Soluble misfolded subfractions of mutant superoxide dismutase-1s are enriched in spinal cords throughout life in murine ALS models

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Mutants of superoxide dismutase-1 (SOD1) cause ALS by an unidentified cytotoxic mechanism. We have previously shown that the stable SOD1 mutants D90A and G93A are abundant and show the highest levels in liver and kidney in transgenic murine ALS models, whereas the unstable G85R and G127X mutants are scarce but enriched in the CNS. These data indicated that minute amounts of misfolded SOD1 enriched in the motor areas might exert the ALS-causing cytotoxicity. A hydrophobic interaction chromatography (HIC) protocol was developed with the aim to determine the abundance of soluble misfolded SOD1 in tissues *in vivo*. Most G85R and G127X mutant SOD1s bound in the assay, but only minute subfractions of the D90A and G93A mutants. The absolute levels of HIC-binding SOD1 were, however, similar and broadly inversely related to lifespans in the models. They were generally enriched in the susceptible spinal cord. The HIC-binding SOD1 was composed of disulfide-reduced subunits lacking metal ions and also subunits that apparently carried nonnative intrasubunit disulfide bonds. The levels were high from birth until death and were comparable to the amounts of SOD1 that become sequestered in aggregates in the terminal stage. The HIC-binding SOD1 species ranged from monomeric to trimeric in size. These species form a least common denominator amongst SOD1 mutants with widely different molecular characteristics and might be involved in the cytotoxicity that causes ALS.

disulfide bond | motor neuron | neurodegeneration

Amyotrophic lateral sclerosis (ALS) is characterized by motor neuron degeneration, resulting in progressive paralysis, and death from respiratory failure. Approximately 10% of ALS cases are familial (1) and in some of these the disease is linked to mutations in the CuZn-superoxide dismutase (SOD1) gene (2). Overall, $\approx 6\%$ of all cases with ALS show SOD1 mutations, and more than 100 different such mutations have been identified (3). The mutations confer a cytotoxic gain of function to the enzyme (4, 5). SOD1 is ubiquitously expressed, and in several organs at higher levels than in the motor areas in the CNS (6, 7). Both the nature of the cytotoxicity, and the reasons for the particular susceptibility of some parts of the CNS remain unexplained.

It is likely that the different mutant SOD1s cause ALS by essentially the same mechanism. Still, the levels of different mutant SOD1s in the human CNS differ by more than 200-fold (7). Similar large differences are observed in the murine ALS models. In spinal cords from mice expressing the D90A, G93A, G85R and G127X (G127X) mutant human SOD1s (hSOD1s), the levels are 20, 14, 0.9, and 0.45 times as large, respectively, as the levels of the endogenous murine SOD1 (mSOD1) (8). In the high-level models, D90A and G93A, most hSOD1 lacks enzymatic activity owing to Cu-deficiency, and significant proportions lack the stabilizing intrasubunit disulfide bond. The G85R and G127X hSOD1s lack both enzymatic

activity and the disulfide bond. Whereas the liver and kidney contain the highest levels of hSOD1 in the D90A and G93A models, the hSOD1 levels are highest in the CNS in the G85R and G127X hSOD1s models. From these data we have previously hypothesized that ALS is caused by misfolded SOD1 species enriched in the susceptible spinal cord. Such species would constitute minute subfractions of the high-level stable SOD1s and larger proportions of the G85R and G127X hSOD1s (8).

Misfolded proteins are likely to externalize parts of the hydrophobic interior, and may therefore show affinity in hydrophobic interaction chromatography (HIC). Destabilized mutant SOD1 species have previously been found to show affinity to hydrophobic matrices (9). Here we devised an HIC protocol with the aim to detect soluble hydrophobic forms of SOD1 that are present in the tissues. Most of the G85R and G127X mutants were found to bind, and minute subfractions of the D90A and G93A hSODs. The hydrophobic hSOD1 species are enriched in the spinal cords throughout the lifetime of the mice and mostly lack the native disulfide bond.

Results

Notes on the HIC Protocol. The aim was to reflect the molecular composition of hSOD1 *in vivo*. After sacrifice, the tissues were rapidly excised, put on ice, homogenized, centrifuged at $20,000 \times g$, and the supernatants applied to the HIC columns. The lapse of time between sacrifice and HIC was ≈ 90 min. The binding time on the column was short, 5 min. Studies with different binding times suggested that the HIC-binding hSOD1 species were present in the applied extracts and not formed during chromatography [supporting information (SI) Fig. 4]. To block thiols and preserve disulfide bonds, iodoacetamide was added to all solutions. Native hSOD1 is very hydrophilic and only binds to Octyl-Sepharose under high salting-out conditions [≥ 1.4 M $(\text{NH}_4)_2\text{SO}_4$]. No bound hSOD1 was eluted in the second step by the reduced salting-out effect of PBS diluted 1:10. High concentrations of ethylene glycol (60%) or guanidinium chloride (6.7 M) caused incomplete elution. Only with the use of 4% of SDS could all bound hSOD1 be eluted. Thus, the hSOD1 that binds to Octyl-Sepharose is either initially very hydrophobic, or

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Abbreviations: DTPA, diethylenetriamine pentaacetic acid; HIC, hydrophobic interaction chromatography; SOD1, CuZn-superoxide dismutase.

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Fig. 1. Reduced immunoblots of soluble HIC-binding hSOD1 fractions in transgenic mice. (A) Total hSOD1 and the HIC-binding subfractions in tissue extracts from a 34-day-old G93A transgenic mouse. The figures indicate the fractions (percentages) of total hSOD1 that bound. (B) HIC-binding hSOD1 fractions of tissue extracts from 100-day-old G127X, G85R, and wild-type hSOD1 mice. In addition to the monomer band at ≈ 17 kDa, tissues from G127X mice contain a prominent band at 33 kDa (7). In quantifications of G127X hSOD1, both bands were included. (C) HIC-binding hSOD1 subfractions in tissues of G93A mice of different ages (in days). Two spinal cords were pooled from 1-day-old and 10-day-old mice; the other data are from individual mice. The percentages and absolute amounts bound ($\mu\text{g/g}$ of wet weight) in the spinal cords were, respectively, 5.3% and 11 at day 1, 3.5% and 19 at day 10, 3.2% and 44 at day 19, 4.8% and 50 at day 34, 3.8% and 43 at day 50, 2.8% and 42 at day 100, and 4.0% and 45 in the terminal mouse. (D) HIC-binding hSOD1 in tissues from D90A mice of different ages. The corresponding data for the D90A mice were, respectively, 0.49% and 4.1 at day 100, 0.33% and 4.8 at day 200, 0.43% and 6.2 at day 300, and 2.6% and 25 in the terminal mouse. Sp.c., spinal cord; Kidn., kidney.

becomes even more unfolded and strongly interacting after the primary binding.

The HIC-Binding hSOD1 Is Enriched in the Spinal Cords in the Different Transgenic ALS Models. The upper row of Fig. 1A shows an immunoblot of equal amounts of extracts of tissues from a 34-day-old G93A mouse. Liver and kidney contained consider-

ably more hSOD1 than spinal cord and brain. Only minute subfractions of the hSOD1 bound in the HIC-assay, but both the absolute amount and the proportion was by far the greatest in the susceptible spinal cord (Fig. 1A Lower). Because there is evidence for early commencing noxious effects of mutant hSOD1s in spinal cords (7, 10–12), the G93A transgenic mice were examined from birth until death. The high proportion ($\approx 3\%$) of HIC-binding hSOD1 in spinal cord was seen from birth throughout life. On the first day, similar absolute levels of HIC-binding hSOD1 were also found in the other organs, but the “adult” pattern with less misfolded enzyme is reached already at 10 days. There was a slow rise in absolute levels in HIC-binding hSOD1 with the greatest amounts in terminally ill mice (Fig. 1C, Table 1, and SI Fig. 5).

Tissue extracts of mice from the other transgenic models were also analyzed in the HIC assay (Fig. 1B and D and Table 1). All G127X mutant hSOD1 in the extracts bound, and in the G85R mice the major proportion (Table 1). Only minute subfractions of the hSODs in D90A and wild-type hSOD1 transgenic mice bound. The greatest proportions and absolute amounts were seen in spinal cords, except in the G127X mice where spinal cord, brain and liver contained similar amounts. There was broadly an inverse relation between absolute levels of HIC-binding hSOD1 and life-spans of the models; they were highest in G93A mice and lowest in D90A mice. In the latter there was also a distinct rise in the terminal stage (Fig. 1D and Table 1). In all of the ALS models the spinal cord levels of HIC-binding hSOD1 were significantly greater than in mice expressing wild-type hSOD1 (Table 1). Skeletal muscle contained considerably less HIC-binding hSOD1 than the other tissues tested (data not shown). In Fig. 1 only the parts of the gels that contain hSOD1 subunits are shown. There are, however, no other significant bands present in the immunoblots (SI Fig. 5A).

Next to spinal cord, the highest levels of HIC-binding hSOD1 were generally found in the brain (Table 1). Evidence for mutant SOD1-induced damage to brain has also been reported both in the murine ALS models (13) and in humans (14, 15).

The HIC-binding of the endogenous mSOD1 was examined in a G93A mouse and the proportions were found to be 0.2% in spinal cord and brain, and less than 0.05% in liver and kidney, thus similar to the binding of wild-type hSOD1 in transgenic mice (Table 1).

In this study we expressed the absolute amounts of HIC-binding hSOD1 per g wet weight with the intention to reflect the concentration in the tissue. In five 100-day-old control mice the protein levels in spinal cord, brain, liver and kidney extracts corresponded to 24.3 ± 1.0 , 28.9 ± 1.3 , 102 ± 10 , and 90 ± 4 mg/g of wet weight (mean $\pm$ SD) resp. Thus, expressed per amount of total protein extracted, the enrichment of HIC-binding hSOD1 in the CNS would appear even clearer.

Table 1. Relative and absolute amounts of HIC-binding hSOD1 in tissues

Murine model	n	Spinal Cord		Brain		Liver		Kidney	
		Relative, %	$\mu\text{g/g ww}$	Relative, %	$\mu\text{g/g ww}$	Relative, %	$\mu\text{g/g ww}$	Relative, %	$\mu\text{g/g ww}$
G127X	4	100	$12 \pm 4^*$	100	14 ± 4	100	15 ± 5	100	4.8 ± 1.4
G85R	4	79 ± 19	$17 \pm 6^*$	69 ± 13	13 ± 5	48 ± 11	9.2 ± 3.7	56 ± 20	5.5 ± 3.1
G93A	7	2.6 ± 1.1	$35 \pm 12^{\dagger}$	1.5 ± 0.8	10 ± 5	0.22 ± 0.08	7.5 ± 1.8	0.28 ± 0.15	3.5 ± 1.6
G93A term	4	3.7 ± 0.7	$48 \pm 7^{\dagger}$	2.1 ± 1.0	20 ± 8	0.26 ± 0.19	8.9 ± 4.8	0.35 ± 0.44	6.1 ± 5.4
D90A	5	0.47 ± 0.15	$3.4 \pm 1.1^{\dagger}$	0.26 ± 0.12	1.6 ± 0.9	0.14 ± 0.06	2.2 ± 1.3	0.14 ± 0.08	1.4 ± 0.8
D90A term	2	$2.6/1.7$	$25/17$	$0.33/0.21$	$2.5/1.9$	$0.14/0.09$	$2.0/1.0$	$0.28/0.19$	$2.7/1.2$
wtTg	4	0.13 ± 0.03	1.5 ± 0.4	0.08 ± 0.03	0.72 ± 0.16	0.07 ± 0.03	2.3 ± 0.8	0.07 ± 0.06	1.5 ± 1.2
wtTg 600 d	4	0.17 ± 0.06	3.3 ± 1.7	0.16 ± 0.06	2.5 ± 1.2	0.09 ± 0.05	2.9 ± 1.6	0.08 ± 0.04	1.3 ± 0.6

The means $\pm$ SD are shown. n, number. The mice were 100-day-old unless otherwise indicated. ww, wet weight. *, $P < 0.05$; $\dagger$, $P < 0.01$; $\ddagger$, $P = 0.064$ compared with 100-day-old wild-type hSOD1 transgenic mice (Mann–Whitney U test).

affinity as long as Zn was liganded (SI Fig. 7*A* and *B*). Thus Zn appears more important for structural integrity than the disulfide bond, as previously found (16). We conclude that only species with a major degree of unfolding or destabilization are captured in the HIC assay.

The disulfide-reduced hSOD1 in the tissue extracts was found to be monomeric (Fig. 2). In the D90A, G93A and wild-type hSOD1 models the major proportion of these monomers lacked HIC affinity, most likely because of liganding of Zn. The disulfide-reduced hSOD1 in the homogenates probably lacked Cu (8). Monomers are, however, on the unfolding pathway of SOD1 (20, 21), suggesting that loss of the disulfide bond could be a key precursor step in a sequence that leads to the formation of cytotoxic species. Notably, the native C57-C146 disulfide bond was mostly absent in the HIC-binding hSOD1 species detected in the spinal cords (Fig. 3*A* and *B*).

A fraction of the species that bound in the HIC assay lacked disulfide bond and should not, according to the analyses on model variants, coordinate prosthetic metals (SI Fig. 7*A* and *B*). Notably, Zn-deficient SOD1 has been shown to exert cytotoxic effects in cultured motor neurons (22). Many additional HIC-binding hSOD1 species were also detected (Fig. 3*A* and *B*). Comparison with spontaneously oxidized recombinant cysteine-mutated hSOD1 variants, suggested that the HIC-binding fractions might include species carrying illicit intrasubunit disulfide bonds (Fig. 3*C*). Such species would be locked in nonnative conformations, leading to exposure of internal hydrophobic structures. In the model that was most closely examined, G93A, these forms appeared to predominate, and were present throughout life. The hSOD1 that is present as insoluble aggregates in terminal spinal cords is partially composed of disulfide-linked dimers and multimers (23, 24). Because SOD1 is mainly present in the reducing cytosol, the presence of the oxidized species is surprising. It is tempting to speculate that the species carrying nonnative intrasubunit and intersubunit disulfide bonds are formed under the same circumstances. We found no evidence, however, for the presence of the HIC-binding nonnatively oxidized subunits in the oxidizing mitochondrial compartment.

Aggregates or Soluble Misfolded SOD1 as ALS-Inducing Species.

SOD1-containing aggregates/inclusions can be found both in ALS patients carrying SOD1 mutations (7, 25), and in transgenic models (8). In the latter, they accumulate in the final phase concomitantly with the appearance of overt symptoms, and are commonly suggested to cause the disease (24, 26). Their appearance probably reflects a terminal disruption of the protein surveillance and degrading systems. The HIC-binding hSOD1 species are likely to be precursors of the aggregates. Indeed, many of the recombinant hSOD1 variants found to bind in the HIC assay were sticky and tended to oligomerize (SI Fig. 7*A* and *B*). The G127X hSOD1 is so aggregation-prone that 20% is continuously present as detergent-soluble protoaggregates (7). In the G93A model there was a slight and in the D90A mice a more distinct increase with time in the spinal cord levels of HIC-binding hSOD1 (Fig. 1*C* and *D* and Table 1). In both cases the increases were far less marked than the terminal surge seen in dense SOD1 aggregates (8).

The total amount of SOD1 sequestered in the terminal aggregates is, however, small. In the high-level D90A and G93A models, aggregated hSOD1 accounts for less than 10% of the hSOD1 in the spinal cords. In the G85R and G127X models the hSOD1 levels in the terminal aggregates are just below the small amounts of soluble protein (8, 18). The amount of detergent-resistant aggregated hSOD1 in terminal G93A mice is 18 $\mu\text{g/g}$ of wet weight (8), thus comparable to the amount of soluble HIC-binding SOD1 present throughout life in this model (Fig. 1*C* and Table 1). In a patient carrying the G127X mutation, the levels in ventral horn aggregates were 2–4% of the level of total

SOD1 in controls (7) and in ALS cases homozygous for the D90A mutation the levels are below 0.5% (K.S.G., P.A.J., S.L.M., T.B. and P.M.A., unpublished data). The absolute levels of aggregated SOD1 are thus very low, because SOD1 accounts for only 0.1% of the tissue protein in ventral horns of human controls (7). Although whole tissue was analyzed in these studies, motor neuron somas and neurites have been estimated to account for 20% of the volume of spinal cord ventral horns (27). Thus, the levels must be low in motor neurons also. Blockage of general protein-handling systems such as chaperones and proteasomes are thus unlikely mechanisms of SOD1 aggregate-induced cytotoxicity. If the aggregates are causative, more specific mechanisms must be involved.

Numerous studies have documented early commencement of noxious effects of mutant hSOD1s in murine spinal cords, long before the appearance of aggregates (7, 10–12). In the most closely examined model, G93A, we have now shown that HIC-binding hSOD1 is enriched in the spinal cord already from birth, preceding signs of injury. These species could thus be the proximate initiators of damage to the motor areas. The misfolded SOD1 might mislocalize, or expose internal structures that interact with essential cellular factors in ways that cause cytotoxicity. Soluble misfolded SOD1 should be more amenable to engagement in such reactions than misfolded SOD1 sequestered in large aggregates.

One limitation of the present study is that whole tissue is analyzed, whereas primarily motor neurons are injured in ALS. There is, however, evidence that the motor neuron demise is caused by effects of mutant SOD1s in multiple cell types in the motor areas which supports the present approach, reviewed in (28).

Conclusions

Herein we demonstrate a life-long enrichment of soluble HIC-binding hSOD1 in the susceptible spinal cord. The levels are comparable to the levels of hSOD1 that are sequestered in terminal aggregates. The HIC-binding hSOD1 appears mainly to be composed of species that lack disulfide bond and liganded metal ions, or are conformationally locked by nonnative intrasubunit disulfide bonds. Common to these species is increased exposure of hydrophobic moieties that are normally protected in the interior of the protein. They form a least common denominator for ALS-associated hSOD1 mutations, ranging from point mutations with widely different molecular characteristics to a defective C-terminally truncated variant.

Materials and Methods

Mice. Homozygous line 716 G127X mice (7), homozygous line 134 D90A mice (29), and heterozygous G85R (30), G93AGur (G1H) and wild-type hSOD1 (N1029) transgenic mice (4) backcrossed 10–30 generations in C57BL/6 mice were studied. The mean lifespans of the mouse lines carrying mutant hSOD1s were 216, 447, 370, and 135 days, respectively. The time from first symptom to death were 12, 50, 31, and 50 days, respectively. C57BL/6 mice were controls.

Preparation of Tissue Homogenates. After sacrifice tissues were rapidly excised, carefully weighed, put on ice, and homogenized in 25 ml per g of wet weight of ice-cold PBS (10 mM K phosphate, pH 7.0, in 0.15 M NaCl) containing EDTA-free Complete (Roche Diagnostics, Mannheim, Germany) antiproteolytic mixture and 20 mM iodoacetamide, by using an Ultraturrax (IKA, Staufen, Germany) for two minutes and sonication for 1 min. The homogenates were centrifuged at 20,000 $\times g$ for 20 min at 4°C. The supernatants were directly subjected to HIC or gel chromatography.

Hydrophobic Interaction Chromatography. One milliliter of Octyl-Sepharose CL-4B (GE Health Care, Uppsala, Sweden) was

packed in 0.8×10 -cm columns. The columns were run stepwise at 22°C and allowed to empty spontaneously. The eluents were bubbled with argon and contained 5 mM iodoacetamide. A 250- μ l sample was added, followed by 250 μ l of PBS. After 5 min, the columns were eluted twice with 2.5 ml $\times$ PBS followed by 2×2.5 ml $\times$ PBS diluted 1:10. Finally, the columns were eluted with 2.5 ml $\times$ 4% SDS in 50 mM Tris-HCl, pH 6.8.

Preparation and Treatment of Recombinant hSOD1 Variants. The variants were prepared as described (31). Preparation of apo forms was carried out as described previously (20). To allow formation of nonnative disulfide bonds, hSOD1 variants were incubated for 7 h at 22°C in 3.5 M guanidinium chloride, 10 mM Tris-(2-carboxyethyl)phosphine hydrochloride (TCEP) and 5 mM diethylenetriamine pentaacetic acid (DTPA) at pH 7.0. The samples were then dialyzed against pH 7.0 PBS containing 1 mM DTPA, centrifuged at $20,000 \times g$ to remove aggregates, and the supernatants collected.

Gel Chromatography. The samples (250 μ l) were applied to a 1 cm $\times$ 30 cm Superdex 75 column (GE Healthcare, Uppsala, Sweden), eluted at 22°C with argon-bubbled PBS containing 5 mM iodoacetamide at 45 ml/hour, and collected in 0.3-ml fractions.

Immunoblotting, Quantification of SOD1 Protein, Determination of Disulfide-Reduced SOD1, In-Gel Reduction, and Total Protein Analysis. The Western blots were carried out as previously described by using species-specific antibodies raised against peptides corresponding to amino acids 24–39 and 24–36 of the human and murine SOD1 sequences, respectively (7). The chemiluminescence of the blots was recorded in a ChemiDoc apparatus and analyzed by Quantity One Software (Bio-Rad). To determine HIC-binding percentages, the SDS-eluted fractions were run in reduced immunoblots together with appropriate dilutions of the

tissue extracts as standards. The hSOD1 concentrations of the extracts were in turn determined in immunoblots by using wild-type hSOD1 with the concentration determined by quantitative amino acid analysis as original standard (17). The absolute amounts of HIC-binding hSOD1 was calculated from these two assays. The proportion (%) disulfide-reduced SOD1 was determined by nonreduced western immunoblotting (omission of reductant in the sample buffer) as previously detailed (8). 20 mM iodoacetamide was mostly added to the sample buffer. In some experiments, after electrophoresis the nonreducing gels were immersed for 10 min at 22°C in transfer buffer containing 2% mercaptoethanol before electroblotting onto PVDF membranes (in-gel reduction).

Total protein in extracts was determined with the Bio-Rad Protein Assay standardized with BSA.

Partitioning of Hydrophobic hSOD1 to Mitochondria. A spinal cord was gently homogenized in a Dounce glass-pestle homogenizer at 4°C by using a procedure optimized for recovery of intact mitochondria (18). The homogenate was centrifuged at $20,000 \times g$ for 30 min, and this primary supernatant was collected. The pellet was sonicated in half the original volume of PBS and then centrifuged at $20,000 \times g$. 250 μ l of the primary and secondary supernatants were subjected to HIC.

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