Reactions of Hydrogen Peroxide with Familial Amyotrophic Lateral Sclerosis Mutant Human Copper-Zinc Superoxide Dismutases Studied by Pulse Radiolysis*

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Mutations in copper-zinc superoxide dismutase (CuZn-SOD) have been implicated in the familial form of the motor neuron disease amyotrophic lateral sclerosis (Lou Gehrig’s disease). We have expressed and purified recombinant human wild type (hWT) and G93A (hG93A) CuZn-SOD, and we have used pulse radiolysis to measure their superoxide dismutase activities and their rates of deactivation upon exposure to hydrogen peroxide or heat. Both hG93A and hWT CuZn-SOD were found to have high SOD activities in their copper and zinc containing as-isolated forms as well as when remetallated entirely with copper (CuCu). Rates of deactivation by hydrogen peroxide of the as-isolated hWT and hG93A enzymes were determined and were found to be similar, suggesting that the FALS mutant enzyme is not inactivated at a higher rate than wild type by generation of and subsequent reaction with hydroxyl radical, ‘OH, when it is in the CuZn form. However, rates of deactivation by hydrogen peroxide of the CuCu derivatives of both hWT and hG93A were significantly greater than those of the copper and zinc containing as-isolated enzymes. Rates of thermal deactivation were also similar for the mutant and hWT as-isolated CuZn forms but were greater for the CuCu derivatives of both enzymes. Reactions of hydrogen peroxide with the CuCu CuCu derivative of the WT enzyme demonstrate that the copper ion in the copper site is reduced much more rapidly than the copper ion in the zinc site, leading to the conclusion that reaction of hydrogen peroxide with Cu(I) in the copper site is the source of deactivation in the CuCu as well as the CuZn enzymes.

Amyotrophic lateral sclerosis (ALS, Lou Gehrig’s disease)† is a neurodegenerative disease characterized by a progressive loss of motor neurons in the spinal cord and brain. The disease is inherited in ~10% of cases, and of those cases, ~20% have been linked to mutations in sod1, the gene that encodes human copper-zinc superoxide dismutase (CuZn-SOD) (1). To date, more than 60 different dominantly inherited point mutations have been found.

FALS mutations in CuZn-SOD are dominant and are currently believed to exert their effects because of a gain of function rather than a loss of activity. The most convincing evidence for such a gain of toxic function is the observation that expression of FALS mutant human CuZn-SODs in transgenic mice causes motor neuron disease while expression of wild type (WT) human CuZn-SOD does not (2–4). Knockout mice that express no CuZn-SOD also do not develop motor neuron disease (5). The gain of function hypothesis is also supported by the observation that expression of FALS mutant human CuZn-SODs is pro-apoptotic in cultured neuronal cells, while WT human CuZn-SOD is anti-apoptotic (6). The expression of the FALS mutant G93A but not WT human CuZn-SOD in a human neuroblastoma cell line induced a loss of mitochondrial membrane potential and an increase in cytosolic calcium concentration (7).

CuZn-SOD is a cytosolic antioxidant enzyme that lowers concentrations of superoxide by catalyzing its disproportionation to give hydrogen peroxide and dioxygen. The mechanism involves alternate reduction and reoxidation of the copper ion at the active site of the enzyme (Reactions 1–3).

The rate constants of Reactions 1 and 2 have been measured and are both 2 × 10⁷ M⁻¹ s⁻¹ and pH-independent over the pH range of 5.0–9.5 (8–13).

\[ \text{O}_2 + \text{Cu(II)Zn-SOD} \rightarrow \text{O}_2 + \text{Cu(II)Zn-SOD} \]  
\[ \text{O}_2 + \text{Cu(I)Zn-SOD} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{Cu(II)Zn-SOD} \]

**This work was supported by NIGMS, National Institutes of Health, Grant GM28222 (to J. S. V.). Pulse radiolysis studies were supported by a grant from the ALS Association (to D. E. C.) and carried out at Brookhaven National Laboratory under contract DE-AC02-98CH10881 with the U.S. Department of Energy and supported by its Division of Chemical Sciences, Office of Basic Energy Sciences. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: ALS, amyotrophic lateral sclerosis (Lou Gehrig’s disease); FALS, familial ALS; CuZn-SOD, copper-zinc superoxide dismutase; WT, wild type; hWT, human WT; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; CuE, CuCu, and CuZn, remetallated derivatives of CuZn-SOD with copper in the native copper site and the native zinc site either empty (CuE) or containing copper (CuCu) or zinc (CuZn).
a reaction of the enzyme itself that results in self-inactivation (14, 15). The mechanism for this peroxidative activity of CuZn-SOD again involves alternate reduction and reoxidation of the copper ion in the enzyme, but this time by hydrogen peroxide (Reactions 4–7).

$$\text{H}_2\text{O}_2 + \text{Cu(II)}\text{Zn-SOD} \rightarrow \text{O}_2^- + 2\text{H}^+ + \text{Cu(II)}\text{Zn-SOD}$$  

**REACTION 4**

$$\text{H}_2\text{O}_2 + \text{Cu(II)}\text{Zn-SOD} \rightarrow \text{OH}^- + \cdot \text{OH} + \text{Cu(II)}\text{Zn-SOD}$$  

**REACTION 5**

Substrate + (·OH)/Cu(II)/Zn-SOD → Oxidized substrate

$$(·\text{OH})/\text{Cu(II)}\text{Zn-SOD}$$  

**REACTION 6**

(·OH)/Cu(II)/Zn-SOD → Inactive CuZn-SOD

$$(·\text{OH})/\text{Cu(II)}\text{Zn-SOD}$$  

**REACTION 7**

Self-inactivation of the enzyme is believed to involve the direct attack of copper-bound hydroxyl radical on the enzyme itself (Reaction 7) rather than on a substrate present in the active site channel of the enzyme (Reaction 6). In support of this conclusion, CuZn-SOD-catalyzed oxidation of substrates by hydrogen peroxide has been shown to occur in competition with enzyme self-inactivation by hydrogen peroxide (16).

Ethanol, formate, and dimethyl sulfoxide are some of the substrates whose oxidation by hydrogen peroxide is catalyzed by CuZn-SOD, and, to varying degrees, each of these substrates inhibits the inactivation of the enzyme (17, 18). The spin trap DMPO (5,5′-dimethyl-1-pyrroline N-oxide) is another example of a substrate that is oxidized by hydrogen peroxide in a reaction catalyzed by CuZn-SOD (Reactions 4, 5, and 8). DMPO-OH, the product that is formed upon reaction of DMPO with hydroxyl radical, is readily detected by EPR spectroscopy.

$$\text{DMPO} + (·\text{OH})/\text{Cu(II)}\text{Zn-SOD} \rightarrow \text{DMPO-OH} + \text{Cu(II)}\text{Zn-SOD}$$  

**REACTION 8**

Solving the puzzling mystery of why CuZn-SOD, a presumably good protein and hero of the cell, has turned into a villain in causing FALS is a major goal of current research on this enzyme. The copper in SOD has been implicated based on the beneficial effects of copper chelators in a neural cell culture model of SOD-associated FALS (6) and in the FALS SOD-expressing transgenic mice (19). Three hypotheses have been proposed to account for the possible involvement of copper in the gain of function of the FALS mutant human CuZn-SODs: 1) copper ions could be leaching out of the mutant proteins and inducing toxic effects at another site in the cell; 2) copper ions in the mutant enzymes could be catalyzing nitration of tyrosine residues by peroxynitrite (20–22); and 3) copper ions in the mutant enzymes could be catalyzing deleterious oxidation reactions of substrates by hydrogen peroxide (17, 18). The latter hypothesis for the gain of a toxic function resulting from the FALS mutations in CuZn-SOD is that they cause a change in the structure of the enzyme that results in an enhancement of the ability of the enzyme-bound copper ion to catalyze destructive oxidative reactions of substrates with peroxides (Reaction 6). In partial support of the feasibility of this hypothetical mechanism, the FALS mutant human CuZn-SOD enzymes A4V and G93A were shown to have an enhanced ability to catalyze the oxidation of DMPO by hydrogen peroxide to give DMPO-OH (23–25).

Determination of the rate of enzyme inactivation in the presence of hydrogen peroxide is another method that can be used to measure the ability of CuZn-SOD and its mutants to function as catalysts of peroxidation reactions. This approach has the advantage that the protein itself is the substrate for the oxidation reaction, uncomplicated by the question of substrate accessibility to the active site. Thus, the relative rates of inactivation of CuZn-SOD and its mutants by hydrogen peroxide are expected to reflect exactly the relative ability of these enzymes to catalyze peroxidative reactions of hydrogen peroxide.

**EXPERIMENTAL PROCEDURES**

Materials—All solutions were prepared using distilled water that had been passed through a Millipore ultrapurification system. EDTA and sodium formate were purchased from Sigma. Solution pH was adjusted by the addition of H₂SO₄ (double distilled from Vycor, GPC Chem. Co.) and NaOH (Puratronic, Alfa/Ventron Chem.) Monobasic potassium phosphate buffer (Ultraz, J. T. Baker Inc.), adjusted to the proper pH, was used in all activity measurements. Hydrogen peroxide was of the highest purity (The Olin Corporation) and titrated for determination of the concentration using published procedures as well as the absorbance at 230 nm ($e_{230} = 61 \text{ M}^{-1} \text{ cm}^{-1}$) (26).

Protein Expression, Purification, and Preparation of Derivatives—The proteins were expressed and purified according to a published procedure (23) with the addition of a final purification step on a Pharmacia Resource Q XR26 ion exchange column (26 × 20 cm), equilibrated with 2.5 mM phosphate buffer, pH 7.0. The protein was eluted from the column using a salt gradient of the same equilibration buffer containing 160 mM NaCl. Protein samples specified as “as-isolated” were purified but were not subjected to demetallation and remetallation after purification. Demetallation of the as-isolated protein samples to obtain the apoproteins was carried out as described previously (27).

Protein concentrations were determined using an extinction coefficient of 1.08 × 10⁴ M⁻¹ cm⁻¹ at 280 nm for the purified enzyme. SDS-polyacrylamide gel electrophoresis was used to estimate purity. Mass spectrometry was also used to determine purity by measuring the exact molecular weight of the monomer for each of the proteins. In some cases, the measured molecular weight of the isolated protein indicated the presence of an extra cysteine bond to the protein. Treatment of those protein samples with dithiothreitol to remove the excess cysteine produced proteins whose measured molecular weights corresponded to the calculated molecular weight for the mutant and WT proteins, within experimental error. The absence or presence of this cysteine made no difference in the measured overall metal binding ability of the SOD.

The protein samples were washed with 3–5 times the total volume of sample, several times, in a Centricon-10 (Amicon) microconcentrator with either pH 5.5 potassium phosphate buffer, for the as-isolated proteins, or pH 7.0 sodium acetate buffer, for the remetallated proteins, in order to remove any excess metal ions that might have been present in the solution and to ensure that the activity of the enzyme originated only from copper ions tightly bound to the protein, i.e. that none of the measured SOD activity was due to free or loosely bound copper ions. Protein concentrations were remeasured after the washing procedure and immediately prior to determination of SOD activities and deactivation measurements.

Atomic absorption spectroscopy was carried out using a Pye-Unicam atomic absorption instrument to determine copper and, where applicable, zinc concentrations. Measurements were made in triplicate with an experimental error of <5%. Copper and zinc content was determined for both the as-isolated and the remetallated proteins.

Pulse Radiolysis and SOD Activity—Pulse radiolysis experiments were carried out using the 2 MeV Van de Graaff accelerator at Brookhaven National Laboratory. Dosimetry was established using the KSCN dosimeter, assuming that (SCN)₂⁻ has a G value of 6.13 and a molar absorptivity of 7950 M⁻¹ cm⁻¹ at 472 nm. Irradiation of water by an electron beam generates the primary radicals, ·OH, eaq, and H. These radicals are efficiently converted into O₂ in the presence of formate and oxygen via the following reactions: ·OH + HCO₂⁻ → CO₂ + H₂O, where CO₂ + O₂ → H₂O + O₂. The decay of O₂ was monitored at 250–270 nm. Determinations of SOD activities were carried out in air-saturated 10 mM formate and 10 mM phosphate aqueous solutions at either pH 5.5 or pH 7.0 at 25 °C, with 30 μM EDTA present to complex any adventitious metal ions that might be present in trace amounts.

An observed first order rate for the catalytic dismutation of O₂ in the
presence of SOD was extracted from the observed change in absorbance (at 260 nm) with respect to time. The reported rate constants for the CuZn-SOD studies were calculated by dividing the observed rates by one-half or one-fourth of the total concentration of copper bound to the enzyme in solution for CuZn-SOD and CuCu-SOD, respectively. This calculation takes into account the fact that the two copper ions that are bound per protein dimer in the CuZn derivative are in the copper sites and both are therefore expected to be catalytically active in the SOD reaction, while only two of the four copper ions per protein dimer in the CuCu derivative are expected to be active since copper in the zinc sites of CuZn-SOD derivatives does not have SOD activity. The amount of bound copper was determined by first measuring the activity of the enzyme at pH 5.5–5.6 both in the presence and absence of EDTA. The amount of free copper in the solution was determined by extrapolating the difference in these observed rates in that pH range from a previously determined pH versus rate constant calibration curve for free copper in the same buffer conditions as used in the experiment. The amount of free copper was then subtracted from the total copper concentration as determined by atomic absorption spectroscopy to give the total amount of copper bound to the enzyme and therefore the active enzyme concentration (after the correction described above). The data was an average of five measurements for each sample for every pH data point.

**Hydrogen Peroxide Deactivation**—Hydrogen peroxide (4.90, 9.80, 19.5, 47.0, and 94.0 mM final concentrations after mixing) was added to solutions of the proteins (0.4–1.2 μM final concentration) in 10 mM potassium phosphate buffer, pH 7.0. Aliquots of 0.5 ml were withdrawn at set time points, mixed with 0.5 ml of 20 mM formate and 10 mM phosphate solution, and immediately pulse-irradiated for determination of the SOD activity. The solutions assayed thus remained 10 mM in phosphate, while the concentrations of CuZn-SOD, H$_2$O$_2$, and formate were diluted by a factor of 2 relative to the initial concentrations. It should be noted here that formate, itself an OH radical scavenger, was added after the timed exposure to hydrogen peroxide and immediately prior to pulsing and thus did not interfere with the deactivation reaction.

The rate constants for deactivation with hydrogen peroxide were measured under first order conditions with peroxide in large excess over the enzyme. The second order rate constants ($k_{obs}$) were calculated by dividing the first order rate by the peroxide concentrations. Therefore, the absolute concentration of enzyme was not important for the calculation.

**Thermal Inactivation**—Thermal inactivation of the proteins was studied at temperatures ranging from 25 to 85 °C in the same buffers used for the hydrogen peroxide inactivation experiments described above. To conserve protein, samples were reused as follows. A sample was heated to the indicated temperature, and the SOD activity was determined. The sample was then heated to a higher temperature, and the SOD activity was determined again. This procedure was repeated until the activity was observed to decrease, at which point a new sample was substituted and heated to the temperature at which the previous sample had lost activity for subsequent activity determinations. The change in activity of the enzyme with temperature was reversible in the range of 25–70 °C. At higher temperatures, the deactivation was essentially irreversible.

**Rates of Reduction**—The reduction of CuCu hWT (0.3 mM dimer enzyme) with H$_2$O$_2$ (1.0, 5.0, and 10.0 mM final concentrations) was followed by UV-visible light. The hydrogen peroxide was added directly to the protein in the cuvette and then immediately monitored using a Cary3 UV-visible spectrometer (Varian, Sunnyvale, CA). The complete spectra (230–900 nm) were obtained every 3 min for approximately 30 min. The particular wavelength absorbance, corresponding to the Cu(II) d-d transitions in the copper site (~680 nm) and Cu(II) in the zinc site (~810 nm) was followed with respect to time. All experiments were carried out in air-saturated solutions.

**RESULTS**

**Metal Ion Content of As-Isolated Human CuZn-SODs**—Wild type human CuZn-SOD was expressed in Saccharomycyes cerevisiae as described previously. Metal analyses of the as-isolated proteins varied according to the exact conditions of the expression, but the zinc-to-copper ratio was consistently high. Proteins that had been properly metallated in vitro would be expected to contain identical amounts of copper and zinc ions, but we found ratios of 2–5 zinc ions per copper. The total metal ion content, i.e. ([Cu] + [Zn])/[protein dimer], would normally be expected to be 4.0, but, for both the as-isolated WT and the mutant proteins, it was found to fall in the range 4.4–5.0. The CuCu derivatives, prepared by the addition of four equivalents of Cu(II) to the apoprotein dimer followed by washing with buffer, retained 3.64 equivalents per dimer (91%) of its copper in the case of the WT protein and 2.69 equivalents per dimer (67%) in the case of G93A; they each were found to contain <0.40 equivalents (<10% of the total metal bound to the enzyme) of zinc per dimer.

**Superoxide Dismutase Activity**—The rate constants as a function of pH for dismutation of superoxide catalyzed by CuZn-SOD derivatives were determined as described under “Experimental Procedures” for: 1) as-isolated WT CuZn-SOD, 2) as-isolated G93A CuZn-SOD, 3) remetallated CuCu-WT SOD, and 4) remetallated CuCu G93A. Solutions of the enzyme (0.4–1.2 μM) were pulse-irradiated, and the disappearance of superoxide was monitored at 250–270 nm. The catalytic rate constants of the as-isolated WT CuZn-SOD and as-isolated G93A CuZn-SOD as a function of pH are shown in Fig. 1. (Note the rate constant calculations are based on the concentration of copper-bound enzyme rather than the concentration of the proteins themselves, since only copper-containing enzymes are active.) Both as-isolated WT and G93A CuZn-SOD had relatively high SOD activities (1–2 × 10$^8$ M$^{-1}$s$^{-1}$) that were pH-independent over the range of pH 5.0–9.5 (see Fig. 1).

The remetallated CuCu derivatives of both WT and G93A CuZn-SOD, prepared by the addition of 4.0 equivalents of Cu(II) per apoprotein dimer followed by washing, also gave full SOD activity (Fig. 1). Analogous studies were carried out using remetallated samples of WT and G93A CuZn-SOD that were prepared from the apoprotein by the sequential addition of Zn$^{2+}$ followed by Cu$^{2+}$. The SOD activity per protein-bound copper of the remetallated WT CuZn-SOD was identical to that of the as-isolated WT CuZn-SOD. By contrast, the SOD activity of the remetallated G93A CuZn-SOD was much reduced, suggesting improper placement of metal ions in the remetallated mutant protein, although it was reconstructed by the same method that was successful for remetallation of the WT apoprotein. Therefore we used as-isolated G93A protein for the CuZn derivative in these experiments.

**Deactivation of CuZn-SOD by Hydrogen Peroxide**—The SOD catalytic rate constants are given in Fig. 2, A–D as a function of the time of exposure to hydrogen peroxide at the indicated concentrations. The rate constants for hydrogen peroxide-in-
duced inactivation were identical for the WT CuZn-SOD and as-isolated FALS mutant G93A enzymes (Fig. 2, A and B). The same phenomenon was observed for the remetallated CuCu derivatives of WT CuZn-SOD and G93A, i.e. the rates of loss of activity in the presence of hydrogen peroxide were identical for the FALS mutant and the WT enzymes (Fig. 2, C and D). However, comparison of the rates for the as-isolated derivatives that contain both Cu$^{2+}$ and Zn$^{2+}$ with those that contain only Cu$^{2+}$ reveals that the rate of deactivation in the presence of hydrogen peroxide is significantly enhanced when copper ions are bound to the zinc site as well as to the copper site for either the WT or FALS mutant enzymes (Fig. 2, compare A with C and B with D).

Plots of the log of the observed first order rate constants for catalytic dismutation of superoxide by the enzyme versus peroxide concentrations were linear, indicating that the deactivation reaction was first order in hydrogen peroxide concentration. A regression line was fitted to the data ($r^2 = 0.95$–0.99), and the half-life ($t_{1/2}$) for deactivation was extracted.

The rate constants for deactivation were calculated from the half-lives using the following relationship: $k_{obs} (s^{-1}) = 0.693/ t_{1/2}$ and are given in Table I. The bimolecular rate constant was calculated by dividing the first order rate by the peroxide concentration: $k_{bimolec} (M^{-1} s^{-1}) = k_{obs} /[H_2O_2]$. If all of the first order rate constants are essentially identical, then the simple reaction mechanism given above (Reactions 4, 5, and 7) is a reasonable description. However, the results are such as to give a hint that at high peroxide concentration, saturation may be occurring, possibly by the mechanism shown in Reaction 9. This result is not unprecedented; previous work by others in the field showed similar indications (24, 25, 28).

Cu(I)Zn-SOD + H$_2$O$_2$

\[ \text{Cu(II)Zn-SOD} + \text{H}_2\text{O}_2 \rightarrow \text{H}^+ + \text{Cu(II)Zn-SOD} \ldots \text{"OH + OH"} \]

**Reaction 9**

**Thermal Deactivation of CuZn-SOD**—The loss of SOD activity upon heating the as-isolated WT and G93A and the remetallated CuCu derivatives was also investigated. Once again, the as-isolated WT and the FALS mutant behave similarly to each other, as did the remetallated CuCu derivatives of those same proteins, but both of the remetallated CuCu derivatives were inactivated at a lower temperature ($\sim 65$–75 °C) than both of the as-isolated copper- and zinc-containing proteins ($\sim 75$–85 °C) (Fig. 3).

**Stoichiometric Reaction of CuCu-SOD with Hydrogen Peroxide**—Preferential reduction of Cu(II) in the copper site of CuCu WT had been observed previously for the CuCu derivative of bovine CuZn-SOD (14, 29), and similar behavior was expected for the human WT enzyme and was in fact observed. Accordingly, the rate of reduction for Cu(II) in the copper site was found to be significantly faster than for the Cu(II) in the zinc site (data not shown).

**DISCUSSION**

The evidence obtained thus far indicates that FALS mutant CuZn-SOD enzymes have acquired an as yet unidentified property that is deleterious to motor neurons. One hypothesis as to the identity of this property is that mutant enzymes catalyze deleterious oxidation reactions, enhancing production of OH radicals from hydrogen peroxide. In support of this hypothesis, EPR spin trapping experiments demonstrated an enhanced production of DMPO-OH from the reaction between the enzyme and H$_2$O$_2$. However, our current studies show that there is no difference in the rates of deactivation between the WT and
There is no exogenous substrate; the substrate is the enzyme itself. Isolated wild type human CuZn-SOD has usually been found to contain copper and zinc in nearly equal amounts. Extensive structural and spectroscopic studies have led to the conclusion that copper is bound predominantly to the copper site and zinc to the zinc site in the wild type, but the mechanism of metal ion insertion and the basis for the high degree of selectivity of each site for its respective metal ion is not well understood. Studies of metal-substituted derivatives of CuZn-SOD have demonstrated that a variety of different metal ions can be bound in place of the native metal ions at either or both of the metal binding sites, but only copper ions in the copper site of the mutant enzymes when reconstituted with copper and zinc in vitro in the absence of a copper chaperone, appeared to bind much of the copper ion in the zinc site, where it was SOD-inactive. Thus, the copper site could be fully loaded with copper to give full SOD activity only when copper ions were used to fill the zinc sites as well.

Oxidative damage occurring as a result of the peroxidative activity of FALS mutant CuZn-SOD can thus be hypothesized to arise from either or both of these two mechanisms: 1) increased access of a substrate to the active site, leading to increased oxidation of a substrate (Reaction 6) and 2) increased formation of 'OH radical (Reaction 5) due to the presence of copper rather than zinc in the zinc site leading to a subpopulation of CuCu-SOD that is more reactive. An intriguing third possibility is that increased production of 'OH radical could lead to damaged inactivating metal ions that could slow release free copper ions that could catalyze deleterious Fenton-type oxidation reactions at or near the sites where they are released. It should nevertheless be noted that the concentration of hydrogen peroxide used in these experiments is undoubtedly greater than that normally encountered by the mutant enzymes in vivo and that the significance of this type of peroxidative reactivity in ALS is therefore difficult to judge, especially in the absence of any knowledge of the nature of potential targets of oxidative damage in or near the motor neuron.
Future experiments are planned to test the ability of a variety of hydroxyl radical scavengers to slow the inactivation of the WT and mutant enzymes in their CuCu and CuZn forms by reaction with hydrogen peroxide. A preferential reduction in the abilities of the metal sites of the mutant enzymes to discriminate between copper and zinc ions in binding to the individual metal binding sites in an effort to judge if they are related to the disease-causing properties of these mutant enzymes.

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