Cutting Off Functional Loops from Homodimeric Enzyme Superoxide Dismutase 1 (SOD1) Leaves Monomeric \(\beta\)-Barrels\(^*\)

Jens Daniëllson, Martin Kurnik, Lisa Lang, and Mikael Oliveberg

From the Department of Biochemistry and Biophysics, Arrhenius Laboratories of Natural Sciences, Stockholm University S-106 91 Stockholm, Sweden

Demetallation of the homodimeric enzyme Cu/Zn-superoxide dismutase (SOD1) is known to unleash pronounced dynamic motions in the long active-site loops that comprise almost a third of the folded structure. The resulting apo species, which shows increased propensity to aggregate, stands out as the prime disease precursor in amyotrophic lateral sclerosis (ALS). Even so, the detailed structural properties of the apoSOD1 framework have remained elusive and controversial. In this study, we examine the structural interplay between the central apoSOD1 barrel and the active-site loops by simply cutting them off; loops IV and VII were substituted with short Gly-Ala-Gly linkers. The results show that loop removal breaks the dimer interface and leads to soluble, monomeric \(\beta\)-barrels with high structural integrity. NMR-detected nuclear Overhauser effects are found between all of the constituent \(\beta\)-strands, confirming ordered interactions across the whole barrel. Moreover, the breathing motions of the SOD1 barrel are overall insensitive to loop removal and yield hydrogen/deuterium protection factors typical for cooperatively folded proteins (i.e. the active-site loops act as a “bolt-on” domain with little dynamic influence on its structural foundation). The sole exceptions are the relatively low protection factors in \(\beta\)-strand 5 and the turn around Gly-93, a hot spot for ALS-provoking mutations, which decrease even further upon loop removal. Taken together, these data suggest that the cytotoxic function of apoSOD1 does not emerge from its folded ground state but from a high energy intermediate or even from the denatured ensemble.

\(*\) This work was supported by grants from Swedish Research Council, the Knut and Alice Wallenberg Foundation, the Bertil Hållsten Foundation, and Hjärnfonden. This work was also supported by the Access to Research Infrastructures Activity in the 6th Framework Program of the EC (Contract RI3–026145, EU-NMR) for conducting the research at CERM.

\(1\) To whom correspondence should be addressed. Tel.: 46-8-162459; E-mail: mikael.oliveberg@dbb.su.se.

\(2\) The abbreviations used are: ALS, amyotrophic lateral sclerosis; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; H/D exchange, hydrogen/deuterium exchange; H-bond, hydrogen bond.

Cu/Zn-superoxide dismutase (SOD1) is a ubiquitous radical scavenger that is observed to misfold and aggregate into small intracellular inclusions in the motor neurons of patients with both familial and sporadic amyotrophic lateral sclerosis (ALS)\(^2\) (1–3). As with other protein misfolding diseases (e.g. Alzheimer disease, Parkinson disease, and the prion diseases), the structural events underlying the pathological aggregation (4, 5) and also the role of this aggregation in the neurodegeneration are still obscure (6). Native SOD1 is a thermodynamically stable (7), long lived (8, 9), and structurally robust (10) homodimer that coordinates one redox-active Cu\(^{1+}/2+\) ion and one structural Zn\(^{2+}\) ion in each subunit (7, 11–13). The Cu\(^{1+}/2+\) ion is coordinated directly to the protein’s immunoglobulin-like scaffold, whereas the neighboring Zn\(^{2+}\) ion is coordinated more peripherally by the long loop IV (Fig. 1). Loop IV also stretches across the SOD1 surface to support the dimer interface, contains the conserved disulfide bond between Cys-57 and Cys-146, and forms together with loop VII the rigid encasement of the metallated active site (Fig. 1). Upon dissociation of the metal ions, however, the active-site loops loosen up and become more dynamic (14–18), coupled to a radical loss of protein stability (7, 9, 18) and increased propensity to aggregate (19–22). Consistently, several in vivo studies (23) and folding analysis of ALS-associated SOD1 mutations (24) implicate apoSOD1 as the precursor for pathological misfolding and aggregation. Moreover, there is congruent evidence that the core of the SOD1 aggregates involves sequence elements from the \(\beta\)-barrel, at least as they occur in aggregation assays in vitro (25) and during over-expression in bacteria and transgenic mice (26). The question is then from which state of the apoSOD1 molecule the misfolding commences. To shed light on this issue, we examine here the role of the long functional loops in modulating the stability and structure of the apoSOD1 barrel. Experimentally, we replace loops IV and VII with short Gly-Ala-Gly linkers and follow the structural consequences by NMR and folding analysis. The results show that the loop removal is energetically favorable in the sense that it increases the thermodynamic stability of the apoSOD1 monomer but has limited effect on the structure and dynamic motions of the barrel to which they are anchored. In essence, the apoSOD1 barrel retains ordered structure and cooperative folding transition in both the presence and absence of loops IV and VII. Together with the low thermodynamic stability of the immature apoSOD1 monomers, this intrinsic two-state nature of the SOD1 barrel points at the globally unfolded state as the starting material for gain of toxic function in ALS.

EXPERIMENTAL PROCEDURES

**Gene Design**—The monomeric reference protein, SOD1\(^{\text{pwt}}\), was obtained from the human SOD1 wild-type sequence by introduction of the following mutations: C6A/C111A, which...
The Structural Properties of the Loop-depleted SOD1 Barrel

prevents intermolecular cross-linking, and the dimer-splitting substitutions F50E/G51E (27). SOD1IIIV,AVII was designed from the wild-type sequence by replacement of residues 49–81 (loop IV) and 124–139 (loop VII) with Gly-Ala-Gly tripeptide linkers. As a result of loop IV removal, a significant portion of the dimer interface, including Phe-50 and Gly-51 (Fig. 1), as well as the disulfide bond between Cys-57 and Cys-146, were eliminated. Further, to avoid intermolecular disulfide cross-linking, we removed the remaining free cysteines by the mutations C6A/ C111S/C146S. The resulting nucleotide sequence is ATGCCACAAAGCGGTGCTAGCTGAAAGGTGTGAT- GGTCGGTGACGGGCAATCAATCTGGAAACAAAGG- GAGGACCCAGGTCCGTCATAAGGTGTTTACCTC- GAAATGTCATACGGAGTCTGAGTAATGAACTGC- GGACAAAGCAGGTTGTTGCTGACGTATCCATCGAGG- ACTCTGTATATTACCTTCTGTGTCACACGTACATC- ATGGTCCTAGCCTGGTGTCTGTATCATGACAGCAG- CAGGTTGCTGTTCTGCTGACTTCGTTATATCGGAT- TATTGGCAGCTGAAGTATTGCGCAGTAA.

The reason for using C111S, rather than C111A, in SOD1IIIV,AVII gene is that this mutation yields an unfolding rate constant indistinguishable from that of the parent protein SOD1pw, which offers a considerable advantage in the interpretation of H/D exchange data (see below).

Overexpression and Protein Purification—The SOD1IIIV,AVII gene was purchased from Entelechon GmbH (Regensburg, Germany) and subcloned into pET3a from Novagen, EMD Chemicals (Gibbstown, NJ). After transformation into Escherichia coli strain BL21(DE3) by heat shock, protein overexpression was induced at 37 °C in LB medium in the presence of 100 μg/ml carbenicillin, 1 g/liter 15NH4Cl, and 4 g/liter 13C6 glucose, and induced using 0.5 mM isopropyl 1-thio-D-galactopyranoside. Growth was continued for 5 h before harvesting by centrifugation at 5000 rpm in a Beckman Avanti J-25 centrifuge, JA-25.50 rotor, followed by incubation in 1% (v/v) cation, and centrifuged at 15,000 rpm in a Beckman Avanti J-25 centrifuge, JA-25.50 rotor, and resuspended in 50 mM Tris-HCl, pH 7.5, before application of the supernatant to a Q-Sepharose system from Bio-Rad and by electrospray ionization mass spectrometry performed at the Protein Analysis Center (Karolinska Institute, Solna, Sweden) after dialysis against milliQ H2O and centrifugation at 18,000 rpm in a Beckman Avanti J-25 centrifuge, JA-25.50 rotor. For the variant SOD1IIIV,AVII S111A and S111A/S146C, mutagenesis was performed on the SOD1IIIV,AVII gene using the QuikChange™ site-directed mutagenesis kit from Stratagene (Agilent Technologies, Santa Clara, CA) with primers from Eurofins MWG Operon (Ebersberg, Germany).

Equilibrium and Kinetic Measurements—Fast refolding and unfolding kinetics (log kobs > −2.5) were measured in 10 mM BisTris, pH 6.3, from Sigma-Aldrich at 25 °C by Trp fluorescence using an Applied Photophysics PiStar-180 stopped-flow spectrometer (Leatherhead, UK). The excitation wavelength was 280 nm, and emission was collected with a 320-nm cut-off filter. The final protein concentration was 4 μM. Equilibrium unfolding and slow kinetics (log kobs < −2.5) were measured on a Varian Cary Eclipse spectrophotometer (Santa Clara, CA) with excitation at 280 nm and emission collected at 360 nm. Ultrapure urea from MP Biomedicals Inc. (Solon, OH) was used in all denaturation experiments. The SOD1 monomers were assumed to display two-state behavior, yielding the expression,

\[
K_{UF} = \frac{[U]}{[F]} = \frac{k_f}{k_u} \tag{Eq. 1}
\]

where U and F represent the unfolded and folded monomers, respectively, and ku and kf are the unfolding and refolding rate constants, respectively (28, 29). Protein stability, ΔG_{U/F} = −2.3RT log K_{UF}, was further assumed to depend linearly on [urea], yielding the following,

\[
\log K_{UF} = \log k^{H_2O} + m_{UF}[urea] \tag{Eq. 2}
\]

and

\[
\log k_u = \log k^{H_2O}_u + m_u[urea] \tag{Eq. 3}
\]

\[
\log k_f = \log k^{H_2O}_f + m_f[urea] \tag{Eq. 4}
\]

where \(k^{H_2O}_u\) and \(k^{H_2O}_f\) represent the rate constants extrapolated to 0 M denaturant, and \(m_u\) and \(m_f\) are constants that reflect the sensitivity to the denaturant, commonly perceived as a measure of the change in solvent-accessible surface area in the activation process of unfolding or refolding, respectively (30).

The chevron plots of observed rate constants obtained from stopped-flow experiments (i.e. log kobs = log(\(k_f + k_u\)) (Fig. 3) were then fitted to the standard two-state expression (29),

\[
\log k_{obs} = \log(k_f + k_u) = \log(10^{\log k^{H_2O}_f + m_f[urea]} + 10^{\log k^{H_2O}_u + m_u[urea]}) \tag{Eq. 5}
\]

where \(k^{H_2O}_f\) and \(k^{H_2O}_u\) represent the rate constants at 0 M urea, and \(m_f\) and \(m_u\) are the slopes of the refolding and unfolding limbs, respectively. Data analysis was performed using the Applied Photophysics Pro-Data Viewer (Leatherhead, UK) and Kaleidagraph (Abelbeck Software). Equilibrium unfolding data (Fig. 3) were fitted according to the following.
The Structural Properties of the Loop-depleted SOD1 Barrel

FIGURE 1. The structure of the native SOD1 dimer (Protein Data Bank entry 1HL5) and the loop regions removed by protein engineering. A, in the native SOD1 dimer, the long loops IV and VII adapt a compact and highly ordered structure around the active site, where loop IV also forms part of the dimer interface (green). The left-hand monomer is shown as accessible surface (1.4 Å probe radius), whereas the right-hand monomer is represented as a schematic diagram. Highlighted are the residues coordinating the active-site Cu$^{1+}$/Zn$^{2+}$ ions and the Cys-57-Cys-146 disulfide linkage between loop IV and the central β-barrel. B, to examine the structural interplay between the active-site loops and the SOD1 barrel, we substituted loops IV and VII with short Gly-Ala-Gly linkers. The figure shows the energy-minimized structure of the loop-free construct apoSOD1$^{IV, VII}$.

\[
I_{obs} = \frac{a[\text{urea}] + b[\text{urea}] + h_{\text{dil}}}{1 + 10^{\frac{\text{MPeq}[\text{urea}]-\text{MPeq}}{10}}} \\
\text{(Eq. 6)}
\]

where \(I_{obs}\) represents the observed fluorescence intensity, \(I_F\) and \(I_U\) are the fluorescence intensities of folded and unfolded protein, respectively, \(a\) and \(b\) are the base-line slopes, and \(\text{MPeq}\) is the denaturation midpoint. Data were analyzed as described (31).

NMR Spectroscopy—All experiments were performed at 25 °C in 10 mM BisTris buffer. Chemical shifts of 13C- and 15N-labeled apoSOD1$^{IV, VII}$ were determined at ~1 mM protein concentration using standard 15N-1H HSQC (32–34), HNCA (35, 36), HN(CO)CA (35, 36), CBCANH (37), C(CA)CNH (38), HNCACB (39), HNCO (35, 36, 40), 15N-edited NOESY (41), and total correlation spectroscopy (32, 41, 42) experiments on a Bruker 700 MHz spectrometer (Bruker Avance, Karlsruhe, Germany) equipped with a cryogenically cooled triple resonance probe. Spectra were transformed using nmrPipe and analyzed using the program Sparky (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco).

\(T_1\), \(T_2\), and steady state heteronuclear NOE experiments were performed on a Bruker 600-MHz spectrometer equipped with a triple resonance probe. Spectra were transformed using nmrPipe and analyzed using the program Sparky. In the \(T_1\) and \(T_2\) experiments, the signal attenuation, from 10 different relaxation delays, was fitted to a single exponential decay, and the relaxation rates were determined. The fitting routine was performed using MATLAB (Mathworks, Natick, MA).

Diffusion experiments were performed using a pulse field gradient longitudinal encoding-decoding (PFG-LED) sequence (43) with a gradient prepulse on a Bruker 600-MHz spectrometer. The 1H signal intensity was determined at 32 linearly spaced gradient strengths, and the attenuating intensity was fitted to the Stejskal-Tanner equation. The diffusion delay was 150 ms, and the gradient pulses were 5 ms. The gradient strength was calibrated using the water and α-cyclodextrin diffusion at 25 °C.

H/D exchange was initiated by diluting the protein sample with D$_2$O to 50% final D$_2$O concentration. The exchange rate was detected as the attenuation of the peak intensity in 15N-1H HSQC experiments. The decay of the peak intensities was fitted to a single exponential decay with base line with a linear slope.

RESULTS AND DISCUSSION

Loop Removal Diminishes the Dimer Interface and Leads to Soluble Monomers

To examine how the active-site loops influence the structural properties of the apoSOD1 molecule, we truncated them by protein engineering (Fig. 1). Loop IV was replaced with a short Gly-Ala-Gly linker between His-48 and Gly-82, removing 49 of the 153 residues comprising the wild-type monomer. In addition, to avoid aggregation by disulfide cross-linking, we substituted Cys-6 with Ala, and Cys-111 and the leftover Cys-146 with Ser. The energy-minimized structure of the loop-depleted protein (SOD1$^{IV, VII}$) is shown in Fig. 1. Essentially, SOD1$^{IV, VII}$ comprises nothing but the naked, barrel scaffold of the wild-type monomer, including the poorly structured loop VII comprises nothing but the naked, barrel scaffold of the wild-type monomer, including the poorly structured loop VI (15) that links β6 and β7 (Fig. 1). SOD1$^{IV, VII}$ was subsequently cloned and overexpressed with high yields in E. coli.

The first notable effect of loop removal is that SOD1$^{IV, VII}$ migrates as a monomer in size exclusion chromatography (Fig. 2). Splitting of the dimer is fully consistent with structural predictions; excision of loop IV substantially reduces the dimer
The Structural Properties of the Loop-depleted SOD1 Barrel

interface area (Fig. 1). Moreover, purification of SOD1\(^{\text{IV, VII}}\) yields apoprotein without coordinated metals. As an additional control of the solution state of apoSOD1\(^{\text{IV, VII}}\), we measured the protein’s translational diffusion coefficient (\(D_t\)), which reports directly on the hydrodynamic dimensions and overall fold of the structure (44, 45). The analysis was done by PFG-NMR diffusion experiments. As references for monomeric and dimeric SOD1, we used the hydrodynamic radius of 19.7 Å (Fig. 2). This value is slightly lower than \(R_H\) for the wild-type monomer (11) but is in good agreement with the predicted radius of 18.6 Å for a globularly folded protein of 110 residues (45). Accordingly, the solution state of apoSOD1\(^{\text{IV, VII}}\) seems to be a folded monomer, fully consistent with the dimensions of the naked SOD1 barrel in Fig. 1. The conclusion is further supported by the spectral difference between apoSOD1\(^{\text{IV, VII}}\) and the apoSOD1\(^{\text{pwt}}\) monomer as measured by CD, which shows a coil-like component consistent with the x-ray structure of the active-site loops (see supplemental material S1).

Chevron Data Show That ApoSOD1\(^{\text{IV, VII}}\) Is a Two-state Folder with Enhanced Protein Stability

Stopped-flow analysis (28, 29) shows that the refolding (\(k_f\)) and unfolding (\(k_u\)) rate constants of apoSOD1\(^{\text{IV, VII}}\) yield a v-shaped chevron plot (Fig. 3). Moreover, the values of \(k_f\) and \(k_u\) match the equilibrium constant \((K_{U/F})\) obtained from equilibrium denaturation data according to Equation 1 (Table 1 and Fig. 3). The chevron plot of apoSOD1\(^{\text{IV, VII}}\) lacks further the

![Figure 2](image1)

**FIGURE 2.** Removal of loops IV and VII from apoSOD1 reduces the dimer interface and leads to soluble apoSOD1\(^{\text{IV, VII}}\) monomers. A, size exclusion chromatography (Sephacryl S-100) shows that apoSOD1\(^{\text{IV, VII}}\) elutes as a monomer directly after apoSOD1\(^{\text{pwt}}\). The elution profile of a mixture of apoSOD1\(^{\text{IV, VII}}\) and apoSOD1\(^{\text{pwt}}\) (solid line) can be deconvoluted into two separate peaks corresponding to the elution of apoSOD1\(^{\text{pwt}}\) (gray line) and apoSOD1\(^{\text{IV, VII}}\) (dotted line). The identities of proteins in the partly overlapping elution peaks were confirmed by SDS-PAGE. B, NMR diffusion data for apoSOD1\(^{\text{IV, VII}}\) showing PFG-NMR signal intensity attenuation consistent with free monomers. The data are fitted to the Stejskal-Tanners equation, yielding a hydrodynamic radius of 19.7 Å according to Stokes-Einstein’s relationship. For comparison, the expected intensity attenuation for a SOD1 dimer with hydrodynamic radius of 28 Å is shown as a dotted line.

![Figure 3](image2)

**FIGURE 3.** Chevron plots (A) and equilibrium unfolding transitions (B) of apoSOD1\(^{\text{IV, VII}}\) and apoSOD1\(^{\text{pwt}}\) showing that loop removal increases the refolding rate constant (\(k_f\)) and decreases the unfolding rate constant (\(k_u\)). Data are from oxidizing conditions where the disulfide linkage Cys-57-Cys-146 of apoSOD1\(^{\text{pwt}}\) is intact. As a consequence of these kinetic changes, SOD1\(^{\text{IV, VII}}\) becomes more stable than SOD1\(^{\text{pwt}}\) and requires higher urea concentration to unfold. The chevron data are fitted to Equation 6 (Table 1). a.u. denotes arbitrary units. ApoSOD1\(^{\text{pwt}}\) data are from Ref. 48.

**TABLE 1**

| Kinetic and thermodynamic parameters of the apoSOD1\(^{\text{IV, VII}}\) and apoSOD1\(^{\text{pwt}}\) monomers |
|---------------------------------------------------------------|
| ApoSOD1\(^{\text{pwt}}\)                                      | ApoSOD1\(^{\text{IV, VII}}\)                        |
| \(\log k_f^\text{G270} (s^{-1})\)                           | \(-1.12 \pm 0.10\)                                  |
| \(\log k_f^\text{G270} (s^{-1})\)                           | \(-0.28 \pm 0.03\)                                  |
| \(m_{\text{G270}} (s^{-1})\)                                 | \(-3.91 \pm 0.05\)                                  |
| \(m_{\text{G270}} (s^{-1})\)                                 | \(-4.03 \pm 0.04\)                                  |
| \(m_{\text{G270}} (s^{-1})\)                                 | \(-1.08 \pm 0.10\)                                  |
| \(m_{\text{G270}} (s^{-1})\)                                 | \(-0.78 \pm 0.01\)                                  |
| \(m_{\text{G270}} (s^{-1})\)                                 | \(0.46 \pm 0.01\)                                  |
| \(m_{\text{G270}} (s^{-1})\)                                 | \(0.27 \pm 0.01\)                                  |
| MP(U/F) (m^2/g)                                              | \(1.54 \pm 0.10\)                                  |
| MP(U/F) (m^2/g)                                              | \(1.05 \pm 0.01\)                                  |
| MP(U/F) (m^2/g)                                              | \(1.81 \pm 0.08\)                                  |
| MP(U/F) (m^2/g)                                              | \(3.57 \pm 0.04\)                                  |
| MP(U/F) (m^2/g)                                              | \(3.79 \pm 0.15\)                                  |
| MP(U/F) (m^2/g)                                              | \(5.1 \pm 0.07\)                                  |
| MP(U/F) (m^2/g)                                              | \(1.17 \pm 0.10\)                                  |
| MP(U/F) (m^2/g)                                              | \(0.99 \pm 0.10\)                                  |
| MP(U/F) (m^2/g)                                              | \(2.12 \pm 0.06\)                                  |
| MP(U/F) (m^2/g)                                              | \(3.64 \pm 0.05\)                                  |
| MP(U/F) (m^2/g)                                              | \(3.38 \pm 0.30\)                                  |
| MP(U/F) (m^2/g)                                              | \(4.91 \pm 0.48\)                                  |

\(^{a}\) Equilibrium unfolding and chevron data from Ref. 31.

\(^{b}\) Derived from fitting of Equation 5 to kinetic data in Fig. 3.

\(^{c}\) Derived from fitting of Equation 6 to equilibrum unfolding data in Fig. 3 (31).
The Structural Properties of the Loop-depleted SOD1 Barrel

curved unfolding limb characteristic for the dimeric protein (24, 29) and is qualitatively the same as that for the apoSOD1\textsuperscript{pwt} monomer (46) (Fig. 3). Such folding behavior constitutes the hallmark for globular proteins that obtain their structures in cooperative two-state transitions (28, 29),

\[ \frac{k_f}{k_{down}} \]

where \( U \) represents the unfolded state, \( F \) is the folded protein, and \( k_{down} \) is the downhill rate constant. As an independent test of two-state folding, we have recorded the NMR HSQC spectrum at the midpoint of the urea equilibrium transition. Consistent with Scheme 1, these data show only a clean mixture of random coil and folded state cross-peaks and no trace of populated intermediates (data not shown). Removal of loops IV and VII thus seems to obliterate dimerization without affecting the two-state folding behavior of the SOD1 monomers. Even so, the chevron plots of apoSOD1\textsuperscript{IV, VII} and the apoSOD1\textsuperscript{pwt} monomer display two interesting differences. First, loop removal induces a nearly 10-fold increase of the folding rate constant; \( \log k_{H2O} \) increases from \(-1.12 \pm 0.1 \) to \(-0.28 \pm 0.03 \) (Table 1). The corresponding effect on the unfolding rate constant, however, is much smaller, with extrapolated \( \log k_{H2O} \) values of \(-3.91 \pm 0.05 \) and \(-4.03 \pm 0.04 \) for apoSOD1\textsuperscript{IV, VII} and apoSOD1\textsuperscript{pwt}, respectively (Table 1). Coupled to these changes of \( k_f \) and \( k_u \), is a gain in protein stability of \( \Delta G_{U/F} = -1.31 \pm 0.17 \) kcal/mol (Equations 2–4 and Table 1). The stability gain is mainly due to the replacement \( \Delta S_{IV, VII} \), which accounts for \(-1.29 \) kcal/mol (supplemental Fig. S2 and Table S1). The source of this stability gain seems to be better space filling at the protein surface around position 111; Ser mimics more closely the native Cys than the smaller Ala. The contribution from the mutation C146A in SOD1\textsuperscript{IV, VII}, on the other hand, seems relatively small, judged by the double mutation SOD1\textsuperscript{IV, VII, C146A} (Table 1). The effects of mutation in positions 111 and 146 of SOD1\textsuperscript{IV, VII} are in good agreement with previous results for the wild type-like apo monomer (29). On this basis, we conclude that loops IV and VII contribute relatively little to the stability of the apo monomer, despite being an integral part of the native SOD1 structure. More detailed interpretation of the energetics of loop removal is currently precluded by the unknown impact of the Gly-Ala-Gly linkers; in addition to reducing the sequence separation between the anchoring strands, these could destabilize the SOD1\textsuperscript{IV, VII} by steric strain. The second effect of loop removal is a pronounced decrease of the slope of the unfolding limb (\( m_u \)); the area dependence of \( \log k_u \), goes down (Table 1). Such a selective decrease of the \( m_u \) value indicates that the folded state (F) has reduced in size (i.e. loop removal leads to smaller exposure of surface area in the global unfolding process) (47, 48). Consequently, an identical change of the \( m_u \) value is observed for a SOD1 variant in which loops IV and VII are locally unfolded due to mis-
Evidence for Ordered Barrel Structure

Extensive Network of Long Range NOEs—In earlier studies, it has been suggested that the β-sheet facing the active-site loops gains increased conformational freedom and becomes partly disordered upon demetallation of the SOD1 dimer (14). To see whether such structural loosening is triggered also by loop removal, we mapped out the sequential and long range NOEs within the apoSOD1\(^{\text{IV, VII}}\) structure. The presence of long range NOEs indicates that the contacts mediating the dipolar couplings are close, relatively long lived, homogeneous, and stable (52). Such robust long range contacts constitute the hallmark for ordered tertiary structure in globular proteins (53). As expected, sequential NOEs are found throughout the backbone of the apoSOD1\(^{\text{IV, VII}}\) structure. In addition, we observe an extensive network of long range NOEs joining together all strands of the β-barrel into a native-like tertiary topology (Fig. 6). The only gap in this pattern is the absence of NOEs between the ends of β5 and β6 that splay apart in the crystallographic structure of the wild-type protein. This slit in the barrel hydrogen bonding closes up toward the short linkage between β5 and β6, which contains the “torsioned” residue Gly-93, a hot spot for ALS-associated mutations. Another notable region is the interface between β4 and β5, which comprises relatively few NOEs. These NOEs are mainly between the adjacent pairs Gly-41–Ala-89 and His-43–Val-87 at the top of the barrel, whereas the lower interface between β4 and β5 lacks NOEs. The pattern is, again, consistent with the slight divergence of β4 and β3 at these positions seen in the x-ray structure. Moreover, it can be noted that the Zn\(^{2+}\) ligand Asp-83 imposes a twist to the C-terminal end of β5, which could further compromise the interactions with β4. Strand 5 can thus be seen as moving away from the edge of the active-site sheet to partly fill the slit to β6. It is possible that this frustrated arrangement of β5 compromises its structural rigidity in the absence of coordinated metals. Overall, these NOE data match the subset of NOEs observed for the β-barrel of the apoSOD1\(^{\text{pwt}}\) monomer (15), showing that the truncation of loops IV and VII has no major effect on the SOD1 scaffold. On this basis, we conclude that the solution structure of apoSOD1\(^{\text{IV, VII}}\) is a rigid β barrel, in good accord with its two-state folding behavior (Fig. 3).

\(^{15}\text{N} \text{NMR Relaxation Data}—\text{Evidence for an ordered SOD1 barrel is further provided by data from }^{15}\text{N} \text{NMR relaxation measurements (54, 55) (supplemental material S5). The heteronuclear }^{15}\text{N}–{^1}\text{H} \text{NOE values are overall high (0.81 ± 0.1) and constant along the apoSOD1\(^{\text{IV, VII}}\) backbone, with only slight dips in the loop regions connecting the β strands. Such high order of the backbone is characteristic for well packed, folded structures (56). For comparison, the more dynamic loops IV and VII of apoSOD1\(^{\text{pwt}}\) show average NOE values of 0.44 and 0.27, respectively (supplemental material S5). As a final test of the conformational state of apoSOD1\(^{\text{IV, VII}}\), we used the ratio of the relaxation rates \(R_2/R_1\) to estimate the rotational correlation time (\(\tau_c\)) (57), which constitutes a sensitive measure of the protein’s hydrodynamic dimensions (supplemental material S5). By omitting the relaxation rates from the flexible loop regions, we calculated \(\tau_c\) of the apoSOD1\(^{\text{pwt}}\) monomer to 9 ns, corresponding to a globular protein with a hydrodynamic radius \(R_h = 21.5\) Å. This estimate is in good agreement with the previously published value of 22.5 Å (11). Correspondingly, the \(\tau_c\) and \(R_h\) values of apoSOD1\(^{\text{IV, VII}}\) were determined to be 6.7 ns and 19.5 Å, respectively, which are in excellent agreement with the results from the pulse field gradient-NMR diffusion experiments in Fig. 2. Taken
together, these data provide strong evidence that the solution structure of apoSOD1\textsuperscript{I\!V} is a rigid \(-\)barrel, in good accord with its two-state folding behavior (Fig. 3).

Detection of Rare Structural Fluctuations by Hydrogen Exchange Experiments

A common method for studying rare fluctuations in protein structures is native state H/D exchange of the backbone amides (58, 59). The method is based on the observation that amide protons are protected from H/D exchange when they are fixed in stable intramolecular H-bonds. As long as an individual backbone H-bond is protected in this manner, it is considered to be in its closed state (C). For exchange to occur, the bond has to open up transiently to interact with the solvent water molecules that carry the deuterons. It needs to undergo a transition to the open state (O) as follows,

\[ C \xrightarrow{k_{\text{open}}} O \xrightarrow{k_{\text{ex}} \text{int}} O \xrightarrow{k_{\text{close}}} C \]  

\textbf{SCHEME 2}

where \( k_{\text{open}} \) and \( k_{\text{close}} \) are the rate constants for structural opening and closing, respectively, and \( k_{\text{ex}} \text{int} \) is the intrinsic rate of exchange in the open state (58). At steady state, the observed rate constant for H/D exchange (\( k_{\text{ex}} \text{obs} \)) is then given by the following,

\[ k_{\text{ex}} \text{obs} = k_{\text{open}} k_{\text{ex}} \text{int}/(k_{\text{close}} + k_{\text{ex}} \text{int}) \]  

(Eq. 7)

assuming that \( k_{\text{open}} \ll k_{\text{close}} \) (i.e. the occupancy of the open state [O]/[C] is relatively low) (58). If the probability of exchange during each opening transition is high (\( k_{\text{close}} \ll k_{\text{ex}} \text{int} \)), Equation 7 simplifies further to the following,

\[ k_{\text{ex}} \text{obs} = k_{\text{ex}} \text{int} \]  

(Eq. 8)

This is known as the EX1 limit, and exchange through this mechanism occurs typically from the unfolded state following global unfolding (58). Alternatively, if closing is rapid and only a fraction of the opening transitions lead to exchange (\( k_{\text{close}} > k_{\text{ex}} \text{int} \)), Equation 7 can be rewritten as follows,

\[ k_{\text{ex}} \text{obs} = k_{\text{ex}} \text{int}(k_{\text{open}}/k_{\text{close}}) = k_{\text{ex}} \text{int}[O]/[C] = k_{\text{ex}} \text{int} K_{O/C} \]  

(Eq. 9)

where the stability of the open state estimated from \( \Delta G_{O/C} \) is \(-RT \ln K_{O/C}\). This is known as the EX2 regime, and exchange through this mechanism is often found for local fluctuations on the native side of the folding barrier (58). Following standard procedures, we measured in this study \( k_{\text{ex}} \text{obs} \) by HSQC NMR (supplemental material S6) and calculated the values of \( k_{\text{ex}} \text{int} \) from model peptide data using the protocols by Englander and co-
The Structural Properties of the Loop-depleted SOD1 Barrel

The results for apoSOD1pwt and apoSOD1ΔIV,ΔVII are listed in Table 2.

Data Define Three Levels of H/D Exchange Rates

The three levels are (i) slowly exchanging amide protons with $k_{\text{ex}}$ values similar to or somewhat slower than the rate constant for global unfolding $k_u$ ($-4.9 < \log k_{\text{ex}}^{\text{obs}} < -3.9$); (ii) amide protons that exchange on intermediate time scales ($-3.9 < \log k_{\text{ex}}^{\text{obs}} < -2.8$); and (iii) amides protons that exchange rapidly in the dead time of the experiment ($\log k_{\text{ex}}^{\text{obs}} > -2.8$). The slowly exchanging amides, we conclude, represent stable H-bonds that exchange through global unfolding close to the EX1 limit where $k_{\text{ex}}^{\text{obs}} \approx k_u$. Consistent with this, the $k_{\text{ex}}^{\text{obs}}$ values for these positions are overall lower for apoSOD1ΔIV,ΔVII than for apoSOD1pwt (Table 2). The reason for this difference is 2-fold. First, apoSOD1ΔIV,ΔVII unfolds somewhat slower than apoSOD1pwt with $\log k_u^{\text{H}_{2}\text{O}}$ values of $-4$ and $-3.9$, respectively. Second, the higher refolding rate of apoSOD1ΔIV,ΔVII, $\log k_u^{\text{H}_{2}\text{O}} = -0.28$ versus $\log k_u^{\text{H}_{2}\text{O}} = -1.12$ for apoSOD1pwt, ensues at several positions a kinetic competition between $k_{\text{close}}$ and $k_{\text{int}}$ (Table 2). This decreased probability of exchange during the globally unfolded state decreases $k_{\text{ex}}^{\text{obs}}$ and biases the system toward the EX2 regime (Equation 7 and supplemental material S6). Correspondingly, the amides with intermediate $k_{\text{ex}}^{\text{obs}}$ values ($-3.9 < \log k_{\text{ex}}^{\text{obs}} < -2.8$) indicate backbone positions that exchange by local fluctuations in the clean EX2 regime. Finally, the amide protons that exchange rapidly ($\log k_{\text{ex}}^{\text{obs}} > -2.8$) are either exposed to the solvent or involved in relatively weak hydrogen bonds, which offer little protection. As observed for other proteins (61), the exchange behavior of SOD1 under close to physiological conditions is thus a mix between EX1 and EX2.

As an independent test of EX1 and EX2 behavior, we determined the exchange rates at three different pH values, pH 5.4, 6.3, and 7.2 (supplemental Table S3). Amide protons exchanging in the EX2 regime are expected to display exchange rate constants that follow the pH dependence of $k_{\text{int}}^{\text{H}_{2}\text{O}}$ (Equation 9) (i.e. they should increase by a factor of 10 per increased pH unit (60). The results show that, consistently, the amide protons with fast and intermediate exchange at pH 6.3 exchange rapidly in the experimental dead time at pH 7.2, whereas they are considerably slowed down at pH 5.4 (supplemental Table S3). At pH 5.4, we can also resolve some of the amide protons that exchange too rapidly for detection at pH 6.3 (e.g. 41, 43, and 74–76) (supplemental Table S3). The amide protons exchanging with rate constants close to global unfolding, on the other hand, show much weaker dependence on pH, corroborating the idea that these positions are in the EX1 regime (supplemental Table S3). Phenylalanine 45 is also detected at low pH, whereas the cross-peak is not detectable at higher pH. The measured exchange rate at pH 5 is similar to that of His-46, suggesting that Phe-45 forms a hydrogen bond with Ala-55 and exchanges by the same dynamic events as His-46. Toward a final test of the slowly exchanging positions, we determined the pH dependence of $\log k_u^{\text{H}_{2}\text{O}}$ from chevron data. The results show that $\log k_u^{\text{H}_{2}\text{O}}$ has a maximum around pH 6.3 and decreases slightly both upon raising and lowering the pH (supplemental Fig. S6 and Table S4). This pH dependence matches that of the average of the slowly exchanging amides (i.e. the pH dependence of $\log k_u^{\text{H}_{2}\text{O}}$) follows that of $\log k_{\text{ex}}^{\text{obs}}$ as is expected for EX1 exchange rate-limited by global unfolding (supplemental Fig. S7).

The structural locations of the amide protons with slow, intermediate, and rapid exchange are shown in Figs. 7 and 8. From these data, it is apparent that the exchange patterns of apoSOD1ΔIV,ΔVII and apoSOD1pwt are very similar; most of the slowly exchanging amides are confined to the H-bond network defining the central $\beta$ barrel, whereas the rapidly exchanging amides are mainly in the loops connecting the $\beta$ strands. However, there are two regions of the SOD1 barrel that stand out as structurally deviant. Below is a detailed description of these regions and how they respond to loop removal and metallation.

The effects of metallation are deduced by comparison with previously published H/D exchange data from the holoSOD1 dimer (10).

Weak Protection of $\beta_2$ at the Center of the Major Sheet

For both apoSOD1ΔIV,ΔVII and apoSOD1pwt, the amide protons of Gly-16$_{\beta_2}$ and Ile-17$_{\beta_2}$ exchange rapidly in the dead time of the experiment ($\log k_{\text{ex}}^{\text{obs}} > -2.8$), despite forming H-bonds with the neighboring strands in the crystal structure (Table 2 and Figs. 7 and 8). Next to Ile-17$_{\beta_2}$ is the rapidly exchanging Val-7$_{\beta_1}$ facing a local gap in the H-bonding to Val-148$_{\beta_3}$ which yields a contiguous region of weakly protected H-bonds at the center of the main sheet. Interestingly, a similar exchange pattern is seen in the holoSOD1 dimer (10), suggesting that the weak protection of the N-terminal end of $\beta_2$ is a built-in feature of the SOD1 barrel rather than an effect of metal loss or dimer splitting. The explanation for the weak protection could be the notably long H-bond distances in this region coupled to increased backbone flexibility (10). In the x-ray structure of holoSOD1pwt (Protein Data Bank entry 2XJK), the distance between the Ile-17$_{\beta_2}$ amide and the Ser-34$_{\beta_3}$ carbonyl is 2.46 Å compared with an average of 1.9 ± 0.2 Å for the rest of the sheet. The apparent flexibility of $\beta_2$ disappears at the highly protected residue Ile-18$_{\beta_3}$ which anchors the strand into the core at the packing layer of the folding nucleus (46) (Table 2 and Figs. 7 and 8). Such closely packed layers of hydrophobic side chains are not unique for the core of SOD1 but appear to be a general feature of immunoglobulin-like folds (62). Before this position, $\beta_2$ is poorly anchored to the SOD1 core, mainly because the conserved position Gly-16$_{\beta_2}$ lacks side chain. The role of this seemingly conserved “imperfection” in the SOD1 barrel, if any, is not yet clear. Although data from the holoSOD1 dimer indicate that the protection of $\beta_2$ undergoes a small change upon mutation G93A (10), we do not detect any corresponding effects upon loop removal in the apo monomer (Table 2 and Figs. 7 and 8). We note, however, that $\beta_2$ is one of the few segments in the SOD1 sequence with a propensity to fibrillate in vitro (25, 26). In combination with its low protection factors, this local sequence property underlines $\beta_2$ as a putative aggregation hot spot. Also, this rapidly exchanging region of the sheet borders in the wild-type protein the buried Cys-6$_{\beta_1}$.
The Structural Properties of the Loop-depleted SOD1 Barrel

Table 2

| aa  | H-bond partner | log $k_{ox}$ | Error$^c$ | log $k_{red}$ | log PF$^a$ |
|-----|----------------|-------------|-----------|--------------|-----------|
| Ala-1 | MA             |             |           |              |           |
| Thr-2 |                | >2.8        | 1.41      | <4.21        |           |
| Lys-3 |                | >2.8        | 1.08      | 3.75         |           |
| Ala-4 | 20             | >2.8        | 0.03      | >4.00        |           |
| Val-5 | 150            | >2.8        | 0.01      | 3.25         |           |
| Ala-6 | 18             | >2.8        | 0.03      | 4.00         |           |
| Val-$\text{H}$ | 148   | >2.8        | 0.05      | <1.72        |           |
| Lys-9 | 146            | >2.8        | 0.05      | <2.18        |           |
| Gly-10 |              | >2.8        | 0.05      | >2.81        |           |
| Asp-11 |               | >2.8        | 0.01      | >2.46        |           |
| Gly-12 |              | >2.8        | 0.01      | >2.55        |           |
| Pro-13 |              | >2.8        | 0.02      | >2.8         |           |
| Val-14 |                | >2.8        | 0.03      | >1.48        |           |
| Glu-$\text{H}$ | 36   | >2.8        | 0.03      | 3.28         |           |
| Gly-16 | 8              | >2.8        | 0.09      | 3.34         |           |
| Ile-$\text{H}$ | 34   | >2.8        | 0.09      | >2.89        |           |
| Ile-$\text{H}$ | 6           | >2.8        | 0.13      | >1.87        |           |
| Asn-$\text{H}$ | 32  | >2.8        | 0.08      | 3.44         |           |
| Phe-$\text{H}$ | 4     | >2.8        | 0.07      | >4.32        |           |
| Glu-$\text{H}$ | 30   | >2.8        | 0.06      | >3.87        |           |
| Gly-$\text{H}$ | 21 | >2.8        | 0.06      | >2.38        |           |
| Lys-$\text{H}$ | 24  | >2.8        | 0.02      | >2.29        |           |
| Ser-$\text{H}$ | 25  | >2.8        | 0.02      | >2.69        |           |
| Asp-$\text{H}$ | 26  | >2.8        | 0.02      | >2.32        |           |
| Gly-$\text{H}$ | 27  | >2.8        | 0.02      | >3.01        |           |
| Pro-$\text{H}$ | 28  | >2.8        | 0.02      | >1.32        |           |
| Ala-$\text{H}$ | 101 | >2.8        | 0.04      | >3.34        |           |
| Glu-$\text{H}$ | 93  | >2.8        | 0.05      | >4.01        |           |
| Thr-39 |                | >2.8        | 0.06      | >2.15        |           |
| Glu-$\text{H}$ | 40  | >2.8        | 0.05      | >2.20        |           |
| Gly-$\text{H}$ | 89  | >2.8        | 0.05      | >2.29        |           |
| Leu-$\text{H}$ | 82  | >2.8        | 0.05      | >2.18        |           |
| His-$\text{H}$ | 18 | >2.8        | 0.05      | >2.20        |           |
| Gly-$\text{H}$ | 118 | >2.8        | 0.04      | >3.86        |           |
| Gly-$\text{H}$ | 56  | >2.8        | 0.04      | >2.93        |           |
| Gly-$\text{H}$ | 57  | >2.8        | 0.04      | >2.77        |           |
| Thr-58 |                | >2.8        | 0.04      | >2.68        |           |
| Ser-$\text{H}$ | 59  | >2.8        | 0.04      | >2.69        |           |
| Ala-$\text{H}$ | 60  | >2.8        | 0.04      | >2.69        |           |
| Pro-$\text{H}$ | 61  | >2.8        | 0.04      | >2.69        |           |
| His-$\text{H}$ | 62  | >2.8        | 0.04      | >2.69        |           |
| Phe-$\text{H}$ | 63  | >2.8        | 0.04      | >2.69        |           |
| His-$\text{H}$ | 64  | >2.8        | 0.04      | >2.69        |           |
| Asn-$\text{H}$ | 65  | >2.8        | 0.04      | >2.69        |           |
| Pro-$\text{H}$ | 66  | >2.8        | 0.04      | >2.69        |           |
| Lys-$\text{H}$ | 67  | >2.8        | 0.04      | >2.69        |           |
| Ser-$\text{H}$ | 68  | >2.8        | 0.04      | >2.69        |           |
| Ala-$\text{H}$ | 69  | >2.8        | 0.04      | >2.69        |           |
| Pro-$\text{H}$ | 70  | >2.8        | 0.04      | >2.69        |           |
| His-$\text{H}$ | 71  | >2.8        | 0.04      | >2.69        |           |
| Gly-$\text{H}$ | 72  | >2.8        | 0.04      | >2.69        |           |
| Gly-$\text{H}$ | 73  | >2.8        | 0.04      | >2.69        |           |
| Pro-$\text{H}$ | 74  | >2.8        | 0.04      | >2.69        |           |
| Lys-$\text{H}$ | 75  | >2.8        | 0.04      | >2.69        |           |
| Asp-$\text{H}$ | 76  | >2.8        | 0.04      | >2.69        |           |
| Glu-$\text{H}$ | 77  | >2.8        | 0.04      | >2.69        |           |
| Gln-$\text{H}$ | 78  | >2.8        | 0.04      | >2.69        |           |
| Arg-$\text{H}$ | 79  | >2.8        | 0.04      | >2.69        |           |
| His-$\text{H}$ | 80  | >2.8        | 0.04      | >2.69        |           |
| Val-$\text{H}$ | 81  | >2.8        | 0.04      | >2.69        |           |

Note: $^a$ Rate constants are in units of s$^{-1}$. $^b$ MA, missing assignment.

The Structural Properties of the Loop-depleted SOD1 Barrel

TABLE 2

Determined H/D exchange rates per residue for apoSOD1$^{\text{IIV,AVII}}$ and apoSOD1$^{\text{Pwrt}}$

Rate constants are in units of s$^{-1}$. MA, missing assignment.
### The Structural Properties of the Loop-depleted SOD1 Barrel

#### TABLE 2—continued

| aa$^c$ | H-bond partner | $\log k_{on}^e$ | $\log k_{out}^c$ | $\log \text{PP}^a$ |
|-------|----------------|--------------|-----------------|----------------|
| Gly-82 | MA | -0.25 | -0.44 | -0.50 |
| Asp-94 | MA | -0.10 | -0.32 | -0.19 |
| Leu-94$_{p}$ | 45/82 | -1.10 | -3.29 | -3.09 |
| Gly-95$_{ps}$ | >-2.8 | 0.29 | <3.09 | |
| Asn-96$_{p}$ | 43 | 0.04 | -0.56 | 0.24 |
| Val-97$_{p}$ | 43 | -0.58 | 2.22 | |
| Thr-98$_{p}$ | 41 | -0.49 | 2.31 | |
| Ala-99$_{p}$ | 94 | 0.07 | 2.76 | |
| Lys-91 | >-2.8 | -0.56 | <2.24 | |
| Asp-92 | >-2.8 | -0.49 | 2.31 | |
| Val-94$_{p}$ | 90 | -0.40 | 3.77 | |
| Ala-95$_{p}$ | 35 | -0.46 | 3.96 | |
| Asp-90$_{p}$ | >-2.8 | -0.61 | <2.19 | |
| Val-97 | 33 | 0.09 | 3.57 | |
| Ser-98$_{p}$ | >-2.8 | -0.14 | <2.66 | |
| Ile-99$_{p}$ | 31 | -0.45 | 3.77 | |
| Glu-100 | >-2.8 | 1.03 | <1.77 | |
| Asp-101 | 29 | >-2.8$^b$ | -0.72 | <2.08 |
| Ser-102 | >-2.8 | -0.15 | <2.65 | |
| Thr-103 | >-2.8 | -0.78 | <2.02 | |
| Ile-104 | >-2.8 | -1.24 | <1.56 | |
| Ser-105 | 112 | -0.23 | <2.57 | |
| Leu-106 | >-2.8 | -0.65 | <2.15 | |
| Ser-107 | >-2.8 | 0.21 | <2.59 | |
| Gly-108 | >-2.8 | 0.19 | <2.99 | |
| Asp-109 | >-2.8 | -0.44 | 2.36 | |
| His-110 | >-2.8 | 0.23 | <3.03 | |
| Ala-111 | >-2.8 | 0.41 | <3.21 | |
| Ile-112 | 105 | -0.39 | 2.39 | |
| Ile-113 | 110 | -0.04 | 1.33 | |
| Gly-114$_{p}$ | 149 | -0.61 | 3.82 | |
| Arg-115$_{p}$ | 112 | -0.13 | 4.45 | |
| Thr-116$_{p}$ | 48 | 0.58 | 3.36 | |
| Leu-117$_{p}$ | 47 | -0.75 | 3.74 | |
| Val-118$_{p}$ | 46 | 1.31 | 3.39 | |
| Val-119$_{p}$ | 145 | -1.22 | 3.30 | |
| His-120$_{p}$ | 44 | 0.57 | 2.30 | |
| Glu-121 | >-2.8 | -0.02 | <2.78 | |
| Lys-122 | >-2.8 | -0.52 | <2.28 | |
| Ala-123 | >-2.8 | 0.32 | <2.55 | |
| Asp-124 | >-2.8 | -0.01 | <2.48 | |
| Asp-125 | >-2.8 | -0.76 | <2.04 | |
| Leu-126 | >-2.8 | -1.10 | <1.70 | |
| Gly-127 | >-2.8 | -0.32 | <2.48 | |
| Lys-128 | >-2.8 | -0.24 | <2.56 | |
| Gly-129 | 0.01 | <2.81 | |
| Gly-130 | >-2.8 | 0.06 | <2.86 | |
| Asn-131 | >-2.8 | 0.21 | <3.09 | |
| Glu-132 | >-2.8 | -0.48 | <2.32 | |
| Gln-133 | >-2.8 | 0.41 | <2.38 | |
| Ser-134 | >-2.8 | 0.20 | <3.00 | |
| Thr-135 | 0.24 | <2.66 | |
| Lys-136 | >-2.8 | -0.21 | <2.59 | |
| Thr-137 | >-2.8 | -0.32 | <2.48 | |
| Gly-138 | 0.09 | <2.89 | |
| Asn-139 | >-2.8 | 0.29 | <3.09 | |
| Ala-140 | >-2.8 | -0.05 | <2.75 | |
| Gly-141 | >-2.8 | -0.11 | <2.69 | |
| Asp-142 | >-0.32 | <2.48 | |
| Arg-143 | >-2.8 | -0.24 | <2.56 | |
| Leu-144 | >-2.8 | -0.24 | <2.32 | |
| Ala-145$_{p}$ | 119 | -0.58 | <3.05 | |
| Cys-146$_{p}$ | 9 | >-2.8$^b$ | 0.25 | <3.05 | |
| Gly-147$_{p}$ | 117 | 0.48 | 4.92 | |
| Val-148$_{p}$ | 7 | 0.00 | <2.80 | |
| Ile-149$_{p}$ | 115 | -0.91 | <1.89 | |
| G150$_{p}$ | 5 | -0.34 | 4.19 | |
| Ile-151 | >-2.8 | -0.93 | <1.87 | |
| Ala-152 | >-2.8 | 0.16 | <2.20 | |
| Gln-153 | >-2.8 | -0.21 | <2.09 | |

$^a$ Amino acid; the subscript $\beta$ denotes the secondary structure element in which the amino acid residue is located.

$^b$ Calculated from the fit of $I = I_0 \exp(-k_{on}/k_{out})$ to the time dependence of the attenuation of NH cross-peak intensity.

$^c$ Error is the S.D. in the fitted $k_{on}$ from 103 fits with randomized normal distributed noise estimated from the spectra.

$^d$ Intrinsic exchange rates were calculated from model-peptide data using the protocols by Engler and co-workers (60).

$^e$ Log PP = log $k_{on}^c$ - log $k_{out}^c$.

$^f$ Numbering of the SOD1$_{1A}$ sequence.

$^g$ Not completely exchanged during the experimental dead time but too few data points for reliable fitting.

$^h$ The pH dependence of the exchange rate suggests that these amide protons exchange in the EX2 regime.

$^i$ Substitution of the loop IV residues 49–81 to Gly-Ala-Gly.

$^j$ Substitution of the loop IV residues 124–139 to Gly-Ala-Gly.
FIGURE 7. Schematic outlines of the apoSOD1pwt and apoSOD1ΔIV,ΔVII structures showing the results from H/D exchange NMR. The arrows indicate the amide proton to carbonyl direction in the analyzed backbone H-bonds. Red, positions that exchange slowly through global unfolding (log \( k_{\text{ex}} < -4 \)). Yellow, positions with intermediate exchange rates (\(-4 < \log k_{\text{ex}} < -2.8\)). White, positions that exchange in the dead time of the experiment (\(\log k_{\text{ex}} > -2.8\)). Black, positions missing assignment. A, data for apoSOD1pwt; B, data for apoSOD1ΔIV,ΔVII. The highlighted positions show where \( k_{\text{ex}} \) increases upon loop removal. The similar H/D exchange kinetics of apoSOD1pwt and apoSOD1ΔIV,ΔVII shows that the lower regions of the folding energy landscape remain largely unaffected by loop removal. This does not exclude, however, that the introduced changes in sequence separation between native contacts lead to alterations of the folding trajectory at transition state level, as observed upon circular permutation of the ribosomal protein S6 (78).
which is implicated in aggregation by disulfide cross-linking (14).

**Weak Protection of β4, β5, and β7 at the Interface to the Active Site**

The second region of the SOD1 barrel with poorly protected H-bonds is the segment comprising β4, β5, and the beginning of β7. From a structural perspective, these strands are interesting because they anchor the functional loop IV (β4 and β5) and harbor several of the metal-binding ligands (i.e. the Cu$^{1+/-2+}$ ligands His-46$_{β4}$, His-48$_{β4}$, and His-120$_{β7}$ and the Zn$^{2+}$ ligand Asp-83$_{β5}$) (Fig. 7). Although β4 stretches between Leu-42$_{β4}$ and His-48$_{β4}$ in the crystal structure, its only position with slowly exchanging amide is His-46$_{β4}$ (log $k_{ex}^{obs} = -4.42$), which forms an H-bond with the carbonyl of V118$_{β7}$ (Table 2 and Figs. 6 and 7). This is also the only position with backbone NOEs between β4 and β7 in apoSOD1$^{ΔIV,ΔVII}$ (Fig. 6). The amides of β4 facing the carbonyls in β5 all exchange rapidly (log $k_{ex}^{obs} > -2.8$). Even so, the amides of Val-87$_{β5}$ and Ala-89$_{β5}$, which bind back to the carbonyls of His-43$_{β4}$ and Gly-41$_{β4}$, display relatively slow exchange rates (Fig. 7). In apoSOD1$^{pwt}$, the log $k_{ex}^{obs}$ values of Val-87$_{β5}$ and Ala-89$_{β5}$ are $-4.13$ and $-3.53$, respectively (Table 2). This large variation of the $k_{ex}^{obs}$ values in the sheet, taken together with the intermediate exchange rate of Ala-89$_{β5}$, suggests that β4 and β5 exchange protons by local fluctuations in, or close to, the EX2 regime (Equation 9). The extent of structural opening required for such local exchange is believed to be small (63), ranging from backbone breathing sufficient to allow the penetration of individual water molecules (64, 65) to local unfolding of a few neighboring H-bonds (66, 67). In apoSOD1$^{ΔIV,ΔVII}$, the exchange rates of Val-87$_{β5}$ and Ala-89$_{β5}$ increase to log $k_{ex}^{obs} = -3.36$ and log $k_{ex}^{obs} > -2.8$, respectively (Table 2). The increase supports not only the interpretation that β4 and β5 exchange by local fluctuations but shows further that these fluctuations are facilitated by loop removal. Following loop removal, we observe also increased exchange rates for Asp-90, Gly-93, and Val-94 (loop V) and for Thr-116$_{β7}$ that H-bonds to His-48$_{β4}$ next to the Gly-Ala-Gly insert (Table 2 and Figs. 7 and 8B). Structurally, the increased flexibility of the β4-β5 region could have several causes. It could arise from loss of the loop-packing interface, loss of backbone H-bonding between Lys-122 (loop VII) and the twisted Gly-44$_{β4}$, or conformational strain introduced by the short Gly-Ala-Gly linker between His-48$_{β4}$ and Gly-82. Comparison with the H/D exchange data from the holoSOD1 dimer suggests that the high exchange rates of β4, β5, and β7 in apoSOD1$^{pwt}$ and apoSOD1$^{ΔIV,ΔVII}$ are induced by demetallation (Fig. 7). In the holoSOD1 dimer, the majority of positions in this barrel segment as well as around the Zn$^{2+}$-binding site display slow exchange rates (10). We conclude from these results that the loss of metal ions from the SOD1 structure not only sets free rapid dynamic motions of the functional loops IV and VII (14–18) but also enhances the slower breathing motions of the underlying β sheet and loop V. However, these breathing motions of the apoSOD1 barrel are relatively rare and are not in conflict with the minimal two-state folding behavior depicted in Scheme 1. For example, the intermediate exchange rate of Ala-89$_{β5}$ in apoSOD1$^{pwt}$ corresponds to an open state that is populated only $1/2300$ of the time ($K_{O/C} = 4.4 \times 10^{-3}$, Equation 9), yet the interaction between Ala-89$_{β5}$ and Gly-41$_{β4}$ yields strong backbone NOEs in apoSOD1$^{ΔIV,ΔVII}$, where the H/D exchange rate is even faster (Fig. 6).

**Stable Stacking of Loops 3 and 5 Constitutes a Hot Spot for ALS-associated Mutations**

The amide of Leu-38 (loop III) forms a highly protected H-bond to the carbonyl of Gly-93 (loop V). This indicates high structural rigidity in the stacking of loops III and V, which is also a hot spot for ALS-associated SOD1 mutations (2). An analogous, but not identical, strong protection pattern of this region is reported for the holoSOD1 dimer by Jonsson and co-workers (10), who observe further that the local ALS mutation...
G93A, which strains loop V sterically, has long range effects on the metal binding sites (10) at the other ends of β4 and β5. In this study, we find reciprocally that substitution of the metal-supporting loops IV and VII leads to increased H/D exchange rates in loop V (Fig. 7). An explanation for this apparent allosteric effect could be that the stacked loops III and V play a role in supporting the structure of the bridging and relatively dynamic strands β4 and β5. Whether the observed phenomenon is just a trivial effect of loop removal, stems from intrinsic structural frustration in the active-site region (31, 68), or reflects a functional feature of the SOD1 molecule is not yet clear.

**Loops IV and VII Have Modest Influence on the Barrel Stability in the Absence of Coordinated Zn^{2+}**

The immunoglobulin-like barrel represents one of the most common folds in living organisms (69), indicating viable and useful structural properties. In the case of SOD1, it provides a scaffold for biological function by anchoring the active-site metal ion. It is possible that this complex functional optimization of the SOD1 loop region has occurred, at least partly, in conflict with the structural properties of the barrel scaffold (31, 72, 73). The existence of such a conflict could have direct bearing on the susceptibility of SOD1 to misfold and the involvement of SOD1 in ALS (31). Consistent with the idea that the functional features exert a burden on the SOD1 scaffold, apoSOD1 has been implicated as a misfolding hot spot in previous NMR (15, 17) and folding (46) studies. Given the cooperative folding behavior of the SOD1 barrel, however, a more reductionist conclusion would be that the precursor for misfolding is the denatured ensemble (24, 77) (cf. U in Scheme 1). At physiological temperatures, where the disulfide-reduced apo monomers are already in the unfolding transition region because of their low thermodynamic stability (29, 77), the occupancy of globally denatured species is likely to exceed that of any high energy intermediate on the native side of the folding barrier. Under the same conditions, some of the severely destabilizing ALS-associated SOD1 mutations (e.g. G41D, H43R, and G93A) will even populate the denatured ensemble close to 100% in the absence of coordinated metal ions (24). The aggregation precursor of SOD1 could then be structurally similar to those of the Aβ peptide associated with Alzheimer disease and the disordered protein α-synuclein associated with Parkinson disease (i.e. an overall flexible chain that samples in a dynamic way aggregation-prone microstates) (6). Such a scenario would also explain how so many destabilizing, but structurally disparate, SOD1 mutations can still result in an overall ALS phenotype; they all shift the folding equilibrium toward the denatured ensemble (24).

**REFERENCES**

1. Doucette, P. A., Whitson, L. J., Cao, X., Schirf, V., Demeler, B., Valentine, J. S., Hansen, J. C., and Hart, P. J. (2004) *J. Biol. Chem.* 279, 54558–54566
2. Valentine, J. S., Doucette, P. A., and Zittin Potter, S. (2005) *Annu. Rev. Biochem.* 74, 563–593
3. Forsberg, K., Jonsson, P. A., Andersen, P. M., Bergemalm, D., Graffmo, K. S., Hultdin, M., Jacobsson, J., Rosquist, R., Marklund, S. L., and Bränström, T. (2010) *PLoS One* 5, e11552
4. Jahn, T. R., and Radford, S. E. (2008) *Arch. Biochem. Biophys.* 469, 100–117
5. Daggett, V. (2009) *Protein Eng. Des. Sel.* 22, 445
6. Chiti, F., and Dobson, C. M. (2006) *Annu. Rev. Biochem.* 75, 333–366
7. Forman, H. J., and Fridovich, I. (1973) *J. Biol. Chem.* 248, 2645–2649
8. Lynch, S. M., Boswell, S. A., and Colón, W. (2004) *Biochemistry* 43, 16525–16531
9. Kayatekin, C., Zitzewitz, J. A., and Matthews, C. R. (2008) *J. Mol. Biol.* 384, 540–555
