Published in final edited form as:

Arch Biochem Biophys. 2009 December ; 492(1-2): 40–47. doi:10.1016/j.abb.2009.09.020.

Structural and Biophysical Properties of Metal-Free Pathogenic SOD1 Mutants A4V and G93A

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal, progressive neurodegenerative disease characterized by the destruction of motor neurons in the spinal cord and brain. A subset of ALS cases are linked to dominant mutations in copper-zinc superoxide dismutase (SOD1). The pathogenic SOD1 variants A4V and G93A have been the foci of multiple studies aimed at understanding the molecular basis for SOD1-linked ALS. The A4V variant is responsible for the majority of familial ALS cases in North America, causing rapidly progressing paralysis once symptoms begin and the G93A SOD1 variant is overexpressed in often studied murine models of the disease. Here we report the three-dimensional structures of metal-free A4V and of metal-bound and metal-free G93A SOD1. In the metal-free structures, the metal-binding loop elements are observed to be severely disordered, suggesting that these variants may share mechanisms of aggregation proposed previously for other pathogenic SOD1 proteins.

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that affects motor neurons in the spinal cord and brain, eventually leading to paralysis and death [1]. Approximately 90% of cases are termed “sporadic” (sALS), arising without family history or known cause. About 10% of cases have a genetic component and are termed “familial” (fALS), with approximately 20% of fALS cases being linked to dominant mutations in the gene encoding the cytosolic antioxidant enzyme copper-zinc superoxide dismutase (SOD1) [2,3]. To date, over 100 different pathogenic SOD1 mutations have been reported (reviewed in [4–9]). A steadily increasing list of mutations can be monitored at <http://alsod.iop.kcl.ac.uk/Als>.

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Atomic coordinates and structure factors have been deposited in the Protein Data Bank with accession numbers 3GZO, 2WKO (metal-bound G93A), 3GZP (metal-free G93A), and 3GZQ (metal-free A4V).

Studies in transgenic mice have established that pathogenic SOD1 proteins elicit motor neuron dysfunction through a gain of a toxic property and not a loss of enzymatic function. Mice expressing human ALS SOD1 polypeptides in addition to their endogenous SOD1 (thereby retaining normal SOD1 activity) become paralyzed [10–13], while SOD1 knockout mice do not [14]. Although the exact nature of this toxicity is not well understood, the observation that insoluble proteinaceous inclusions enriched in pathogenic SOD1 appear concomitant with the onset of paralytic symptoms in these animals suggests that SOD1-linked ALS is a protein misfolding disease [4–9].

Human SOD1 is a 32-kDa homodimeric enzyme that catalyzes the detoxification of superoxide anion (O_2^-) to molecular oxygen and hydrogen peroxide ($2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$) [15, 16]. Figure 1 shows that each subunit folds as an eight-stranded Greek key β -barrel, binds one copper and one zinc ion, and contains an intrasubunit disulfide bond [17]. The protein-bound copper ion is essential for catalysis, while the protein-bound zinc ion is important to maintain the structural integrity of the enzyme and for the proper positioning of charged residues that assist in the electrostatic guidance of the superoxide substrate into the active site [18,19]. SOD1 is abundant in brain and spinal cord, presumably because these highly respiring tissues produce copious levels of superoxide as a normal byproduct of respiration.

The binding of metal ions and the oxidation of the intrasubunit disulfide bond by nascent SOD1 have been demonstrated to favor dimerization [20–22] and are also known to impart enormous thermal stability to the enzyme. The importance of these posttranslational modifications to the stability of SOD1 in the context of pathogenic SOD1 misfolding and aggregation has focused attention on the copper chaperone for SOD1 (CCS), a helper protein that recognizes and binds to newly translated SOD1, inserts the catalytic copper ion, and catalyzes the oxidation of the intrasubunit disulfide bond in each SOD1 monomer [23,24]. Hindering these posttranslational modifications results in immature, metal-deficient and/or disulfide-reduced forms of SOD1 that are increasingly observed as components of the higher order oligomers believed to be relevant in ALS toxicity [25–28]. However, whether the oligomers of pathogenic SOD1 mutants are the causative agents or are symptomatic of motor neuron dysfunction is not yet clear.

A rapidly progressing form of ALS arises from mutations at position A4 (A4V, A4T, and A4S), of which A4V is the most common in North American patients. ALS patients harboring the A4V mutation have a mean survival time of only about 1.5 years post diagnosis [29]. Figure 1 shows that A4 is located in the first β -strand that forms part of the dimer interface. Mutations at the A4 position are predicted to affect SOD1 dimerization [30] and/or to destabilize the Greek key β -barrel because A4 packs into the hydrophobic core of the SOD1 monomer. There are six known pathogenic substitutions at G93 (G93A, G93C, G93D, G93R, G93S, and G93V). As shown in Figure 1, G93 is positioned in the short loop V connecting the anti-parallel β -strands 5 and 6, and which is near residues 37 – 42 of loop III. Substitution of G93 is also predicted to destabilize the β -barrel by interfering with the packing of residues that form the “apolar plug” found at one end of the β -barrel [31].

Here, we describe crystal structures of metal-free A4V and metal-bound and metal-free G93A pathogenic SOD1 variants. The structures reveal the destabilizing effects of metal ion deficiency on the conformations of two major loop elements of the enzyme and illuminate intriguing posttranslational modifications of C111 residues in A4V. These data, taken together with data coming from transgenic mouse and cell culture experiments, suggest the hypothesis that SOD1-linked ALS may arise from a general failure of a subset of newly translated pathogenic SOD1 mutants to mature.

MATERIALS AND METHODS

Protein Expression, Purification, and Demetallation

DNA fragments encoding the human A4V and G93A mutations were amplified by the polymerase chain reaction (PCR) and ligated into the YEP351 plasmid, where expression of the human SOD1 is directed under the control of its own promoter. A4V and G93A SOD1 proteins were expressed, purified, and when appropriate, stripped of their metals as previously described [21]. The metal contents of all SOD1 proteins used in this study were determined using inductively coupled plasma mass spectrometry (ICP-MS) at the mass spectrometry facility in the Department of Chemistry and Biochemistry at the University of California, Los Angeles as described previously [21].

Crystallization and Data Collection

Two distinct metal-containing SOD1 samples were used in crystallization experiments. The “metal-bound” G93A SOD1 sample contained the complement of metal ions present after purification, while the “metal-reconstituted” G93A SOD1 sample contained the complement of metal ions present after the purified G93A SOD1 protein is incubated with 1 mM copper and zinc sulfate overnight, followed by dialysis against 2.25 mM potassium phosphate pH 7.5, 150 mM sodium chloride. All crystals were grown using the hanging-drop vapor diffusion method [32] at room temperature. Metal-free A4V SOD1 crystals in space group $P2_12_12_1$ were grown from a drop containing equal volumes of protein solution (protein concentration of 17 mg/ml in 10 mM sodium acetate pH 5.5, 125 mM sodium chloride) and reservoir solution (1.6 M ammonium sulfate, 0.1 M MES at pH 6, and 5 mM tris(2-carboxyethyl)phosphine (TCEP)). Block-like prisms appeared within two weeks. Metal-free G93A crystals in space group C2 were grown by mixing a 1:1 ratio of protein solution (protein concentration of 5 mg/ml in 10 mM sodium acetate, pH 5.5, 125 mM sodium chloride) and reservoir solution (2.4 M ammonium sulfate, 0.1 M HEPES, pH 7). Slender rod-like clusters appeared in approximately one month. The metal-bound G93A crystals in space group $C222_1$ were obtained using the same conditions as metal-free G93A, except the reservoir solution contained 2.4 M sodium malonate, pH 6. Large trapezoids appeared after about three days. Crystals of metal-reconstituted G93A in space group $P2_1$ were grown from a drop containing 2 μ L of protein solution (protein concentration 9 mg/mL in 2.25 mM potassium phosphate pH 7.5, 150 mM sodium chloride) and 2 μ L of reservoir solution (20% PEG3350, 0.2 M sodium iodide). Rectangular prisms appeared within two weeks.

Crystals of metal-free A4V, metal-free G93A and metal-bound G93A in space group $C222_1$ were quickly swiped through well solution made 20% (v/v) in glycerol as a cryoprotectant. The metal-reconstituted monoclinic G93A crystals were soaked for 10 s in reservoir solution made 25% (w/v) in sorbitol as a cryoprotectant. All cryoprotected crystals were flash-cooled by plunging into liquid nitrogen prior to X-ray data collection. Diffraction data from metal-free G93A crystals in space group C2 were collected at the Advanced Photon Source beam line 19-BM (Argonne, IL) on a custom charge-coupled device (CCD) detector. Diffraction data from metal-free A4V crystals in space group $P2_12_12_1$ were collected at the Advanced Light Source beam line 8.2.2 (Berkeley, CA) on an Area Detector Systems Corporation Quantum 315 CCD detector. Diffraction data from metal-bound G93A crystals in space group $C222_1$ were collected at the *X-ray Crystallography Core Laboratory* at the University of Texas Health Science Center at San Antonio using a Rigaku FR-D high flux X-ray generator equipped with Osmic High-flux mirrors on a Rigaku R-AXIS HTC imaging plate detector. Diffraction data from metal-reconstituted G93A crystals in space group $P2_1$ were collected at the Synchrotron Radiation Source beam line 9.6 (Daresbury, U.K.) on an Area Detector Systems Corporation CCD detector. All data sets were indexed, scaled, and merged using HKL2000 [33]. Data collection statistics are presented in Table 1.

Structure Determination and Refinement

The structure of metal-free A4V was determined by molecular replacement using the program MOLREP [34] with the S134N SOD1 variant (PDB code 1ozu) as the search model [35]. Initial refinement was performed using the maximum likelihood method as implemented in REFMAC5 [36] as part of the CCP4 program suite. Minor adjustments to the model were made using σ_A -weighted electron density maps [37] in the program O [38]. Prior to refinement, 5% of the reflections were excluded for use in cross-validation for the calculation of R_{free} [39]. Solvent molecules were added with the aid of the program ARP/WARP [40]. Individual anisotropic B-factor refinement was performed using the program PHENIX [41,42]. In the later stages of structure refinement the graphics program COOT [43] was used for model adjustment.

The structure of metal-free G93A was determined by molecular replacement using the program MOLREP and wild type SOD1 subunits A and H (PDB code 1hl5 [44]) as the search model. The structure obtained from molecular replacement was subsequently refined using REFMAC5 and PHENIX with non-crystallographic-symmetry restraints. Fine tuning of the model proceeded using the program COOT.

The structure of metal-bound G93A SOD1 in space group C222₁ was determined by molecular replacement using the program MOLREP and wild type SOD1 subunits A and F (PDB code 1sos [45]) as the search model. The molecular replacement solution was subjected to several rounds of refinement using PHENIX. All ten subunits (five dimers) in the asymmetric unit were refined independently and no stereochemical restraints were applied to metal-ligand distances or bond angles. Water molecules were included in the later stages of refinement by automatic water picking routines within PHENIX and COOT, and some of them were positioned manually after visual inspection of σ_A -weighted electron density maps.

The structure of metal-reconstituted G93A SOD1 in space group P2₁ was determined by molecular replacement using one dimer from wild type SOD1 subunits A and F (PDB code 2c9v [43]) as the search model. The molecular replacement solution was subjected to several rounds of refinement using REFMAC5. The two monomers were refined independently and no stereochemical restraints were applied to metal-ligand distances or bond angles. Water molecules were included in the later stages of refinement by automatic water picking routines in ARP/WARP and COOT. Three iodine anions from the crystallization medium were added manually to the model after visual inspection of 2(Fo-Fc) and (Fo-Fc) electron density maps.

Analytical Ultracentrifugation

Sedimentation velocity experiments were performed using a Beckman Optima XL-I centrifuge at the *Center for Analytical Ultracentrifugation of Macromolecular Assemblies*, University of Texas Health Science Center at San Antonio. A4V and G93A SOD1 variants at a concentration of 2.4 mg/ml in sodium acetate buffer pH 5.5 with and without the reducing agent TCEP were used in the experiments, which were conducted at 20 °C and at 50,000 rpm. Sedimentation velocity data were analyzed using the method of van Holde and Weischet [46] as implemented in the ULTRASCAN 6.0 software [47]. This analysis method effectively removes the contributions of diffusion to boundary spreading to yield the integral distribution of $s_{20,w}$ of all species in the sample, $G(s)$. Consequently, a $G(s)$ plot of boundary fraction versus $s_{20,w}$ will be vertical if the sample is homogeneous, and will have a positive slope if the sample is heterogeneous [48,49].

RESULTS

Structures of Metal-Free A4V and G93A SOD1

X-ray diffraction data collection and protein structure refinement statistics are given in Table 1. Metal-free A4V SOD1 crystallized in space group $P2_12_12_1$ with one dimer (subunits A/B) per asymmetric unit. Metal-free G93A crystallized in space group C2 with two dimers (subunits A/B and C/D) per asymmetric unit. Figure 2A shows a ribbon representation of the dimeric human wild type holoenzyme (Protein Data Bank code 2c9v [50]). Each SOD1 subunit of the homodimer folds as an eight-stranded Greek key β -barrel, binds one copper and one zinc ion, and possesses an intrasubunit disulfide bond [17]. Two rather lengthy loop elements protrude from the β -barrel to form the walls of the active site and to participate in metal binding. The first, termed the “zinc loop” (loop IV, residues 50–83) holds most of the zinc-binding ligands. The second, termed the “electrostatic loop” (loop VII, residues 121–142), contains charged amino acid residues that help guide the negatively charged superoxide substrate to the active site entrance while simultaneously excluding larger nonsubstrate anions [19,51]. A third, smaller element, the “disulfide loop” (residues 50–62, a substructure of loop IV), is covalently linked to the β -barrel through a disulfide bond between C57 and C146. The disulfide loop and the eighth strand of the β -barrel from each subunit interact reciprocally and comprise most of the SOD1 dimer interface [17,21].

Figures 2B and 2C show ribbon representations of metal-free A4V and G93A SOD1, respectively, in the same orientation as the wild type enzyme, revealing conformational disorder in the electrostatic and zinc loop elements (detailed in Table 2). Figure 3A and Figure 4A show the A4V substitution site and the copper- and zinc-binding sites in subunit A of the metal-free A4V SOD1 structure, respectively, superimposed on σ_A -weighted electron density contoured at 1.2 σ .

Structures of Metal-Bound and Metal-Reconstituted G93A SOD1

Metal-bound G93A SOD1 crystallized in space group $C222_1$ with five dimers (subunits A/B, C/D, E/F, G/H, and I/J) per asymmetric unit, while metal-reconstituted G93A SOD1 crystallized in space group $P2_1$ with one dimer (subunits A/F) in the asymmetric unit. The overall structures of all subunits in the metal-bound and metal-reconstituted G93A SOD1 structures are quite similar to that of the wild type enzyme. Although zinc ions are bound in the zinc-binding sites with full occupancy in all subunits, the occupancy of copper in the copper-binding sites was variable, ranging from 0.17 to 1.0, depending on the crystal form. Figure 3B and Figure 4B show the G93A substitution site and the copper- and zinc-binding sites, respectively, in subunit A of the monoclinic, metal-reconstituted G93A structure superimposed on σ_A -weighted electron density contoured at 1.2 σ .

Analytical Ultracentrifugation

Figure 5 shows the results of analytical ultracentrifugation sedimentation velocity runs of the A4V and G93A SOD1 protein samples used for crystallization in this study. Metal-bound G93A SOD1 sedimented at ~ 2.8 S, which is in agreement with previously reported values for the dimeric wild type enzyme [21]. Metal-free A4V and G93A SOD1 proteins sedimented between 2.7 and 2.8 S at high concentrations (high boundary fraction %), but at smaller S values at lower concentrations (low boundary fraction %). The shape of the distributions of S for the metal-free A4V and G93A SOD1 proteins is suggestive of a self-associating system (i.e. an equilibrium between monomeric and dimeric forms of the protein). In the presence of the reducing agent TCEP, the distributions for metal-free A4V and G93A SOD1 proteins shift almost entirely to S values approaching 1.2, which is the value previously reported for monomeric SOD1 [21].

DISCUSSION

Metal-Binding Influences Loop Structure

As expected, crystal structures of metal-bound forms of A4V SOD1 [30] and metal-bound and metal-reconstituted forms of G93A SOD1 (this study) are similar to that of the wild type enzyme in that they possess well-ordered zinc loop (loop IV, residues 50–83) and electrostatic loop (loop VII, residues 121–142) elements. The molecular basis for why the binding of metal ions (particularly zinc) exerts a stabilizing effect on the conformation of these loop elements is shown in Figure 1. First, in the Cu(II) form of the enzyme, H63 of the zinc loop bridges the copper and zinc ions. This “primary bridge”, also known as the “bridging imidazolate”, is a metal ion coordinating feature unique to SOD1 [51]. Second, the binding of zinc to the zinc-binding site by amino acid residues H63, H71, H80, and D83, induces the zinc loop to adopt a structure in which the proline residues at positions 62, 66, and 74 adopt the *trans* conformation [17,52]. Third, when copper and zinc occupy their respective binding sites, they orient the nonliganding imidazole nitrogen atoms of copper ligand H46 and zinc ligand H71 such that they form hydrogen bonds with the side chain oxygen atoms of D124 coming from the electrostatic loop. Because D124 also directly links the copper- and zinc-binding sites, it has been termed the “secondary bridge” [17]. Fourth, when zinc occupies the zinc-binding site, it sits precisely on an axis running down the center of the short α -helix consisting of residues 131–136 of the electrostatic loop, thereby capping the helix dipole at the C-terminus of this helix. Figure 1 shows that this electrostatic helix-capping interaction, plus the two hydrogen bonds of the secondary bridge, together with a hydrogen bond between the amide nitrogen of H71 and the carbonyl oxygen of T135, are the only non-van der Waals interactions linking the zinc and electrostatic loop elements. Thus, the conformation of the electrostatic loop appears to be strictly dependent on the conformation of the zinc loop, which is in turn dependent on the binding of zinc.

Recent studies reveal that the binding of metal ions to the copper-binding site alone is insufficient to induce order in the zinc and electrostatic loop elements. For example, structures of the pathogenic SOD1 variants D124V and H80R [SVS and PJH under review], of a human SOD1 variant engineered to be monomeric and to have an ablated zinc-binding site [53], and of tomato SOD1 [AG and PJH in preparation] all reveal zinc ions bound in the copper-binding sites, empty zinc-binding sites, and disordered zinc and electrostatic loop elements. In contrast, wild type and mutant forms of SOD1 with empty copper-binding sites, but containing zinc in the zinc-binding site, reveal well-ordered zinc and electrostatic loop elements [54,55]. Taken together, the structural data indicate that the binding of metal ions to the zinc-binding site (but not the copper-binding site) is the critical factor dictating whether or not the zinc and electrostatic loop elements will be ordered or disordered. Consistent with this observation, Figure 2 shows that metal-free forms of A4V and G93A pathogenic SOD1 variants possess disordered zinc and electrostatic loop elements. Disordered zinc and electrostatic loop elements in metal-deficient pathogenic SOD1 dimers have been observed to permit non-native SOD1-SOD1 interactions at deprotected edge strands in the Greek key β -barrel, leading to the formation of linear filamentous arrays [35] that are similar in appearance to the fibrillar SOD1 aggregates found in various murine models of ALS [56].

Dissociation of A4V and G93A SOD1 Dimers

Although Figure 3 shows that the regions local to the A4V and G93A substitutions impart only relatively small displacements of surrounding residues in the Greek key β -barrels of these proteins, these two pathogenic substitutions are clearly destabilizing to both dimeric and monomeric forms of SOD1. For example, in previous analytical ultracentrifugation sedimentation velocity and sedimentation equilibrium experiments, disulfide-oxidized, wild type SOD1 proteins were observed to sediment as homogeneous dimers, whether metal-bound

or metal-free. The wild type enzyme did not dissociate to monomers until the metal ions were removed *and* the intrasubunit disulfide bond was reduced [21]. In contrast, as shown in the sedimentation velocity experiments in Figure 5, metal-free A4V and G93A SOD1 variants are prone to monomerization even though they still retain the intrasubunit disulfide bond.

The notion that monomeric pathogenic SOD1 proteins might be prone to aggregation in SOD1-linked ALS is gaining acceptance. Monomeric SOD1 was found to be a common intermediate in oxidation models of SOD1 aggregation [57]. In biochemical studies on A4V SOD1, the introduction of an intersubunit disulfide bond designed to prevent dimer dissociation completely abolished the aggregation observed in A4V SOD1 proteins without this engineered crosslink [58]. Small molecules designed to bind in a pocket at the SOD1 dimeric interface, thereby stabilizing the SOD1 dimer, were observed to protect A4V and G93A SOD1 from aggregation even when these proteins were metal-free [59]. In studies probing the oligomeric state of SOD1 in murine models of ALS in which transgenic mice overexpressed G37R, G85R, and G93A SOD1, the presence of monomeric pathogenic SOD1 variants in the insoluble aggregates was established using an antibody that specifically recognizes monomeric, misfolded forms of SOD1 [60]. Although all of the above-mentioned studies focused on the dissociation of pathogenic SOD1 dimers to monomers, the observations that SOD1 monomers are prone to aggregation are also consistent with the hypothesis that newly translated, monomeric pathogenic SOD1 proteins fail to mature to their dimeric forms via posttranslational modification by CCS [8,9,28,55,61] (see below).

Destabilizing Effects of A4V and G93A Pathogenic Substitutions

In a previous solution NMR study on metal-bound G93A, loops III and V exhibited higher mobility than wild type SOD1 on a time scale faster than the rate of protein tumbling. This increased mobility was interpreted as an indication that the β -barrel may be more easily opened than the wild type, especially when the protein is metal-free, potentially leading to fibrillogenesis through non-native SOD1-SOD1 interactions [62]. In spite of the relatively small displacements of surrounding residues shown in Figure 3, the A4V and G93A pathogenic substitutions have dramatic destabilizing effects on metal-deficient, monomeric forms of these variants as has been demonstrated in differential scanning calorimetry (DSC) experiments [63,64]. Disulfide-oxidized, dimeric, holo-wild type SOD1 has a melting temperature (T_m) of $\sim 93^\circ\text{C}$. Upon metal ion removal, disulfide-oxidized, dimeric, wild type SOD1 has a melting temperature of $\sim 52^\circ\text{C}$ and upon reduction and monomerization, metal-free wild type SOD1 melts at $\sim 42^\circ\text{C}$. In contrast, metal-free, disulfide-oxidized A4V melts at $\sim 40.5^\circ\text{C}$ and upon reduction, exhibits no endotherm in the DSC experiment, suggesting that it is predominantly unfolded. Although G93A was not tested in these DSC experiments, the disulfide-oxidized, metal-free pathogenic G93R variant melted at 44.3°C and also exhibited no endotherm upon reduction [63,64]. The unfolded state of these metal-free, monomeric pathogenic SOD1 variants suggested by the DSC experiments might hinder the ability of the copper chaperone for SOD1 to act upon these molecules (see below).

Hindered CCS-mediated Posttranslational Modification and Pathogenic SOD1 Aggregation

The metal-free, disulfide-reduced A4V and G93A/G93R pathogenic SOD1 variants that appear unfolded in the DSC experiments described above presumably correspond to the state in which these molecules exist when newly translated. These observations, combined with previous observations that CCS is unable to posttranslationally modify pathogenic SOD1 variants H46R/H48Q, D124V, and H80R, suggest the hypothesis that hindered CCS-mediated posttranslational modification of pathogenic SOD1 variants leads to metal-deficient, disulfide-reduced, immature pathogenic SOD1 molecules that are aggregation-prone. In support of this hypothesis, recent studies in cell culture and in transgenic mice reveal that much of the pathogenic SOD1 protein in the soluble fraction is metal-deficient, disulfide-reduced, and/or

monomeric [25,27,60,65]. Interestingly, in a pair of recent studies, it was found that overexpression of CCS greatly accelerated disease in a G93A SOD1 mouse model in the absence of visible proteinaceous inclusions [66,67]. However, CCS overexpression failed to enhance oxidation of the G93A SOD1 disulfide bond and in fact, elevated the population of disulfide-reduced G93A SOD1 in the soluble fraction of brain and spinal cord of these animals [66]. These data suggest that CCS may be interacting with nascent G93A, keeping it in the soluble fraction, but in a non-productive way in terms of the posttranslational insertion of copper and oxidation of the disulfide bond. However, it remains unclear why CCS overexpression in these animals results in elevated rather than diminished levels of disulfide reduced G93A SOD1, but this observation suggests that, like H46R/H48Q SOD1, CCS binding alone is not sufficient to convert nascent G93A SOD1 into its mature holo form. A more detailed description of how the various pathogenic SOD1 substitutions might interfere with CCS-mediated SOD1 maturation can be found in [8,9,55,66].

In summary, the results presented here on the structure and biophysical properties of metal-bound and metal-free A4V and G93A SOD1 variants, together with data on pathogenic SOD1 proteins coming from cell culture and in transgenic mice, suggest that incomplete posttranslational modification of nascent SOD1 polypeptides may be a characteristic shared by fALS SOD1 mutants, resulting in a population of destabilized, folding intermediates that are toxic to motor neurons.

Acknowledgments

This work was supported by grants NIH-NINDS R01-NS39112 (P.J.H.), the Motor Neuron Disease Association, U.K. (S.S.H., R.W.S.), and in part by NIH T32AG021890-03 (J.P.S.), the American Foundation for Aging Research (L.J.W.), the William and Ella Owens Medical Research Foundation and the Judith and Jean Pape Adams Charitable Foundation (A.G.). The U.K. group is grateful to BBSRC, MRC, EPSRC, NWDA and STFC for financial support and to STFC Daresbury Laboratory for provision of facilities and also thanks Peter A. Doucette and Joan S. Valentine for the gift of G93A SOD1. Support for the *X-ray Crystallography Core Laboratory* and the *Center for Analytical Ultracentrifugation of Macromolecular Assemblies* by the UTHSCSA Executive Research Committee and the San Antonio Cancer Institute is also gratefully acknowledged.

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Figure 1.

The wild type SOD1 monomer (pdb code 2c9v [50]). A) The Greek key β -barrel is shown in yellow, the zinc loop (loop IV, residues 50–83) is shown in green, and the electrostatic loop (loop VII, residues 121–142) is shown in red. Copper and zinc ions are shown as blue and orange spheres, respectively. Sulfur atoms forming the intrasubunit disulfide bond between residues 57 and 146 are shown as cyan spheres. The position of the A4V and G93A substitutions are shown as magenta and black spheres, respectively. The rotation symbol indicates the approximate axis around which the second monomer of the dimer would be positioned. B) The view is rotated approximately 90° from that in panel A). The positive charge of the zinc ion caps the C-terminal helix dipole.

Figure 2.

Structural comparison of human wild type and metal-free pathogenic SOD1 mutants A4V and G93A. A) wild type human SOD1 [pdb code 2c9v [50]]. The color scheme is the same as in Figure 1. B) Metal-free human A4V SOD1. The Greek key β -barrel is shown in light gold. The zinc and electrostatic loop elements are colored as in Figure 1 and panel A) The C positions of last visible residues in the zinc and electrostatic loop elements in the electron density are shown as green and red spheres, respectively (also see Table 2). C) Metal-free human G93A SOD1. The Greek key β -barrel is shown in light blue. The zinc and electrostatic loop elements are colored as in panels A) and B) The C α positions of last visible residues in the zinc and electrostatic loop elements in the electron density are shown as green and red spheres, respectively (also see Table 2).

electrostatic loop elements in the electron density are shown as green and red spheres, respectively (also see Table 2).

Figure 3.

The site of substitution in the pathogenic SOD1 variants A4V and G93A superimposed on σ_A -weighted electron density with coefficients $2mFo-DFc$ contoured at $1.2 \cdot \sigma$. A) Residues forming part of the apolar core of subunit A of the metal-free A4V structure (light gold) superimposed on subunit A of the wild type enzyme (pdb code 2c9v [50]). The largest displacements due to the A4V substitution occur in L106 and the C $\delta 1$ atom of I113. B) The site of pathogenic substitution in the C222₁ metal-bound G93A structure (blue) superimposed on subunit A of the wild type enzyme (pdb code 2c9v [38]). The largest displacements due to the G93A substitution occur due to a rotation of the peptide bond in residues 92–93 and in the carbonyl oxygen atom of L38.

Figure 4.

Divergent stereo view of the metal-binding sites in metal-free A4V and in metal-reconstituted G93A SOD1 in space group P2₁ superimposed on σ_A -weighted electron density with coefficients $2mFo-DFc$ contoured at 1.2 σ . A) Metal-free A4V SOD1. Residues of the Greek key β -barrel, zinc loop, and electrostatic loops are color coded as in Figure 2B. The copper-ligand H120 exists in two rotameric forms and a sulfate from the crystallization medium occupies the anion binding site [68]. Residues 67–79 and 126–141 are disordered and not visible in the electron density. B) Metal-reconstituted G93A SOD1 subunit A in space group P2₁. Copper and zinc ions are shown as blue and orange spheres, respectively. The structure

of the metal-reconstituted G93A metal-binding sites is quite similar to that of the wild type enzyme.

Figure 5.

Sedimentation velocity analysis of metal-free A4V and metal-bound and metal-free forms of G93A SOD1 in 100 mM acetate buffer, pH 5.5. The loading concentration was 0.8 A_{280} , corresponding to a protein concentration of ~2.4 mg/mL. The G(s) plot is obtained after analysis of the sedimentation boundaries by the method of van Holde and Weischet [46]. Filled triangles: metal-bound G93A SOD1; open diamonds: metal-free G93A SOD1; filled diamonds: metal-free G93A SOD1 in 10 mM TCEP (reducing agent); open squares: metal-free G93A SOD1 in 27 mM TCEP; filled crosses: metal-free A4V SOD1.

Table 1

X-ray diffraction data and refinement statistics for the four crystals used in this study.

	Metal-free A4V P2₁2₁2₁ pdb code 3GZQ	Metal-free G93A C2 pdb code 3GZP	Metal-bound G93A C222₁ pdb code 3GZO	Metal-reconstituted G93A P2₁ pdb code 2WKO
Crystal Parameters				
Unit cell				
<i>a</i> (Å)	40.5	113.8	166.1	51.5
<i>b</i> (Å)	58.8	67.9	203.3	47.2
<i>c</i> (Å)	104.9	81.0	143.3	56.4
β (degrees)		110.9		90.4
X-ray Diffraction Data				
λ (Å)	1.0	1.03	1.54	0.87
Resolution Range (Å)	50–1.40	50–3.1	50–2.1	23.5–1.97
Observations				
Total (including partials)	833,685	307,796	2,489,386	47,697
Unique Reflections	49,502	11,514	140,272	18,073
Redundancy ^a	6.7(4.4)	3.2(2.7)	3.5(3.2)	2.6 (2.4)
Completeness (%) ^a	99.0 (93.2)	99.3 (95.7)	99.8(99.6)	93.6 (87.9)
<i>R</i> _{sym} (on <i>I</i>) (%) ^a	4.5 (58)	14.5 (44.9)	12.1(51)	7.4 (20)
Refinement				
SOD1 Dimers/AU	1	2	5	1
<i>R</i> _{cryst} (%)	16.4	20.1	17.2	17.4
<i>R</i> _{free} (%) ^b	19.3	24.8	21.4	22.6
<i>F</i> / σ <i>F</i>	>0	>0	>0	>0
Geometry				
RMS Bonds (Å)	0.006	0.006	0.009	0.020
RMS Angles (°)	1.012	0.958	1.159	1.693
Atoms/(Average B)				
Protein	1816 (22.2)	4,044 (30.4)	11,150 (24.4)	2221 (29.4)
Water	251 (39.2)	0	1621 (33.7)	293 (25.4)
Copper	0	0	10 (20.3)	2 (27.9)
Zinc	0	0	10 (20.9)	2(24.8)
Glycerol	0	0	2 (34.6)	0
Malonate	0	0	1 (24.1)	0
Sulfate	2 (27.4)	0	0	0
Iodide ions	0	0	0	3 (32.1)

^a values in parentheses are for the highest resolution shell^b 5 % of reflections were chosen randomly in each resolution shell for cross-validation.

Table 2

Summary of structural observations in the four different crystal systems.

<u>Structure</u>	<u>Disordered region(s)</u>	<u>Metal Content (occupancy)</u>	
		<u>Copper site</u>	<u>Zinc site</u>
Metal-Free A4V			
Subunit			
A	67–79, 126–141	empty	empty
B	68–79, 125–141	empty	empty
Metal-Free G93A			
Subunit			
A	--	empty	empty
B	--	empty	empty
C	67–78, 125–139	empty	empty
D	67–78, 125–139	empty	empty
Metal-Bound G93A C222₁			
Subunit			
A,B,C,D,E,F,G,H,I,J	--	~0.2 copper	1.0 zinc
Metal-Reconstituted G93A P2₁			
Subunit			
A	--	1.0 copper	1.0 zinc
F		1.0 copper	1.0 zinc