Conformational Disorder of the Most Immature Cu, Zn-Superoxide Dismutase Leading to Amyotrophic Lateral Sclerosis*

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Yoshiaki Furukawa‡, Itsuki Anzai‡, Shuji Akiyama‡*, Mizue Imai†, Fatima Joy C. Cruz‡, Tomohide Saio‡**, Kenichi Nagasawa‡, Takao Nomura‡, and Koichiro Ishimori‡***

From the ‡Laboratory for Mechanistic Chemistry of Biomolecules, Department of Chemistry, Keio University, Yokohama 223-8522, §Research Center of Integrative Molecular Systems (CIMoS), Institute for Moleculer Science, Okazaki 444–8585, ‡Department of Functional Molecular Science, SOKENDAI (The Graduate University for Advanced Studies), Okazaki 444-8585, ‡Graduate School of Chemical Sciences and Engineering, Hokkaido University, Sapporo 060-8628, and **Department of Chemistry, Faculty of Science, Hokkaido University, Sapporo 060-0810, Japan

Misfolding of Cu,Zn-superoxide dismutase (SOD1) is a pathological change in the familial form of amyotrophic lateral sclerosis caused by mutations in the SOD1 gene. SOD1 is an enzyme that matures through the binding of copper and zinc ions and the formation of an intramolecular disulfide bond. Pathogenic mutations are proposed to retard the post-translational maturation, decrease the structural stability, and hence trigger the misfolding of SOD1 proteins. Despite this, a misfolded and potentially pathogenic conformation of immature SOD1 remains obscure. Here, we show significant and distinct conformational changes of apoSOD1 that occur only upon reduction of a disulfide bond in solution. In particular, loop regions in SOD1 lose their restraint and become significantly disordered upon dissociation of metal ions and reduction of the disulfide bond. Such drastic changes in the solution structure of SOD1 may trigger misfolding and fibrillar aggregation observed as pathological changes in the familial form of amyotrophic lateral sclerosis.

Mutations in Cu,Zn-superoxide dismutase (SOD1) are linked to familial forms of amyotrophic lateral sclerosis (fALS) (1). A major pathological change observed in SOD1-related fALS is the abnormal accumulation of misfolded mutant SOD1 proteins in affected motor neurons (2). Actually, many in vivo as well as in vitro studies have supported that pathogenic mutations facilitate the misfolding of SOD1 proteins (3); however, the molecular mechanism triggering the misfolding of SOD1 remains controversial.

SOD1 is known as one of the most stable proteins to the extent that its melting temperature (Tm) is >90 °C (4); therefore, a misfolding event appears quite unlikely for SOD1. Nonetheless, SOD1 was found to have acquired such high stability through several post-translational processes including copper and zinc binding and disulfide bond formation (Fig. 1A). Actually, disulfide-reduced apoSOD1 exhibits significantly decreased stability (Tm ~ 42 °C) and is susceptible to unfolding/misfolding at physiological temperatures (5, 6). Intracellular deregulation of metal binding and/or disulfide formation will, hence, be a key event triggering the misfolding of SOD1.

Notably, many pathogenic mutations are found to disturb the post-translational control of SOD1 maturation (7, 8) and thereby increase intracellular fractions of the apo- (9) and/or disulfide-reduced state (10). Only when both metal ions and disulfide bond are absent, SOD1 forms fibrillar aggregates (11). Given that SOD1 fibrillation is a pathological hallmark in SOD1-related fALS patients (12) as well as model mice (13), the most immature form of SOD1 will provide a clue to understand the molecular pathomechanism of this devastating disease. In a number of previous studies, the roles of metal binding and disulfide formation in the misfolding of SOD1 have been suggested by a variety of experimental methods (6, 8, 11, 14–19); however, conformational information on SOD1 in solution lacking both metal ions and the disulfide bond, which is the only state accessible to fibrillar aggregates (11), is still limited. Also, a crystal structure of apoSOD1 lacking the disulfide bond has implied little impacts of metal binding and disulfide formation on its overall folding pattern albeit with increased disorder at loop regions (19). This is probably because the crystallization process sorts the folded conformation out of many other conformations adopted by immature SOD1 in solution. Indeed, significant changes in chemical shifts were concentrated on loop regions (loops IV and VII; Fig. 1A) between the solid state and solution NMR spectra of apoSOD1 with a disulfide bond in crystalline and solution state, respectively (20). Nonetheless, the degree of disorder in the loop regions of immature SOD1 is still ambiguous. To understand the molecular mechanism of...
SOD1 misfolding in SOD1-related fALS, conformational features of apoSOD1 lacking a disulfide bond, which is prone to fibrillation, therefore need to be clarified in more detail.

Here, we have investigated conformational features of the most immature SOD1 lacking both metal ions and the disulfide bond by using spectroscopic and scattering methods. Although metallated SOD1 with a disulfide bond exists as a homodimer, apoSOD1 without the disulfide bond has been shown to favor a monomeric state and is considered to adopt a protomer conformation (21). In this study we nonetheless found that a conformation of disulfide-null apoSOD1 in solution was significantly distinct from that of the SOD1 protomer; in particular, loop regions usually connected via bound metal ions and a disulfide bond (loops IV and VII; Fig. 1A) lost their restraint and were significantly disordered upon both dissociation of metal ions and reduction of the disulfide bond. Such distinct conformational disorder of SOD1 realized only in its most immature state will be discussed in relation to the pathological changes observed in SOD1-related fALS cases.

**Experimental Procedures**

**Preparation of SOD1 Proteins—Escherichia coli SHuffle™** (New England BioLabs) was transformed with a pET15b plasmid containing cDNA of human SOD1 with an N-terminal His<sub>6</sub> tag, and the expression of His-tagged SOD1 proteins was induced by cultivating the transformed cells in the presence of 0.1 mM isopropyl-β-D-1-thiogalactopyranoside at 20 °C for 16 h. His-tagged SOD1 proteins in the lysates were purified with a HisTrap HP (1 ml, GE Healthcare) and dialyzed against a buffer without EDTA, and then an equimolar amount of ZnSO₄ of apoSOD1 samples was first exchanged to a Chelex-treated preparation of Zn²⁺ Nacalai Tesque) with NNE buffer as the running buffer. For filtration column chromatography (Cosmosil 5Diol-300-II, Healthcare), and the purified apoSOD1 was obtained using gel the sample solution using HiTrap benzamidine (1 ml, GE Health care), and then measured as a reference. For hydrogen-deuterium exchange experiments, the 15N-labeled proteins (3 mM) were diluted 10× with NN buffer containing 10% D₂O, and the 1H, 15N heteronuclear single quantum correlation (HMQC) spectra were then measured as a reference. For hydrogen-deuterium exchange experiments, the 15N-labeled proteins (~3 mM) were diluted 10× with D₂O buffer containing 10% D₂O, and the 1H, 15N heteronuclear single quantum correlation (HMQC) spectra were then measured as a reference.
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FIGURE 1. The most immature state of SOD1 is monomeric, but its conformation is significantly different from that of an SOD1 protomer. A, a crystal structure of SOD1 homodimer (PDB ID 1HLS). Loops IV (cyan) and VII (pink) of SOD1 are indicated in the crystal structure, and the disulfide bond between Cys-57 and Cys-146 is shown in yellow. Ligands for binding copper and zinc ions are also shown: gray, His-46, His-48, and His-120 for copper binding; red, His-71, His-80, Asp-83 for zinc binding; purple, His-63 for copper and zinc binding. B and C, gel filtration chromatograms of E,E-SOD1noCys (red open circles), E,E-SOD1(57/146)S-S (black open circles), and E,E-SOD1(F50E/G51E)S-S (blue open circles) (B) and E,Zn-SOD1noCys (red-filled circles), E,Zn-SOD1(57/146)S-S (black-filled circles), and E,Zn-SOD1(F50E/G51E)S-S (blue-filled circles) (C) were shown. A sample containing 60 μM protein in 100 mM Na-Pi, 100 mM NaCl at pH 7.0 was loaded on the gel filtration column, and the elution profile was monitored with absorbance (Abs.) change at 280 nm (left axis). Molecular mass obtained by MALS analysis is also shown in each chromatogram (right axis).

Results and Discussion

SOD1 has four Cys residues in total, among which Cys-57 and Cys-146 form an intramolecular disulfide bond (Fig. 1A). The other two Cys residues (Cys-6 and Cys-111) do not form disulfide bonds in native SOD1 but are susceptible to aberrant...

\[
\text{Ln}(I(Q)) = \ln(I(0)) - \frac{R_g^2}{3} \cdot Q^2 \quad \text{(Eq. 1)}
\]

where \( I(0) \) and \( R_g \) are the forward scattering intensity \((Q = 0)\) and the radius of gyration, respectively. Experimental SAXS data were manipulated using PRIMUS (28) and GNOM (29). The theoretical \( R_g \) value of the crystal structure of SOD1 (PDB ID 2C9V) was calculated using CRYSOL (30).

Shape Reconstructions—Low-resolution shapes of E,E-SOD1noCys were restored from SAXS data using GASBOR (31), scored with DAMAVER (32), and visualized with the SITUS package (33). The rigid-body and ensemble models of E,E-SOD1noCys were refined by using BUNCH (34) and EOM (35), respectively. During both refinements the \( β \)-barrel scaffold (residues 5–52 and 84–124) of E,E-SOD1noCys was treated as a rigid body harboring three flexible loops in its N terminus (residues 1–4), a region including loop IV (residues 53–83), and a region from loop VII to the C terminus (residues 125–157). Coordinates of the rigid body were taken from an x-ray crystal structure of SOD1 (PDB ID 2C9V). The flexible loops were first assumed as dummy residues, and the conformations were then refined against the experimental SAXS data while keeping the rigid body structure intact. In the ensemble modeling, 10,000 hypothetical rigid-body models were randomly generated, from which a plausible ensemble was suggested by refining populations and compositions of the hypothetical structures.

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SOD1 Becomes a Monomer with Decreased Contents of Secondary Structures upon Losing Both Metal Ions and the Disulfide Bond—To understand conformational features of the most immature SOD1, a quaternary structure of apoSOD1noCys (E,E-SOD1noCys) was examined by SEC-MALS. Native SOD1 is a homodimeric protein (Fig. 1A), but the monomer-dimer equilibrium has been known to be regulated by the binding of metal ions and the formation of the disulfide bond (21, 37). Indeed, SOD1 in which all four Cys residues were mutated to Ser (SOD1noCys) was used as the model of SOD1 lacking the disulfide bond.

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FIGURE 2. Contents of secondary structures were decreased in the most immature and aggregation-prone state of SOD1. Far-UV CD spectra (A), near-UV CD spectra (B), FTIR spectra (in second derivatives) (C), and aggregation kinetics of E,E-SOD1noCys (red open circles), E,Zn-SOD1noCys (red-filled circles), E,E-SOD1(57/146)S-S (black open circles), and E,Zn-SOD1(57/146)S-S (black-filled circles) (D) are shown. The samples for CD measurements contained 10 μM (far UV) and 400 μM (near UV) proteins in 20 mM Na-Pi, 50 mM NaCl at pH 8.0, whereas FTIR spectra were measured by using 1 mM protein in 100 mM Tris, 100 mM NaCl, pH 7.0. Aggregation kinetics was examined by using 20 μM SOD1 and monitored by the increase of solution turbidity. Abs., absorbance.

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troscopy. Matured SOD1 (i.e. a copper- and zinc-bound state with a disulfide bond, Cu,Zn-SOD1(57/146)S-S) exhibits a negative CD signal at 208 nm with a distinct shoulder around 230 nm, consistent with the /H9252-barrel structure of SOD1 (data not shown and Ref. 39). Removing copper ion from Cu,Zn-SOD1(57/146)S-S did not change the CD spectrum (E,Zn-SOD1(57/146)S-S), suggesting limited effects of copper ion to the secondary structure of SOD1 (data not shown and Fig. 2A). In contrast, the binding of a zinc ion and the formation of a disulfide bond were found to play significant roles in attaining the secondary structures (Fig. 2A); upon dissociation of a zinc ion, the peak around 230 nm became vague, and a negative CD signal around 200 nm increased its intensity. In E,E-SOD1noCys (red open circles, Fig. 2A) in particular, a negative CD signal at 208 nm that was evident in other SOD1 species examined in this study was lost, and an additional signal was observed around 200 nm, implying the presence of random coils (40). We also measured the CD spectra in the near-UV region and thereby attempted to characterize the effects of disulfide formation and Zn$^{2+}$ binding on the tertiary structure of SOD1. A near-UV CD spectrum reflects the environments around aromatic side chains (Trp, Phe, Tyr) and disulfide bonds (41). As shown in Fig. 2B, significant differences in spectral shape were observed between SOD1(57/146)S-S and SOD1noCys, which probably reflects the contribution of the disulfide bond to CD signals in this region (240–290 nm) (41). On the other hand, the effects of Zn$^{2+}$ ion on the spectrum were minimal in SOD1(57/146)S-S and SOD1noCys. Although interpretation of near-UV CD spectra has not been well established (41), the results would support no drastic changes of SOD1 in the regions near Trp and Phe residues (no Tyr residue in SOD1) upon losing a Zn$^{2+}$ ion.

The presence of random coils in E,E-SOD1noCys was further confirmed with FTIR spectroscopy. As shown in Fig. 2C (red open circles), the second derivative of an IR spectrum of E,E-SOD1noCys showed an absorption peak at 1642 cm$^{-1}$, which is characteristic to random coils (42). Upon the addition of an equimolar Zn$^{2+}$ ion to E,E-SOD1noCys, an absorption peak at 1630 cm$^{-1}$ that corresponds to /H9252-sheet structures (42) emerged, whereas random coil structures indicated by an absorption peak at 1643 cm$^{-1}$ still remained (Fig. 2C, red-filled circles). In contrast, both apo- and Zn$^{2+}$-bound SOD1 with its disulfide bond exhibited an absorption peak at 1630 cm$^{-1}$ but not 1642 cm$^{-1}$, suggesting the formation of /H9252-sheet structures (Fig. 2C, black-filled and open circles). Although SOD1 has already been shown to become fibrillated only in the apo- and disulfide-reduced state (11), we have confirmed again in this study that E,E-SOD1noCys but not E,E-SOD1(57/146)S-S is fibrillogenic and also that the addition of excess Zn$^{2+}$ ions suppresses the fibrillation of SOD1 proteins (Fig. 2D). Accordingly, a distinct conformational disorder of apoSOD1 that is realized only in the absence of the disulfide bond may have the potential to trigger the fibrillation of SOD1.

The /H9252-Barrel-like Scaffold of SOD1 Is Not Significantly Affected in the Most Immature State— Whereas increased fractions of random coils were evident in apoSOD1 upon losing the disulfide bond, it is notable that the absorption peak at 1673
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FIGURE 4. SAXS experiments support distinct conformations between E,E-SOD1\textsuperscript{noCys} and E,E-SOD1(57/146)\textsuperscript{S-S}. A, SAXS curves of E,E-SOD1\textsuperscript{noCys} (filled circles) and E,E-SOD1(57/146)\textsuperscript{S-S} (open circles). Scattering intensity, I(Q), is plotted against scattering angular momentum, Q, in a logarithmic scale. The indicated curves were obtained by merging the two curves taken with different camera lengths, one at infinite dilution (0.02592 Å\(^{-1}\) < \(Q < 0.70975\) Å\(^{-1}\)) and the other at finite concentration (0.06994 Å\(^{-1}\) < \(Q < 1.48845\) Å\(^{-1}\)). B, Guinier plots of the scattering curves obtained in E,E-SOD1\textsuperscript{noCys} and E,E-SOD1(57/146)\textsuperscript{S-S} (open circles) at infinite dilution. Each line shows the linear fit to ln I(Q) using the Q range from 0.02588 to \(Q_{\text{max}} < 1.3/R_g\) (black data points), which gave the estimated values of \(R_g\) and ln I(0) (Table 1). C, The pair-distance distribution function, \(P(r)\), from the scattering profile is shown: E,E-SOD1\textsuperscript{noCys} (filled circles) and E,E-SOD1(57/146)\textsuperscript{S-S} (open circles). \(D_{\text{max}}\) values obtained from \(P(r)\) are summarized in Table 1.

| TABLE 1 | SAXS structural parameters |
|---------|---------------------------|
| Proteins | \(R_g\) | \(R_g\) | \(I(0)\) | \(I(0)\) | \(D_{\text{max}}\) | \(V_p\) | \(M_r\) from \(I(0)\) | \(M_r\) from \(V_p\) |
|---------|--------|--------|--------|--------|-------------|-------|----------------|----------------|
| E,E-SOD1(57/146)\textsuperscript{S-S} | 21.5 ± 0.2 | 21.2 ± 0.1 | 4.67 ± 0.03 | 4.63 ± 0.01 | 68 | 5.85 \times 10^4 | 38.7 | 35.4 | 16.2 |
| E,E-SOD1\textsuperscript{noCys} | 18.4 ± 0.2 | 17.7 ± 0.1 | 2.31 ± 0.02 | 2.26 ± 0.01 | 58 | 3.00 \times 10^4 | 19.1 | 18.2 | 16.2 |
| BSA\(^\text{a}\) | 27.6 ± 0.3 | ND. \(^\text{b}\) | 8.01 ± 0.05 | ND. \(^\text{c}\) | ND\(^d\) | ND\(^d\) | ND\(^d\) | ND\(^d\) | 66.4 |

\(^{a}\) Guinier analysis using the Q range from 0.02588 to \(Q_{\text{max}} < 1.3/R_g\).

\(^{b}\) Estimates in real space upon \(P(r)\) determination.

\(^{c}\) Maximum dimension estimated by using GNOM package.

\(^{d}\) Porod volume.

\(^{e}\) \(M_r\) molecular mass calculated by using the \(I(0)\) value for BSA as the standard.

\(^{f}\) \(M_r\) calculated according to an empirical relationship (\(M_r = V_p/1.65\)) (53).

\(^{g}\) Theoretical \(M_r\) calculated according to amino acid sequences.

\(^{h}\) Extrapolated to infinite dilution.

\(^{i}\) At finite concentration of 3.06 mg/ml (54).

\(^{j}\) Not determined.

...corresponding to \(\beta\)-sheet structures, were still observed in E.E-SOD1\textsuperscript{noCys} (Fig. 2C, red open circles). The structure of SOD1 consists of a \(\beta\)-barrel-like fold with two major loop regions (loop IV and loop VII; also see Fig. 1A). As suggested by previous studies (14, 19, 25–27), upon dissociation of metal ions and/or reduction of the disulfide bond, the two major loops appear to increasingly fluctuate with a mostly retained structure of the \(\beta\)-barrel-like core region.

To probe the effects of the disulfide reduction on the protein folding of our apoSOD1 samples, we took advantage of the fact that SOD1 has a single Trp residue in the \(\beta\)-strand of the \(\beta\)-barrel-like scaffold and examined its fluorescence properties in E,E-SOD1(57/146)\textsuperscript{S-S} and E,E-SOD1\textsuperscript{noCys}. In particular, the wavelength of maximum fluorescence emission of Trp has been known to be an indicator of changes in the environment surrounding Trp residues (43). Actually, the fluorescence peak was red-shifted (~6 nm) when E.E-SOD1\textsuperscript{noCys} and E.E-SOD1(57/146)\textsuperscript{S-S} were unfolded in the presence of guanidine hydrochloride (data not shown); nonetheless, the spectra were almost completely overlapped between E.E-SOD1(57/146)\textsuperscript{S-S} and E.E-SOD1\textsuperscript{noCys} (data not shown). These results hence further support that the \(\beta\)-barrel structure in apoSOD1 is not largely affected upon reduction of the disulfide bond.

Actually, previous NMR studies on several immature states of SOD1 have shown that the \(\beta\)-barrel structure is well maintained and that the flexible loop regions (loop IV and VII) experience extensive mobilities upon removal of metal ions and/or reduction of the disulfide bond (14, 25–27). To confirm the distinct behaviors of the \(\beta\)-barrel structure and the loop regions, we performed a hydrogen-deuterium (H/D) exchange analysis on the \(^1\)H, \(^15\)N HSQC spectra of our E.E-SOD1\textsuperscript{noCys} and E.E-SOD1(57/146)\textsuperscript{S-S} proteins at 283 K. After dilution of the protein sample with deuterated buffer, intensities of the resonances from amide protons decreased due to the H/D exchange. Fig. 3 represents time-dependent and heterogeneous changes of the resonance intensities in E.E-SOD1\textsuperscript{noCys} and E.E-SOD1(57/146)\textsuperscript{S-S} over the entire proteins. In this study, 20\% of resonance intensities relative to those before H/D exchange were set as a threshold; namely, the resonances of the residues colored red almost disappeared with <20\% of the original intensities before dilution, whereas significant intensities (>20\%) of resonances were maintained in the residues colored...
In both E,E-SOD1noCys and E,E-SOD1(57/146)S-S, most of the residues with relatively slow H/D exchange (colored blue) are found in β-strands, consistent with a robust β-barrel scaffold of SOD1 (Fig. 3). Moreover, all of the observed resonances from the residues in loop IV and VII already disappeared in E,E-SOD1noCys at 4 h after H/D exchange (Fig. 3A), but notably, resonances from some of the residues in loop IV and VII of E,E-SOD1(57/146)S-S (Gly-61, Arg-69, His-71, Asp-76, Glu-78 in loop IV; Thr-135 and Ser-142 in loop VII) retained significant intensities even after 36 h of exchange (Fig. 3B). These data are thus consistent with the flexible nature of those loop regions and also imply that the loop regions in E,E-SOD1 would become more disordered upon losing the disulfide bond. To get more insight into the effects of disulfide reduction on the conformation of demetallated SOD1 species, we further evaluated their molecular size and shape by utilizing SAXS.

The Most Immature SOD1 Has a Significantly Distinct Conformation from That of Dimeric SOD1 in Solution—Fig. 4A shows scattering curves of E,E-SOD1(57/146)S-S and E,E-SOD1noCys at infinite dilution. From the Guinier plot of the scattering curve, forward scattering intensity, I(0), was first determined from the intercept of the linear fit (Fig. 4B) and then used for estimation of a relative molecular mass (Table 1). Based upon the estimates, E,E-SOD1(57/146)S-S and E,E-SOD1noCys were found to be a dimer and a monomer in solution, respectively. These estimated masses are also consistent with the ones empirically calculated from the Porod volume (Vp, Table 1), further strengthening the fact that apoSOD1 became a monomer upon reduction of the disulfide bond (Fig. 1, B and C).

Furthermore, in the Guinier plot (Fig. 4B) the slope of the linear fit contained information on the radius of gyration, Rg, of a molecule. As summarized in Table 1, the Rg value of E,E-SOD1(57/146)S-S was estimated from the plot as 21.5 Å, which is comparable with the one calculated based upon the crystal structure of matured human SOD1 (PDB ID 2C9V, 20.9 Å) (30). In contrast, E,E-SOD1noCys showed an Rg of 18.4 Å, which was significantly larger than the one calculated from the protomer in the crystal structure of SOD1 dimer (15.5 Å) (30). A less compact structure in E,E-SOD1noCys was further supported by the pair-distance distribution function, P(r); E,E-SOD1noCys and E,E-SOD1(57/146)S-S had a maximum dimension, Dmax, of 58 and 68 Å, respectively (Fig. 4C, Table 1). The observed Dmax of E,E-SOD1(57/146)S-S was comparable with the one calcu-
lateral from the crystal structure of matured SOD1 dimer (70.7 Å), but again, E,E-SOD1noCys in solution had a $D_{\text{max}}$ that was larger than that of a protomer of SOD1 in its crystal structure (47.7 Å). Collectively, these results clearly indicate that monomeric E,E-SOD1noCys adopts a relatively extended conformation compared with a monomer unit of the dimer.

Actually, a low resolution model of E,E-SOD1noCys, which was restored from the scattering curve using GASBOR (31), could not be superimposed well to a monomer unit of the native SOD1 dimer and was more ellipsoidal (Fig. 5A, Table 2). More precisely speaking, our experimental SAXS curve of E,E-SOD1noCys was significantly deviated from the theoretical one calculated using a protomer of the SOD1 crystal structure ($\chi = 7.29$; Fig. 5B). These observations may be reconciled with our results to point to the conformational disorder of loops IV and VII upon losing the disulfide bond in apoSOD1. To confirm this, we refined the conformations of loops IV and VII in the SOD1 crystal structure against the experimental SAXS curve of E,E-SOD1noCys while keeping the $\beta$-barrel scaffold intact. As shown in Fig. 5, B and C, a rigid body model of disulfide-null apoSOD1, in which loops IV and VII were “peeled” off from the $\beta$-barrel scaffold, gave a scattering curve quite similar to the experimentally observed one ($\chi = 1.98$).

Nevertheless, loops IV and VII in the rigid body model are highly extended (Fig. 5C) and thus seem to be inappropriate for the description of a single distinct conformer. Rather, taking into account that previous studies showed significant fluctuations at loops IV and VII in immature forms of SOD1 (14, 25–27), E,E-SOD1noCys is considered to adopt multiple conformations with highly mobile loops IV and VII. Indeed, compared with the rigid body model, the experimental SAXS curve of E,E-SOD1noCys was a better fit to the theoretical scattering curve from an ensemble of conformations carrying the flexed loops IV and VII ($\chi = 1.14$) (Fig. 6A, Table 2). Most of the simulated conformations exhibited $R_g$ of 16–19 Å and $D_{\text{max}}$ of 40–70 Å, whereas minor conformations also populated at $R_g$ 21–27 Å and $D_{\text{max}}$ 70–100 Å (Fig. 6, B and C). It is, therefore, possible that the most immature apoSOD1 without the disulfide bond is monomeric with significant fluctuations in loops IV and VII (Fig. 6D), resulting in a conformation distinct from that of a protomer of the native SOD1 dimer.

**Table 2**

| Shape reconstruction | E,E-SOD1noCys |
|----------------------|---------------|
| GASBOR2.2i           | 0.02618–1.110 |
| Q range (Å$^{-1}$)   |               |
| Symmetry             | P1            |
| Total number of residues | 157 |
| Dummy residues       | 4–53, 83, 125–157 |
| Total number of known residues | 89 |
| Number of models reconstructed | 10 |
| DAMAVER NSD (mean ± S.D.) | 0.980–1.061 (1.015 ± 0.023) |

**BUNCH08**

| Q range (Å$^{-1}$)   | 0.02618–0.49974 |
| Symmetry             | P1             |
| Total number of residues | 157 |
| Dummy residues       | 4–53, 83, 125–157 |
| Total number of known residues | 89 |
| Number of models reconstructed | 10 |
| DAMAVER NSD (mean ± S.D.) | 1.049–1.166 (1.103 ± 0.033) |

**EOM2.0**

| Q range (Å$^{-1}$)   | 0.02618–0.49974 |
| Symmetry             | P1             |
| Total number of residues | 157 |
| Dummy residues       | 4–53, 83, 125–157 |
| Total number of known residues | 89 |
| Number of models reconstructed | 10 |
| DAMAVER NSD (mean ± S.D.) | 1.138–1.147 (1.143 ± 0.002) |

**Pathological Significance of the Most Immature SOD1—Misfolding/aggregation of SOD1 proteins is a major pathological change in SOD1-related fALS patients (44). It has been suggested that demetallation and/or disulfide reduction are involved in misfolding of SOD1 for the formation of insoluble aggregates (3). Actually, only the most immature state of SOD1 (modeled by E,E-SOD1noCys in this study) was accessible to the formation of fibrillar aggregates (Fig. 2D) (11). In other words, E,E-SOD1noCys can be regarded as a precursor for fibrillation, and we have found here that its structural conformation is unique and distinct from that of the dimeric SOD1 in the metalated and/or disulfide-bonded states. In particular, severe disorder at loops IV and VII is considered to be responsible for such a unique conformation of fibrillation-prone E,E-SOD1noCys (Figs. 5 and 6).

Given that loop IV contains all three zinc ligands (His-71, His-80, Asp-83) and the copper-zinc bridging ligand (His-63) (Fig. 1A), demetallation is expected to cause increased disorder around the loop. Indeed, it has been suggested that in the $^{1}H,^{15}N$ HSQC spectra of apoSOD1SH/S-S, the amino acid residues at loops IV and VII exhibit significant changes in their chemical shifts upon binding of a $Zn^{2+}$ ion (25, 26). Many of the amino acid residues in loop IV have not been identified both in the crystal and solution structures of metal-deficient SOD1SH/S-S due to severe thermal fluctuations (14, 19, 45). Moreover, the disulfide bond tethers loop IV (Cys-57) to the $\beta$-strand after loop VII (Cys-146) (Fig. 1A); therefore, loss of the disulfide bond is also a significant contribution to the increased fluctuation of the loops. Actually, the absence of the disulfide bond facilitated the H/D exchange of amide protons at loops IV and VII of apoSOD1 (Fig. 3). Given that either demetallation or disulfide reduction alone is not sufficient for triggering fibrillation of SOD1 proteins (Fig. 2D) (11), the increased disorder of loops IV and VII, which are realized only in the most immature state of SOD1, could facilitate fibrillation.

As described above, loop IV includes both the disulfide bonding Cys residue (Cys-57) and the $Zn^{2+}$ binding ligands (Fig. 1A); therefore, fluctuations in loop IV caused by the removal of metal ions would affect the conformation around the disulfide bond. Indeed, lesser amounts of dithiothreitol were required to reduce the disulfide bond in E,E-SOD1S-S compared with its $Zn^{2+}$-bound form, E,Zn-SOD1S-S (Fig. 7A), suggesting that the disulfide bond becomes increasingly accessible upon losing the metal ions. Also, the chemical modification of Cys-57 and -146 in the disulfide-reduced state (SOD1SH) was found to proceed more efficiently in the apo form than in its $Zn^{2+}$-bound form (Fig. 7B); certain amounts of the unmodified state remained in
E,Zn-SOD1SH, but the modification was almost completed in E,E-SOD1SH. This is also supported by the NMR-derived solution structures of apo- and Zn2+/H11001-bound SOD1 with a disulfide bond (14, 46) in which Cys-57 and Cys-146 are found to become more exposed to the solvent upon dissociation of a Zn2+/H11001 ion (47) (Fig. 7C). Collectively, therefore, these data show that the disulfide bond as well as its constituents, Cys-57 and -146, are more exposed to the solvent upon losing metal ions. SOD1 is localized mostly in the cytoplasm, where the redox environment is highly reducing; therefore, exposure of the disulfide bond to the solvent would increase the chance of being attacked by endogenous reductants such as glutathione. Once the disulfide bond is reduced, apoSOD1 became a monomer with an ellipsoidal conformation in which loops IV and VII are possibly wide-open (Figs. 5 and 6).

Although both concentration and specific activities of SOD1 were decreased in erythrocytes from affected SOD1-related fALS patients (48), it is notable that enzymatic activity of SOD1 is almost fully retained in transgenic mice expressing human SOD1 with pathogenic mutations (49). In physiological conditions, therefore, mutant SOD1 could exist initially as a matured state: i.e. a copper, zinc-bound state with a disulfide bond. Given that affinity for Zn2+/H11001 ion has been shown to decrease in mutant SOD1 proteins in vitro (7), mutant SOD1 in the matured state would nevertheless gradually lose its bound metal ions in the cytoplasm with high chelating capacity for metal ions. In motor neurons with a meter-long axon in particular, more than a year will be necessary for SOD1 to be anterogradely transported to the nerve termini (50, 51); therefore, it may be difficult for

**FIGURE 6.** An experimental SAXS curve of E,E-SOD1noCys was reasonably fit with an ensemble of conformations. From 10,000 hypothetical rigid-body models with the randomly flexed loops IV and VII, 10 independent ensembles of conformations were reconstructed to fit the experimental SAXS curve of E,E-SOD1noCys (Fig. 4A). A theoretical curve obtained from a representative ensemble of conformations with the flexed loops IV and VII is shown in red and well matched with the observed scattering curve (open squares, $\chi = 1.14$). A scattering curve calculated from a monomer unit of the SOD1 crystal structure is again shown for comparison (blue, $\chi = 7.29$). A distribution of $R_g$ (B) and $D_{\text{max}}$ of conformations (C) in each of those 10 ensembles is shown. D, representative conformations in a population with (left) 17.9 Å and (right) 24.2 Å of $R_g$ are shown. Regions allowed for conformational changes during refinements are colored cyan (loop IV) and pink (from loop VII to the C terminus).
mutant SOD1 to survive in a matured form during the axonal transport. Losing metal ions from the matured state of mutant SOD1 could gradually occur in a year-long scale and contribute to the accumulation of the most immature SOD1 proteins (Fig. 7D).

Actually, the amount of SOD1-positive inclusions formed in model mice appear to be negatively correlated with the affinity of mutant SOD1 for metal ions. For example, human SOD1 with mutation abrogating (H46R) or significantly reducing (G85R) metal binding have been found to accumulate significant amounts of inclusions, whereas formation of inclusions was quite limited in the model mice expressing mutant SOD1 retaining the metal binding ability (e.g. G37R, G93A) (24, 52).

These pathological observations are thus consistent with our proposed mechanism where failure of metal acquisition in SOD1 increased the amounts of the disulfide-reduced state in the cytoplasm (Fig. 7D), and a unique conformation of the most immature SOD1 will be a promising target for controlling the pathogenicity of mutant SOD1 in ALS.

Author Contributions—Y. F. directed the project, performed the experiments, analyzed most of the data, and wrote the manuscript. S. A. performed the SAXS experiments and analyzed the data. M. I., F. J. C. C., T. S., and K. I. performed the NMR experiments and analyzed the data. K. N., I. A., and T. N. contributed to the preparation and measurements of protein samples for MALS, SAXS, fluorescence, and NMR. All authors review the manuscript.

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