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Characterization of a Covalent Polysulfane Bridge in Cu, Zn Superoxide Dismutase ^{1,&}

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Abstract

In the course of studies on human copper-zinc superoxide dismutase (SOD1) we observed a modified form of the protein whose mass was increased by 158 mass units. The covalent modification was characterized, and we established that it is a novel heptasulfane bridge connecting the two Cys111 residues in the SOD1 homodimer. The heptasulfane bridge was visualized directly in the crystal structure of a recombinant human mutant SOD1, H46R/H48Q, produced in yeast. The modification is reversible, with the bridge being cleaved by thiols, cyanide, and by unfolding of the protein to expose the polysulfane. The polysulfane bridge can be introduced *in vitro* by incubation of purified SOD1 with elemental sulfur, even under anaerobic conditions and in the presence of a metal chelator. Because polysulfanes and polysulfides can catalyze the generation of reactive oxygen and sulfur species, the modification may endow SOD1 with a toxic gain of function.

Human copper, zinc superoxide dismutase (SOD1) is a homodimeric protein which catalyzes the dismutation of the superoxide radical to hydrogen peroxide and oxygen (1). Each subunit contains 1 zinc atom, 1 copper atom, 2 free cysteines (Cys6 and Cys111), and 2 cysteines in a disulfide linkage (Cys57 and Cys146). As an intracellular protein, mature SOD1 is unusual in possessing a stable disulfide bond which is thought to be important for the high tertiary and quaternary structural stability of SOD1 (2;3). Disulfide bond formation is catalyzed by the copper chaperone for SOD1 (CCS) which also facilitates insertion of copper into the newly translated enzyme (4;5). One of the free cysteine residues, Cys111, is a "reactive cysteine", that is, its pK_a is relatively low leading to ionization at physiological pH which facilitates its oxidation (6;7) and post-translational modification (6;8;9). A number of investigators have reported changes in cysteine behavior associated with SOD1 mutations which cause amyotrophic lateral sclerosis (ALS) (10). These changes alter the biochemical and biophysical properties of the protein and have been hypothesized to play a role in the pathophysiology of ALS through a variety of proposed effects (2;11-17). During investigations of recombinant

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wild-type and mutant human SOD1 proteins, we noted a form with increased mass. Here we establish that the increased mass is a consequence of the introduction of a polysulfane² bridge between the Cys111 in each subunit of the SOD1 dimer.

MATERIALS AND METHODS

Materials

Chemicals, CM-cellulose, and DEAE-resin were purchased from Sigma-Aldrich or Fluka (St. Louis, MO). Spectra/Por[®]3 dialysis membrane was obtained from Spectrum Laboratories (Rancho Dominguez, CA). Outdated and fresh human red blood cells were obtained from the blood bank of the National Institutes of Health (Bethesda, MD).

Production and Analysis of SOD1—Recombinant wild-type and mutant SOD1 proteins were expressed in yeast and purified as described (13). The proteins were stored in 2.25 mM potassium phosphate buffer, pH 7.0, containing 160 mM NaCl. Metal-free or “apo” proteins were prepared as previously described (13). For isolation from human red blood cells, 10 mL whole blood was centrifuged at $2,000 \times g$ for 15 min. The cell pellet was washed with 10 mL of 0.9% cold saline three times. Cells were then lysed by suspension in 15 mL of chilled deionized water with gentle rocking at 4° C for 1 h. Cell debris was removed by centrifugation at $20,000 \times g$ for 30 min. The supernatant was mixed with 30 g CM-cellulose (pre-equilibrated with 5 mM NaH₂PO₄ at pH 6.0) and the pH was adjusted to 6.0. The suspension was stirred at 4° C for 35 min, which removed most of the hemoglobin. CM-cellulose was then removed by filtration. A portion of the filtrate was concentrated in a Centriprep YM-10 spin tube (Millipore, Billerica, MA) for analysis by HPLC-MS, and the rest of the solution was adjusted to pH 7.8 and stirred with 12.5 g of pre-equilibrated DEAE resin overnight. After filtration, the DEAE resin was washed with 5 mM NaH₂PO₄ buffer, and the bound protein was eluted with 0.2 M NaH₂PO₄ buffer at pH 7.8. A portion of the eluant was concentrated for HPLC-MS analysis, and the remainder was subjected to 60% (NH₄)₂SO₄ precipitation. The precipitated protein was removed by centrifugation, leaving SOD1 in the supernatant. A portion of the supernatant was concentrated for HPLC-MS analysis, and the rest was desalted by dialysis against 10 mM NaH₂PO₄ at pH 7.0. Before use, the dialysis membrane was rinsed in deionized water as recommended by the manufacturer. A more rigorous cleaning to remove sulfur was followed where noted, again following a procedure recommended by the manufacturer (19): The membrane was washed in 0.3% Na₂SO₃ at 80° C for 2 min, then placed in 100 mL deionized water at 60 °C for 2 min, followed by the addition of 3.4 mL 0.4% H₂SO₄ and incubation for another 2 min. The membrane was then rinsed with deionized water thoroughly before use.

Reverse phase HPLC-MS analyses were carried out with an Agilent 1100 series HPLC with electrospray and detected by a model G1946 mass spectrometer equipped with either a quadrupole or time-of-flight detector (Agilent, Palo Alto, CA) using columns and conditions as described (13;20). For direct-infusion without a column, the protein was introduced at the electrospray needle without addition of acetic acid.

The double SOD1 mutant P62A/P66A was studied after various chemical treatments. For denaturation by guanidine, 3 μ L of protein (0.5 mg/mL) was mixed with 10 μ L of 8 M guanidine hydrochloride. The pH of the solution remained 7.0, checked by pH paper. It was held at room temperature for 1 h before analysis. For treatment with dithiothreitol or glutathione the protein at 1 mg/mL was made 10 mM in dithiothreitol or in 5 mM glutathione, held for 2 h at room

²R-(S)_n-H should be referred to as a persulfide or polysulfide and R-(S)_n-R as a polysulfane (18). In practice the biochemical literature tends to refer to both as polysulfide, which can be confusing.

temperature, and then analyzed by HPLC-mass spectrometry. Sodium borohydride reduction was performed for 2 h at 20 mM. Cyanolysis was effected by treating protein at 0.5 mg/mL with 2 or 10 mM KCN for 90 min before analysis. Incubation of the same mutant with 7 mM sodium sulfide was performed for 20 min.

Crystallization, Structure Determination and Refinement of Polysulfane- Linked H46R/H48Q SOD1—Automated screening for crystallization was carried out using the sitting drop vapour-diffusion method with an Art Robbins Instruments Phoenix pipetting robot as described previously (21). Suitable crystals were obtained from the Qiagen Cryos Suite in condition #86 (0.17 M ammonium sulfate, 0.085 M sodium acetate pH 4.6, 25.5% w/v polyethylene glycol 4000, and 15% v/v glycerol). Crystals were flash-cooled in liquid nitrogen prior to data collection using a Rigaku MicroMax 007HF X-ray generator equipped with VariMax High-Flux optics, RAXIS-HTC image plate detectors, and X-Stream cryogenic crystal cooling systems. Data were processed with HKL-2000 software (Charlottesville, VA) (22). Initial phases for *apo* H46R/H48Q SOD1 were taken from the human S134N SOD1 structure, PDB 1OZU (23), since the crystals of these SOD1 variants are isomorphous. Coordinates were refined against the data using PHENIX (24), including simulated annealing, alternating with manual rebuilding using COOT (25). The heptasulfane bridge linking the Cys111 residues of each subunit of the single SOD1 dimer in the asymmetric unit was included in the final rounds of refinement. A composite annealed omit map was calculated using CNS (26) to verify the heptasulfane model. Data collection and refinement statistics are shown in Table 1. The coordinates and structure factors have been deposited in the Protein Data Bank with accession 3K91.

RESULTS

A Stable Dimer with Increased Mass

The HPLC-MS conditions routinely utilized in our laboratory are denaturing [(0.05% trifluoroacetic acid and an acetonitrile gradient with the effluent made 50% in acetic acid before infusion into the mass spectrometer (27)]. These conditions typically dissociate non-covalently bound multimeric proteins to monomers and strip non-covalently bound metals and cofactors. In various purified preparations of recombinant wild-type SOD1 produced in *S. cerevisiae*, we did observe the expected monomer, but its mass was 30-31 Da higher than calculated. Notably, we also found a form whose mass was that calculated for the dimer plus an additional 158 Da. Examples are shown in Figure 1. The calculated monomeric mass of wild-type SOD1 is 15,844.6 Da, including the N-terminal acetylation and the intramolecular disulfide bond, but the two forms observed had masses of 15,876.0 and 31,847.5 which are 31 and 158 Da higher than the calculated masses for the monomer (15,844.6) and the dimer (31,689.2), respectively. The accuracy of this time-of-flight mass spectrometer is such that there should be no more than a 0.5 Da error in mass determination for SOD1. Mutant SOD1 proteins consistently showed a similar pattern. Figure 1B shows the results for the double proline mutant P62A/P66A, being studied in order to understand the role of these proline residues in the action of the copper chaperone for SOD1. The major form had an observed mass of 31,743.0 (+158 from calculated 31,585.0) and the monomer an observed mass of 15,823.5 Da (+31 from calculated 15,792.5). ALS-associated mutants G93A, A4V, G93A/C6A, and H46R/H48Q, and the proline mutants P62A, P62A/P66A, P28A/P62A/P66A, and P62A/P66A/C6A all exhibited mass increases of 158 Da from the calculated dimer mass and except for H46R/H48Q, increases of 30-31 Da from the calculated monomer mass (Table 2). The relative ratio between the modified dimer and monomer varied from preparation to preparation. The modification is not unique to recombinant protein produced in yeast, as it was also observed in a commercial product purified from human red blood cells (Sigma). The persistence of the dimeric form under the harsh

conditions of the reverse phase HPLC-MS suggests a covalent linkage between the two subunits of the dimer.

Bound Cations Do Not Account for the Mass Increase

As mentioned above, the conditions used for the HPLC-MS analysis typically monomerize non-covalently bound oligomeric proteins and strip off cofactors and bound metals. Thus, the observed mass increase of 158 Da in the dimer might result in part from retention of Cu or Zn. In addition, if other moieties are bound to the native protein but stripped by exposure to the HPLC-MS, then +158 is an underestimate the mass increase in the preparation. In an effort to obtain the mass of SOD1 in its native form, we infused the sample in buffer directly to the electrospray needle, avoiding exposure to trifluoroacetic acid, acetonitrile, and acetic acid. With direct infusion, the measured mass of G93A under this condition was 32,127.4 Da (Figure 2A), a 410.2 Da increase over the expected dimer mass without bound metals and a 158.4 Da increase for the dimer with one Cu¹⁺ and one Zn²⁺ bound to each subunit³. The measured mass for the double proline mutant P62A/P66A was 31,996.7 Da, 411.6 Da higher than that calculated for the apo dimer and 159.8 Da higher than the dimer with bound metals (Figure 2B). When acetic acid was added to 50% (v/v) by mixing in a tee located just before the electrospray needle, the masses of these SODs decreased to that of the dimer plus 158 Da, due to the stripping of Cu and Zn from the protein. Metals can also be stripped from SOD1 very effectively by dialysis against EDTA (13). HPLC-MS analysis of such apo proteins demonstrated the presence of the dimer form plus 158 Da in the 3 SOD1s examined, wild-type, G93A, and A4V. We conclude that metals are not required to observe the modification which adds 158 Da and that the moiety added is stable to our HPLC-MS.

Characterization of the Covalent Modification

The UV detector of our HPLC system records spectra of peaks as they are detected. Inspection of spectra of the SOD1 peak revealed a broad absorption band from 300 to 350 nm (not shown), reminiscent of the spectrum reported by Calabrese and colleagues of SOD1 (8). They established that the chromophore was due to the presence of labile sulfur which they proposed was a persulfide, R-S-S-H. We note that 158 Da is precisely the additional mass which would be contributed by 5 sulfurs linked to two cysteine residues to form a heptasulfane. We therefore investigated the characteristics of the modification with emphasis on whether it matched those reported for polysulfides and polysulfanes. They are generally quite sensitive to thiol reducing agents which cause elimination of the bridge, leaving the cysteines in their thiol form. Exposure of the modified SOD1 to dithiothreitol, borohydride, or glutathione as described in Materials and Methods converted them to the monomer with masses now matching that calculated from the sequence (not shown).

In general polysulfurs within folded proteins are more stable than low molecular weight polysulfides and denaturation causes loss of the modification (8). When we exposed dimeric P62A/P66A SOD1 to 6M guanidine we observed a time-dependent decrease in mass and conversion of the dimer to the monomer (Figure 3A). Expansion of the scale revealed a sequential loss of 32 Da, consistent with loss of sulfur atoms (Figure 3B). In the region of the dimeric mass, one can observe masses differing by 32 Da and equal to the calculated masses

³Protein mass is calculated by deconvolution of the mass spectrum. The deconvolution algorithm assumes that every positive charge on a protein ion is contributed by H⁺, and its mass is subtracted when calculating the protein mass. However, if the charge is actually contributed by a bound metal cation, a correction must be made to the value reported by the algorithm because it underestimates the true mass of the metalated protein. The error will be 1 Da for each monovalent cation and 2 Da for each divalent cation. Thus, bound Zn²⁺ would be reported by the algorithm as having a mass of 63.4 instead of 65.4. The situation with copper varies since the charge could be +1 or +2, but due to the reducing conditions of the electrospray mass spectrometer, it is expected to be +1 (28). This would be reported as having a mass of 62.5 instead of the correct 63.5. The observed increase of 158 Da in the dimer was determined from the corrected deconvoluted mass.

of an SOD with heptasulfane (31,743.1), hexasulfane (31,710.0), pentasulfane (31,678.7), tetrasulfane (31,646.0), trisulfane (31,614.6), and disulfane (31,582.5). A similar ladder of masses differing by 32 Da was observed in the monomer (Figure 3C).

Polysulfur compounds react readily with cyanide to generate thiocyanate (30). Reaction at alkaline pH is rapid while reaction at neutral pH is relatively slow, allowing the observation of intermediates during cyanolysis. Treatment of modified P62A/P66A at neutral pH with 2 mM cyanide for 90 min resulted in shortening of the polysulfane with the mass of the dimer shifting from that of the heptasulfane to the hexasulfane with the monomer remaining as the trisulfane. Treatment with a higher concentration of cyanide, 10 mM, gave primarily monomer along with a small dimer peak of 31,647.3 corresponding to the tetrasulfane (Figure 4). Inorganic sulfide will also attack polysulfanes and exposure of modified P62A/P66A converted it to the monomer (not shown). De Beus and colleagues found a trisulfide modification on Cys111 upon trypsin digestion of the Sigma preparation, likely due to hydrolysis of the heptasulfane by solvent exposure during trypsin exposure (31).

Visualization of the Heptasulfane-Bridge by X-Ray Crystallography

Extra electron density positioned between the two Cys111 residues at the dimer interface was observed in a crystal of apo-H46R/H48Q SOD1. The density was virtually a perfect fit for the heptasulfane linking the two subunits (Figure 5). This is the first polysulfane modification observed in an SOD1 structure. This heptasulfane was not observed in several other crystals screened from wild-type and mutant SOD1s, perhaps because they were prepared before we understood the labile nature of the modification. However, we have observed increased densities on Cys111 consistent with shorter sulfane modifications, for example Figure 1 in (21).

Introduction of the Polysulfane Bridge In Vitro

As noted above, millimolar concentrations of glutathione remove the polysulfane from the modified SOD1. Given the high concentration of glutathione in red blood cells, we were surprised to find the heptasulfane form in the commercial preparation from Sigma since it is prepared from human red blood cells⁴, although Okado-Matsumoto and Fridovich found a trisulfane linkage in their Sigma lot (32). They did not find modified forms in human and bovine SOD1 purified by several different methods, including one which had been proposed by some investigators to artifactually introduce the modification (6). In an attempt to clarify these conflicting reports, we purified SOD1 from freshly drawn human red cells and checked its mass at each step of the purification by HPLC-MS analysis. The expected monomeric mass was found at each step of the purification (Figure 6). Following the final step, the purified SOD1 was dialyzed overnight to remove residual ammonium sulfate. Following dialysis, the +158 Da modification *was* observed, whether dialyzing into Tris or phosphate buffers. Until the relatively recent introduction of higher quality dialysis membranes, it was standard practice to clean dialysis tubing before use, typically by boiling in EDTA during which one typically smelled a strong sulfurous odor. The manufacturer of the dialysis membrane used in our experiments recommends that the membrane still be treated to remove residual sulfur and trace metals if they are of concern to the investigator⁵. Precleaning of the membrane with sulfite followed by 0.01% sulfuric acid (19) did prevent introduction of the polysulfane during dialysis. Without precleaning, polysulfanylation still occurred when 5 mM EDTA was included

⁴While outdated cells may have low glutathione content, the modification should not have been present in the cells when collected. Briggs and Fee reported that labile sulfur can be introduced into SOD1 when the purification method includes use of chloroform and ethanol to precipitate hemoglobin (6). Although Sigma-Aldrich informed us that they do not use such an organic solvent step in their preparation, we were careful to avoid it and instead use the chromatographic methods which Briggs and Fee determined did not have that effect.

⁵The manufacturer's analysis gives a sulfur content of 0.1% and an iron content of 20-60 ppm (19).

in the dialysis buffer and also when the dialysis was conducted in an anaerobic hood (< 1 ppm O_2). Thus neither catalysis by redox cations such as iron nor the production of superoxide is required for the insertion of the polysulfane. This result suggested that elemental sulfur alone can form the polysulfane, as noted by Calabrese and colleagues (8). We then incubated wild-type SOD1 in phosphate buffered saline, pH 7.4, with a suspension of elemental sulfur at 7 mg/mL overnight. Most of the SOD1 was converted to the heptasulfane form⁶. The amount of elemental sulfur in the suspension is not critical for the yield of heptasulfane form, which was the same for a sulfur suspension from 0.05 – 5.0 mg/mL.

DISCUSSION

We have established that human SOD1 can form a heptasulfane bridge (Cys-S₅-Cys) between the Cys111 of its two identical subunits. Polysulfur linkages in proteins are not common but are clearly documented and some are involved in the catalytic mechanism of enzymes (8; 33-38). Sulfide can add to disulfide bonds within proteins to yield a polysulfur (39). Calabrese and colleagues noted in passing that they could sub-stoichiometrically modify wild-type human SOD1 by incubation with colloidal sulfur or thiosulfate (8) while Briggs and Fee had a higher yield with cysteine trisulfide or glutathione polysulfide (6). Searcy and colleagues reported that bovine SOD1 catalyzed the reaction of sulfide and superoxide to produce zero valence sulfur as either elemental sulfur or a polysulfide (40). This reaction requires a catalytically active SOD1 so it could not explain the ready introduction of a sulfane into catalytically inactive mutants such as H46R/H48Q (Figure 5).

There are four cysteine residues in each subunit. Cys57 and Cys146 within the subunit are linked in a stable disulfide bond, Cys6 is buried and generally not reactive, while Cys111 is positioned at the subunit interface and is readily accessible, for example, reacting fully with the thiol reagent N-ethylmaleimide (32). The reactivity of Cys111 has been documented in many studies, including several which provided evidence for the presence of a persulfide on that residue (6-8;11;42-43). Various investigators have proposed that the chemical and structural characteristics conferred by Cys residues contribute to the pathophysiology of ALS caused by mutant SOD1s. Liu and colleagues demonstrated that Cu^{2+} binds strongly to Cys111 and suggested that solvent exposure of Cys111 may be important to the mechanism of neurodegeneration in ALS (11). Tiwari and Hayward showed that the disulfide bond of mutant SOD1s was more susceptible to reduction, which leads to a marked decrease in stability of the protein including dissociation of the dimer to the monomer (2;12;13). In the mouse model of ALS, mutant SOD1s lacking the disulfide bond accumulate in the central nervous system and are prone to aggregation (14), including amyloid-like aggregates (16). Both wild-type and mutant SOD1 can form non-physiological intermolecular sulfide bonds between Cys6 and Cys111, but the mutant forms do so more readily (15). The formation of such sulfide linkages would likely be hastened by the presence of a polysulfane on Cys111.

Elemental sulfur readily formed the heptasulfane when incubated with unmodified SOD1. The distance between the Cys111 residues in the SOD1 dimer is ~ 8.2 Å. Elemental sulfur exists as a cyclic octamer whose structure resembles a crown (44). The longest distance between atoms is ~ 4.6 Å so that the sulfur allotrope would fit comfortably between the Cys111 residues, one of which could attack the ring and open it through formation of a polysulfide, followed by formation of the heptasulfane by the other Cys111.

Organic polysulfur compounds are found in a variety of organisms including algae, bacteria, and plants (45) as well as in hydrolysates of wool (46). Some are known to be cytotoxic,

⁶The incubation must be done in glass. The reaction did not go when performed in a plastic Eppendorf tube in which the sulfur adhered to the walls of the tube.

possessing antifungal, antibacterial, or anticancer properties (45;47;48). Formation of a persulfide at the active site of rhodanese is central to its catalytic mechanism (49). The polysulfide-sulfur transferase from *Wolinella succinogens* has a hexasulfide at its active site cysteine, visualized in the NMR structure (50).

If polysulfane modification occurs *in vivo*, might it be relevant to the pathophysiology of ALS caused by mutant SOD1? That wild-type human SOD1 can also be modified does not rule out the relevance of the polysulfane because wild-type SOD1 can cause disease if expressed at sufficiently high levels, and it can worsen the severity of disease caused by mutant SOD1 (51-54). Oxidative stress occurs in motor neuron cells of patients and mice expressing mutant SOD, although whether this is causal or secondary is unsettled (55). Wild-type SOD1 is capable of generating reactive oxygen species with hydrogen peroxide as a substrate, and mutant SOD1s have an enhanced ability to do so leading to the proposal that this is the toxic gain of function which causes ALS (56-60). However, the attractiveness of this proposal has diminished somewhat because production of reactive oxygen species requires the presence of copper at the active site of SOD1 and mutants with a disrupted copper binding site can still cause ALS (61;62).

We propose that the polysulfane at Cys111 can generate reactive oxygen species. This suggestion is supported by investigations which demonstrate that low molecular weight polysulfur compounds catalyze the formation of reactive oxygen species and radical production through redox cycling (63-68). Nucleophilic attack on polysulfides also occurs, with generation of H₂S (67;70), now recognized as a cellular signaling molecule similar to NO (71). If either radical generation or sulfide production is confirmed experimentally, actual relevance to causality in ALS will require demonstration of polysulfanylation *in vivo* and that the modification occurs preferentially with mutant SOD1 compared to wild-type. There are two forms of sulfur stores in cells (72;73). One is as iron-sulfur clusters which are found primarily in mitochondria and which can be released non-enzymatically by severely acidic conditions (73). The other is referred to as “bound sulfur”, is almost exclusively cytosolic, is richer in neurons than astrocytes, and can be released by the mild alkalinization of the cytosol which occurs upon neuronal excitation. Thus, polysulfanylation of SOD1 and other proteins may occur *in vivo* in the brain.

Abbreviations

ALS	amyotrophic lateral sclerosis
CCS	copper chaperone for superoxide dismutase
HPLC-MS	high pressure liquid chromatography-mass spectrometry
SOD1	copper-zinc superoxide dismutase
WT	wild-type

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Figure 1.

A. The mass spectrum of wild-type SOD1; B. the mass spectrum of the double mutant P62A/P66A.

Figure 2.
Mass spectra of SOD1 mutants obtained by the direct infusion method. A) G93A; B). P62A/
P66A.

A **B**

C **Figure 3.**

Mass spectra of P62A/P66A after exposure to 6M guanidine. A) The full spectrum B) Expanded view of the dimer region showing different forms of covalent dimeric P62A/P66A with varying length of polysulfanes; C) Expanded view of the monomer region showing different forms of monomer P62A/P66A with varying length of polysulfanes.

Figure 4.
P62A/P66A after treatment with 10 mM KCN for 90 min.

Figure 5. Composite annealed omit electron density calculated at 1.75 Å resolution with coefficients 2Fo-Fc of the dimer interface region of *apo*-H46R/H48Q (contoured at 1 σ). The polysulfane chain is shown in yellow. Water molecules have been removed from the image for clarity.

Figure 6.
Human SOD1 purified from red blood cells through the DEAE elution step.

Table 1

Data collection and refinement statistics

Data collection	
Space group	$P2_12_12_1$
Cell dimensions	
a, b, c (Å)	40.0, 57.9, 105.0
α, β, γ (°)	90, 90, 90
Wavelength	1.5418
Resolution (Å)	30 – 1.75
R_{sym}^*	0.059 (0.351)
$I/\sigma I$	15.8 (3.0)
Completeness (%)	95.8 (94.9)
Redundancy	2.8 (2.6)
Refinement	
Resolution (Å)	27.9 – 1.75
No. reflections	24,337
$R_{\text{work}} / R_{\text{free}}$	0.188/0.218
No. atoms	
Protein	2016
Ligand	5
Solvent	255
B -factors	
Protein	24.8
Ligand	35.5
Solvent	33.3
R.m.s deviations	
Bond lengths (Å)	0.010
Bond angles (°)	0.925

* Values in parentheses are for the highest-resolution shell.

Table 2

Masses of SOD1 Preparations

SOD1 proteins *	Measured mass (monomer/dimer)	Calculated mass (monomer/dimer)	Mass increase (monomer/dimer)
Wild-type	15,876.0/31,847.5	15,844.6/31,689.2	31 /158.0
P62A/P66A	15,823.5/31,743.0	15,792.6/31,585.1	30.4/ 157.9
P62A	15,849.6/31,795.2	15,818.6/31,637.1	31 /158.1
P28A/P62A/P66A	15,797.2/31,691.2	15,766.5/31533.0	30.7 /158.2
P62A/P66A/C6A	15,791.6/31,679.0	15,760.4/31520.8	31.2/158.2
G93A	15,889.6/31,875.5	15,858.6/31,717.2	31/158.3
G93A/C6A	15,857.8/31,811.1	15,826.6/31,653.1	31.2/158.0
A4V	15,903.9/31,903.5	15,872.6/31,745.2	31.3/158.3
H46R/H48Q	15854.2/31867.3	15854.6/31709.2	0/158.1
Wild-type (Sigma)	15,875.9/31,847.5	15,844.6/31,689.2	31.3 /158.3

* Except for the Sigma preparation shown in the last row, all proteins were produced in yeast.