Local Unfolding in a Destabilized, Pathogenic Variant of Superoxide Dismutase 1 Observed with H/D Exchange and Mass Spectrometry*

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Hydrogen exchange monitored by mass spectrometry has been used to study the structural behavior of the pathogenic A4V variant of superoxide dismutase 1 (SOD1) in the metal-free (apo) form. Mass spectrometric data revealed that in the disulfide-intact (S-S) form, the A4V variant is destabilized at residues 50–53, in the disulfide subloop of the dimer interface, but many other regions of the A4V protein exhibited hydrogen exchange properties identical to that of the wild type protein. Additionally, mass spectrometry revealed that A4V apoSOD1 undergoes slow localized unfolding in a large segment of the β-barrel that included β3, β4, and loops II and III. In the disulfide-reduced form, A4V apoSOD1 exchanged like a “random coil” polypeptide at 20 °C and began to populate folded states at 4 °C. These local and global unfolding events could facilitate intermolecular protein-protein interactions that cause the aggregation or neurotoxicity of A4V SOD1.

Mutations in the gene encoding copper-zinc superoxide dismutase (SOD1) are one of the few known causes of amyotrophic lateral sclerosis (ALS) (1, 2) and account for ~2% of all ALS cases. These mutations cause the neurodegenerative disease by imparting an additional function or property to the SOD1 protein (3, 4), and a large amount of research suggests that this toxic property is an increased propensity for the pathogenic SOD1 to aggregate relative to the wild-type (WT) protein (5–8). Yet, as is the case with proteins that cause other familial protein aggregation diseases, this increased aggregation propensity can be explained solely by the global stability of the ALS mutant SOD1 proteins (9–11). Although it is true that the A4V substitution, one of the most frequently occurring mutations in SOD1-related familial ALS, results in a significantly destabilized SOD1 protein and causes an especially severe form of the disease (12), several other ALS mutant SOD1 proteins are very close in stability to the WT protein while also having WT-like metallation properties and SOD activities (13).

Global instability is only one of the properties known to cause protein aggregation (14); local unfolding of the polypeptide may be sufficient to cause irreversible aggregation with or without causing global destabilization of the native state (15, 16). In many proteins this local unfolding can be identified by the rate and mechanism of solvent exchange at the backbone amide hydrogen, as was observed with mass spectrometry for hematopoietic cell kinase (17) and pathogenic variants of lysozyme (18). Such local or global unfolding events by mutant SOD1 may contribute to the pathogenesis of some ALS-SOD1 mutations (19), and the identification of unfolded regions could provide ideal therapeutic targets for the treatment of ALS.

The metal-free and disulfide-oxidized forms (apoSOD1S−5−S) of A4V and WT SOD1 are well structured, having Tm values of 42 and 52 °C, respectively (13, 20). By contrast, the disulfide reduced form of apo-A4V (apoSOD1S−5−S) appears to be globally unfolded, according to differential scanning calorimetry studies, whereas WT apoSOD1S−5−S appears to be well folded and has a Tm of 42 °C (13, 20). X-ray crystallography of the metallated and disulfide-oxidized forms of A4V and WT SOD1 has revealed that the backbone of the A4V and WT polypeptide are superimposable (21, 22), although small perturbations at the dimer interface and the turning of one subunit caused a different orientation of A4V subunits relative to each other (22).

Site-specific HX-MS measures the backbone amide H/D exchange rate in different regions of a protein by subjecting the protein to rapid protein denaturation followed by LC-MS under conditions where isotopic exchange is quenched (18, 23–28). We report here the results of studies using HX-MS to study the structure and dynamics of the pathogenic A4V variant of SOD1 in both the apoSOD1S−5−S and apoSOD1S−5−S states. In accordance with our previous work, we are interested in studying the ALS-linked SOD1 apoproteins with and without the intramolecular disulfide bond. These two demetallated forms of the polypeptide may be particularly relevant to ALS because they represent the most destabilized form of the protein (the Tm of Cu2Zn2 SOD1 is >90 °C), and demetallation may be necessary before aggregation occurs (29). We have found that the A4V mutation causes perturbations to apoSOD1S−5−S that are far from the Val-4 substitution, particularly at residues 50–53, whereas many other regions of the A4V variant, such as β1 and the Greek-key loop VI, are unaffected by the mutation, as measured by HX-MS. Additionally, the A4V mutation promotes the formation of an intermediate apoSOD1S−5−S species that is locally unfolded at residues 21–53. We also report that at 20°C, A4V apoSOD1S−5−S undergoes rapid exchange similar to a completely disordered polypeptide but begins to populate folded states at 4 °C.

EXPERIMENTAL PROCEDURES

WT and A4V apoSOD1S−5−S Proteins—Recombinant human WT and A4V SOD1 were purified from yeast, demetallated, and analyzed with inductively coupled plasma-emission spectroscopy as previously described (30, 31). ApoSOD1 proteins contained <0.09 eq of either copper or zinc per dimer. Aliquots of apoSOD1S−5−S (80 μM) were pre-

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The abbreviations used are: SOD1, superoxide dismutase 1; ALS, amyotrophic lateral sclerosis; WT, wild type; HX-MS, hydrogen exchange monitored by mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; HPLC, high performance liquid chromatography.
pared, flash-frozen in N₂ (liquid) and stored at −70 °C. Mass spectral analysis of each protein confirmed >95% purity, with experimental molecular masses of 15872.92 and 15844.60 Da for A4V and WT apoSOD1, respectively (see the supplemental data). For each day of experiments, a fresh aliquot of SOD1 protein was thawed and kept on ice. All buffers and protein solutions were prepared using deionized water passed through a Millipore® ultrapureification system.

Deuterium Exchange—Because the unstable A4V apoSOD1 protein readily aggregates at room temperature after extended time periods (hours), we chose to conduct site-specific HX-MS experiments at 4 °C. 10 mM deuterated phosphate buffer (pD 7.4) was prepared by SpeedVac evaporation of H₂O from multiple 500-μl aliquots of 10 mM phosphate buffer (pH 7.0) in microcentrifuge tubes. D₂O (500 μL; 99.9% D, Sigma-Aldrich®) was then added back to each dry tube. This process was repeated two additional times. Aliquots of deuterated phosphate buffer were sealed with Parafilm and stored at −20 °C; a fresh aliquot was thawed and used for each day of experiments. Before H/D exchange and mass spectrometry, apoSOD1 samples were transferred to a low salt buffer (10 mM phosphate (pH 7.4)) and concentrated to 800 μL using Microcon® centrifugal filtration devices (molecular weight cutoff, 10 kDa, Millipore®). Concentrated apoSOD1 samples and deuterated buffer were then equilibrated at 4 °C in thin-walled PCR tubes using a Minicycler PCR machine (MJ Research). H/D exchange was initiated by diluting concentrated apoSOD1 samples 1:10 (v/v) into deuterated phosphate buffer at 4 °C. Isotopic exchange was quenched at time points varying from 0.25 to 325 min by the addition of a low pH pepsin solution (see below). Identical experiments were also conducted with proptic buffer to provide a zero time point.

ApoSOD1 S-S-Proteolysis and Electrospray Ionization-LC/MS—Lysophilized pepsin (Sigma-Aldrich®) was weighed and dissolved (5 mg/mL) into low pH buffer (100 mM phosphate, pH 2.5). Fresh aliquots of pepsin solutions were flash-frozen in N₂ (liquid) and stored at −70 °C. A few minutes before each proteolysis, a pepsin aliquot was thawed and kept on ice. H/D exchange was quenched, and proteolysis was initiated at various time points by the addition of 40 μL of low pH pepsin (0 °C) solution and 7 μL of a solution containing 500 mM TCEP (Tris-2-carboxyethyl phosphine hydrochloride; 0 °C) to 20 μL of apoSOD1 S-S-deuterated samples (pepsin:SOD1 ~ 2:1). After 10 min of proteolysis, 20 μL of this solution was injected into the low temperature liquid chromatography apparatus coupled to the electrospray ionization-MS using an ice-chilled, gas-tight syringe. The low temperature HPLC apparatus consisted of a Rheodyne injector (20-μL injection loop) and HPLC reverse phase column (Thermo-electron Bio-Basic 8, 50 × 1 mm, 5-μm particle size) packed in ice and insulated with a foam box. A precolumn micro-filter (Upchurch Scientific) containing a 0.5-μm frit was placed in-line between the injector and column. A fused silica line led from the column outlet directly to the electrospray source. HPLC pumps (Applied Biosystems 120A), which were at room temperature, were connected to the Rheodyne injector with PEEK (polyetherether ketone) tubing.

All H/D exchange mass measurements were made using a PerkinElmer Life Sciences Sciex® (Thornhill, Canada) API III triple quadrupole mass spectrometer with a scanning range of 350–1800 m/z. Reverse phase separation of peptides was done with an increasing, non-linear gradient of acetonitrile in water (2–18% in 1 min, 18–25% in 8 min, 25–29% in 2 min, 29–60% in 1 min) both containing 0.1% trifluoroacetic acid, with a flow rate of 80 μL/min. Calculation of molecular masses from at least 2 charge states and deconvolution of the ion series into a molecular mass spectrum was achieved with MacSpec® software (Version 3.3).

Back-exchange and In-exchange Determination—Although low pH (2.5) and low temperature (0 °C) quenches H/D exchange by ~5 orders of magnitude (32, 33), there is still slow back-exchange of amide hydrogens with solvent protons during proteolysis and LC-MS. To measure and correct for this deuterium loss, the mass of peptides from completely deuterated SOD1 were measured and compared with the theoretical masses of completely deuterated peptides in 90% D₂O. Completely deuterated SOD1 peptides were prepared by heating A4V and WT apoSOD11-35 at 70°C (well above the Tₘ value (13) for apoSOD1) for ~5 min in deuterated phosphate buffer followed by cooling (back down to 4 °C) and proteolysis. Quenching, proteolysis, and mass measurements were carried out as described above. From this mass, the maximum number of exchangeable protons in WT or mutant SOD1 peptides was determined. To determine the amount of deuterium that is incorporated into A4V and WT SOD1 during the 10-min proteolysis (referred to as in-exchange), identical proteolysis experiments were performed with concentrated SOD1 proteins in protic buffer, and upon mixing with low pH pepsin solution, an appropriate volume of D₂O was added immediately to the digest. An average in-exchange value of −7% was determined, and in-exchange contributions were ignored during data analysis.

Peptide Sequencing—Peptides that were generated from the proteolysis of SOD1 were sequenced by reverse-phase liquid chromatography and information-dependent acquisition tandem mass spectrometry (LC-MS/MS) using a Thermo-Finnigan LCQ ion-trap mass spectrometer. Similar reverse phase separation and acetonitrile/water gradients were used for peptide separation. The fragmentation patterns were analyzed with Sequest® software. Peptide identities that possessed cross-correlation values above 3.0 were considered reliable.

Kinetic Analysis of Deuterium Exchange—Deuterium incorporation was plotted as a function of time using SigmaPlot 9.0 (see Figs. 1–3 and 5). The value of unexchanged protons for each peptide was calculated as the difference between the measured mass of partially deuterated SOD1 peptides at various time points (Mₑ) and the mass of the same SOD1 peptides that were completely deuterated (Mₒ). For peptides that exhibited protection from complete exchange, the following sum of three exponentials was fit to the data set,

$$M_{D,t} = M_{D,∞} - Ae^{-kt} - Be^{-kt} - Ce^{-kt} \quad (Eq \ 1)$$

where Mₒ is the peptide mass at each time point in deuterated buffer, Mₑ is the peptide mass in completely exchanged protein, and A, B, and C denote the number of amide hydrogens undergoing exchange with fast (k₁ > 1 min⁻¹), intermediate (0.01 min⁻¹ < k₂ < 1 min⁻¹), and slow (k₃ < 0.01 min⁻¹) rate constants, respectively. The presence of the slow exchanging amide hydrogen atoms proved troublesome in the curve fitting. To make the fittings converge, an artificial “infinity” time and mass point (t = 43,200 min, mass = Mₒ) was added to each data set. For peptides that exchanged completely within the 325 min range, the sum of two exponentials of the following form was fit to that data set, with variables similar to above.

$$M_{D,t} = M_{D,∞} - Ae^{-kt} - Be^{-kt} \quad (Eq \ 2)$$

The theoretical kinetic profiles representing the intrinsic H/D exchange rate for intact SOD1 and SOD1 peptides were calculated according to Bai et al. (33) and are plotted as dashed lines in each kinetic profile (see Figs. 1–3, 5, 6). These curves represent the backbone amide H/D exchange profile of completely unstructured, random coil polypeptides. Intramolecular Disulfide Reduction and Global H/D Exchange of ApoSOD1291—Global H/D exchange experiments at 20 °C and 4 °C (pD 7.4) on the disulfide intact and disulfide broken form of WT and A4V
apoSOD1 were carried out as previously described, as was disulfide reduction of apoSOD1\textsuperscript{1+5} in 100 mM 1,4-dithiothreitol (pH 5.5, 4°C, 24 h) (13).

RESULTS

\textit{ApoSOD1\textsuperscript{1+5} Proteolysis—}The complete proteolysis of apoSOD1\textsuperscript{1+5} required 10 min (pepsin:SOD1 \sim 2:1 per SOD1 dimer), as measured by the absence of multiply charged ions corresponding to the mass of full-length SOD1. The peptides that were identified with LC-MS/MS to be products of SOD1 proteolysis are listed in Table 1. The proteolysis pattern of A4V was identical to WT SOD1; these peptides represent 60% of the SOD1 amino acid sequence. The time required for complete SOD1 proteolysis (10 min) and LC separation of peptides (<10 min), allowed all peptides to be separated and measured by the mass spectrometer in <20 min after the quenching of H/D exchange and the beginning of proteolysis. The values for the amide deuteron back-exchange that occurred during this time period are listed in Table 1 and calculated as the difference between the experimental mass of completely deuterated peptides and the theoretical mass of completely deuterated peptides in 90% D\textsubscript{2}O; these values ranged from 17 to 34% with an average of 24%.

\textit{H/D Exchange at Residues 7–20—}The peptide that contained residue 4 in WT and A4V SOD1 was not sequenced by LC-MS/MS, and although low intensity ions corresponding to the correct mass of these peptides were detected during HX-MS experiments, the mass values were highly variable and considered unreliable in both the mutant and WT protein. However, two peptides comprising residues 7–20 and 9–20 co-eluted in LC-MS and were identified with LC-MS/MS (Table 1, Fig. 1). Residues 7 and 8 are located in the same \textit{\beta}-strand as alanine and valine 4 (B1), and residues 9–20 include loop I, a majority of residues 7–20 co-eluted in LC-MS and were identified with LC-MS/MS (Table 1, Fig. 1).
level of unexchanged protons for A4V (filled circle) and WT (open circle) apoSOD1\textsuperscript{S-S} are superimposable and yield similar kinetic parameters (Fig. 1, Table 1), suggesting that the backbone of residues 7–20 is not altered in A4V apoSOD1\textsuperscript{S-S}. The \( \Delta m/z \) of the doubly charged ions of peptide 7–20 (denoted [MH\textsuperscript{+}]\textsuperscript{2+} or (7–20)\textsuperscript{2+}) from A4V (red) and WT (blue) are shown in Fig. 1C, with the bottom plot representing the fully deuterated peptide, derived from SOD1 that was heated at 70°C before protein exchanges via EX2 kinetics in both A4V and WT apoSOD1\textsuperscript{S-S} (see “Discussion”) (13, 24, 25). H/D exchange for peptide 21–53 also have different values in A4V and WT apoSOD1\textsuperscript{S-S} (Table 1). For example, WT apoSOD1\textsuperscript{S-S} had a \( \kappa \) value of 11.57 ± 0.41, indicating that ~11 protons exchange slowly, with a \( \kappa \) \(< 0.01 \) min\textsuperscript{−1}; A4V had a \( \kappa \) value of 8.75 ± 0.57. Because these differences in H/D exchange between A4V and WT apoSOD1\textsuperscript{S-S} were not detected in peptides 21–42, 21–45, or 21–49 and were only detected in the 21–53 peptide, the structural perturbations responsible for this increased H/D exchange in A4V apoSOD1\textsuperscript{S-S} can be localized to residues 50–53.

The shape of the MH\textsuperscript{3+} peak for peptides 21–42, 45, 49, and 53 in A4V apoSOD1\textsuperscript{S-S} (Fig. 4, A–D) suggests that A4V undergoes correlated exchange throughout residues 21–53 (24, 25, 27). For example, unlike the unimodal mass spectra for A4V peptides 7–20 (Fig. 1C) or 104–116 (Fig. 2C), the mass spectra for A4V peptides in Fig. 4 were bimodal in shape. This correlated exchange can be considered good evidence that residues 21–53 undergo local exchange via an EX1 mechanism (see “Discussion”). The more intense, lower \( m/z \) peak for the four A4V peptides in Fig. 4, A–D, represents SOD1 species that exchange by an EX2 mechanism (the masses derived from these more intense signals were used for the kinetic exchange profiles in Fig. 3, A–D). The lower intensity, higher \( m/z \) shoulder in the four A4V MH\textsuperscript{3+} ion traces represents a species whose mass is approximately equal to the mass of the completely deuterated peptide (Fig. 4, A–D), and this signal represents an A4V SOD1 species that is locally unfolded at these residues. However, the signal representing this fully deuterated species increases slowly and does not become prominent relative to the lower \( m/z \) peak during the experimental time course, suggesting that there is a slow conversion between the native state of A4V apoSOD1\textsuperscript{S-S} and this locally destabilized state. In contrast, the unimodal shape of the (21–53)\textsuperscript{3+} peak from WT apoSOD1\textsuperscript{S-S} suggests that this region undergoes H/D exchange in WT via a predominantly EX2 mechanism (Fig. 4, A–D; see also “Discussion”).

H/D Exchange at Residues 117–144—Both A4V and WT apoSOD1\textsuperscript{S-S} exhibited similar H/D exchange rates at residues 117–144, which include \( \beta \) (residues 116–120) and loop VII (residues 121–142; called the electrostatic loop). In both A4V and WT, these residues exchanged very rapidly. For example, by the second time point (15 s) both peptides exchanged most amide hydrogens with solvent (Fig. 5). Moreover the exchange profiles for these peptides are nearly superimposable with the theoretical curve representing the exchange of a completely disordered
polypeptide with the same amino acid sequence of peptide 117–144 (Fig. 5). The rapid exchange and lack of protection indicates that residues 117–144 are highly disordered in both A4V and WT apoSOD1S-S, even at 4°C.

**Global H/D Exchange of Disulfide-reduced WT and A4V ApoSOD1**—Global H/D exchange was used to study the structure of A4V and WT apoSOD1 in the disulfide-reduced state at 20 and 4°C. WT and A4V SOD1 have 252 labile hydrogen atoms with 146 located on backbone amides, 104 on amino acid side chains, and 2 on the termini. Most of these labile protons (∼190) exchange with D₂O before the first time point (t ≈ 30 s; Fig. 6A). After an 80-min exposure to D₂O (20°C, pH 7.4), WT apoSOD1S-S retained ∼34 unexchanged protons, whereas A4V apoSOD1S-S retained ∼25 (Fig. 6A). Presumably, these unexchanged protons are protected from exchange because they are engaged in hydrogen bonding and/or are solvent-inaccessible.

Relative to the disulfide intact state, WT apoSOD12SH possessed ∼10 fewer unexchanged protons in the disulfide-reduced state after 80 min in D₂O (Fig. 6, A and B). This decrease in protected protons can be attributed to increased solvent exposure of backbone amides and/or the breaking of hydrogen bonds at the dimer interface after the monomerization of apoSOD1, which occurs upon disulfide reduction (34–36). In contrast, A4V apoSOD12SH exchanged rapidly and completely with D₂O, retaining zero unexchanged protons, and the kinetic exchange profile is nearly identical to the calculated profile for a random coil SOD1 polypeptide (33).

H/D exchange experiments on A4V apoSOD12SH at 4°C revealed a bimodal mass distribution with signal intensities changing over time (Fig. 6C). The higher mass signal in Fig. 6C increased rapidly in mass, similar to unstructured A4V apoSOD12SH at 20°C, and had a mass consistent with a fully deuterated A4V SOD1 polypeptide that had zero unexchanged protons. The lower mass signal for A4V apoSOD12SH increased in mass at a rate characteristic of a structured protein and correlated to 26 unexchanged protons at 80 min. The low mass peak also decreased in intensity, whereas the higher mass peak intensity increased over time. This bimodal distribution is best explained by a two-state unfolding-refolding process for apo-A4V2SH, with correlated H/D exchange occurring most likely by an EX1 mechanism (24). The prevalence of a single peak that gradually increases in mass as well as previous temperature-dependent HX-MS data (13) suggests that H/D exchange occurs in WT apoSOD12SH via an EX2 (Fig. 6D; see also "Discussion"). Whereas A4V apoSOD12SH samples the folded and unfolded state at 4°C, WT apoSOD12SH does not populate the unfolded state sufficiently for complete exchange to occur and retains 32 unexchanged protons at 80 min (Fig. 6D).

**DISCUSSION**

**H/D Exchange Near Residue 4 in WT and A4V ApoSOD1S-S**—As discussed earlier, the peptide-containing residue 4 was not identified during the data-dependent LC-MS/MS sequencing experiments for WT or A4V apoSOD1S-S. However, a peptide containing residues 7–20...
(as well as another cleavage product containing 9–20) was sequenced, and this peptide contained the C-terminal end of H92521 and most of H92522 (Fig. 1D). The x-ray crystal structure of metallated human WT SOD1 has shown that residues 20–22 from H92522 are hydrogen-bonded with residues 2–5 of H92521 in metallated WT SOD1 (22) and that in metallated A4V SOD1 the hydrogen bond between Thr-2 and Gln-22 is broken (22). This loss of hydrogen bonding may partly explain the local unfolding of residues 21–53. Other crystallographic work on metallated A4V SOD1 showed that Phe-20 was slightly displaced in comparison to its position in WT SOD1 (21). In the current study residues 7–20 underwent identical exchange in A4V and WT apoSOD1-S, suggesting that no differences in backbone structure are present for these residues in solution (Fig. 1, A–D).

**Residues 50–53 Are De-protected from H/D Exchange in A4V ApoSOD1-S**—The additional exchange of ~2 amide protons seen in the A4V peptide 21–53, but not in 21–49, suggests that residues 50–53 are the site of the backbone perturbations that allow this additional exchange (Figs. 3 and 4). Because we failed to identify peptides beyond residue 53, we have no information about the accessibility of the region beyond 53. Residues 50–53 are located in the disulfide subloop that, along with the zinc subloop, compose the whole of loop IV (Fig. 3E). In x-ray crystal structures of metallated WT and A4V SOD1, the main dimer contacts are residues 50–53, 114, 148, and 150–153 (22); NMR has shown the dimer contacts in solution to be residues 49–54, 113–115, and 148–153 (37). Adding to their known role in SOD1 dimerization, residues Phe-50 and Gly-51 were also found to be an optimal site for engineering a monomeric variant of SOD1 (38). Residues 50–53 are quite far away from residue 4 in the three-dimensional structure, and residue 4 is nearer to residues 50–53 on the adjoining monomer than for its own chain (Fig. 3E). Therefore, it is possible that the perturbations in residues 50–53, caused by the A4V substitution, are intermolecular in nature and that Val4 perturbs residues 50–53 on the adjoining monomer as opposed to residues 50–53 in its own polypeptide chain.

**Disordered β7 and Loop VII in WT and A4V ApoSOD1-S**—The rapid exchange of peptides 117–132 and 117–144 clearly shows that these regions of apoSOD1-S possess very little structure. Peptide 117–144 contains most of β7 (made up of residues 116–120) and all of loop VII (made up of residues 121–142) and the beginning of β8 (residues 143 and 144). Peptide 117–144, therefore, contains ~5 backbone amide protons that are involved in secondary structure hydrogen bonding in...
apoSOD1, yet the exchange profile of this peptide is nearly identical to that of a random coil polypeptide (shown as a dotted line in Fig. 5, A and B). Disorder in part of loop VII has been observed with x-ray crystallography, where residues 125–140 in WT apoSOD1 lack electron density (39). NMR studies showed that residues 121–143 are disordered in the thermostable, monomeric variant of WT apoSOD1 at 25 °C (40). However, these HX-MS data suggests that the disorder around residues 121–142 extends to residues 117–120 in β7 even at the low experimental temperature of 4 °C.

The disorder of loop VII may also explain why ALS-linked amino acid substitutions in this loop do not destabilize the apoprotein (13). For example, the melting temperature (T_m) values and global H/D exchange properties of four loop VII variants (i.e., D124V, D125H, S134N, and N139K) are similar to the WT protein, and the D124V
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variant has a slightly higher $T_m$ compared with WT (13). Although when SOD1 is metallated, loop VII and $\beta_7$ become important determinants of structure and stability, because they are involved, secondarily, in the coordination of copper and zinc. For instance, loop VII contains Asp-124, which hydrogen bonds to the copper and zinc ligands His-46 and His-71; Gly-141 forms hydrogen bonds to the copper ligand His-120, and Arg-143 forms a secondary hydrogen bond to copper ligand His-48 via Gly-61 (2). Consequently, ALS mutations in this loop can diminish the coordination of both copper and zinc, as seen with the low metal content of the MBR mutants D124V, D125H, and S134N (2). Residues in loop VII (125–131) were also shown with x-ray crystallography to be involved in interdimeric contacts between pathogenic SOD1 dimers, and these interactions facilitated the formation of cross-$\beta$ fibrillar structures (41). It is tempting to hypothesize that this large disordered region of SOD1 could facilitate aggregation, but intermolecular interactions involving residues 125–131 could not exist with all ALS mutants because the ALS truncation mutant L126Z lacks most of these residues (8).

Slow, Local Unfolding in A4V ApoSOD1$^{1-5}$—The bimodal mass spectra of peptides from A4V apoSOD1$^{1-5}$ in Fig. 4 indicate that, in addition to the destabilization at residues 50–53, A4V apoSOD1$^{1-5}$ is also destabilized throughout residues 21–53. For example, the bimodal mass distribution for peptide 21–53 in A4V apoSOD1$^{1-5}$ indicates that these residues have undergone correlated exchange; that is, they have exchanged simultaneously with solvent. This correlated exchange observed in the four A4V peptides derived from residues 21–53 indicates that these residues (expressed in A4V apoSOD1$^{1-5}$) are simultaneously deprotected from solvent exchange. Such correlated exchange processes are highly suggestive of exchange via a local EX1 mechanism (42); EX1 exchange processes occur when locally or globally disordered states are sufficiently populated for exchange to occur, and exchange becomes limited by the unfolding rate of the polypeptide.

A4V and WT apoSOD1$^{1-5}$ were previously shown to exchange globally at 10°C, pH 7.4, by an EX2 mechanism (13), indicating that unfolded states are not populated long enough for complete exchange to occur (24, 25). EX2 isotopic exchange processes usually result in a gradually increasing mass signal with a constant, unimodal shape (for a full-length polypeptide or for a peptide fragment) as opposed to a dynamic bimodal mass distribution (27, 42). Similar to the previous global HX-MS results (13), these site-specific HX-MS results show that the WT protein exchanges by an EX2 mechanism throughout all observed residues of the polypeptide as shown by the unimodal mass spectra in Figs. 1C, 2C, and 4C. Likewise, A4V appears to exchange via EX2 kinetics throughout the whole polypeptide, the exception being the subpopulation that underwent correlated exchange at residues 21–53.

Surprisingly, the higher $m/z$ signal in Fig. 4 representing locally unfolded A4V increases in intensity slowly, and this signal never becomes prominent, as would be the case as more and more members of the native ensemble undergo local unfolding and isotopic exchange. This nearly static, bimodal mass distribution suggests that the conversion of the fully native A4V apoSOD1$^{1-5}$ to the locally disordered conformation is slow and that the majority of the native ensemble did not fully convert during the time course of the experiment (5 h). Of course we cannot estimate how long-lived this intermediate is, except to say that it is populated sufficiently for solvent exchange to occur locally. It must be remembered that the closing of this locally open state (i.e. refolding or reordering) cannot be measured with HX-MS because deuterium incorporation is an additive process. Therefore, we cannot determine the relative amount of native and locally unfolded A4V apoSOD1 species present from the relative intensity of the two signals of the bimodal mass spectra in Fig. 4. Zhang et al. (27) have observed similar bimodal mass distributions with static intensities in their HX-MS studies of rabbit muscle aldolase.

Previous work has shown that residues 21–53 contain residues of SOD1 that are especially susceptible to local destabilization in the WT protein. For example, NMR folding studies on the metallated, monomeric variant of WT SOD1 (Cu,Zn-E133QM2 SOD1) demonstrated that the $\beta_3$ (residues 29–36) in addition to all seven loops (residues 23–28 and 37–40) make up loops II and III) were the first regions of SOD1 to be affected during guanidinium hydrochloride unfolding experiments. Residues in the other $\beta$-strands were only affected at significantly higher concentrations of guanidinium hydrochloride (43).

Local unfolding has been previously observed with HX-MS in the $\beta$-domain of pathogenic lysozyme variants that cause amyloidosis (18); local unfolding and EX1 exchange can also be thermally or chemically induced and observed with HX-MS (28) as was recently reported for the heat shock transcription factor $\sigma^{17}$ (44).

Disulfide-reduced A4V ApoSOD1 Exchanges Like a Random Coil Polypeptide—Whereas the disulfide intact state of A4V apoSOD1 is well structured at 20°C, the disulfide-reduced form of apo-A4V exchanged with solvent rapidly at 20°C, suggesting that A4V apoSOD1$^{1-5}$ is globally disordered or unfolded (Fig. 6B). At lower temperatures (4°C) A4V apoSOD1$^{1-5}$ partially populated folded states, as shown by the bimodal mass distribution (Fig. 6C), where the lower mass represents folded SOD1 that is protected from complete exchange. The higher mass peak in Fig. 6C represents unfolded A4V SOD1 that has undergone correlated exchange, presumably through an EX1 mechanism. Site-specific H/D exchange was not carried out on A4V apoSOD1$^{1-5}$ simply because the protein is globally disordered at 20°C and experiences no protection from exchange. The existence of a globally disordered A4V apoSOD1$^{1-5}$ is corroborated by recent differential scanning calorimetry work on apoSOD1$^{1-5}$ showing that A4V apoSOD1$^{1-5}$ lacks observable changes in heat capacity (13, 20). However, WT and other ALS mutants such as H48Q and D101N apoSOD1$^{1-5}$ exhibit endothermic transitions that yield similar $T_m$ values (42°C) (13).

Two of the aggregating proteins implicated in Parkinson disease, Alzheimer disease, and several other neurodegenerative disorders, $\alpha$-synuclein and tau, are natively unfolded (45). It is intriguing to note that the A4V mutation causes apoSOD1$^{1-5}$ to be similarly disordered well below physiological temperature. Nevertheless, this global disorder is not a property common for all ALS SOD1 variants, such as H48Q and D101N apoSOD1$^{1-5}$ (13). Hence, if globally disordered SOD1 is necessary for pathogenesis in SOD1-linked ALS, other biochemical or physiological factors must be required to induce the disordering of the reduced apoSOD1 variants whose properties are more similar to the WT protein.

Can Dimer Destabilization Explain A4V SOD1 Pathogenesis?—We have shown that the subunit interface of A4V apoSOD1$^{1-5}$ is partially disrupted in solution, with backbone amide protons at residues 50–53 unprotected from exchange. Moreover, the slow, local unfolding at residues 21–53 of A4V apoSOD1$^{1-5}$ may be initiated by the destabilizations at residues 50–53. These results seem to coincide with various reports suggesting that the A4V substitution promotes SOD1 aggregation by perturbing the dimer interface (22, 29, 46–48). Indeed, protein stabilizing Effectors such as intersubunit cross-links (46) and small organic molecules (49) were recently shown to slow A4V SOD1 aggregation via dimer stabilization. It should be noted however, that the most destabilized form of A4V SOD1 is the metal-free, disulfide-reduced form, which is a globally disordered polypeptide and only begins to populate

**Figure 4**. Zhang et al. (27) have observed similar bimodal mass distributions with static intensities in their HX-MS studies of rabbit muscle aldolase.
folded states at low, physiologically irrelevant temperatures (i.e. at 4 °C, Fig. 6C). Therefore, it is feasible that the pathogenic SOD1 species could be a newly synthesized polypeptide that misfolds early on, as opposed to SOD1 polypeptides that are protected from aggregation long enough to fold properly into stable (Tm > 75 °C), active dimers (13, 31).

**Similarly Destabilized Residues in A4V and G93A SOD1**—Previous NMR studies have shown that the fully metallated (Cu2Zn5) G93A SOD1 variant is structurally perturbed at residues 92–95 (loop V) and at residues 35–42 (β4) as compared with the WT protein (50). As discussed above, residues 35–42 are included in the segment of A4V apoSOD1S-S that is locally destabilized. Considering that metal coordination orders β4, lengthening it from residues 41–44 in apoSOD1 to include residues 41–46 in metallated SOD1 (37, 40, 51), it is possible that the G93A substitution would destabilize the metal-free form of the protein to include more of the same residues that are destabilized in A4V apoSOD1S-S (13, 31). Moreover, the similarly destabilized residues in A4V and G93A SOD1 may represent aggregation prone residues of SOD1; residues 35–42 are included in the segment of A4V apoSOD1S-S that is locally destabilized. Considering that metal coordination orders β4, lengthening it from residues 41–44 in apoSOD1 to include residues 41–46 in metallated SOD1 (37, 40, 51), it is possible that the G93A substitution would destabilize the metal-free form of the protein to include more of the same residues that are destabilized in A4V apoSOD1S-S (13, 31). Moreover, the similarly destabilized residues in A4V and G93A SOD1 may represent aggregation prone residues of SOD1; residues 35–42 were recently reported to be 1 of 4 regions of the SOD1 sequence that are “hot spots” for amyloidogenesis (52).

Finding areas of local destabilization that are common to more ALS-SOD1 mutant proteins may provide insight into the cytotoxicity of ALS-variant SOD1 (15). However, a commonly perturbed region may not be found with all ALS-causing apoSOD1 proteins because destabilization of the native state is not common to all pathogenic SOD1 variants.

**Conclusion**—We can only speculate whether the locally unfolded A4V apoSOD1S-S species described here represents a unique SOD1 conformer that is required for the neurodegenerative effects of SOD1. In addition, this local destabilization will probably not be found with all ALS variants such as D101N and E100K that have similar global H/D exchange kinetics and Tm values compared with the WT protein (13). These extremely wild type-like proteins are also not completely unfolded in their disulfide-reduced metal-free state (13). Hence, other parameters such as charge, amino acid sequence, or processes leading to SOD1 demetallation may explain the increased aggregation propensity of these very stable proteins. Nevertheless, in the case of A4V SOD1, the pathogenic substructure causes the unfolding of a large segment of the β-barrel, and this local unfolding may be sufficient for SOD1 aggregation.

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