed on the bands due to Co^{2+} in the zinc site, which decreased slightly in intensity and shifted to lower energy. This red shift of the *d–d* bands of Co^{2+} has been attributed to the fact that the imidazole side chain of His63 (numbered as in the human and yeast proteins) binds to Co^{2+} as imidazole in the case of ECoSOD but is deprotonated to form the imidazolate bridge between Cu^{2+} and Co^{2+} in $Cu^{II}CoSOD$ (27). Similar red shifts upon anion binding are characteristic of $Co^{II}(His)_3$ centers and have been seen for cobalt-substituted carbonic anhydrase and cobalt-substituted hemocyanin (28, 29).

Although the details of the spectral changes differed from mutant to mutant, none of the mutants tested herein were identical to WT SOD with respect to substitution of Zn^{2+} with Co^{2+}. For example, addition of Co^{2+} to apo yG85R gave a relatively low intensity peak (ε = 160 $M^{-1}{\cdot}cm^{-1}$) at 550 nm with little fine structure apparent (compare Fig. 1 *D*, spectrum d, with *C*, spectrum d) (9). The intermediate extinction coefficient of this band indicates that Co^{2+} is bound in a different geometry than in WT ECoSOD and suggests that it is five-coordinate. Such a geometry could result from binding of the Co^{2+} ion to either the copper or the zinc site, possibly with additional ligands provided by water or buffer ions.

Addition of one equivalent per subunit of Cu^{2+} to yG85R ECoSOD gave results dramatically different from those observed for the WT protein (compare Fig. 1 *D*, spectrum e, with *C*, spectrum e) (9). The resulting visible spectrum contained no apparent visible absorption bands attributable to Co^{2+}, suggesting that Co^{2+} had been released from the protein to form $Co(H_2O)_6{}^{2+}$ or remained bound to the protein but adopted an octahedral rather than tetrahedral geometry. In either case, the intensity of the *d–d* bands in the spectrum would be too low to detect. In some other cases, the *d–d* bands of the $Cu^{II}CoSOD$ derivatives were detectable but inequivalent to those found in the WT $Cu^{II}CoSOD$ proteins. For hG93A the intensity of Co^{II} absorption is $\varepsilon_{586\ nm}$ = 160 $M^{-1}{\cdot}cm^{-1}$, indicating possible five-coordination at the site; for hG37R that same Co^{II} band has an extinction coefficient of 370 $M^{-1}{\cdot}cm^{-1}$, which is normal except that this mutant only binds one Co^{2+} per dimer.

EPR spectroscopy also revealed marked differences between the metal binding properties of yWT and yG85R. In the case of yWT $Cu^{II}CoSOD$, the Cu^{II} EPR signal intensity was substantially weaker than that expected for the total concentration of Cu^{2+} in the sample. The signal reduction results from the interaction of the Cu^{2+} and Co^{2+} centers across the imidazolate bridge (Fig. 2, spectrum a). The observed signal is due to a small amount of residual uncoupled Cu^{II} (13, 30). By contrast, the EPR spectrum of yG85R $Cu^{II}CoSOD$ was typical of a mononuclear copper protein, with no evidence of perturbation of the Cu^{II} center by the Co^{2+} ion, indicating that the Cu^{2+}–imidazolate–Co^{2+} configuration was not present (see Fig. 2, spectrum b). This result is thus consistent with our conclusion based on the visible spectral results that Co^{2+} had either been released from the protein to form $Co(H_2O)_6{}^{2+}$ or remained bound to the protein but in a highly altered geometry.

As described above, the spectroscopic differences between yWT and the ALS mutant yG85R $Cu^{II}CoSOD$ were particularly dramatic. Much to our surprise, these differences vanished upon reduction of the copper centers from Cu^{II} to Cu^{I}. In the case of the WT proteins, reduction of the $Cu^{II}Co$ protein derivatives causes the imidazolate bridge to break and become protonated; the resulting $Cu^{I}Co$ form has cobalt *d–d* bands identical to those of WT ECoSOD (compare Fig. 1*C*, spectra d and f). In the case of yG85R, reduction of the Cu^{II} to Cu^{I} resulted in the appearance of high-intensity Co^{2+} *d–d* bands (see Fig. 1*D*, spectrum f), indicating that the geometry of the Co^{II} center had been radically altered from octahedral to tetrahedral and suggesting that the structure is similar to that found for the $Cu^{I}Co$ derivatives of the WT proteins. Additionally, in the case of hH46R, the visible spectral bands due to Co^{2+} were not seen when one equivalent per subunit each of Cu^{2+} and Co^{2+} were present, but upon reduction of the Cu^{2+} to Cu^{+} high-intensity *d–d* bands characteristic of tetrahedral Co^{2+} appeared (Fig. 1*E*, spectra e and f).

The studies of CuCo G85R described above suggested that the availability of His-63 as a ligand played an essential role in determining the nature of the binding of Co^{2+} to the zinc site. To investigate further the role of His-63, we carried out a similar study of the CuCoSOD derivatives of yH63A and yH63E, non-ALS mutants in which the bridging His-63 was changed to Ala and Glu, respectively (31). Titration of the

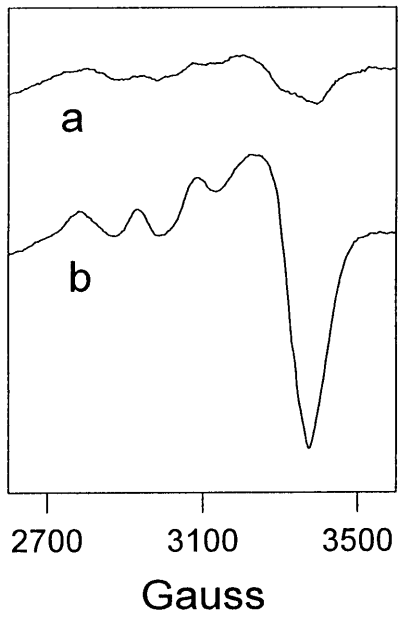

FIG. 2. EPR spectra at 90 K. a, yeast WT $Cu^{II}CoSOD$, pH 5.5; b, yeast G85R with 1 Cu^{2+} and 1 Co^{2+} per subunit, pH 5.5.

apoproteins of both mutants with one equivalent per subunit of Co^{2+} resulted in low-intensity peaks due to *d–d* bands of Co^{2+}, possibly in a five-coordinate geometry (Fig. 1 *F*, spectrum d, and *G*, spectrum d). In both cases, subsequent addition of one equivalent per subunit of Cu^{2+} resulted in the appearance of a Cu^{2+} *d–d* band at 670 nm and the simultaneous disappearance of the bands due to Co^{2+} (Fig. 1 *F*, spectrum e, and *G*, spectrum e). The reduction of yH63A did not induce the return of peaks due to Co^{2+} (Fig. 1*F*, spectrum f). By contrast, reduction of Cu^{II}CoH63E resulted in the appearance of a strongly red-shifted tetrahedral Co^{2+} peak (Fig. 1*G*, spectrum f). Thus CuCoH63E, but not CuCoH63A, behaved similarly to CuCoG85R, presumably because a glutamate side chain can act as a ligand and an alanine cannot.

Effects of pH. The SOD activities and spectroscopic properties of yeast and human WT CuZnSOD and CuCoSOD are invariant with pH over a wide range, i.e., pH 5–9 (32, 33). Thus the visible and EPR spectroscopic properties of WT CuCoSOD described above are the same at pH 7 as at pH 5.5 (e.g., for yWT; Figs. 1*C*, spectra d and e, and 2, spectrum a). By contrast, the *d–d* bands due to Co^{2+} in yG85R to which one equivalent per subunit each of Cu^{2+} and Co^{2+} have been added were markedly pH-dependent (compare Fig. 1*D*, spectra e and g). Similar behavior was seen for hH46R (compare Fig. 1*E*, spectra e and g).

Redox Behavior. In addition to the metal-binding properties of the ALS mutant proteins, their redox behavior is also markedly altered. Reduction of 100 μM WT human and yeast Cu^{II}ZnSOD in the presence of 3 mM ascorbate, was found to be very slow and was only approximately 65% complete after 2 h. Reduction of yWT Cu^{II}Co SOD by ascorbate was likewise very slow. By contrast, hG93C, hA4V, and yG85R CuZnSOD and yG85R CuCoSOD were completely reduced in less than 30 min. Similar behavior was observed for yWT Cu^{II}ESOD (zinc site empty): complete reduction in less than 10 min.

DISCUSSION

Although none of the mutant CuZnSOD proteins studied had identical metal-binding behavior or redox reactivities, some common patterns did emerge. Most importantly, the ALS proteins studied showed marked differences in their metal binding properties relative to the WT proteins, particularly with respect to the properties of the native zinc site. This defect is likely related to the enzymatic gain of function that is causative in fALS.

One particularly striking type of behavior was seen for the ALS mutant protein yG85R, and similar behavior was seen for the non-ALS mutant yH63E. The apoproteins derived from these two mutants bound one equivalent of Co^{2+} per subunit, but subsequent addition of one equivalent per subunit of Cu^{2+} caused the Co^{2+} to adopt a greatly altered nontetrahedral geometry. The inability of these mutant proteins to bind Co^{2+} in a tetrahedral geometry in the zinc site was remedied when Cu^{2+} in the copper sites was reduced to Cu^{+}. Thus the $Cu^{I}Co^{II}$ derivatives of these mutant proteins bind Co^{2+} in a tetrahedral geometry similar to WT $Cu^{I}Co^{II}$SOD.

The observation that yH63A $Cu^{I}Co^{II}$SOD does not have a tetrahedral Co^{2+} site suggests to us that maintaining a tetrahedral Co^{2+} in the zinc site of these proteins depends upon the availability of a liganding residue in position 63 of the protein. For WT Cu^{II}CoSOD, that ligand is the imidazolate of His-63 that bridges the Cu^{2+} and Co^{2+} ions. Apparently yG85R Cu^{II}CoSOD cannot form the imidazolate bridge leaving His-63 bound only to Cu^{II}. For WT and yG85R Cu^{I}CoSOD, the copper ion becomes three-coordinate, and His-63 is released and thus made available to bind to Co^{2+} in the zinc site. These conclusions are summarized in Fig. 3. The carboxylate side chain of Glu-63 appears to substitute for the imidazole side chain of His-63 in allowing tetrahedral binding of Co^{2+} in yH63E Cu^{I}CoSOD; such behavior is not seen for yH63A, which lacks a liganding amino acid in position 63.

The pH dependence of the CuCo derivatives of yG85R and hH46R are also in sharp contrast to the pH-independent properties of WT CuCoSOD. However, this is not unexpected since a conformational change of the zinc site, resulting in the expulsion of the bound metal ion, has been proposed to occur for bovine CuZnSOD when the pH is lowered to 4.5 (34). It is thus possible that the ALS mutant proteins are undergoing a similar conformational change in the zinc binding site but at a higher pH.

Another common pattern we have observed for the ALS mutant proteins is the rapid rates of reduction by ascorbate. It has been noted in the past that WT Cu^{II}ZnSOD proteins are reduced remarkably slowly by ascorbate ion and other reducing agents unable to penetrate the active site channel of the enzyme (35). This slow rate of reduction by ascorbate and other cellular reducing agents may have functional significance since it enhances the specificity of these proteins for reduction by superoxide. It is likely that the rapid rate of reduction by ascorbate is related to the abnormality in zinc site metal binding since the zinc-free derivative yWT Cu^{II}ESOD shows similar behavior (see *Results*).

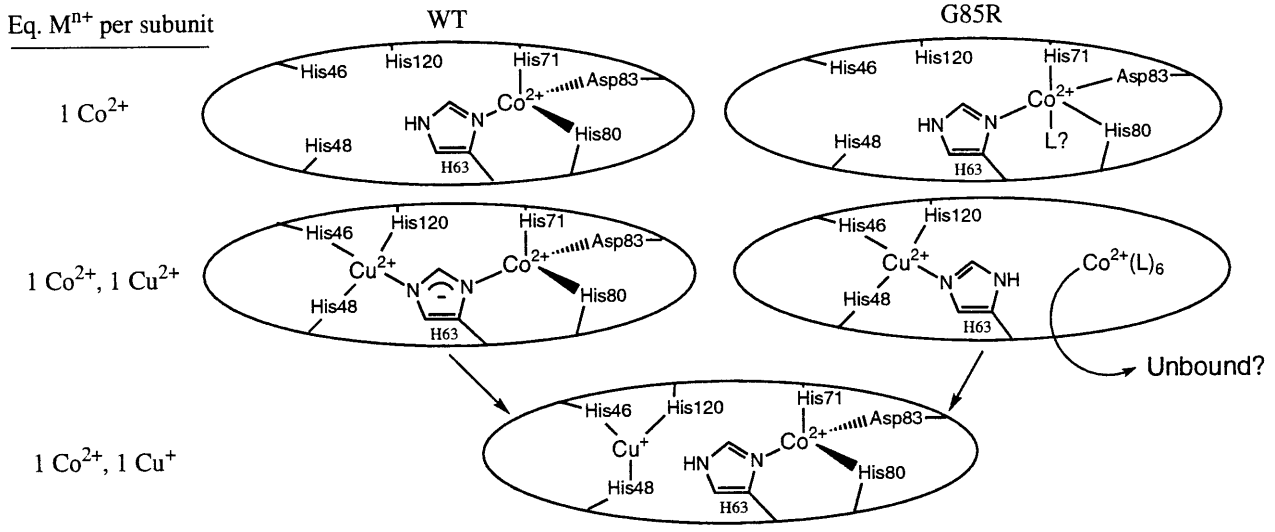

FIG. 3. Proposed metal binding scheme for copper- and cobalt-substituted derivatives of WT SOD and the fALS mutant yG85R.

There is now overwhelming evidence that the effect of the ALS mutations in CuZnSOD is to confer a new and toxic property on this enzyme (3, 5–8). One of the most puzzling aspects of this problem is determining how such a diverse set of mutations can result in the same gain of function. Our results demonstrate that this same diverse set of mutations results in similar disruptions of the metal-binding properties of the zinc site. For this reason, we believe that the structural changes that are associated with the altered properties of the zinc site are directly linked to the gain of function.

Multiple hypothetical mechanisms have recently been proposed as possible explanations for the function gained by ALS mutant CuZnSODs (8). These include (*i*) alterations in copper or zinc metabolism, (*ii*) catalysis of tyrosine nitration by peroxynitrite, (*iii*) catalysis of oxidation by hydrogen peroxide, (*iv*) alterations in oxidative homeostasis resulting in enhanced apoptosis, and (*v*) protein aggregation. Each of these mechanisms requires that the mutations cause a marked alteration in the reactivity of the WT enzyme. The structural changes associated with the alterations in the properties of the zinc site could produce such an alteration. For example, a change in the metal binding affinity of the zinc sites will undoubtedly alter the metal binding affinity of the copper site as well (36), almost certainly affecting copper or zinc metabolism. Moreover, the strict segregation whereby copper binds preferentially to the copper site and zinc to the zinc site may be disrupted resulting in catalytically active copper in the wrong site. The abnormally rapid reduction of the ALS mutant superoxide dismutases may also augment their reactivity as a catalyst of oxidations by hydrogen peroxide, since cellular reducing agent could increase the concentration of the Cu^{I} form and thus enhance its availability for Fenton-type chemistry (4). Interestingly, WT CuESOD also reacts quickly with ascorbate, suggesting a similarity between the ALS mutants and the Zn-deficient derivative. One possibility is that the mutations have decreased the affinity of the site for zinc, which may result in a loosening of the protein structure. Alternatively, they may sever the communication between the two metal sites, which normally limits the reactivity of the copper. Either way, a more open structure could make the copper ion more accessible to alternative substrates and/or the protein more prone to aggregation and precipitation (36, 37).

Previous studies have demonstrated that the zinc ion in CuZnSOD plays an important role in both its structure and enzymatic activity. For example, binding of zinc ions to the zinc site of the apoprotein causes the protein to adopt a native-like structure, even in the absence of Cu^{2+} (38), and to increase the overall stability of the protein (36). In addition, removal of zinc ions from WT CuZnSOD to form the zinc-free derivative, Cu^{II}ESOD, causes the SOD activity to become pH dependent (32).

In conclusion, these current studies have shown that the FALS mutations have dramatically altered both the metal binding and redox behavior of the human and yeast CuZnSOD. Further investigations are necessary to elucidate the link between the altered physical behavior and the disease itself.

Helpful discussions with Nancy L. Ogihara and P. John Hart are gratefully acknowledged. We also thank D.C. Ferris for his insightful editorial suggestions. This research was supported by grants to J.S.V. from the National Institutes of Health (GM28222 and DK46828), to E.B.G. from The ALS Association, and to D.E.B. from the ALS Association, the Joseph Drown Foundation, and the National Institutes of Health (AG12282 and NS25554).

1. Deng, H.-X., Hentati, A., Tainer, J. A., Iqbal, Z., Cayabyab, A., *et al.* (1993) *Science* **261,** 1047–1051.
2. Rosen, D. R., Siddique, T., Patterson, D., Figlewicz, D. A., Sapp, P., *et al.* (1993) *Nature (London)* **362,** 59–62.
3. Reaume, A. G., Elliott, J. L., Hoffman, E. K., Kowall, N. W., Ferrante, R. J., Siwek, D. F., Wilcox, H. M., Flood, D. G., Beal, M. F., Brown, R. H., Scott, R. W. & Snider, W. D. (1996) *Nat. Genet.* **13,** 43–47.
4. Wiedau-Pazos, M., Goto, J. J., Rabizadeh, S., Gralla, E. B., Roe, J. A., Lee, M. K., Valentine, J. S. & Bredesen, D. E. (1996) *Science* **271,** 515–518.
5. Rabizadeh, S., Gralla, E. B., Borchelt, D. R., Gwinn, R., Valentine, J. S., Sisodia, S., Wong, P., Lee, M., Hahn, H. & Bredesen, D. E. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 3024–3028.
6. Gurney, M. E., Cutting, F. B., Zhai, P., Doble, A., Taylor, C. P., Andrus, P. K. & Hall, E. D. (1996) *Ann. Neurol.* **39,** 147–165.
7. Gurney, M. E., Pu, H., Chiu, A. Y., Dal Canto, M. C., Polchow, C. Y., Alexander, D. D., Caliendo, J., Hentati, A., Kwon, Y. W., Deng, H.-X., Chen, W., Zhai, P., Sufit, R. L. & Siddique, T. (1994) *Science* **264,** 1772–1775.
8. Brown, R. H., Jr. (1995) *Curr. Opin. Neurobiol.* **5,** 841–846.
9. Nishida, C R., Gralla, E. B. & Valentine, J. S. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9906–9910.
10. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 488–492.
11. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. & Pease, L. R. (1989) *Gene* **77,** 51–59.
12. Valentine, J. S. & Pantoliano, M. W. (1981) in *Copper Proteins*, ed. Spiro, T. G. (Wiley, New York), pp. 291–358.
13. Bertini, I., Banci, L., Piccioli, M. & Luchinat, C. (1990) *Coord. Chem. Rev.* **100,** 67–103.
14. Donaire, A., Salgado, J., Jimenez, H. R. & Moratal, J. M. (1995) *NATO ASI Ser. Ser. C* **457,** 213–244.
15. Fee, J. A. & Briggs, R. G. (1975) *Biochim. Biophys. Acta* **400,** 439–450.
16. Djinovic, K., Gatti, G., Coda, A., Antolini, L., Pelosi, G., Desideri, A., Falconi, M., Marmocchi, F., Rotilio, G. & Bolognesi, M. (1992) *J. Mol. Biol.* **225,** 791–809.
17. Parge, H. E., Getzoff, E. D., Scandella, C. S., Hallewell, R. A. & Tainer, J. A. (1986) *J. Biol. Chem.* **261,** 16215–16218.
18. Pantoliano, M. W., Valentine, J. S. & Nafie, L. A. (1982) *J. Am. Chem. Soc.* **104,** 6310–6317.
19. Maret, W. & Vallee, B. L. (1993) *Methods Enzymol.* **226,** 52–71.
20. O'Neill, P., Fielden, E. M., Cocco, D., Rotilio, G. & Calabrese, L. (1982) *Biochem. J.* **205,** 181–187.
21. Ogihara, N. L., Parge, H. E., Hart, P. J., Weiss, M. S., Goto, J. J., Crane, B. R., Tsang, J., Slater, K., Roe, J. A., Valentine, J. S., Eisenberg, D. & Tainer, J. A. (1996) *Biochemistry* **35,** 2316–2321.
22. Merli, A., Rossi, G., Djinovic-Carugo, K., Bolognesi, M., Desideri, A. & Rotilio, G. (1995) *Biochem. Biophys. Res. Commun.* **210,** 1040–1044.
23. Djinovic, K., Coda, A., Antolini, L., Pelosi, G., Desideri, A., Falconi, M., Rotilio, G. & Bolognesi, M. (1992) *J. Mol. Biol.* **226,** 227–238.
24. Bertini, I., Luchinat, C., Piccioli, M., Oliver, M. V., & Viezzoli, M. S. (1991) *Eur. Biophys J* **20,** 269–279.
25. Bertini, I., Luchinat, C. & Monnanni, R. (1985) *J. Am. Chem. Soc.* **107,** 2178–2179.
26. Lindskog, S. (1970) *Struct. Bonding* **8,** 153–196.
27. Cupane, A., Leone, M., Militello, V., Stroppolo, M. E., Polticelli, F. & Desideri, A. (1995) *Biochemistry* **34,** 16313–16319.
28. Bertini, I., Luchinat, C. & Scozzafava, A. (1982) *Struct. Bonding* **48,** 45–92.
29. Dutton, T. J., Baumann, T. F. & Larrabee, J. A. (1990) *Inorg. Chem.* **29,** 2272–2278.
30. Moss, T. H. & Fee, J. A. (1975) *Biochem. Biophys. Res. Commun.* **66,** 799–808.
31. Graden, J. A., Ellerby, L. M., Roe, J. A. & Valentine, J. S. (1994) *J. Am. Chem. Soc.* **116,** 9743–9744.
32. Ellerby, L. M., Cabelli, D. E., Graden, J. A. & Valentine, J. S. (1996) *J. Am. Chem. Soc.* **118,** 6556–6561.
33. Pantoliano, M. W., McDonnell, P. J. & Valentine, J. S. (1979) *J. Am. Chem. Soc.* **101,** 6454–6456.
34. Pantoliano, M. W., Valentine, J. S., Mammone, R. J. & Scholler, D. M. (1982) *J. Am. Chem. Soc.* **104,** 1717–1723.
35. St. Clair, C. S., Gray, H. B. & Valentine, J. S. (1992) *Inorg. Chem.* **31,** 925–927.
36. Roe, J. A., Peoples, R., Scholler, D. M. & Valentine, J. S. (1990) *J. Am. Chem. Soc.* **112,** 1538–1545.
37. Winterbourn, C. C., Domigan, N. M. & Broom, J. K. (1995) *FEBS Lett.* **368,** 449–451.
38. Lippard, S. J., Burger, A. R., Ugurbil, K., Pantoliano, M. W. & Valentine, J. S. (1977) *Biochemistry* **16,** 1136–1141.