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Post-Translational Modification by Cysteine Protects Cu/Zn-Superoxide Dismutase From Oxidative Damage

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Abstract

Reactive oxygen species (ROS) are cytotoxic. To remove ROS, cells have developed ROS-specific defense mechanisms, including the enzyme Cu/Zn superoxide dismutase (SOD1), which catalyzes the disproportionation of superoxide anions into molecular oxygen and hydrogen peroxide. Although hydrogen peroxide is less reactive than superoxide, it is still capable of oxidizing, unfolding, and inactivating SOD1, at least in vitro. To explore the relevance of post-translational modification (PTM) of SOD1, including peroxide-related modifications, SOD1 was purified from post-mortem human nervous tissue. As much as half of all purified SOD1 protein contained non-native post-translational modifications (PTMs), the most prevalent modifications being cysteinylolation and peroxide-related oxidations. Many PTMs targeted a single reactive SOD1 cysteine, Cys₁₁₁. An intriguing observation was that unlike native SOD1, cysteinylolated SOD1 was not oxidized. To further characterize how cysteinylolation may protect SOD1 from oxidation, cysteine modified SOD1 was prepared in vitro and exposed to peroxide. Cysteinylolation conferred nearly complete protection from peroxide-induced oxidation of SOD1. Moreover, SOD1 that has been cysteinylolated and peroxide oxidized in vitro comprised a set of PTMs that bear a striking resemblance to the myriad of PTMs observed in SOD1 purified from human tissue.

Introduction

Reactive oxygen species (ROS) are by-products of aerobic metabolism and are also the primary products of certain oxidoreductases. For example, the incomplete reduction of oxygen to water during mitochondrial respiration can create both hydrogen peroxide (H₂O₂) and superoxide anion (O₂^{•-}). These by-products are harmful to cells because they can alter protein conformation, disrupt enzyme function, and mutate DNA, amongst other things¹⁻³. A testament to the toxicity of ROS is the monocyte-resident oxidoreductase, NADPH oxidase (NOX), which generates superoxide enzymatically to kill targeted cells, including

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Supporting Information

Additional figures further characterizing cysteinylolation are available as supporting information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

microorganisms. Cells combat harmful ROS species with a multi-faceted anti-oxidant defense mechanism that includes the metalloenzyme Cu/Zn superoxide dismutase (SOD1). SOD1 catalyzes the disproportionation of the superoxide anion as follows ⁴:

Loss of SOD1 function leads to an increase in superoxide anions causing negative effects, including cell death under conditions of oxygen stress. One potential mechanism for inactivation of SOD1 is via oxidation by its own reaction product, hydrogen peroxide. Indeed, modification by two (sulfinic acid) or three (sulfonic acid) oxygen atoms on SOD1 Cys₁₁₁ are well-established peroxide-mediated modifications in vitro ⁵ and in vivo ⁶. Residue 111 is situated at the SOD1 dimer interface ⁵ and is highly conserved, commonly serine. In humans, great apes, and a few other species residue 111 is a cysteine. Cys₁₁₁ is highly reactive and has been shown to be modified by oxygen, copper ⁷, glutathione ⁸⁻¹⁰, and potentially cysteine ^{7, 11, 12}.

Modification by an oxygen atom can be detrimental to SOD1 structure and function ¹⁻³ and has been implicated in diseases such as Amyotrophic Lateral Sclerosis ¹³. In fact, sulfonic acid modified SOD1 (3 oxygen atoms) is the same form of SOD1 that Bosco *et al* showed inhibits fast axonal transport in a similar fashion to SOD1 familial Amyotrophic Lateral Sclerosis (FALS) variants ¹³. Here, we characterize post-translational modifications (PTMs) of SOD1 in situ, including peroxide- and cysteine-related modifications, and provide in vitro evidence that cysteinylolation protects SOD1 from oxidative damage.

Methods

SOD1 Purification from Human Tissue

Two purification protocols using distinct elution buffers and antibodies were used to investigate PTMs and their relative amounts in human tissue. The first purification protocol was previously described and used polyclonal rabbit antibodies raised in house against a mixture of native and modified (by both oxygen and sulfur adducts on Cys₁₁₁) SOD1 purified from human erythrocytes and elution with 5 % acetic acid ¹⁴. This protocol provides protein that can be directly infused into a mass spectrometer, avoiding lengthy liquid chromatography. In the second purification SOD1 was isolated from human nervous tissue as previously described ¹³, using a sheep polyclonal antibody raised against SOD1 from human erythrocytes and Gentle Elution Buffer[®] (reportedly 3M MgCl₂ at roughly neutral pH). Frozen human nervous tissue was homogenized in lysis buffer (25 mM Tris, pH 7.8, supplemented with protease inhibitor cocktail (Roche)) at 4 °C followed by centrifugation at 14,000 RPM and this supernatant was applied to an individual immunoaffinity column. Columns were washed four times with 600 µl (~20 column volumes total) wash buffer (25 mM Tris, 100 mM NaCl, pH 7.8). SOD1 proteins were eluted with 2 × 500 µl of either 5 % acetic acid (purification 1) or gentle antibody elution buffer (GEB), pH 6.6 (Pierce, 21027) (purification 2). To ensure that the purified samples contained a representative sampling of native and modified SOD1, we verified that SOD1 was immunodepleted from the homogenates. Following the first immunopurification the column was re-equilibrated in lysis buffer, the depleted homogenates (flow-through) were re-applied, and the purifications were repeated in this way a total of 3 times. If protein was detected in a repeat purification (using MALDI-TOF MS, only the second purification occasionally contained minor amounts of SOD1) that purification was pooled with the first. Proteins eluted with GEB were buffer exchanged into 25 mM HEPES, pH 7.4 and concentrated to ~100 µl, and the concentrations were determined by western blot and densitometry (ImageJ) analyses with recombinant wild-type SOD1 standards. Proteins

eluted with 5 % acetic acid were used as purified. In addition, SOD1 was purified anaerobically, in the presence or absence of iodoacetamide (10mM), iodoacetic acid (4mM) and S-methyl methanethiosulfonate (MTS) (0.5 mM) to block any unreacted cysteine residues and to scavenge any free cysteine, using an MBraun Unilab glove box with oxygen levels below 10 ppm, monitored by zinc ethane.

Recombinant SOD1 expression and purification

In vitro studies we used SOD1 overexpressed and purified from *S. cerevisiae*. The construct for expression of human SOD1 in *S. cerevisiae* was obtained through the generous gift of Dr. P. John Hart, Ph.D. (University of Texas Health Science Center, San Antonio). Expression and purification was carried out as previously described^{15, 16}. Briefly, each construct in the yeast expression vector YEp-351 was transformed into EGY118ΔSOD1 yeast and grown at 30 °C for 36–48 hours. Cultures were pelleted, lysed using 0.5 mm glass beads and a blender, and subjected to a 60 % ammonium sulfate cut. After ammonium sulfate precipitation the sample was pelleted and the supernatant was diluted with 0.19 volumes of a low salt buffer (50 mM sodium phosphate, 150 mM sodium chloride, 0.1 M EDTA, 0.25 mM DTT, pH 7.0) to a final concentration of 2.0 M ammonium sulfate. This sample was then purified using a phenyl-sepharose 6 fast flow (high sub) hydrophobic interaction chromatography column (GE Life Sciences) using a 300 mL linearly decreasing salt gradient from a high salt buffer (2.0 M ammonium sulfate, 50 mM sodium phosphate, 150 mM sodium chloride, 0.1 M EDTA, 0.25 mM DTT, pH 7.0) to the low salt buffer. Samples containing SOD1 were eluted between 1.6–1.1 M ammonium sulfate, pooled and buffer exchanged to a 10 mM Tris, pH 8.0 buffer using Amicon Ultra-15 centrifugal filter units (Millipore). The protein was then loaded onto a Mono Q 10/100 anion exchange chromatography column (GE Life Sciences) and eluted using a 200 mL linearly increasing salt gradient from a low salt buffer (10 mM Tris, pH 8.0) to a high salt buffer (10 mM Tris, pH 8.0, 1 M sodium chloride). The gradient was run from 0–30 % 10 mM Tris, pH 8.0, 1 M sodium chloride and SOD1 eluted between 5–12 % 10 mM Tris, pH 8.0, 1 M sodium chloride. SOD1 protein was quantified using the Bradford assay with yields of 6 mg/8L (0.75 mg/L) and confirmed by MALDI-TOF and FTMS analysis.

Direct infusion ESI-FTMS and ESI-ion trap MS

Samples in 5 % acetic acid were analyzed by direct infusion (Figure 1 and 2A), and were compared to LC-MS results (Figure 2B). For ESI-FTMS infusion experiments SOD1 was diluted to approximately 1 μM concentration in 50 % acetonitrile (ACN)/49.9 % HPLC grade water/0.1 % formic acid and infused (sprayed directly) into the FTMS using similar instrument acquisition parameters as described below. For ESI-ion trap infusion experiments SOD1 was also diluted to approximately 1 μM concentration in 40 % ACN and infused (sprayed directly) into the Bruker Daltonics HCT Ultra ion trap with capillary voltage = –4000V, Skimmer 1 = 40V, ICC on with a maximum accumulation time of 200,000 μs.

RP-HPLC and FTMS

Purified SOD1 protein in GEB was analyzed using reverse-phase liquid chromatography and Fourier transform mass spectrometry as previously described¹³. Briefly, reversed-phase liquid chromatography was performed using a two-dimensional nano-flow rate liquid chromatography (Eksigent), a 5-mm, 300-μm ID guard column (LC Packings, Part Number 160454) and a self-packed 14-cm, 100-μm ID column with 5-μm C₁₈ beads (unpacked from a larger Targa column). Buffer A consisted of 0.1 % formic acid (vol/vol) in HPLC grade water and buffer B consisted of 0.1 % formic acid (vol/vol) in 100 % HPLC grade acetonitrile (vol/vol). Samples were diluted to a final formic acid concentration of 0.1 % (v/v) and injected. Following injection, samples were washed on the guard column with 160

column volumes of buffer A ($8 \mu\text{l min}^{-1}$), and eluted at 650 nl min^{-1} using a 0–40 % gradient over 30 min. Samples were introduced via a nanospray ion source with a dual ion funnel (Apollo II) connected to a 9.4 T hybrid quadrupole Fourier transform ion cyclotron resonance (FT-ICR, FT-MS) mass spectrometer (apex Q_e-94, Bruker Daltonics). External calibration of m/z scale was performed using electrospray tuning mix (Agilent, G2431A) using peaks at m/z 622, 922, 1522, and 2122.

After desolvation, the ions were transferred from a source hexapole to the quadrupole mass filter where isolation could occur in a second hexapole (collision cell). Ions accumulated in the second hexapole were then transferred through the ion optics region of the instrument to the ICR cell. Frequency sweep excitation was followed by image charge detection. Important instrument operation parameters include source declustering potential = 40 V, hexapole 1 accumulation time = 0.1 ms, collision cell accumulation time = 1 s, time of flight = 1.8 ms (D2), sidekick extraction voltages = -1.0 V (EV1, EV2, DEV2), RF excitation voltage = 130 V and ICR trapping potential = 1.2 V. Intact protein masses were reconstructed using the deconvolution function from DataAnalysis (Bruker Daltonics, version 3.4), and monoisotopic masses were determined using the Snap II algorithm (Bruker Daltonics).

In vitro cysteinylolation and oxidation

Human SOD1 overexpressed and purified from *S. cerevisiae*, at either $0.1 \mu\text{M}$ or $1 \mu\text{M}$ concentration, was incubated with $40 \mu\text{M}$ L-cysteine overnight at room temperature and analyzed using direct infusion into the Fourier transform mass spectrometer. Oxidation was performed at room temperature for four hours using $100 \mu\text{M}$ or 10 mM hydrogen peroxide. Time points were collected every hour and analyzed using direct infusion into the FTMS using similar parameters as described above. All samples were desalted using C₁₈-containing micropipette tips according to the manufacturer's protocol (Millipore) and diluted 1:3 in HPLC water prior to injection into the mass spectrometer.

Identification of site of cysteinylolation using funnel skimmer dissociation (FSD)

Cysteinylated SOD1 ($1 \mu\text{M}$) in 5 % acetonitrile, 0.1 % formic acid was directly infused into a 9.4 T Bruker Daltonics Fourier transform mass spectrometry in nanospray mode. Skimmer 1 voltage was then increased from 40 V–120 V in ten volt increments in order to fragment the protein using diverse fragmentation channels (at low voltage primarily mobile proton-directed, for example at proline residues, and at higher voltages primarily charge remote, for example at acidic residues¹⁷). Data were analyzed using Bruker Daltonics's DataAnalysis software.

Results

SOD1 purified from human brain and spinal cord is modified by cysteine and oxygen

SOD1 was first immunopurified using rabbit polyclonal anti-SOD1 (each respective sample individually), from ten non-diseased human nervous tissue samples and mice (8 from human and 2 from mice) overexpressing human SOD1 (brain and spinal cord), eluted using 5 % acetic acid and analyzed by direct infusion-Fourier transform mass spectrometry (Figure 1, human) or by direct infusion-ion trap mass spectrometry (Figure S1, mouse). Cysteinylolation of SOD1 was consistently the most prevalent PTM observed as peaks (*e.g.* the 14+ charge state at m/z 1141.586) corresponding to a deconvoluted and deisotoped monoisotopic mass of 15955.11 Da, which is 119.01 Da larger than unmodified SOD1 (15836.10 Da). A 119.01 Da modification is consistent with the molecular weight of cysteine with the two sulfhydryl hydrogen atoms liberated during disulfide bond formation (theoretical mass 119.01) between Cys₁₁₁ of SOD1 and free cysteine (Figure 1). In addition to cysteinylolation, we observed

modifications we putatively assigned as modification with one (15851.40 Da) and two oxygen atoms (15866.38 Da). The abundance of these putative oxidative modifications was too low to permit further characterization - however, oxidative modifications of Cys₁₁₁ and Trp₃₂ have been described previously¹⁴.

To further investigate PTMs by cysteine and oxygen, as well as their relative amounts, an additional four post-mortem human brain samples were analyzed using a different purification method (different homogenization buffer, SOD1 antibodies, wash buffer, and elution buffer) and different preparative conditions (reverse-phase liquid chromatography mass spectrometry [LC-MS], (Figure 2)). Cysteinylation and oxidation were observed in all four samples (Figure 2B), although the percentage of total of Cys modified SOD1 appeared slightly lower in RP-LC-MS analysis ($38 \% \pm 7 \%$ via RPLC-MS versus $48 \% \pm 6 \%$ via direct infusion). Reverse phase chromatography generally increases the dynamic range of MS analysis, and as a result additional modifications were detected in these samples. Therefore, the overall average cysteinylation observed in human nervous tissue (both direct infusion and RP-LC-MS) was 41 % (maximum cysteinylation observed was 62 %; minimum cysteinylation observed was 22 %). Notably, cysteinylation was not a prevalent modification in SOD1 purified from human blood by the first method¹⁴ but was reportedly observed in other purifications^{11, 12}.

To determine if the extent of cysteine modification or oxidation depended upon purification methods, for example if oxidative addition of free cysteine or thiol disulfide exchanged with free cystine, two additional controls were used. First, SOD1 was purified anaerobically. Second, SOD1 was homogenized anaerobically in the presence of iodoacetamide, iodoacetic acid, and S-methyl methane thiosulfonate (MMTS) to alkylate and scavenge any free cysteine as well as unmodified SOD1 Cys₁₁₁. Cysteinylation was observed in SOD1 purified anaerobically, however there was approximately a 2-fold reduction in the amount observed compared to samples purified aerobically. The cysteine alkylators and scavengers removed the majority of the cysteinylation, however a small amount was still present.

In summary, to investigate PTMs by cysteine and oxygen, we performed purifications using (1) different antibodies and different elution conditions; (2) with and without liquid chromatography; (3) aerobically and anaerobically; (4) with and without alkylation agent to block endogenous “free” cysteine from binding SOD1 during homogenization; and (5) from different tissues and organisms (human and mouse spinal cord and brain). Under the conditions tested here SOD1 purified from human tissue, but not from yeast, contained cysteinylated SOD1 (Table 1).

The majority of SOD1 modifications observed in human tissue can be created in vitro using cysteine and peroxide

To determine if both the types and relative amounts of modifications of SOD1 purified from human tissue could be recapitulated in vitro, SOD1 was incubated with 40-fold molar excess cysteine, which approximated the cysteinylation levels of post-mortem SOD1. This sample was incubated with a 100-fold molar excess of peroxide (100 μ M) in a time course experiment. Following incubation with low levels of peroxide (the amount generated in 100 turnovers of peroxide) for 3.5 hours, these in vitro samples resembled SOD1 purified from human tissue, indicating that many of the modifications observed in SOD1 purified from human samples are the result of modification by cysteine and peroxide (Figure 2).

Cysteine modified SOD1 is protected from oxidation

Human SOD1 expressed and purified from yeast cells was analyzed using a Fourier transform mass spectrometer (FTMS), which showed a peak consistent with native SOD1

(Figure 3A, note: acidic buffers and desolvation conditions were such that Cu and Zn could not be detected). Cysteinylation of SOD1 was created in vitro as described above. Both native and cysteinylation SOD1 were observed, and the binding stoichiometry was approximately one cysteine per SOD1 dimer (Figure 3B), indicating that cysteinylation of one Cys₁₁₁ can potentially block cysteinylation of the adjacent Cys₁₁₁, presumably sterically.

To determine the extent to which in vitro cysteine modification protected SOD1 from peroxide-mediated modification, a sample containing both cysteine modified SOD1 and the native protein was oxidized using 10 mM hydrogen peroxide in a time course study. After two hours the majority of native SOD1 protein was modified by two or three oxygen atoms. Conversely, only a small amount of cysteine modified SOD1 was oxidized (Figure 3C; red dotted lines versus blue dotted lines), indicating near complete protection. In addition, four hours after peroxide treatment all the native SOD1 had been oxidized by two or three oxygen atoms (native protein is no longer observed), whereas cysteine modified SOD1 was not oxidized (Figure 3D; red dotted lines versus blue dotted lines).

Cysteinylation occurred specifically upon SOD1 Cys₁₁₁

PTMs are typically localized using endoprotease digestion followed by LC-MS/MS analysis. In both MALDI-TOF fingerprinting and LC-MS/MS experiments, we observed cysteinylation peptides containing Cys₁₁₁ and localized this modification to Cys₁₁₁ using MS/MS data (Supplemental Figures 2–3). This approach, however, was not ideal for cysteinylation. Control experiments revealed that, following endoprotease digestion, rapid scrambling of SOD1 disulfides occurred, including scrambling of the native disulfide (Cys₅₇ and Cys₁₄₆) with Cys₆ and Cys₁₁₁. This necessitated a “top-down” MS approach, whereby fragmentation occurs within the mass spectrometer such that disulfide scrambling cannot normally occur¹⁸. To localize the site of modification by cysteine, SOD1 was cysteinylation in vitro and analyzed via intact protein dissociation within the FTMS (Figure 4A). Cysteinylation SOD1 was fragmented in the FTMS using collisionally-activated dissociation at the funnel-skimmer interface, yielding an abundant b-ion series (Figure 4B). The b₆-ion and larger b-ions (fragments containing the N-terminus and Cys₆) fit the theoretical mass of unmodified Cys₆ (Figure 4C) and no modified peaks were observed (based upon S/N as little as 2% of modified Cys₆ could have been detected). In addition, we observed a y₁₃₉-ion at a mass of 14454.09 Da. This mass is consistent with a cysteinylation SOD1 y-ion containing C-terminal residues 16–153, and containing the intramolecular disulfide bond between residues 57 and 146 (Figure 4D). In summary, cysteine 57 and 146 are in a disulfide bond and are unable to be cysteinylation; we ruled out cysteinylation of cysteine 6 using MS/MS data; and observed a large cysteinylation C-terminal SOD1 fragment, consistent with cysteinylation of Cys₁₁₁. In a sister publication we present the 3-dimensional structure of cysteinylation SOD1, which is consistent with the results presented here, and in addition characterize the binding stoichiometry as 1 cysteinylation per SOD1 dimer.

Discussion

Protein cysteinylation is not well-characterized, partially due to the practice of purifying/treating proteins in the presence of reducing agents such as DTT. It has, however, been observed in transthyretin (TTR), human serum albumin, and the k1 light chain from an amyloid patient^{19, 20}. In addition, cysteinylation has been observed in *Bacillus subtilis* during oxidative stress treatment. Hochgrafe *et al* suggest that cysteinylation may play a role in protecting cysteine residues from oxidation and irreversible damage in *Bacillus subtilis* and possibly other organisms²¹.

More than half of all the SOD1 protein isolated here from post-mortem human nervous tissues contained PTMs, predominantly cysteinylation and oxidation. Cysteine modified

SOD1 was protected from peroxide mediated oxidation in vitro, and cysteinylated SOD1 purified from human tissue appears also to have been protected from oxidation. SOD1 that had been cysteinylated and peroxide oxidized in vitro was composed of a set of PTMs that bear a striking resemblance to the myriad of PTMs observed in SOD1 purified from human tissue (Figure 1 and 2), indicating peroxide and cysteine are amongst the major modifiers of SOD1.

Putative cysteinylolation of SOD1 was observed in vitro⁷ and in preparations from blood^{11, 12} based upon differences in intact protein mass, and in some cases the modification was labile to reductants. Li *et al*⁷ observe a modified SOD1 118 Da heavier than unmodified SOD1, which disappears with DTT treatment and concluded that the modification was cysteine. However, this delta mass of 118 Da is not consistent with the 119 Da increase in mass expected for a cysteine modification. In no previous studies were peptide digest or MSⁿ data provided to confirm that the modification was cysteine or to determine the site of modification. Here, using endoproteinase peptide mapping by MALDI-TOF MS, MSⁿ data of an LC-ESI-ion trap, and top-down MS, cysteinylolation of SOD1 on residue 111 was confirmed. In addition, we suggest a possible role for SOD1 cysteinylolation, namely protection from peroxide-mediated oxidation.

In anaerobic control experiments in the presence or absence of alkylating/thiol scavenging agents we observed a reduction in the amount of cysteinylolation, consistent with some of the modifications we observed from human tissue occurring during the homogenization process. Note that cysteinylolation from cystine occurs via thiol-disulfide exchange and as redox neutral, whereas cysteinylolation from cysteine requires the loss of two protons and two electrons (oxidation). Although treatment with alkylating agents/cysteine scavengers is a harsh treatment with the potential to remove cysteinylolation²², it did not do so in in vitro control experiments. Cysteinylolation remained the most abundant PTM in the anaerobic (with no other chemicals) treatment.

An argument against non-enzymatic cysteinylolation of SOD1 occurring during homogenization can be made based upon the cellular ratios of SOD1 to free cysteine/cystine (CSH/CSSC) and glutathione (GSH/GSSG). Whereas the amount of SOD1 is ~10 μ M, the amount of cysteine inside the cell is approximately 2.5 μ M^{23, 24} and the amount of cystine is approximately 0.25–1.3 μ M^{23, 25–27}. Given similarities in redox potential (E_0) for CSH/CSSC and GSH/GSSG (– 0.22 V and – 0.24 V²⁸ respectively) and the 1000-fold higher concentration of glutathione in the brain (~ 1–2 mM GSH and ~8 μ M GSSG^{29–31}), glutathionylation (not observed) is the most likely artifact of our purification. Furthermore, despite concentrations of cystine and cysteine being 50–100 times higher in plasma³², we do not observe cysteinylated SOD1 in blood preparations¹⁴ (>50 purifications). On the other hand, cysteinylolation was putatively assigned, although without MS/MS or protein digest confirmation, in other purifications^{11, 12}. Thus, based on the protein concentration in the cell, the cysteine concentration, and their redox potential, it is likely cysteinylolation can occur in vivo.

Despite treatment with large excesses of cysteine, only one cysteine was detected to be bound per SOD1 dimer, consistent with cysteinylolation of SOD1 Cys₁₁₁ on one monomer blocking cysteinylolation on the second monomer. This is probably due to the close proximity (~8–10 Å) of both cysteine residues in the dimer interface of SOD1⁷- we were unable to model two cysteine residues per dimer without them overlapping or being strained. A sister publication characterizes the three dimensional structure of cysteinylated SOD1. Although the binding of free cysteine to one of SOD1's cysteine residues blocks the binding of a second cysteine, our data suggested it does not completely protect the adjacent Cys₁₁₁ (non-cysteinylated Cys₁₁₁) from oxidation.

Dimer destabilization of SOD1 has been implicated in disease progression for amyotrophic lateral sclerosis (ALS) ^{15, 33–36}. Oxygen-modified cysteine residues are negatively charged ³⁷ and if both Cys₁₁₁ are oxidized there would be a columbic impetus for destabilizing the SOD1 dimer. We, and others, have shown that oxidation of SOD1 by hydrogen peroxide is capable of destabilizing SOD1 ^{1–3, 13}. In addition, the sulfonic acid modified SOD1 (3 oxygen atoms per cysteine) is the same form of SOD1 that Bosco *et al* show inhibits fast axonal transport in a similar fashion to SOD1 FALS variants ¹³. Thus, by preventing oxidation of Cys₁₁₁, cysteinylated could ameliorate SOD1 dimer destabilization and minimize the formation of toxic SOD1 species. Under conditions of both oxidative stress and aging there is both more cystine, which can cysteinylate SOD1 directly, and more oxygen to promote cysteine-SOD1 oxidative coupling. For example, cystine concentrations double with age (av. 52 μ M at age 26, av. 105 μ M at age 60 ²²). Cysteinylated SOD1 is therefore more likely to occur under conditions of both oxidative stress and aging and may be an adaptive modification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Cysteinylolation is a prevalent post-translational modification of SOD1 in human nervous tissue

SOD1 was isolated from a non-diseased human spinal cord using an SOD1 antibody column, 50 % acetonitrile was added to improve MS signal, and then infused directly into the FTMS¹⁴. Deisotoped and deconvoluted data for the entire mass range analyzed showing the monoisotopic mass for unmodified (15836.10 Da) and modified (15955.11 Da) SOD1, the delta mass being 119.01 Da, which is consistent with cysteinylolation. Note that the solvents and declustering potentials used were such that native metals were not observed. **(Inset)** 14+ charge state showing unmodified (m/z 1132.085) and modified (m/z 1141.586) apo SOD1. This data is representative of the 8 human nervous tissue samples analyzed.

Figure 2. Cysteinylation and peroxide-mediated oxidations accounted for the majority of observed SOD1, purified from human tissue, post-translational modifications
 (A) Spectra of SOD1 modified by cysteine (40 μ M) and hydrogen peroxide (100 μ M); 3.5 hours after oxidation. SOD1 is modified by multiple oxygen atoms at this time point, whereas cysteine modified SOD1 is not (dotted blue box). (B) Spectra of SOD1 purified from a human brain. SOD1 is oxidized with multiple oxygen atoms, whereas cysteine modified SOD1 is not (dotted box). This is representative of the 4 additional samples analyzed using LC-MS.

Figure 3. Cysteinylation protected SOD1 from oxidation

(A) Spectra of unmodified (as-isolated) SOD1 (15835.97 Da) (B) Spectra of SOD1 modified by cysteine (40 μ M) (15954.97 Da), which is consistent with the molecular weight of SOD1-cysteine (less two hydrogen atoms, forming cystine) (C and D) Taken from the time course of 10 mM peroxide-mediated oxidation of cysteinylated SOD1. (C) Spectra of cysteine modified, SOD1, oxidized using hydrogen peroxide; 2 hours after oxidation. Most of the native SOD1 protein has been modified by two (sulfinic acid) (15867.97 Da) or three (sulfonic acid) oxygen atoms (15883.96 Da) (dotted red lines), whereas a small amount of cysteine modified SOD1 is oxidized (dotted blue lines). (D) Spectra of SOD1 modified by cysteine and oxidized using hydrogen peroxide; 4 hours after oxidation. All native SOD1 has been oxidized by two (15867.97 Da) or three oxygen atoms (15883.95 Da) (dotted red lines), whereas cysteine modified SOD1 does not appear to be oxidized (dotted blue lines). In addition, the sulfonic acid modified SOD1 is the same form of SOD1 that Bosco *et al* show inhibits fast axonal transport in a similar fashion to SOD1 FALS variants¹³. These experiments were repeated in triplicate.

Figure 4. SOD1 Cys₁₁₁ is the site of cysteinylation

(A) Mass spectrum of intact cysteinylated SOD1. (B) Fragmentation spectrum of cysteinylated SOD1 using funnel skimmer dissociation with the N-terminal b ion series labeled. (C) Zoom in of the b₆-ion (contains cysteine 6); no peak corresponding to a cysteinylated form of cysteine 6 is observed (approximately m/z 632; dotted blue box). (D) C-terminal fragment (y₁₃₉) of SOD1 (residues 16-153), which is consistent with the mass of a cysteinylated SOD1 fragment. The red lines are the theoretical isotopic distribution modeled using the simulate isotopic pattern parameter in DataAnalysis (Bruker) of cysteinylated y₁₃₉ (mass of y₁₃₉ plus 121.158 Da for cysteine, subtract two hydrogen atoms upon its non-native disulfide formation, and an additional two hydrogen atoms for the intramolecular disulfide).

Table 1

Amount of SOD1 cysteinylated using different purification procedures and from different sources.

<u>Purification Method</u>	<u>% Cysteinylation</u>	
Polyclonal rabbit antibody	48	Figure 1
Polyclonal sheep antibody	38	Figure 2B
<i>Elution</i> : 5% acetic acid	48	Figure 1
<i>Elution</i> : gentle elution buffer	38	Figure 2B
With liquid chromatography	38	Figure 2B
Without liquid chromatography	48	Figure 1
Aerobically	48	Figure 1, 2B
Anaerobically		
With alkylating agent	1	
Without alkylating agent	24	Figure 1, 2B
<u>Protein Source</u>	<u>Cysteinylation</u>	
Human nervous tissue	41	Figure 1, 2B
Mouse nervous tissue	21	Supplemental figure 1
Yeast	0	Figure 2A, 3A