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Characterization of three yeast copper–zinc superoxide dismutase mutants analogous to those coded for in familial amyotrophic lateral sclerosis

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ABSTRACT **Sequences encoding three copper–zinc superoxide dismutase (CuZnSOD) mutant proteins analogous to those coded for in familial amyotrophic lateral sclerosis (fALS) were constructed in the *Saccharomyces cerevisiae* CuZnSOD gene and expressed in yeast lacking CuZnSOD (*sod1*$^-$). Gly^{85} → Arg CuZnSOD failed to rescue the oxygen-sensitive phenotype of *sod1*$^-$ yeast, but Gly^{93} → Ala CuZnSOD and Lys^{100} → Gly CuZnSOD were apparently fully functional *in vivo*. The Gly^{85} → Arg mutant protein was purified and its metal-binding properties and SOD activity were found to be significantly altered relative to wild type. The Gly^{93} → Ala CuZnSOD was likewise purified but, in contrast, demonstrated metal-binding comparable to wild type and activity 80% that of wild type. These results suggest that SOD activity of human fALS mutant CuZnSODs may vary considerably *in vivo*, with at least some of them retaining a considerable amount of activity. Alternative theories to increased free-radical damage should be considered in attempting to explain fALS.**

Amyotrophic lateral sclerosis (ALS), commonly termed Lou Gehrig's disease, is a neurological disease characterized by late onset, degeneration of motor neurons, and death, almost invariably within 6 years (1–3). Approximately 10% of cases are familial (fALS), and genetic linkage studies have therefore been possible. A recent collaborative effort succeeded in tracing the fALS locus to several single point mutations in the gene (*SOD1*) coding for copper–zinc superoxide dismutase (CuZnSOD) (4), providing a means of directly studying the defective gene products associated with fALS.

CuZnSOD (5–8) catalyzes the disproportionation of superoxide anion, O_2^-, to give O_2 and H_2O_2, and it has been demonstrated to play a role in protecting cells against oxygen toxicity (9, 10). CuZnSOD also acts as a major repository for copper ions within virtually all eukaryotic cells, although no role in copper metabolism has been explicitly demonstrated for it. The crystal structure of CuZnSOD is known, and the fALS mutations are localized in regions that have been predicted to affect the stability of the protein (11). It is tempting to jump to the conclusion that loss of SOD activity is causing the disease. However, little is known of mechanisms which would explain how the mutant SODs might cause the accelerated death of motor neurons which is the primary characteristic of the disease.

We report here the results of biological characterization of three fALS proteins expressed in yeast and of physical characterization of the purified proteins from two of the mutants. Our results indicate that decreased SOD activity may not be the determining factor in fALS.

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MATERIALS AND METHODS

DNA Manipulations. fALS mutant yeast CuZnSOD genes were created by site-directed mutagenesis on pBSIIKS(−)-SOD0.5 (12). For expression of SOD in yeast, pBSIIKS(+)-SOD1.1Nco1 (13) was digested with *Eco*RI/*Bam*HI to generate a 1.1-kb fragment containing the wild-type CuZnSOD gene with an *Nco* I site at the translation start site. This was ligated into the *Eco*RI/*Bam*HI sites of YEp351 (14), a yeast multicopy shuttle vector containing a *LEU2* selectable marker, to make YEp600. The mutated genes were removed from plasmids pBSIIKS(−)-SOD0.5-G85R, pBSIIKS(−)-SOD0.5-G93A, and pBSIIKS(−)-SOD0.5-K100G by digestion with *Nco* I and *Sal* I and were ligated into similarly digested YEp600, thus replacing the wild-type coding sequence with the mutant one, to make YEp604, YEp605, and YEp606, respectively. These plasmids and YEp351 were transformed into yeast strain EGy118 (*MATa, leu2-2,11 his3Δ1 trp1-289a ura3-52 sod1ΔA::URA3*) (15), which lacks CuZnSOD, and cell lines expressing the wild-type or mutant CuZnSOD were isolated.

Oxygen and Paraquat Sensitivity Analyses. Yeast transformed with control plasmid or plasmids carrying the *SOD1* gene with the various fALS mutations were streaked on SD − leu plates and grown for 3 days at 30°C. For oxygen growth cells were streaked on plates that were placed in a desiccator filled with 100% O_2. Paraquat sensitivity was measured by streaking on freshly prepared plates containing various amounts of paraquat.

SOD Activity and Protein Levels *in Vivo*. Yeast crude cell extracts of soluble proteins were prepared by glass-bead lysis (16). SOD activity was measured by the 6-hydroxydopamine assay (17), and the level of CuZnSOD protein was estimated from Western immunoblots of equal amounts of crude cell extract.

Expression of Gly^{85} → Arg (G85R) and Gly^{93} → Ala (G93A) CuZnSOD Mutants in *Escherichia coli*. CuZnSOD mutants were prepared as described (12). Briefly, pBluescript II KS(−) (Stratagene) containing the SOD gene served as the template for oligonucleotide-directed mutagenesis (18). Oligonucleotides were synthesized on a Pharmacia LKB Gene Assembler Plus. Following mutagenesis and screening, the SOD gene was subcloned into pET-3d for expression using the bacteriophage T7 RNA polymerase system in *E. coli* BL21(DE3) (19). Each 10-liter culture yielded ≈50 mg of pure CuZnSOD mutant protein after glass-bead lysis and column chromatography on Sepharose CL-6B anion-exchange medium (Pharmacia) and Sephadex G-75 gel filtration medium (Pharmacia).

Preparation of Apoprotein and Metallated Derivatives. G85R CuZnSOD and metal-substituted derivatives were prepared by procedures developed previously for native CuZn-

Abbreviations: fALS, familial amyotrophic lateral sclerosis; SOD, superoxide dismutase.

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SODs (12, 20). Apoprotein was prepared by sequential dialysis against 0.5 M sodium acetate containing 100 mM EDTA (pH 3.8), 0.5 M sodium acetate containing 1 M NaCl (pH 3.8), and 0.5 M sodium acetate (pH 5.5). Metallated derivatives were made by addition of solutions of metal sulfate salts; typically, 150 μl of 0.25 mM apoprotein was titrated with 7.5 mM metal. Protein concentrations were 0.25 mM for the G85R mutant, 0.27 mM (CuZn, CuCu) or 0.18 mM (CuCo) for the G93A mutant, and 0.3 mM (CuZn and CuCu) or 0.2 mM (CuCo) for the wild-type protein. Spectra were obtained on a Cary 3 spectrophotometer (Varian).

EDTA Treatment of CuZnSOD. Solutions (5 μM) of wild-type or G85R protein containing 0.1% bovine plasma γ-globulin in 50 mM sodium phosphate (pH 7.8) were incubated 5 hr at 25°C with the indicated concentration of EDTA and then assayed by the 6-hydroxydopamine method as above.

RESULTS

In order to study the structural and physiological basis for the causation of fALS by mutant CuZnSODs, we engineered point mutations in yeast CuZnSOD that are analogous to the human fALS mutations. Structurally, the human and yeast enzymes are extremely similar, having 54% sequence identity and very similar crystal structures (21, 22). We used the yeast system because the availability of the gene (16) and of yeast cell lines lacking CuZnSOD (*sod1*$^-$) in our lab (15) provides the means not only to characterize purified mutant protein but also to observe *in vivo* the effects of mutant proteins on the native host in a simple system.

Two mutations, G85R and G93A, were chosen in regions where yeast and human CuZnSOD have nearly identical structures. Both residues are conserved in almost all known CuZnSODs (23). Gly^{85} is involved in hydrogen bonding to another β-strand and in forming the β-bulge which holds the zinc-binding Asp^{83} in position. Substitution of the bulky, positively charged arginine side chain at this position, which is in close proximity to the zinc-binding site, was anticipated to disrupt the wild-type structure in a significant fashion. Gly^{93} is located at the tight turn of a β-strand. Substitution of an alanine side chain at this position was anticipated to destroy the β-strand turn uniquely provided by glycine and thus also significantly disrupt the structure. The Lys^{100} → Gly (K100G) mutant was also constructed. This residue is not itself conserved between yeast and human wild-type proteins (it is Glu in human CuZnSOD), but the structure of the region is conserved (21, 22), and we expected that substitution of a glycine for this residue would introduce flexibility into this region of either the yeast or the human protein.

Biological Function of the fALS SOD Mutant Proteins. The yeast fALS *SOD1* mutant genes were expressed in yeast lacking CuZnSOD (*sod1*$^-$) in order to test the ability of the mutant CuZnSODs to provide SOD activity in the native host. Expression plasmids containing wild-type or mutant CuZnSOD genes under the control of the native CuZnSOD promoter were constructed and transformed into *sod1*$^-$ yeast and tested for oxygen sensitivity, paraquat sensitivity, and growth in air (see *Materials and Methods*). The vector control—like the parent strain EGy118, lacking CuZnSOD—is extremely sensitive to oxygen, grows slowly in air, and exhibits several other distinct phenotypes (9, 15) (Table 1). Transformation with the plasmid containing the wild-type gene (YEp600) restored resistance to both oxygen and paraquat, as well as growth in air. Surprisingly, the G93A and K100G CuZnSOD mutants were able to rescue completely the *sod1*$^-$ yeast for growth under oxidative conditions, whereas the G85R mutant protein was not.

SOD activity was measured in soluble protein extracts for each of the strains. Little or no SOD activity was found in the strain carrying the G85R mutant protein. By contrast, a significant level of SOD activity was found in the strains expressing the G93A and K100G proteins, although the wild-type strain showed a higher level of activity (see Table 1). The vector-only control showed no activity. (The gene for the mitochondrial MnSOD is present in these strains but is not highly expressed when cells are grown in dextrose, as they were in this case.) To determine whether the lack of SOD activity was due to the absence of the mutant CuZnSOD protein, Western immunoblot analysis was carried out (data not shown). This analysis demonstrated that the concentrations of the G85R, G93A, and K100G mutant proteins were similar, although somewhat reduced relative to the level of the wild-type CuZnSOD protein in the strain carrying the wild-type CuZnSOD gene. That the CuZnSOD protein levels in the three mutant strains were not noticeably different from one another indicates that the loss of SOD activity in the G85R strain was due to the presence of an inactive CuZnSOD protein rather than to the total absence of the protein. We concluded, therefore, that the SOD activities of the mutant CuZnSOD proteins were markedly different—i.e., that the G85R protein had a significantly lower SOD activity than G93A or K100G CuZnSOD. The same pattern of rescue and SOD activity was also observed in strains which had only a single copy of either the G85R, G93A, or K100G mutant CuZnSOD genes integrated in the chromosome (data not shown).

Physical Characterization of Purified fALS Mutant Proteins. To investigate the markedly different abilities of G85R and G93A CuZnSODs to function *in vivo*, we purified these proteins to homogeneity (12) in order to characterize their SOD activity, thermal stability, and spectroscopic and metal-binding properties *in vitro*. We studied the changes the mutations had on the distinctive and well-studied spectroscopy of the wild-type SOD. We suspected that the metal-binding characteristics of the G85R mutant protein might be significantly altered by the mutation because of the proximity of Gly^{85} to the zinc-binding ligand Asp^{83}.

The UV–visible spectra of wild-type and G85R Cu_2Zn_2SODs are compared in Fig. 1 *A* and *B*. The strong similarity between the spectra of the two proteins suggests that the metal-binding regions are very similar in geometry. The shoulder at 450 nm has previously been assigned in native CuZnSODs to an imidazolate-to-copper charge-transfer transition (24), and its presence in the spectrum of G85R Cu_2Zn_2SOD suggests that the imidazolate bridge that links the copper and zinc ions in the native protein is present in the G85R mutant protein as well.

Table 1. Sensitivity to oxidative stress, SOD activity, and CuZnSOD levels of *sod1*$^-$ yeast transformed with yeast fALS plasmids

Mutation	Plasmid	O_2 growth	Paraquat sensitivity,* μM	SOD activity, units/mg of protein	CuZnSOD protein level
(No SOD)	YEp351	−	10	0.0	−
Wild type	YEp600	++	1250	0.65	++
G85R	YEp604	−	10	0.02	+
G93A	YEp605	++	1250	0.15	+
K100G	YEp606	++	1250	0.16	+

*The indicated concentration is the highest at which the strain would grow.

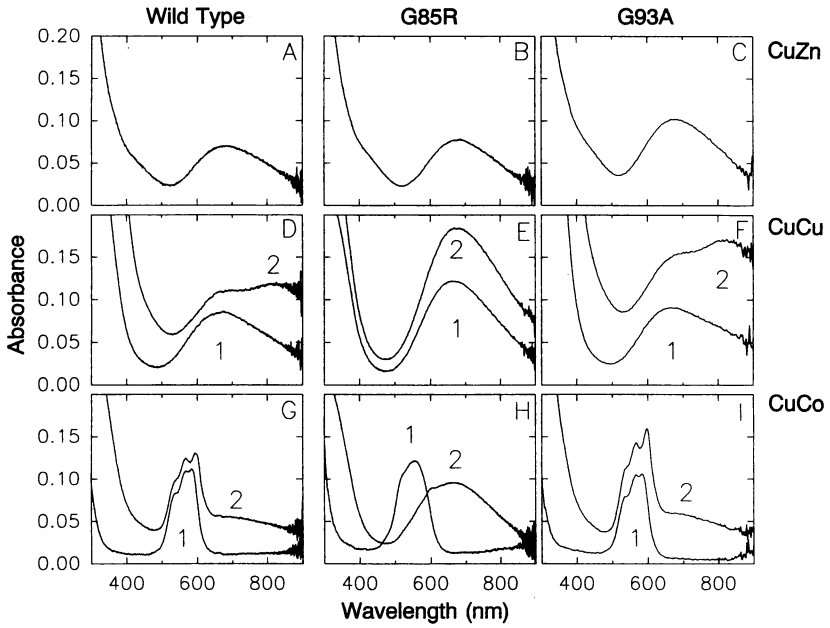

FIG. 1. Electronic absorption spectra of wild-type and mutant CuZnSODs. (*A–C*) Apoprotein plus 2 equivalents (per dimer) of Zn^{2+}, yielding E_2Zn_2 (E, empty) for wild type, followed by 2 equivalents of Cu^{2+} to give Cu_2Zn_2. (*D–F*) Apoprotein plus 2 equivalents of Cu^{2+} (scans 1) giving Cu_2E_2 for wild type, followed by another 2 equivalents (scans 2), which gives Cu_2Cu_2 for wild type. (*G–I*) Apoprotein plus 2 equivalents of Co^{2+} (scans 1), yielding E_2Co_2 for wild type, followed by 2 equivalents of Cu^{2+} (scans 2), giving Cu_2Co_2 for wild type.

Whereas the Cu_2Zn_2 derivative of G85R CuZnSOD appeared to behave similarly to native CuZnSODs, substitution of other metal ions in place of Zn^{2+} gave quite different results. Gradual addition of two equivalents of Cu^{2+} to G85R CuZnSOD apoprotein produced a *d–d* absorption band at about 670 nm, characteristic of Cu^{2+} in the distorted square planar environment of the copper sites, similar to wild type (compare Fig. 1 *D* and *E*, scans 1). However, addition of two more equivalents of Cu^{2+} did not produce the 810-nm band characteristic of the Cu_2Cu_2 derivative of native CuZnSOD (Fig. 1*D*, scan 2) that has been assigned to the *d–d* band of Cu^{2+} in a distorted tetrahedral zinc site. Instead, a further increase in the *d–d* band, with a modest redshift to about 700 nm, was observed for addition of a third and fourth equivalent of Cu^{2+} to G85R CuZnSOD apoprotein (Fig. 1*E*, scan 2). This result indicates that the metal-binding properties of the zinc site of G85R CuZnSOD have been changed substantially by the mutation and that this site is consequently able to adjust to the more nearly tetragonal geometry preferred by Cu^{2+}.

Addition of two equivalents of Co^{2+} to G85R CuZnSOD apoprotein gave a visible spectrum that is virtually identical to that of the E_2Co_2 (E, empty) derivative of the native protein (compare Fig. 1 *G* and *H*, scans 1). This type of spectrum is characteristic of Co^{2+} in a tetrahedral site and is found for many cobalt-substituted zinc proteins (25). Prior addition of Zn^{2+} prevented Co^{2+} binding (data not shown), suggesting that both metals bind at the native zinc-binding site. Ordinarily, addition of two equivalents of Cu^{2+} to E_2Co_2 derivatives of native CuZnSOD results in formation of Cu_2Co_2 derivatives whose visible spectrum represents a superposition of the spectra of the Co^{2+} which remains in the tetrahedral zinc site and the ≈700-nm *d–d* band due to Cu^{2+} in the copper site (Fig. 1*G*, scan 2) (24). However, addition of Cu^{2+} to the Co_2 derivative of G85R CuZnSOD resulted in loss of the spectral band due to tetrahedral Co^{2+} concomitant with the appearance of the spectral band characteristic of Cu^{2+} bound to the protein at the copper site, suggesting either that the Co^{2+} ion is released from the protein upon copper binding or that its coordination geometry is changed to one that gives very weak absorption in the visible spectrum—e.g., octahedral (25). This result also indicates that the metal-binding properties of the zinc site of the G85R CuZnSOD protein have been substantially changed by the mutation.

In sharp contrast to the G85R mutant, the G93A mutant protein bound metals in a manner indistinguishable from wild type. Fig. 1 (*C*, *F*, and *I*) reveals the close similarity between the Cu_2Zn_2 and Cu_2Cu_2 forms of G93A and wild type.

SOD Activity of Purified Protein. We assayed the activity of G85R CuZnSOD, using two different SOD assays at pH 7.4 (6-hydroxydopamine assay) (17) and pH 7.8 (cytochrome *c* assay) (26). As isolated from our expression system, G85R CuZnSOD, unlike wild-type CuZnSOD, did not contain its full complement of Cu^{2+} and Zn^{2+} and had a SOD activity about 20% of that of the native protein. Reconstituted (fully metallated) G85R Cu_2Zn_2SOD, prepared from the apoprotein, possessed 40% of the SOD activity of native Cu_2Zn_2SOD. Because of the apparent alteration of the metal-binding properties of G85R CuZnSOD, we investigated the effect on the SOD activity of adding various concentrations of the metal chelator EDTA (Fig. 2). We found that reconstituted G85R Cu_2Zn_2SOD began losing activity with <1 equivalent of EDTA added, whereas native Cu_2Zn_2SOD tolerated a >2000-fold excess at pH 7.8. This extreme

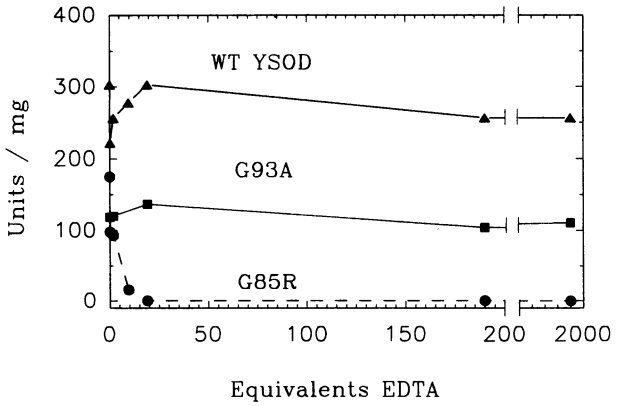

FIG. 2. Effect of EDTA on SOD activity of wild-type (wt) and mutant CuZnSODs.

sensitivity to EDTA testifies further to the altered metal-binding properties of G85R CuZnSOD. We also carried out a SOD assay at pH 10.2 (27) and found that reconstituted G85R Cu_2Zn_2SOD, unlike the native protein, was rapidly inactivated at that pH (data not shown).

Freshly prepared, reconstituted G93A Cu_2Zn_2SOD possessed 80% of the native activity. Consistent with its spectroscopic profile, G93A Cu_2Zn_2SOD, like the native wild-type enzyme, retained SOD activity even in the presence of a 2000-fold excess of EDTA.

DISCUSSION

The prediction has been made, based on the x-ray crystal structure of the human CuZnSOD protein, that the fALS mutations may be destabilizing the protein, resulting in lowered SOD activity in persons carrying one of these genes. In support of this prediction, CuZnSOD isolated from red cells of fALS patients by using the relatively harsh conditions that are commonly used for wild-type CuZnSODs gave preparations with substantially reduced SOD activities relative to wild type (11). Our yeast system enables us to assess the ability of the yeast fALS mutant SODs to function in an *in vivo* system without exposing the protein to the harsh conditions of protein isolation. We then can compare these properties *in vivo* with those of the isolated, purified protein determined *in vitro*.

Our results indicate that fALS mutations of yeast CuZnSOD exhibit markedly different behavior with respect to their *in vivo* and *in vitro* properties. We find that the G85R protein is highly susceptible to inactivation by various processes, suggesting that certain procedures used routinely for the highly stable native CuZnSODs, such as isolation of native CuZnSODs from red-cell extracts by organic solvent extraction (28) or assaying SOD activity at high pH, may be too harsh for the fALS mutant CuZnSODs, which are probably considerably less robust than the native proteins.

The G85R mutation clearly resulted in a structurally altered CuZnSOD protein, as was previously predicted from the x-ray structure (11). However, the protein was stable enough to be expressed and purified in high yield and retained considerable SOD activity when fully metallated. On the other hand, expression of the G85R mutant protein under physiological conditions in its native host did not rescue *sod1*$^-$ yeast. The defect in its *in vivo* activity and the partial defect in its *in vitro* activity may well be due to its altered metal-binding abilities.

The G93A mutant, however, was spectroscopically indistinguishable from wild type and, like wild type, successfully restored the SOD phenotype to *sod1*$^-$ yeast. The fully metallated form possessed 80% of the activity of wild type.

The most obvious possible explanation for the association of mutations in CuZnSOD with fALS is that fALS is caused in some fashion by lowered SOD activity (11), and, in support of this hypothesis, we find that the SOD activities measured in crude extracts of all three of our strains are somewhat reduced relative to strains containing the wild-type gene. However, only one of the mutations, G85R, failed to reverse the *sod1*$^-$ phenotype *in vivo*. By contrast, the other two mutant SODs, G93A and K100G, reversed the oxygen-sensitive *sod1*$^-$ phenotype *in vivo*, and significant levels of SOD activity could be measured in their cell extracts. Results similar to those observed for G93A and K100G have also been obtained with four human fALS SODs expressed in yeast (data not shown), indicating that the effects of the mutations on the properties of the human and yeast fALS SODs are likely to prove similar. These results suggest that some of the human fALS mutant CuZnSODs probably retain significant levels of SOD activity *in vivo* and that it is therefore premature to conclude that fALS is caused simply by a large decrease in SOD activity or, indeed, that SOD activity is always reduced in cases of fALS.

When expressed in yeast, the genes for the two active mutants that we have studied, G93A and K100G, attain a lower level of SOD protein (and thus SOD activity) than does the wild-type gene. In yeast this level is apparently quite sufficient to provide normal antioxidant protection; in human motor neurons it may not be. We thus cannot rule out the possibility that a small decrease in the level of SOD activity plays a key role in fALS. It is likely in the case of G85R that altered metal-binding leads to a loss or lowering of SOD activity and the failure of this mutant to function *in vivo*. However, the other two mutants have a high degree of SOD activity, and yet they also carry the characteristic(s) that cause fALS. We have not yet found conditions that distinguish the SOD-active fALS mutants from wild type physiologically.

We have not been able to identify any physiological effect of the coexpression of fALS mutants and wild-type CuZnSOD in yeast, a situation that more closely approximates the conditions found in fALS patients who carry a wild-type CuZnSOD gene in addition to the mutant gene. Such strains are phenotypically indistinguishable from wild type, so far as we have been able to determine, with respect to their growth rates and resistance to 100% O_2 or to paraquat. It is our hope that full characterization of the various fALS mutants will help to identify the disease-causing aspect(s) of these mutant proteins.

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