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# Gain in Functions of Mutant Cu,Zn-Superoxide Dismutases as a Causative Factor in Familial Amyotrophic Lateral Sclerosis: Less Reactive Oxidant Formation but High Spontaneous Aggregation and Precipitation

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Eight mutant Cu,Zn-superoxide dismutases (SODs) related to familial amyotrophic lateral sclerosis (FALS) were produced in a baculovirus/insect cell expression system and their molecular properties in terms of hydroxyl radical formation and aggregation were compared with the wild-type enzyme. Treatment of the enzymes with Chelex 100 resin decreased Cu contents as well as SOD activities in all mutant Cu,Zn-SODs, indicating that the affinities of the enzymes for copper ion were decreased. Contrary to previous reports, all the mutant Cu,Zn-SODs exhibited less reactive oxidant producing ability in the presence of hydrogen peroxide than the wild-type enzyme. Both SOD activities and their reactive oxidant forming correlated well with the copper ion content of the molecules. In addition, the proteins spontaneously aggregated and were precipitated by simple centrifugation at 12,000*g* for 20 min in keeping their enzyme activities. Since hyaline inclusions found in FALS patients with SOD1 mutations contained components which were reactive to anti-Cu,Zn-SOD antibody, a primary reaction caused by mutant SOD1 may be attributed to their propensity to form aggregates. Aggregated but still active mutant SOD1 would be expected to mediate the formation of reactive oxygen species and nitrosylation in a more condensed state.

*Keywords:* Baculovirus, gain of function, hydroxyl radicals, copper toxicity, protein aggregation

## INTRODUCTION

Superoxide dismutase (SOD; EC 1.15.1.1) is an antioxidative enzyme which scavenges superoxide radicals in the cytoplasm of most mammalian cells.[1] Of the three SOD isozymes, Cu,Zn-SOD is present in the cytoplasm as a dimer with the copper and zinc ions functioning as catalysis and as a part of the protein architecture, respectively. Since defects in the gene which encodes Cu,Zn-SOD (SOD1) have been identified in familial

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amyotrophic lateral sclerosis (FALS),[2] a fatal motor neuron disease, more than 50 additional mutations linked to FALS have been found in 10–15% of the patients.[3]

When mutations were initially discovered in SOD1, it was commonly thought that the elevation in levels of reactive oxygen species (ROS), as a result of the loss of SOD activities, were involved in the process of developing ALS.[4] However, at present, it is now believed that this disease is the result of gain of function and not the loss of SOD activity of these mutant enzymes. This is because mutant SODs have activities which are equivalent to the wild-type enzyme[3,5] and transgenic mice carrying mutant human SOD1,[6,7] which have higher SOD activity than control mice due to the extra genes, develop symptoms which are characteristic of ALS. In addition, SOD1-knockout mice do not exhibit motor neuron dysfunction.[8] Several mechanisms, such as the production of ROS,[9–14] metal toxicity,[15–17] interaction with reactive nitrogen species,[15,18] and protein aggregation,[19–21] have been proposed as possible candidates for the reason for the gain in function. Although the production of reactive oxidant is a known function of wild-type Cu,Zn-SOD,[22,23] the enhanced production of ROS has also been reported for purified mutant enzymes,[9–12] cells transfected mutant gene,[9,13] and transgenic mice.[14]

Our previous papers[5,24] reported on a baculovirus expression system in which a large amount of wild-type and three mutant Cu,Zn-SODs with characteristics similar to human enzymes are produced and preliminary characterization of these enzymes. Here we report further analyses of the enzymatic properties of eight mutant Cu,Zn-SODs including the potency of reactive oxidant production and the tendency to form aggregates in comparison to the wild-type enzyme. Our data are not consistent with the observation that the mutant enzymes are capable of producing more reactive oxidant than the wild-type enzyme.[9–12] However, we cannot rule out the possible involvement of aggregated enzymes in the acceleration of oxidation and nitrotyrosine formation at aggregated locations in motor neurons.

## MATERIALS AND METHODS

### Materials

5,5-Dimethyl-1-pyrroline N-oxide (DMPO) was purchased from Dojindo Laboratories. 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy (4-hydroxy-TEMPOL) were purchased from Aldrich. All other reagents were of the highest grade available.

### Site-Directed Mutagenesis and Expression of Mutant Cu,Zn-SODs

Since the previous papers,[5,24] three new mutant Cu,Zn-SOD cDNAs, designated as G37R, G41S, and I113T, were constructed by site-directed mutagenesis[25] using a uracil template as described previously.[26] The H46R mutant was a generous gift from Prof. Joseph S. Beckmann, University of Alabama at Birmingham. Mutations of the coding sequences were confirmed by the dideoxy chain termination method[27] using Sequenase ver. 2.0 (USB). The DNA fragments were ligated back to a transfer vector pVL1393 (Invitrogen). After purification by cesium chloride ultracentrifugation, 1 μg of the purified plasmid DNA was cotransfected with baculovirus DNA (100 ng Baculogold, Pharmingen) into Sf21 cells using Lipofectin (Gibco/BRL). After 4–5 days of incubation, the viral supernatant was harvested from the cells, and the recombinant viruses were then amplified on a large scale.

### Overproduction of the Cu,Zn-SODs in Sf21 Cells

The culture of Sf21 cells and manipulation of baculovirus were performed according to the

published protocols.[28] For protein production Sf21 cells ($10^6$) in a 10-cm dish were infected with baculovirus carrying Cu,Zn-SOD cDNA at a multiplicity of infection of 10 after which they were incubated for 3 days. $CuSO_4$ and $ZnSO_4$ dissolved in water and sterilized by filtration through Dismic filters (Advantec) were added directly into the medium to a final concentration of 1 mM after viral infection.

### Purification of Cu,Zn-SOD from Infected Sf21 Cells

The purification procedures used have essentially been described previously[5] with some modifications. Harvested cells were washed with PBS three times and lysed in hypotonic buffer containing 2.5 mM potassium phosphate, pH 7.4, 1 mM $CuSO_4$, 1 mM $ZnSO_4$, 1 mM benzamidine, and 0.1 mM (p-amidinophenyl)methanesulfonyl fluoride hydrochloride (Wako) on ice. The cells were homogenized in a Dounce homogenizer, and centrifuged at 100,000*g* for 1 h at 4°C to precipitate insoluble materials. The supernatant was loaded onto a DE52 ion-exchange column (Whatman) preequilibrated with 2.5 mM potassium phosphate, pH 7.4. After washing with 10 volumes of the same buffer, the bound proteins were eluted with a linear gradient of potassium phosphate from 2.5 to 200 mM. To further purify the enzyme, gel-filtration column chromatography on AcA54 (IBF Biotechnics) preequilibrated with 100 mM NaCl and 10 mM potassium phosphate, pH 7.4, was performed. The Cu,Zn-SODs were treated with 100 μM $CuSO_4$ and 100 μM $ZnSO_4$ overnight at 4°C to saturate the copper and zinc sites of the enzyme. The mixture was dialyzed against 10 mM potassium phosphate, pH 7.4, which was pretreated with Chelex 100 resin (Bio Rad), overnight at 4°C to remove free copper and zinc ions. The concentration of purified Cu,Zn-SOD was determined spectrophotometrically using the molar extinction coefficient $A_{280} = 1.74 \times 10^4\,M^{-1}\,cm^{-1}$.

### Quantification of Copper Ions

In most experiments, free and loosely bound copper ions were removed from the enzyme by treatment with Chelex 100 resin. The levels of bound copper ions in the Cu,Zn-SOD was determined by polarized Zeeman atomic absorption spectrophotometer (Hitachi Z-8000). Ten μl of each sample and standard solution was directly loaded into a cuvette, and copper was determined by absorption measurement at 324.8 nm.

### Assay of SOD Activity

The SOD activity of the purified protein was assayed using the xanthine–xanthine oxidase/nitroblue tetrazolium method.[29] Each data point represents the mean and standard deviation for triplicate experiments.

### Detection of DMPO–OH by a EPR Spectroscopy

In the presence of DMPO, the reaction of Cu, Zn-SOD with $H_2O_2$ produces a stable radical adduct, DMPO–OH, which can be determined by EPR spectroscopy. All buffer and water used in this experiment were treated with Chelex 100 resin to remove any trace metals before use. Reactions were initiated by the addition of $H_2O_2$ to the incubation mixtures (total volume of 100 μl) containing wild-type or mutant SODs (1 μM) and DMPO (100 mM) in 25 mM $NaHCO_3/CO_2$ buffer, pH 7.4. Spectral acquisitions began 60 s after initiation of the reaction. The EPR spectra were measured at room temperature using a Bruker ESP300 instrument under the following conditions: microwave power, 19 mW; modulation frequency, 100 kHz; modulation amplitude, 1.035 G; conversion time, 10.24 ms; time constant, 10.24 ms; sweep time, 21 s; sweep width, 100 G with 1024-point resolution. Quantification of the spin adducts were carried out by double integration of the data using 4-hydroxy-TEMPOL as a standard.

### Evaluation of Aggregated SODs

Stored enzymes were centrifuged at 12,000*g* for 20 min at 4°C to remove aggregated SODs before use. The supernatants were supplemented with $CuSO_4$, gently shaken overnight, and then centrifuged at 12,000*g* for 20 min at 4°C to precipitate the SOD aggregates. The protein content of the supernatants were determined by measuring the absorbance at 280 nm. The amounts of aggregated SODs were calculated by subtracting the soluble fraction from total amounts of the proteins.

## RESULTS

### Impaired Cu Binding Abilities and Loss of SOD Activities in Mutant Cu,Zn-SODs

In this work, eight mutant Cu,Zn-SODs were produced, which were found in FALS, as well as the wild-type enzyme in a baculovirus/insect cell system and purified as described previously.[5,24] The proteins, which were more than 98% pure (data not shown), were saturated with copper and zinc ions by incubation with 100 μM $CuSO_4$ and 100 μM $ZnSO_4$ overnight, and then dialyzed to remove free metal ions. When their SOD activities and Cu contents were measured, all but the H46R mutant, which has an amino acid substitution in one of Cu chelating His residues, exhibited nearly equal SOD activities and Cu contents to those of the wild-type enzyme (Table I). When the enzymes were treated with Chelex 100 resin to remove free and loosely bound metal ions in the preparation, levels of the Cu ions bound to the mutant proteins were decreased to various extents. However, essentially no change was observed for the wild-type, indicating that metal binding affinities of the mutant enzymes were impaired due to the single amino acid substitution in the proteins. By this treatment, SOD activities of the mutant enzymes were also decreased and the extents of decrease were correlated with the copper contents.

### DMPO-OH Forming Abilities Correlate with SOD Activities and Cu Contents

Cu,Zn-SOD reacts with $H_2O_2$ to form DMPO–OH[22,23,30] and this ability is reported to be enhanced in the case of mutant enzymes.[9–12] Since the reactive oxidant forming activity has been examined for only few mutant enzymes thus far, we examined this issue for eight mutant enzymes, which were treated with Chelex 100 resin before use, and compared their characteristics with the wild-type enzyme. Figure 1 shows typical time courses for the formation of DMPO–OH obtained by the incubation of purified A4T, G37R, G85R, and wild type enzymes

TABLE I SOD activities of and copper contents in wild-type and mutant SODs before and after treatment with Chelex resin. Data are shown as means of triplicate assays

| Enzyme | Before chelex column | | After chelex column | |
|---|---|---|---|---|
| | Bound Cu (mol/mol SOD) | SOD activity ($\times 10^2$ U/mg) | Bound Cu (mol/mol SOD) | SOD activity ($\times 10^2$ U/mg) |
| WT | 1.00 | 76.1 | 0.99 | 75.1 |
| A4T | 1.00 | 75.8 | 0.48 | 31.1 |
| G37R | 1.00 | 77.9 | 0.17 | 12.1 |
| G41D | 1.00 | 73.9 | 0.48 | 43.4 |
| G41S | 1.00 | 76.1 | 0.86 | 67.1 |
| H43R | 1.00 | 74.3 | 0.57 | 42.4 |
| H46R | 0.38 | 9.4 | 0.0 | 0.1 |
| G85R | 0.99 | 75.1 | 0.14 | 2.3 |
| I113T | 0.99 | 74.2 | 0.73 | 48.7 |

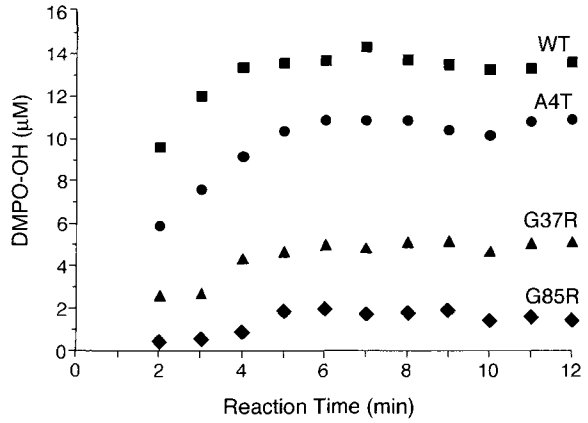


FIGURE 1 Time course for the formation of DMPO–OH during the incubation of wild-type and three mutant SODs with $H_2O_2$. Reactions were initiated by the addition of 40 mM $H_2O_2$ to the incubation mixtures containing wild-type and mutant SODs (1 μM each) and DMPO (100 mM). DMPO–OH concentrations were calculated by double integration of the specific signals using TEMPOL as a standard.

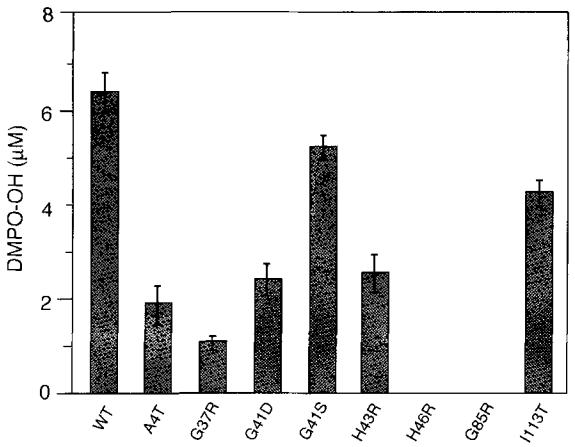


FIGURE 2 Abilities of the wild-type and mutant SODs to form DMPO–OH. All Cu,Zn-SODs were treated with Chelex 100 resin prior to use. The amounts of DMPO–OH produced by the enzymes were quantified at 2 min after $H_2O_2$ addition. Data are shown as means and the standard deviation of triplicate assays.

with 40 mM $H_2O_2$ in the presence of 100 mM DMPO and quantified by double integration of the data using TEMPOL as a standard. As reported by Sato *et al.*,[30] DMPO–OH formation was markedly affected by the buffer used, and, hence, we used bicarbonate buffer in this assay as recommended. The amounts of DMPO–OH increased in a linear fashion within the initial few minutes and then reached a plateau after 6 min. This experiment was also carried out for the other mutant enzymes and the amounts of DMPO–OH produced during the initial 2 min were compared with the wild-type enzyme (Figure 2). The data showed that the DMPO–OH forming abilities of all enzymes were lower than the wild-type enzyme and was in good agreement with their SOD activities.

## Kinetic Parameters for DMPO–OH Formation by Mutant Cu,Zn-SODs

In previous reports,[10,11] higher $V_{max}$ values and lower $K_m$ values for DMPO–OH formation from $H_2O_2$ were reported for the mutant SODs than for the wild-type enzyme. To examine the kinetic parameters for the mutant enzymes,

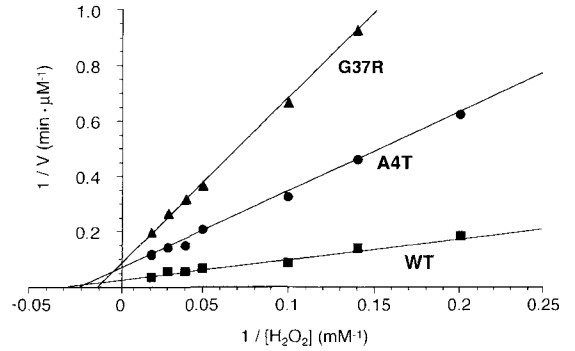


FIGURE 3 Double reciprocal plots of DMPO–OH formation rates versus $H_2O_2$ concentrations. Rates of DMPO–OH formation by the reaction of mutant and wild-type SODs with varying concentrations of $H_2O_2$ were calculated as described in the legend for Figure 1.

double reciprocal plots were carried out for the rate of formation of DMPO–OH at the initial 2 min period, versus $H_2O_2$ concentrations (Figure 3). Although only typical data for A4T, G37R, and wild-type enzymes are shown, they fit simple first order reaction kinetics in this range of $H_2O_2$. The same experiment was performed for all the other enzymes and the $V_{max}$ and $K_m$ values were calculated from the plots (Table II). The $V_{max}$ values for mutant enzymes were all lower and

TABLE II $V_{max}$ and $K_m$ values for formation of DMPO–OH adduct by wild-type and mutant SODs from hydrogen peroxide

| Enzyme[a] | $V_{max}$ | $K_m$ |
|---|---|---|
| WT | 41 | 35 |
| A4T | 18 | 50 |
| G37R | 14 | 85 |
| G41D | 23 | 52 |
| G41S | 20 | 40 |
| H43R | 20 | 51 |
| I113T | 28 | 38 |

[a]Activities of H46R and G85R were too low to be calculated.

the $K_m$ values were all higher than for wild-type enzymes. Thus the data fail to support the view that enhanced radical forming ability is a feature of the mutant Cu,Zn-SODs, contradictory to some reports,[9–12] while they were consistent with several recent reports.[31–33]

### Accelerated Aggregation of SOD Mutants

During course of the work, we found that the mutant enzymes readily formed aggregates during storage at 4°C. Since the stored enzyme solutions contained at different extents of aggregation, they were removed by simple centrifugation at 12,000*g* for 20 min at 4°C and protein concentrations were adjusted to the same levels prior to use. After incubation overnight at 37°C in the presence of 1 or 10 μM $CuSO_4$, purified mutant SODs were centrifuged and the protein contents in the supernatants were evaluated by measuring the absorbance at 280 nm. Figure 4 shows the calculated fractions of aggregated proteins, obtained by subtracting the remaining proteins in the supernatant from the original concentration. About 20–50% of the proteins were precipitated for all mutant enzymes by low gravity centrifugation, but less than 10% was precipitated in the case of the wild-type control enzyme. An equimolar mixture of the wild-type and mutant SODs resulted in average level of the aggregation of them. The physiological relevance of these data is discussed below.

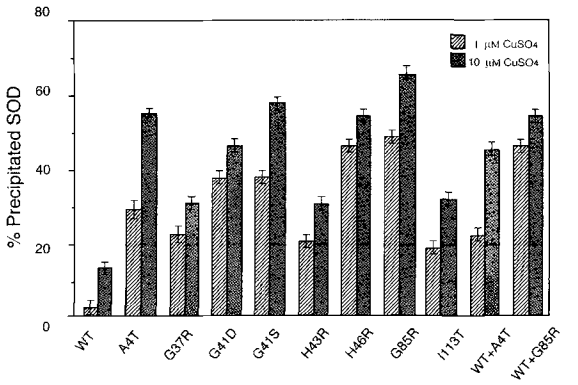


FIGURE 4 Aggregation is accelerated in mutant SODs. Purified SODs were incubated for 18 h in the presence of either 1 or 10 μM $CuSO_4$ and centrifuged at 12,000*g* for 20 min at 4°C to precipitate aggregated fractions. Protein contents in the supernatants were determined by measuring absorbance at 280 nm. Aggregated fractions were calculated by subtracting soluble fraction in the supernatant from the amount of initial proteins.

## DISCUSSION

In this work, we examined properties of the mutant Cu,Zn-SODs which were produced in a baculovirus expression system. When A4T was produced in a bacterial system, the amount of protein expressed was much less than the wild-type enzyme,[24] suggesting that the mutant SOD was structurally unstable, especially in the bacterial system. However, mutant SODs, except for G85R, could be produced at the same extent as the wild-type enzyme in the baculovirus system. In addition, the cells are typically grown under rather hypoxic conditions in tightly capped culture flasks in the baculovirus system, but hyperoxic conditions are used in the case of the bacterial system. Such different oxygen conditions could affect the enzyme properties of the produced proteins. Hence, it is more beneficial to use the baculovirus system than the bacterial system, in order to obtain the mutant SODs in a more physiological state.

Using eight mutant enzymes, we obtained data concerning Cu binding, the production of ROS, and the stability of the enzymes. Affinities for Cu ions were decreased for all the mutant enzymes

as judged by measurement of bound Cu in the enzymes after treatment with Chelex 100 resin (Table I). Thus Cu binding affinities were lower in the mutant enzymes than the wild type, which is consistent with the reported nature of the mutant enzyme.[17] However, in terms of hydroxyl radical formation, our data were not in agreement with previous reports.[9–12] Since we used the mutant Cu,Zn-SODs which had been pretreated with Chelex 100 resin to remove contaminating free Cu in the buffer as well as Cu which is loosely bound to the proteins, contradictory results might be possible, because of the different Cu binding state of the enzymes. Recently, conclusions similar to ours concerning hydroxyl radical formation by mutant enzymes were reported by several other groups.[31–33] The precise reasons for these observational discrepancies are presently unclear.

As another candidate property of the gained function for the mutant Cu,Zn-SODs, we observed the accelerated aggregation of the mutant Cu,Zn-SODs *in vitro*. Since protein aggregations positive to an anti-Cu,Zn-SOD antibody were found in degenerated motor neurons of FALS patients with SOD1 mutation,[34] mutant SOD1-transgenic mice,[35] and mutant cDNA-transfected cells,[20,21] the aggregation forming ability of the mutant enzymes may be of prime importance for such symptoms. In terms of aggregation, the mutant proteins could attach to some cellular components and coprecipitate with them in the cells. As a result, the effective concentration of the components could decrease, leading to dysfunction of the neuron. Elevated nitrotyrosine levels in the suffered lesions may also be explained by the presence of this mutant Cu,Zn-SOD. The mutant enzymes form aggregates, but remain enzymatically active (data not shown), and, therefore, could mediate tyrosine nitration from peroxinitrite.[36] Because hyaline inclusions found in FALS contain both SOD and neurofilaments, in which many tyrosine residues are present, tyrosine nitration occurs in the molecules and consequently destroys their function.

In conclusion, all properties found in the mutant enzymes may be traced back to the folding instability of the enzymes, which accelerate aggregation over a long term frame. Aggregated, but still active Cu,Zn-SODs would efficiently catalyze reactions which are enhanced in transfected cells, transgenic mice, and ALS patients.

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### *Note Added in Proof*

After this paper was accepted, Liochev and Fridovich published a paper (Ref. 37). They proposed that SOD1 reacts with $H_2O_2$ in bicarbonate buffer to form $HCO_3$, which then oxidizes DMPO to $DMPO^+$ and results in forming DMPO-OH. Thus hydroxyl radical is not actually produced by the action of SOD1 with $H_2O_2$. Proper corrections were made on the text according to their proposal.

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