Folding of human superoxide dismutase: Disulfide reduction prevents dimerization and produces marginally stable monomers

Mikael J. Lindberg[†], Johanna Normark[†], Arne Holmgren[§], and Mikael Oliveberg^{†¶}

[†]Department of Biochemistry, Umeå University, S-901 87 Umeå, Sweden; and [§]Department of Medical Biochemistry and Biophysics, Karolinska Institute, S-171 77 Stockholm, Sweden

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The molecular mechanism by which the homodimeric enzyme Cu/Zn superoxide dismutase (SOD) causes neural damage in amyotrophic lateral sclerosis is yet poorly understood. A striking, as well as an unusual, feature of SOD is that it maintains intrasubunit disulfide bonds in the reducing environment of the cytosol. Here, we investigate the role of these disulfide bonds in folding and assembly of the SOD apo protein (apoSOD) homodimer through extensive protein engineering. The results show that apoSOD folds in a simple three-state process by means of two kinetic barriers: $2D \rightleftharpoons 2M \rightleftharpoons M_2$. The early predominant barrier represents folding of the monomers (M), and the late barrier the assembly of the dimer (M_2). Unique for this mechanism is a dependence of protein concentration on the unfolding rate constant under physiological conditions, which disappears above 6 M Urea where the transition state for unfolding shifts to first-order dissociation of the dimer in accordance with Hammond-postulate behavior. Although reduction of the intrasubunit disulfide bond C57–C146 is not critical for folding of the apoSOD monomer, it has a pronounced effect on its stability and abolishes subsequent dimerization. Thus, impaired ability to form, or retain, the C57–C146 bond *in vivo* is predicted to increase the cellular load of marginally stable apoSOD monomers, which may have implications for the amyotrophic lateral sclerosis neuropathology.

protein folding | protein stability | disulfide bond | transition-state shifts | protein engineering

The mechanism by which mutant superoxide dismutase (SOD) leads to neural damage in the familial form of amyotrophic lateral sclerosis (ALS) is yet unknown (1–3). In analogy with other neurodegenerative disorders (4), however, an increasing body of observations (5–16) suggest that the ALS disease mechanism is coupled to destabilization or misfolding of the SOD structure (17), manifested ultimately by cellular inclusions of SOD aggregates (3, 18). From a strictly energetic perspective, the native SOD structure is distinctive by containing one oxidized disulfide bond per monomer (C57–C146) in the reducing environment of the cytosol (19) (Fig. 1). Normally, disulfide bonds are maintained only under oxidizing conditions in the extracellular space (20, 21) where they increase protein stability by confining the configurational entropy of the denatured ensemble (22). Indications that the integrity of the C57–C146 bond may have bearing on the molecular events in ALS were recently provided by Tiwari and Hayward (15), who demonstrated that ALS-associated SOD mutants are more susceptible to chemical disulfide reduction than the wild-type protein. More detailed elucidation of the structural and energetic effects accompanying mutational perturbations and disulfide reduction has so far been prevented by scarce knowledge about how the SOD homodimer folds (23).

In this study, we shed further light on this issue by mapping out the folding and assembly reaction of metal-depleted SOD through kinetic and thermodynamic analysis of mutant protein, with particular focus on the role of the intrasubunit disulfide bond C57–C146. After elimination of artifactual cross-linking by substitution

of the solvent-accessible cysteines 6 and 111, the oxidized SOD apoenzyme (apoSOD) homodimer reveals a conspicuous chevron plot indicative of a three-state process in which the rate-limiting step shifts from the first-order nucleation of the monomers at low-urea concentrations, to the transition state for monomer dissociation at high-urea concentrations. A benefit of this transition-state change is that information about monomer folding and dimer formation can be derived from kinetic data alone. The results show that the C57–C146 bond only marginally affects the folding process of the monomeric protein, but is crucial for its stability and dimerization in the absence of Cu and Zn.

Materials

Proteins. The single and multiple SOD mutations C6A, C111A, C6A/C111A, and C6A/C111A/C57A/C146A and F50E/G51E (monomeric) variants thereof were constructed as described in ref. 12. Samples of apoSOD (molecular mass of 32 kDa for dimer) were prepared in two ways: (i) on a chelate column under mildly acidic conditions (pH 3.5) according to the procedures in ref. 24 and dialyzed against 10 mM EDTA at pH 7; and (ii) by unfolding in 4 M guanidinium chloride in the presence of 50 mM EDTA and then refolding by dialysis against 10 mM Mes (pH 6.3) with 10 mM EDTA (standard buffer). Reducing conditions were obtained by the addition of 2 mM Tris-(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl) in the unfolding buffer and 0.5 mM TCEP-HCl in all other steps.

Measurements. Equilibrium titrations were done on a J-810 CD spectrometer (Jasco, Easton, MD) or a π^* -180 instrument (Applied Photophysics, Surrey, U.K.), and the kinetic measurements were carried out on an SX-17MV stopped-flow fluorimeter (Applied Photophysics) at 25°C. Protein concentration was 4 μ M or 70 μ M monomer.

Data Analysis. In the kinetic analysis, monomeric apoSOD was assumed to display a minimal two-state transition between the denatured state (D) and the fully folded monomer (M), $K_{D-M} = [D]/[M] = k_u/k_f$:

$$\log K_{D-M} = \log K_{D-M}^{\text{H}_2\text{O}} + m_{D-M}^{\log K} [\text{urea}], \quad [1]$$

$$\log k_u = \log k_u^{\text{H}_2\text{O}} + m_u [\text{urea}], \text{ and} \quad [2]$$

$$\log k_f = \log k_f^{\text{H}_2\text{O}} + m_f [\text{urea}], \quad [3]$$

where the slopes $m_{D-M}^{\log K} = m_u - m_f$ are commonly taken as a measure of solvent exposure in the equilibrium unfolding process and the activation process of unfolding and refolding,

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Abbreviations: SOD, superoxide dismutase; apoSOD, SOD apoenzyme; ALS, amyotrophic lateral sclerosis.

[¶]To whom correspondence should be addressed. E-mail: mikael.oliveberg@chem.umu.se.

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Fig. 1. Structural comparison of the SOD homodimer and monomer. (Left) Structure of the human SOD homodimer (1SPD), showing the positions of the cysteines, the loop containing residues 49–84, and the Cu and Zn ions. (Right) Loop displacements upon dissociation of the SOD homodimer (illustrated on the right-hand-side monomer), obtained by structural superposition of holoSOD^{wt} (blue) (51) and holoSOD^{50/51/E133Q} (orange) (41).

respectively (25). The parameters were derived from equilibrium data by using the equation

$$K_{D-M} = [D]/[M] = f_D/(1 - f_D), \quad [4]$$

where f_D is the fraction of D, and protein stability was derived as $\Delta G_{D-M} = -RT \ln K_{D-M} = \Delta G_{D-M}^{\text{H}_2\text{O}} + m_{D-M}^{\Delta G}[\text{urea}]$. The plots of $\log k_f$ and $\log k_u$ vs. [urea] were fitted by

$$\begin{aligned} \log k_{\text{obs}} &= \log(k_f + k_u) \\ &= \log(10^{\log k_f^{\text{H}_2\text{O}} + m_f[\text{urea}]} + 10^{\log k_u^{\text{H}_2\text{O}} + m_u[\text{urea}]}) \end{aligned} \quad [5]$$

and the position of the transition-state ensemble was obtained from

$$\beta^\ddagger = -m_f/m_{D-N} = m_f/(m_f - m_u). \quad [6]$$

The ϕ -values were calculated from wild-type and mutant data by the standard relation (25)

$$\phi = \Delta\Delta G^\ddagger/\Delta\Delta G_{D-N} = \Delta\log k_f/(\Delta\log k_f - \Delta\log k_u), \quad [7]$$

where $\Delta\Delta G^\ddagger$ and $\Delta\Delta G_{D-N}$ are the mutant-induced destabilization of the transition-state ensemble and the folded structure, respectively. A ϕ -value of 1 suggests that the structure around the mutated residue is native-like in the transition state ensemble, whereas a ϕ -value of 0 suggests that it is unfolded. Fractional ϕ -values indicate that the targeted region is partly structured in the transition state ensemble.

The stability of dimeric apoSOD, $\Delta G_{2D-M_2} = -RT \ln K_{2D-M_2}$, was derived from equilibrium denaturation data according to

$$K_{2D-M_2} = [D]^2/[M_2] = 2P f_D^2/(1 - f_D), \quad [8]$$

assuming linear free-energy dependence on [urea]. Data analysis was done by using the KALEIDAGRAPH software (Synergy Software, Reading, PA).

Results

Disulfide Reduction Leads to Dissociation of the apoSOD Dimer. The dimer integrity of oxidized and reduced SOD was analyzed by gel chromatography on a Sephadex S-75 column (Pharmacia). To minimize the effects of local charge differences between the holo- and apo-species, 150 mM NaCl was added to the standard buffers. The concentration of the eluted protein was $\approx 20 \mu\text{M}$ monomer. Oxidized apoSOD^{wt} elutes at 10.5 ml, corresponding to the dimeric species (Fig. 2a). This peak is well separated from that of the monomeric double mutant F50E/G51E (apoSOD^{50/51}) at 12 ml

Fig. 2. Size-exclusion chromatograms showing the dimer/monomer composition of wild-type and mutant SOD with and without the C57–C146 disulfide linkage. Dimers elute at 10–11 ml and folded monomers at 12 ml. (a) Oxidized apoSOD^{wt} (black), reduced apoSOD^{wt} (red), and oxidized apoSOD^{50/51} (blue). (b) Oxidized apoSOD^{50/51} (black), fully cysteine-depleted apoSOD (red), and fully cysteine-depleted holoSOD (blue). (c) Oxidized apoSOD^{6/111} (black) and reduced apoSOD^{6/111} (red). AU, absorbance unit.

(Fig. 2a). Upon reduction of the cysteines, apoSOD^{wt} elutes mainly as a folded monomer with only a small residual of dimers. For comparison, holoSOD^{wt} remains dimeric in both its oxidized and disulfide-reduced state (data not shown). The results suggest that assembly of the SOD homodimer requires either the oxidized disulfide linkage between Cys-57 and Cys-146, or coordinated metal ions; the absence of both promotes dissociation. Consistently, the fully cysteine-depleted mutant C6A/C111A/C57A/C146A (SOD^{CalA}) elutes as a folded monomer in its apo state and as a dimer with coordinated metals, although the dimeric state of this radical construct travels somewhat slower than the wild-type ditto (Fig. 2b). The apo state of the double mutant C6A/C111A (apoSOD^{6/111}), in which only the solvent-accessible cysteines have been replaced, monomerizes upon disulfide reduction in a manner identical to that of the wild-type protein (Fig. 2c).

Monomeric SOD Shows Classical Two-State Folding Behavior. Equilibrium denaturation of the reduced apoSOD^{wt} monomer by urea reveals a sigmoidal unfolding transition consistent with a two-state process (see Scheme 1), where D is the denatured ensemble and M is the folded monomer (data not shown). Fitting of Eq. 4 yields a midpoint of [urea] = 1.51 M and $m_{D-M} = 1.42 \text{ M}^{-1}$, corresponding to $\log K_{D-M}^{\text{H}_2\text{O}} = 2.14$ or a stability of $\Delta G_{D-M}^{\text{H}_2\text{O}} = 2.91 \text{ kcal/mol}$ in the absence of urea (Eq. 1 and Table 1). Compared with other single-domain proteins, this stability is notably low (Fig. 4). Additional evidence for two-state folding is provided by the kinetics. The chevron plot of apoSOD^{wt}, i.e., a plot of $\log k_f$ and $\log k_u$ vs. urea, is characteristically v-shaped with slopes $m_f = -1.05 \text{ M}^{-1}$ and $m_u = 0.48 \text{ M}^{-1}$ (Eqs. 2, 3, and 5) that sum up to $m_{D-M}^{\log K} = 1.5 \text{ M}^{-1}$ from equilibrium data (Fig. 3 and Table 1). Moreover, the sum of $\log k_f^{\text{H}_2\text{O}}$ and $\log k_u^{\text{H}_2\text{O}}$ matches $\log K_{D-M}^{\text{H}_2\text{O}}$, yielding the relation

Scheme 1.

Table 2. Data for the apoSOD^{6/111} dimer

	apoSOD ^{6/111} 4 μ M oxidized	apoSOD ^{6/111} 70 μ M oxidized	apoSOD ^{WT} 4 μ M oxidized*
MP [†] , M	2.72 $\pm$ 0.03	3.06 $\pm$ 0.02	3.55 $\pm$ 0.04
$\Delta G_{2D-M_2}^{H_2O}$, [†] kcal/mol	-15.80 $\pm$ 0.57	-15.6 $\pm$ 0.47	-19.20 $\pm$ 0.54
$m_{2D-M_2}^{\Delta G}$, [†] kcal/mol-M	3.11 $\pm$ 0.25	3.26 $\pm$ 0.15	—
$\Delta G_{D-M}^{H_2O}$, [†] kcal/mol	-3.32 $\pm$ 0.12	-3.32 $\pm$ 0.12	-4.49 $\pm$ 0.13
$m_{D-M}^{\Delta G}$, [†] kcal/mol-M	1.93 $\pm$ 0.08	1.93 $\pm$ 0.08	—
$\Delta G_{2M-M_2}^{H_2O}$, [‡] kcal/mol	-12.48 $\pm$ 0.58	-12.28 $\pm$ 0.48	-14.71 $\pm$ 0.56
$m_{2M-M_2}^{\Delta G}$, [‡]	1.18 $\pm$ 0.26	1.33 $\pm$ 0.17	—
$\log k_f^{\S}$	-1.1 $\pm$ 0.07	-1.1 $\pm$ 0.01	—
$m_f^{\S}$, [¶] M ⁻¹	-0.83 $\pm$ 0.06	-0.83 $\pm$ 0.02	—
$\log k_d^{\S}$	-2.79 $\pm$ 0.02	-2.80 $\pm$ 0.03	—
$m_d^{\S}$, [¶] M ⁻¹	0.09 $\pm$ 0.01	0.09 $\pm$ 0.01	—
$\log k_a^{\S}$	6.39 $\pm$ 0.58	6.23 $\pm$ 0.48	—

*Estimated from apoSOD^{6/111} dimer and difference between reduced apoSOD^{50/51} and apoSOD^{50/51/6/111} (compare Table 1).

[†]Derived from equilibrium data (Eq. 8).

[‡]Estimated from oxidized apoSOD^{50/51/6/111} and differences between apoSOD^{WT} and apoSOD^{50/51} (kinetic data).

[§]Calculated from $\Delta G_{2D-M_2}^{H_2O} = \Delta G_{D-M}^{H_2O} + \Delta G_{2M-M_2}^{H_2O}$.

[¶]Derived from fits with global m_d in Fig. 5.

^{||}Calculated from $K_{2M-M_2}^{H_2O} = k_d/k_a$.

promote monomer stability by decreasing the unfolding rate constant ($\Delta\Delta G_{D-N} = 1.56$ kcal/mol, Table 1 and Fig. 3). The corresponding effect on the transition-state ensemble is notably smaller at $\Delta\Delta G^{\ddagger} = 0.67$ kcal/mol, yielding an apparent ϕ -value of 0.43 (Table 2). At a molecular level, the interpretation of this ϕ -value is somewhat different from that of side-chain truncations (22). In contrast to the enthalpic contribution of side-chain contacts, disulfide linkages stabilize folded structures by reducing the configurational entropy of disordered states. The fractional effect of the C57–C146 disulfide bond on the apoSOD transition-state ensemble, suggests then that the part of the polypeptide chain that is restricted by this linkage is largely disordered in the rate-limiting step for folding. That is, the contacts anchoring loop around position 57 are not required for the early folding events and consolidate mainly on the downhill side the folding barrier. The result is not surprising because it is expected that the correct topological context needs to be firmly established before disulfide-bond formation in the reducing environment of the cytosol.

Folding of the apoSOD Homodimer. As expected, the apparent stability of the oxidized apoSOD^{6/111} homodimer increases at increased protein concentration. The transition midpoint shifts from 2.7 M urea at 4 μ M apoSOD^{6/111} to 3.1 M urea at 70 μ M (Table 2), and the hidden surface area of the dimer interface contributes to an apparent m -value that is ≈ 0.2 units larger than for the monomer. The values of $\Delta G_{2D-M_2}^{6/111}$, as derived from equilibrium unfolding data at 4 and 70 μ M protein (Eq. 8), are -15.8 ± 0.6 kcal/mol and -15.6 ± 0.5 kcal/mol, respectively, in good correspondence with earlier reports on the apo protein (30) (Table 2). The refolding kinetics of dimeric apoSOD^{6/111} resembles closely that of the oxidized monomer apoSOD^{50/51/6/111} (Fig. 5 and Table 1): the values of m_f are within experimental errors, whereas the small offset in $\log k_f$ of 0.26 matches that observed for the dimer-splitting substitutions F50E/G51E. Moreover, the refolding rate constant for dimeric apoSOD^{6/111} shows no dependence on protein concentration: the refolding limbs obtained at 4 μ M and 70 μ M apoSOD^{6/111} are indistinguishable (Fig. 5 and Table 1). On this basis, we conclude that refolding of the SOD homodimer is rate limited by the first-order formation of the monomer. We note, however, that an additional faster phase emerges at high protein concentrations, whose amplitude constitutes $<20\%$ of the total fluorescence change. This minor phase is not resolved with the monomeric protein, and its origin is yet unclear. One possibility is

Scheme 2.

that it stems from transient aggregation of denatured or partly folded protein in the absence of the hydrophilic substitutions F50E/G51E (31). Another possibility is that a subfraction of the SOD molecules assemble before the global refolding transition, or form monomeric intermediates. The major difference between the monomeric and homodimeric protein is seen in the unfolding kinetics: the values of $\log k_u$ are substantially decreased, and the chevron plot shows a pronounced downward kink at around 5 M urea (Fig. 5). Similar kinks have been described for other proteins and are the hallmarks for changes of the rate-limiting step (32, 33); i.e., the transition state shifts closer to the native state along the experimental progress coordinate in accordance with the Hammond postulate (34). In the case of SOD, it is evident that the unfolding reaction proceeds over the transition state for monomer formation at low [urea], to become rate limited by the first-order dissociation of the dimer above 5 M urea (see Scheme 2). In direct support of this folding model, increased protein concentration affects solely the steeper region of the unfolding limb. A qualitative description of the effect is provided by the free-energy profiles in Fig. 5. Below 5 M urea, the unfolding time course is modulated by both the activation barrier for unfolding of M and the second-order equilibrium between M₂ and M. Under these conditions, elevated protein concentrations slow down the unfolding process by shifting the equilibrium toward M₂, i.e., the initial free-energy difference between M₂ and M is increased. Above 5 M urea, the dependence on protein concentration vanishes because the rate-limiting step moves to become the first-order dissociation of M₂. To visualize the dimer folding in a single chevron plot, we have tentatively treated the unfolding kinetics as first-order at all concentrations of denaturant. The second-order component in the unfolding reaction below 5M urea does not cause any detectable deviation from exponential time courses. The $[M]^2/[M_2]$ equilibrium constant at 0 M urea was estimated from the stability difference between dimeric and monomeric protein, $\Delta G_{2M-M_2}^{6/111} = \Delta G_{2D-M_2}^{6/111} - (\Delta G_{D-M}^{50/51/6/111} - \Delta\Delta G_{D-M}^{50/51})$, where $\Delta\Delta G_{D-M}^{50/51}$ is the adjustment for the mutation F50E/G51E (Table 2). The value of $\Delta G_{2M-M_2}^{6/111} \approx -12$ kcal/mol yields a dissociation constant of 1.5 nM, which is in precise agreement with previous estimates (30).

Discussion

Disulfide Reduction Promotes Dimer Dissociation. Compared with other homodimeric proteins, the ratio between interfacial and intramonomer contacts in SOD is relatively small (35). Characteristic for such proteins is that monomer folding precedes dimerization, producing interfaces that are overall less optimized than those of obligatory dimers where folding and association occur in one highly concerted step (35, 36). Consistently, the crystal structures of the ALS mutants A4V and I113T are reported to display marked distortions of the dimer interface although the structural effect on the individual monomers is small (37). Hough *et al.* (37) suggest that the decreased stability of the dimer interface expected to result from this distortion could be an integral part of the ALS disease mechanism. Along the same line, neurodegeneration in Parkinson's disease has been linked to an interface mutation in the homodimeric protein DJ-1 (38), and familial amyloid polyneuropathy has been linked to dissociation of the transthyretin tetramer into alternatively folded monomers (39). In this study, we reveal an additional aspect of the SOD homodimer by showing that its association equilibrium is also critically dependent on the intrasubunit disulfide bond C57–C146. Upon reduction of this disulfide bond, the apo protein completely fails to dimerize at physiological

logical context to allow disulfide bond formation and metal incorporation in the reducing environment of the cytosol. The first folding step of SOD *in vivo* is thus likely to be the slow, cooperative formation of the relatively unstable disulfide-reduced monomer. In support of this conclusion, it was recently reported (40) that disulfide formation *in vivo* takes place in connection with copper loading of the folded SOD monomer through interactions with the copper chaperone (CCS).

Folding of the SOD Homodimer. Folding of the apoSOD homodimer reveals a unique kinetic fingerprint, consistent with a simple three-state process (Scheme 2), where M is a high-energy intermediate at experimentally accessible protein concentrations (Fig. 5). A characteristic feature of the dimer chevron plot is that the folding process is rate-limited by the monomer formation under physiological conditions and that the transition state shifts to dimer dissociation at severely destabilizing conditions at high [urea], allowing full description of the parameters in Scheme 2. The apparent stability of the oxidized apoSOD^{wt} dimer, $\Delta G_{2B-M_2}^{app} = -RT \ln[D]/2[M_2]$, is ≈ 7 kcal/mol at 4 μ M protein and 0 M urea, as estimated from equilibrium unfolding. For the holo protein, the corresponding value of $\Delta G_{2B-M_2}^{app}$ is ≈ 11 kcal/mol, as derived from data in ref. 30. The native SOD homodimer has thus a stability that falls in the typical range of other proteins (Fig. 4), possibly controlled by the *in vivo* requirements for efficient steady-state processing and degradation. It is then conceivable that the unusually low ΔG_{D-M} of the apoSOD monomer is an adaptation to maintain the native dimer at a biologically suitable stability.

Implications for Gain of Function. Taken together, the results in this study show that reduction of the C57–C146 disulfide bond is critical for the integrity of the dimer interface and shifts the equilibrium toward marginally stable monomers. For several of the ALS-associated mutations, these monomers even reach the critical point where they are fractionally unfolded at physiological conditions (M.J.L. and M.O., unpublished results). On this basis, the disulfide-reduced monomer stands out as an interesting candidate for noxious gain of function, either directly through excessive sampling

of folding intermediates or unfolded conformations that are structurally promiscuous or, indirectly, through misfolding and aggregation (14, 17, 18, 44–46). Consistently, the monomeric species has been implicated as a common precursor in SOD aggregation after oxidative damage of His and Phe side chains (47). Moreover, trapping of the dimeric state by the introduction of an intersubunit disulfide bond is reported to completely abolish aggregation of the A4V mutant that otherwise forms pore-like assemblies similar to those of other disease-associated proteins (44, 48). The most compelling evidence that SOD molecules lacking the C57–C146 linkage are powerful inducers of cytotoxic function is found in the list of ALS-associated mutants. C146R and seven different C-terminal truncations (2) provoke disease although they miss one of the key thiol groups. The presence of disulfide-reduced SOD is also found to be the least common denominator in spinal-cord extracts from transgenic mice carrying the ALS-linked mutations G85R, D90A, G93A and the truncation variant G127insTGGG (S. Marklund, personal communication). An attractive feature of disease models linked to an increased load of monomeric protein is that they reconcile the structurally diverse set of mutations associated with ALS. Elevated levels of noxious monomers could arise from mutations that promote disulfide reduction (15), weaken the dimer interface (37), decrease the global stability (11–13, 47) or folding cooperativity (49) of either the monomeric or dimeric species, or adversely affect metal loading through the copper chaperone (CCS) (50). The low stability of the reduced monomer provides also an explanation for sporadic ALS. In these cases, impaired function of the cellular “housekeeping” system, i.e., chaperones, quality control, and degradation mechanisms, might cause the reduced form of wild-type SOD to enter the same cytotoxic pathway as the ALS-associated mutants, consistent with observations of cytosolic SOD-containing aggregates in both familial and sporadic ALS (18). Understanding the roles of the physiological disulfide-reduction systems in the pathology of SOD mutations and ALS is an obvious future goal.

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