Proteasomal Degradation of Mutant Superoxide Dismutases Linked to Amyotrophic Lateral Sclerosis*

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Mutations in copper–zinc superoxide dismutase cause the neurodegenerative disease amyotrophic lateral sclerosis. Many of the mutant proteins have increased turnover in vivo and decreased thermal stability. Here we show that purified, metal-free superoxide dismutases are degraded in vivo by purified 20 S proteasome in the absence of ATP and without ubiquitinylation, whereas their metal-bound counterparts are not. The rate of degradation by the proteasome varied among the mutants studied, and the rate correlated with the in vivo half-life. The monomeric forms of both mutant and wild-type superoxide dismutase are particularly susceptible to degradation by the proteasome. Exposure of hydrophobic regions as a consequence of decreased thermal stability may allow the proteasome to recognize these molecules as non-native.

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that causes death of motor neurons in the spinal cord, brain stem, and cerebral cortex (1). Also known as Lou Gehrig disease after the famous baseball player, it causes progressive loss of motor function leading to death. The age at onset of symptoms varies, but is often in the 40s or later. The duration of the disease is also variable, with the more aggressive forms causing death in 1–2 years. Ninety to 95% of cases of ALS are “sporadic” or non-inherited, but 5–10% are familial. In most cases, inheritance is dominant so that one copy of the mutant gene is sufficient to cause the disease. About 20% of familial ALS cases are caused by mutations in copper-zinc containing superoxide dismutase (SOD1). SOD1 contains 153 residues, and over 100 different mutations are known to occur at 64 positions in the sequence (2). Paralytic disease does not result from loss of superoxide dismutase function but from a toxic gain of function, the nature of which is not yet established (3). These key characteristics can be reproduced in rodent models, where expression of the ALS-associated mutant human SOD1 causes motor neuron disease, whereas expression of wild-type SOD1 does not (4).

Mutant SOD1 proteins have increased turnover rates in vivo, with many of the clinically aggressive mutants having particularly short half-lives (5–10). The in vitro susceptibility of SOD1 proteins to proteinase K does not correlate with in vivo half-life, so turnover cannot be explained by a general increase in susceptibility to proteases (9).

The 26 S proteasome is composed of a 20 S core proteasome, capped at both ends by a 19 S complex that recognizes ubiquitinylated substrates and provides ATP-dependent unfolding of the substrate (11, 12). Dorfin and NEDL1 are two distinct ubiquitin ligases that can ubiquitinylate SOD1 (13, 14), and exposure of cells to proteasome inhibitors increases the intracellular level of mutant SOD1 proteins (15). Thus, it has been thought that degradation of SOD1 proceeds through the ubiquitin-dependent 26 S proteosomal system. However, the 20 S proteasome can distinguish native from non-native proteins and degrade the latter without a requirement for ATP or ubiquitinylation (16–18).

We therefore undertook a study to determine whether purified wild-type or ALS-associated mutant SOD1s could be degraded by purified 20 S proteasome.

EXPERIMENTAL PROCEDURES

Materials—Substrates for fluorimetric assay of proteasome activities (AAF-AMC, AMC, LLE-AMC, and LSTR-AMC), ANSA, DTPA, and lactacystin were purchased from Sigma. Hepes and Tris buffer were from MP Biomedicals (Irvine, CA), and TCEP from Pierce. Sprague-Dawley rat livers were supplied by Harlan Bioproducts (Indianapolis, IN).

Purification of the 20 S Proteasome—Livers were thawed at 4 °C and homogenized in 20 mM Hepes, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.8, using a Potter-Elvehjem glass homogenizer with a Teflon pestle with 4 ml of buffer/g of liver. The homogenizer was rinsed with 20 ml of buffer for each liver, and the homogenate was centrifuged at 25,000 × g for 2 h at 4 °C to remove cell debris. The 20 S proteasome was then purified essentially as described (19, 20). Briefly, the liver homogenate supernatant was subjected to ammonium sulfate cuts (35 and 60% saturation). The 60% ammonium sulfate pellet was resuspended in 10 mM Tris-HCl buffer, 100 mM KCl, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.2, and dialyzed at 4 °C against the same buffer. The first ion-exchange chromatography step was performed on a Tosohaas DEAE-5PW column (Tosoh Bioscience, Montgomeryville, PA) with a Hewlett-Packard 1050 liquid chromatograph. Purification was achieved with two additional chromatographic steps: ion-exchange chromatography on a Mono-Q HR 5/5 column (Amersham Biosciences) and gel filtration chromatography on a Superose 6 column (Amersham Biosciences). The purified proteasome had the same pattern on SDS gels as published (Fig. 1 in Ref. 20), with some variation from preparation to preparation in the amount of associated hsp90.

SOD1 Expression, Purification, and Demetallation—Human wild-type SOD1, “wild-type-like” mutants A4V, G37R and G39A, “metal binding region” mutants H80R, E133A, S134N, and the disulfide incom-
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peptides with masses that indicated the presence of an intramolecular proteasome isolated from rat liver (50 nM) at 37 °C for 120 min in 50 mM Hepes buffer, pH 7.8. When apoproteins were used, pipette tips were pre-washed in 10 mM DTPA, and 2.5 mM DTPA was added to the digestion mixture. The reaction was stopped by adding 1/10 volume of 1% trifluoroacetic acid, giving a pH of 3.5–4.0. Samples were then analyzed by reverse phase HPLC-mass spectrometry with an Agilent 1100 series as described (23). Deconvolution of mass spectra and construction of extracted ion chromatograms were performed with version 10.01 of the Agilent Chemstation software. Control reactions were under the same conditions, but the trifluoroacetic acid was added at the beginning of the incubation. The fraction of digested SOD1 was determined from the loss of area under the intact SOD1 peak in the 210-nm chromatogram. The accuracy of this assay depends on the ability of the reverse phase column to separate the intact SOD1 from all products generated by the proteasome. In our experience, the column and HPLC system used are generally very good at doing this for proteins the size of SOD1. If any products did co-elute with the native SOD1, then the effect would be to underestimate the extent of degradation by the proteasome.

We examined this possibility by mass spectrometric analysis of the peak containing intact SOD1. Deconvolution analysis of the mass spectrum revealed only the expected native mass, with one disulfide bond. If even a single residue were clipped from either the amino or carboxyl terminus, the resulting change in mass of either 131 or 146 mass units would readily be detected. If a single peptide bond were cleaved at any site from Cys57 to Cys146, no peptides would be released because of the disulfide tether. However, hydrolysis of the peptide bond would add a water molecule, and the resulting 18 mass unit increase would also readily be detected.

Protein and Protease Assays—The bicinchoninic acid assay (Pierce) was used for determination of protein concentration using serum albumin as the standard protein. Chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolase activities of the 20 S proteasome were assayed with fluorogenic peptides AAF-MCA, LSTR-MCA, and LLE-MCA, respectively. A typical assay was carried out in a 96-well plate with 1–2 μg of proteasome in a total volume of 200 μl of 50 mM Hepes, pH 7.8, plus the appropriate peptide substrate. Following incubation at 37 °C for 30 min, fluorescence was measured with either a SpectraMax Gemini EM (Molecular Devices, Sunnyvale, CA) or a CytoFluor series 4000 (PerSeptive Biosystems, Framingham, MA). The excitation and emission wavelengths were 360 and 460 nm. A standard curve of the fluorescence for the pure product was used to calculate the concentration of liberated aminomethylcoumarin product in the assays. When lactacystin was tested as a proteasome inhibitor, the proteasome and lactacystin were incubated for 30 min at 37 °C before adding either a fluorogenic peptide or the G93A SOD1.

Protein Surface Hydrophobicity—Surface hydrophobicity of the different SOD1 proteins was determined by incubating the proteins with ANSA at 37 °C as described (24). Briefly 25 μg of SOD1 was incubated at 37 °C for 30 min in 50 mM Hepes, 100 mM KCl, pH 7.2. The change in fluorescence associated with the binding of ANSA to protein surface hydrophobic regions was monitored at 490 nm using 370 nm excitation with a QuantaMaster fluorometer (Photon Technology International, Lawrenceville, NJ). The fluorescence intensity for ANSA binding to the metal-bound, wild-type SOD1 was taken as a point of reference. Results were expressed as the change in fluorescence intensity with respect to this reference.

RESULTS

Apo-SOD1s Are Substrates for the 20 S Proteasome but Metal-bound Forms Are Not—The as isolated SOD1 proteins are metal loaded with copper and zinc, although with varying degrees of fidelity and complete-
The proteasome has three defined proteinase activities, chymotryptic-like, trypsin-like, and caspase-like, also termed peptidyl-glutamyl peptidease activity or V8-like activity. We noted that many of the cleavages sites of SOD1 proteins are on the carboxyl side of aspartyl and glutamyl residues, indicating that the caspase-like subunits are responsible for the observed products, despite the relatively low specific activity of the caspase-like subunits with low molecular weight substrates (TABLE TWO).

Lactacystin is a highly specific inhibitor of the proteasome that binds covalently to the active site threonine of the β5 subunit, which has chymotryptic-like activity (26). Lactacystin thus causes a rapid and irreversible inhibition of chymotryptic-like activity. Trypsin-like activity is also irreversibly inhibited, albeit more slowly than the chymotryptic activity. Caspase-like activity is even more slowly inhibited, and the inhibition is reversible (26). Consistent with this behavior, when 50 μM lactacystin was tested with the low molecular weight fluorogenic substrates, it inhibited chymotryptic-like activity by 100%, tryptic-like by 93%, and caspase-like by 78%. When tested with G93A in a 90-min assay, degradation of SOD1 was inhibited by 63%.

Although the catalytic β subunits of the proteasome require substrate sequences matching their specificity, the presence of those sequences does not determine whether a given protein molecule will be degraded by the proteasome. The molecular mechanisms controlling susceptibility are not well defined, but it is believed that the 100 regulatory subunits of the proteasome control access of proteins into the core of the proteasome (27). The 20 S proteasome allows entry and will degrade protein molecules having exposed hydrophobic patches that are normally buried. For example, oxidatively damaged proteins typically have increased surface hydrophobicity, and the 20 S proteasome is the major pathway by which they are cleared from the cell, without a requirement for ubiquitinylation or ATP hydrolysis (18). The fluorescent probe ANSA can detect changes in surface hydrophobicity (28–30). The three mutant SOD1s with greatest susceptibility to the proteasome showed substantial increase in their surface hydrophobicity (A4V, G37R, and G93A; Fig. 5).

However, an increase in surface-exposed hydrophobic patches might not by itself allow entry of the globular SOD1 protein into the very restricted axial gate of the proteasome formed by the α subunits (12). Threading of the SOD1 into the cavity of the proteasome may require substantial unfolding of the molecule. Wild-type, metal-bound SOD1 is...
an exceptionally stable protein (31) as are many of the metal-bound mutant SOD1 molecules (32). Removal of metals markedly decreases the stability of wild-type SOD1 to heat denaturation and chaotropes, and most, but not all, metal-free mutant SOD1 proteins are much less stable than the wild-type (2, 32). We therefore compared the heat denaturation of apo-SOD1 proteins with their susceptibility to degradation by the 20 S proteasome and found an excellent correlation (Fig. 6). The 3 mutants most susceptible to proteasomal degradation were A4V, G37R, and G93A. The turnover of these mutants and of wild-type human SOD1 has also been determined in transgenic Caenorhabditis elegans; all 3 mutants turned over at a rate approximately double that of the wild-type (10).

The melting temperature \( T_m \) of several mutant metal-free SOD1 proteins is rather close to 37 °C, suggesting that the \( T_m \) fraction of unfolded SOD1 could be substantial. If the melting temperature is a key determinant of susceptibility to degradation by the 20 S proteasome, then one should observe a clear increase in the rate of degradation as the temperature of the assay approaches the \( T_m \), and the effect should be greater than any observed with low molecular weight fluorogenic substrates (TABLE TWO). We studied the effect of temperature on the susceptibility of apo wild-type, G37R, and A4V SOD1 proteins that have melting temperatures of 52, 44, and 40 °C, respectively (2). Degradation of the wild-type SOD1 increased from undetectable levels at 30 through 44 °C, and the increase is similar to that observed for low molecular weight substrates (Fig. 7 and TABLE TWO). Degradation of G37R increased substantially from 30 through 40 °C, but the increase from 40 to 44 °C was modest. However, A4V showed only a small increase from 37 to 40 °C, and none from 40 to 44 °C, consistent with melting temperature being a key determinant in controlling susceptibility to degradation.

SOD1 has one intrasubunit disulfide bond between Cys57 and Cys146, and this linkage substantially increases the stability of the monomeric protein that in turn stabilizes the dimeric form (33). Mutant SOD1 proteins that cause familial ALS are more susceptible to reduction of the disulfide bond than the wild-type (31). Removal of the disulfide bond increases the susceptibility of these proteins to proteasomal degradation (31, 32).

### TABLE ONE

| Elution time \( \text{min} \) | Peptide | Observed mass | Calculated mass | Disulfide |
|----------------------------|---------|---------------|----------------|-----------|
| 13.67                      | P1–76   | 2,621.7       | 2,621.8        | None      |
| 14.57                      | P50–76  | 2,769.0       | 2,769.0        | None      |
| 16.17                      | P122–153| 3,118.4       | 3,118.4        | None      |
| 19.84                      | P1–76   | 3,348.0       | 3,348.0        | None      |
| 19.84                      | P91–153 | 6,363.2       | 6,363.1        | C\(_{111}\)-C\(_{146}\) |
|                             | D91–K91|              |                |           |
| 19.84                      | P21–50  | 7,856.7       | 7,856.6        | C\(_{111}\)-C\(_{146}\) |
|                             | D21–E77|              |                |           |
| 21.75                      | P1–76   | 8,032.2       | 8,032.0        | C\(_{6}\)-C\(_{97}\) |

* From Fig. 3. Peptide masses were determined by deconvolution of the mass spectral peaks. The presence of each peptide was confirmed by analysis of extracted ion chromatograms (Fig. 4).
* The amino acids at each end of the identified peptide are shown in bold, with the preceding or following residue in italics. The carboxyl-terminal residue is Gln\(_{153}\).
* Peptide 20–49 also has mass 3,348.0, but its production would require cleavage on the carboxyl side of Asn\(_{19}\), which is unlikely for the proteasome. Also, analysis of extracted ion chromatograms confirmed the presence of peptide 1–20 but not 1–19 (Fig. 4).
* The mass of peptide 1–76 from the C57S mutant matched that calculated without a disulfide, as expected, because it cannot form a disulfide.

### TABLE TWO

| Activity of the proteasome at different temperatures |
|---------------------------------------------------|
| Cleavage type*                                | 37 °C | 40 °C | 44 °C |
| Chymotryptic-like                             | 100   | 103   | 129   |
| Tryptic-like                                  | 100   | 130   | 155   |
| Caspase-like                                  | 100   | 127   | 179   |

* For each cleavage type, the measured specific activity at 37 °C was set to 100%.

These values were 14.5, 2.30, and 1.11 nmol/min/mg for the chymotryptic-like, tryp tic-like, and caspase-like activities, respectively. Activity was determined with fluorogenic substrates as described under "Experimental Procedures."
their disulfide bonds in vitro, although it is not yet known whether this occurs in vivo (34). The C57S mutant of SOD1 does not form an intramolecular disulfide, and its apo form is a monomer rather than dimer (21). Reduction of the disulfide bond of apo wild-type SOD1 also converts it to a monomeric species (21). Both of these monomeric species were readily degraded by the 20 S proteasome, with all of the C57S being digested during our routine 2-h assay (Fig. 8). Thus, destabilization of SOD1 by either site-specific mutation or reduction of its disulfide bond increases monomerization and susceptibility to the 20 S proteasome.

**DISCUSSION**

Purified, metal-free SOD1 proteins were substrates of the 20 S proteasome and their degradation did not require ATP or ubiquitylation. The susceptibility to proteasomal attack varied among the various ALS-associated mutant SOD1 proteins tested. This susceptibility correlated well with the extent of perturbation of native structure, whether measured by increased accessibility of ANSA to normally buried hydrophobic patches or by melting temperature. The increased exposure of hydrophobic patches may well be the key to recognition by the 20 S proteasome (35–38).

ALS-associated mutant SOD1s have intracellular turnover rates that vary greatly (9, 10). Whereas the number of mutants available for comparison is still limited, the in vitro susceptibility to purified proteasome correlated with in vivo turnover rates.

Binding of 19 S caps to the 20 S proteasome converts it to the 26 S proteasome. The 19 S caps recognize ubiquitylated substrates and provide ATP-dependent unfolding of the substrate with subsequent threading into the core where peptide bond hydrolysis occurs (11, 12). For SOD1 and other substrates degraded by the 20 S proteasome, neither ubiquitylation nor ATP are required, but the mechanisms for unfolding and threading are not understood. In addition, the entrance channel to the euarkotic 20 S proteasome is normally closed and must be opened by activators to allow substrate entry (12, 27, 39–41). Hydrophobic peptides are among the activators that can mediate the opening of the channel to the eukaryotic 20 S proteasome (42). Because apo-SOD1 proteins are degraded by the 20 S proteasome, we suggest that binding of the SOD1 monomer to an subunit causes channel opening and allows threading into the proteasome cavity.

The in vivo metal-binding characteristics of wild-type and the pathogenic mutant SOD1 proteins are not yet well defined, and these may vary among tissues and cellular compartments. Prior to the binding of metal ions and dimerization, nascent SOD1 exists as an apo-meromer. By monitoring 64Cu incorporation by lymphoblasts in the presence or absence of the protein synthesis inhibitor, cycloheximide, Petrovic and colleagues (43) estimated that 35% of the wild-type SOD1 has not bound copper ion. In a similar experiment performed with mouse fibroblasts,
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64Cu was incorporated into an existing SOD1 pool (44). In both studies, the incorporation of 64Cu required the copper chaperone for SOD1. Thus, any pathogenic SOD1 mutation that directly affects metal ion binding or interferes with the interaction of SOD1 and the copper chaperone is predicted to increase the pool of undermetallated SOD1 in vivo. This undermetallated SOD1 has been proposed to protect cells against copper toxicity by providing a high affinity metal-binding pool that can rapidly sequester it as it enters cells (43).

The class of pathogenic SOD1 proteins termed metal-binding region mutants are metal-deficient in vivo because the mutation sites fall at the metal ligands themselves (H46R, H48Q, H80R) or in the loop elements intimately involved in metal binding (D124V, D125H, S134N) (45). Inductively coupled plasma mass spectrometry and high resolution crystal structures of the purified metal-binding region pathogenic mutants confirm this metal ion deficiency (25, 46). In contrast, the class crystal structures of the purified metal-binding region pathogenic metal ligands themselves (H46R, H48Q, H80R) or in the loop elements degenerative diseases, including SOD1-linked amyotrophic lateral sclerosis. A4V, G37R, that facilitate monomerization of SOD1 proteins (21, 34), which our deficiency and absence of the intrasubunit disulfide bond are key factors more readily reduced than that of wild-type SOD1 (34). Metal defi-

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out a requirement for ATP. The monomeric form of the protein is the likely substrate for the proteasome.

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