BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

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

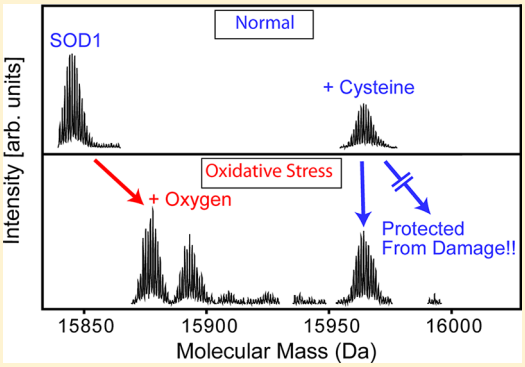
Jared R. Auclair,[†,⊥] Joshua L. Johnson,[†,¶] Qian Liu,[†] Joseph P. Salisbury,[†,⊥] Melissa S. Rotunno,[‡] Gregory A. Petsko,[†] Dagmar Ringe,[†] Robert H. Brown, Jr.,[‡] Daryl A. Bosco,[‡,§] and Jeffrey N. Agar*[,†,⊥]

[†]Departments of Biochemistry and Chemistry and Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, Massachusetts 02454, United States

[‡]Department of Neurology and [§]Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, Massachusetts 01655, United States

Supporting Information

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 postmortem human nervous tissue. As much as half of all purified SOD1 protein contained non-native post-translational modifications (PTMs), the most prevalent modifications being cysteinylation and peroxide-related oxidations. Many PTMs targeted a single reactive SOD1 cysteine, Cys_{111}. An intriguing observation was that unlike native SOD1, cysteinylated SOD1 was not oxidized. To further characterize how cysteinylation may protect SOD1 from oxidation, cysteine-modified SOD1 was prepared in vitro and exposed to peroxide. Cysteinylation conferred nearly complete protection from peroxide-induced oxidation of SOD1. Moreover, SOD1 that has been cysteinylated 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 byproducts 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 (H_2O_2) and superoxide anion ($O_2^{\bullet-}$). These byproducts are harmful to cells because they can alter protein conformation, disrupt enzyme function, and mutate DNA, among other things.[1−3] A testament to the toxicity of ROS is the monocyte-resident oxidoreductase, NADPH oxidase (NOX), which generates the superoxide enzymatically to kill targeted cells, including microorganisms. Cells combat harmful ROS species with a multifaceted antioxidant defense mechanism that includes the metalloenzyme Cu/Zn superoxide dismutase (SOD1). SOD1 catalyzes the disproportionation of the superoxide anion as follows:[4]

$$2O_2^- + 2H^+ \leftrightarrow O_2 + H_2O_2$$

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 Cys_{111} 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. Cys_{111} 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 (three 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 cysteinylation protects SOD1 from oxidative damage.

Received: May 15, 2013
Revised: July 30, 2013
Published: August 8, 2013

   dx.doi.org/10.1021/bi4006122 | *Biochemistry* 2013, 52, 6137−6144

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 Cys_{111}) SOD1 purified from human erythrocytes; elution was 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 3 M $MgCl_2$ at roughly neutral pH). Frozen human nervous tissue was homogenized in lysis buffer (25 mM Tris, pH 7.8, supplemented with a 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) of 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 reapplied, and the purifications were repeated in this way a total of three 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 (10 mM), iodoacetic acid (4 mM), and *S*-methyl methanethiosulfonate (MMTS) (0.5 mM) to block any unreacted cysteine residues and to scavenge any free cysteine using an MBraun Unilab glovebox with oxygen levels below 10 ppm, monitored by diethyl zinc.

Recombinant SOD1 Expression and Purification. In 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, TX). Expression and purification was carried out as previously described.[15,16] Briefly, each construct in the yeast expression vector YEp-351 was transformed into EGy118Δ-SOD1 yeast and grown at 30 °C for 36−48 h. 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 and 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 to 30% 10 mM Tris, pH 8.0, 1 M sodium chloride, and SOD1 eluted between 5 and 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 (Figures 1 and 2A), and the results 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 = −4000 V, skimmer 1 = 40 V, ICC on with a maximum accumulation time of 200 000 μs.

RP-HPLC and FTMS. Purified SOD1 protein in GEB was analyzed using reversed-phase liquid chromatography and Fourier transform mass spectrometry as previously described.[13] Briefly, reversed-phase liquid chromatography was performed using a 2-dimensional nanoflow rate liquid chromatography (Eksigent), a 5 mm, 300 μm i.d. guard column (LC Packings, Part Number 160454), and a self-packed 14 cm, 100 μm ID column with 5 μm C_{18} beads (unpacked from a larger Targa column). Buffer A consisted of 0.1% formic acid (v/v) in HPLC grade water and buffer B consisted of 0.1% formic acid (v/v) in 100% HPLC grade acetonitrile (v/v). 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 μL 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, FTMS) mass spectrometer (Apex Q_e-94, Bruker Daltonics). External calibration of the 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,

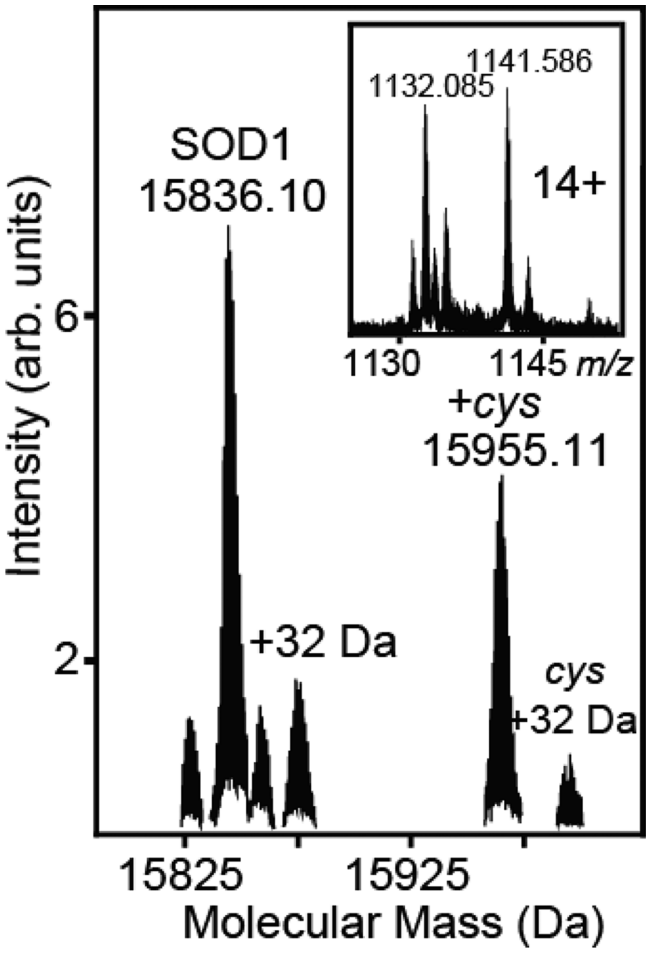


Figure 1. Cysteinylation, a prevalent post-translational modification of SOD1 in human nervous tissue. SOD1 was isolated from a nondiseased human spinal cord using an SOD1 antibody column, 50% acetonitrile was added to improve MS signal, and then the mixture was infused directly into the FTMS.[14] Deisotoped and deconvoluted data for the entire mass range was analyzed, showing the monoisotopic mass for unmodified (15 836.10 Da) and modified (15 955.11 Da) SOD1, the delta mass being 119.01 Da, which is consistent with cysteinylation. 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 eight human nervous tissue samples analyzed.

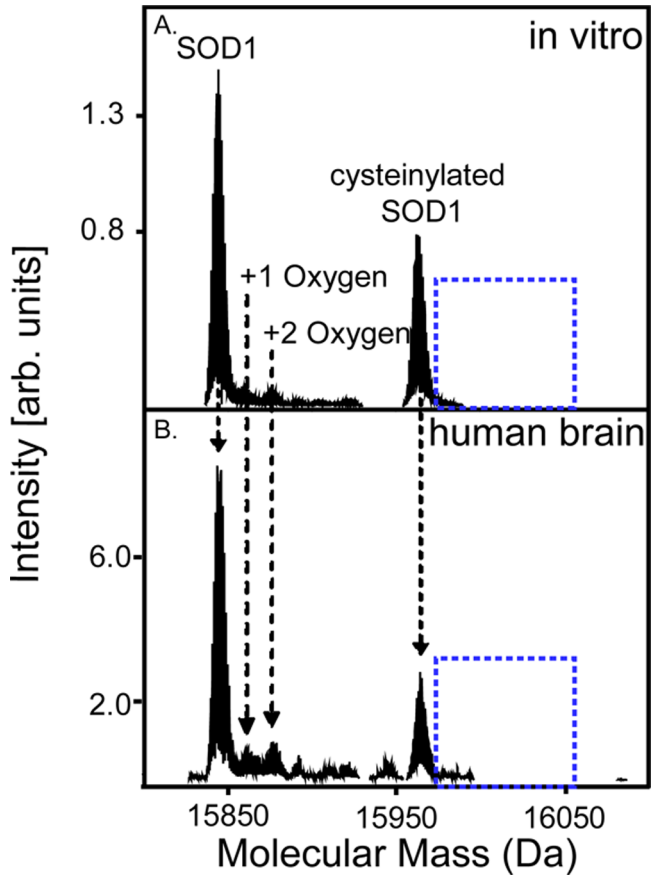


Figure 2. Cysteinylation and peroxide-mediated oxidations accounting for the majority of observed SOD1, purified from human tissue, post-translational modifications. (A) Spectra of SOD1 modified by cysteine (40 μM) and hydrogen peroxide (100 μM) 3.5 h after oxidation. SOD1 is modified by multiple oxygen 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 four additional samples analyzed using LC–MS.

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 Cysteinylation and Oxidation. Human SOD1 overexpressed and purified from *S. cerevisiae*, at either 0.1 or 1 μM concentration, was incubated with 40 μM L-cysteine overnight at room temperature and analyzed using direct infusion into the Fourier transform mass spectrometer. Oxidation was performed at room temperature for 4 h using 100 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 C_{18}-containing micropipet 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 Cysteinylation Using Funnel Skimmer Dissociation (FSD). Cysteinylated SOD1 (1 μM) in a mixture of 5% acetonitrile and 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 to 120 V in 10 V increments in order to fragment the protein using diverse fragmentation channels (at low voltage, primarily mobile proton-directed, for example at proline residues; at higher voltages, primarily charge remote, for example at acidic residues[17]). Data were analyzed using Bruker Daltonic's DataAnalysis software.

RESULTS

SOD1 Purified from Human Brain and Spinal Cord Is Modified by Cysteine and Oxygen. SOD1 was immuno-purified using rabbit polyclonal anti-SOD1 (each respective sample individually), from 10 nondiseased human and mouse nervous tissue samples (8 human and 2 mouse 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 (Supporting Information Figure 1, mouse).

dx.doi.org/10.1021/bi4006122 | *Biochemistry* 2013, 52, 6137–6144

Cysteinylation 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 15 955.11 Da, which is 119.01 Da larger than unmodified SOD1 (15 836.10 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 Cys_{111} of SOD1 and free cysteine (Figure 1). In addition to cysteinylation, we observed modifications we putatively assigned as modification with one (15 851.40 Da) and two oxygen atoms (15 866.38 Da). The abundance of these putative oxidative modifications was too low to permit further characterization; however, oxidative modifications of Cys_{111} and Trp_{32} have been described previously.[14]

To further investigate PTMs by cysteine and oxygen, as well as their relative amounts, an additional four postmortem human brain samples were analyzed using a different purification method (different homogenization buffer, SOD1 antibodies, wash buffer, and elution buffer) and different preparative conditions (reversed-phase liquid chromatography−mass spectrometry [RPLC−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 RPLC−MS analysis (38% ± 7% via RPLC−MS versus 48% ± 6% via direct infusion). Reversed-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 RPLC−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 method[14] 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 exchange 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 methanethiosulfonate (MMTS) to alkylate and scavenge any free cysteine as well as unmodified SOD1 Cys_{111}. Cysteinylation was observed in SOD1 purified anaerobically; however, there was approximately a 2-fold reduction in the amount observed compared to the amount of the samples purified aerobically. The cysteine alkylators and scavengers removed the majority of the cysteinylation, but a small amount was still present.

In summary, to investigate PTMs by cysteine and oxygen, we performed purifications (1) using different antibodies and elution conditions, (2) with and without liquid chromatography, (3) by aerobic and anaerobic methods, (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, the SOD1 purified from human and mouse tissue, but not from yeast, contained cysteinlyated SOD1 (Table 1).

Majority of SOD1 Modifications Observed in Human Tissue Can Be Created In Vitro Using Cysteine and Peroxide. To determine if both the types and the relative amounts of modifications of SOD1 purified from human tissue could be recapitulated in vitro, SOD1 was incubated with a 40-fold molar excess of cysteine, which approximated the cysteinylation levels of postmortem SOD1. This sample was incubated with a 100-fold molar excess of peroxide (100 μM) in a time course experiment. Following incubation with low levels of peroxide (the amount generated in 100 turnovers of peroxide) for 3.5 h, 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).

Table 1. Amount of SOD1 Cysteinylated Using Different Purification Procedures and from Different Sources

purification method	% cysteinylation	figure
polyclonal rabbit antibody	48	Figure 1
polyclonal sheep antibody	38	Figure 2B
elution: 5% acetic acid	48	Figure 1
elution: gentle elution buffer	38	Figure 2B
with liquid chromatography	38	Figure 2B
without liquid chromatography	48	Figure 1
aerobically	48	Figures 1, 2B
anaerobically		
with alkylating agent	1	
without alkylating agent	24	

protein source	% cysteinlyation	figure
human nervous tissue	41	Figures 1, 2B
mouse nervous tissue	21	Supporting Information Figure 1
yeast	0	Figure 3A

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 that acidic buffers and desolvation conditions were such that Cu and Zn could not be detected). Cysteinylated SOD1 was created in vitro as described above. Both native and cysteinylated SOD1 were observed, and the binding stoichiometry was approximately one cysteine per SOD1 dimer (Figure 3B), indicating that cysteinylation of one Cys_{111} can potentially block cysteinylation of the adjacent Cys_{111}, 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 2 h, 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, 4 h after peroxide treatment, all the native SOD1 had been oxidized by two or three oxygen atoms (the 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 Cys_{111}. PTMs are typically localized using endoproteinase digestion followed by LC−MS/MS analysis. In both MALDI-TOF fingerprinting and LC−MS/MS experiments, we observed cysteinylated peptides containing Cys_{111} and localized this modification to Cys_{111} using MS/MS data (Supporting Information Figures 2 and 3). This approach, however, was not

dx.doi.org/10.1021/bi4006122 | *Biochemistry* 2013, 52, 6137−6144

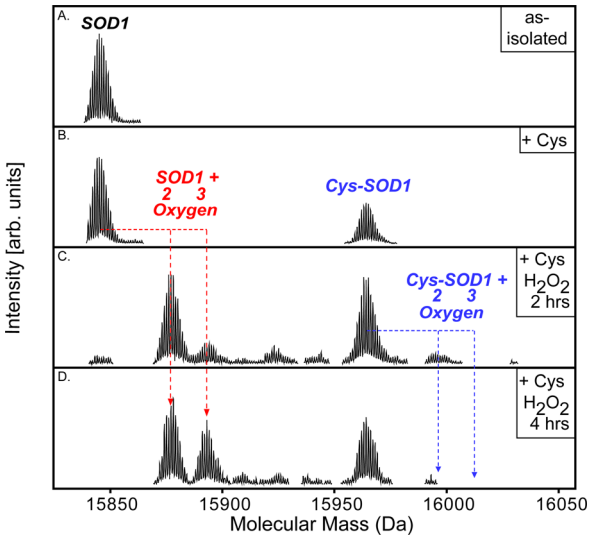


Figure 3. Cysteinylation protecting SOD1 from oxidation. (A) Spectra of unmodified (as isolated) SOD1 (15 835.97 Da). (B) Spectra of SOD1 modified by cysteine (40 μM) (15 954.97 Da), which is consistent with the molecular weight of SOD1-cysteine (less two hydrogen atoms, forming cystine). (C and D) Taken from the time course of 10 mM peroxide-mediated oxidization of cysteinylated SOD1. (C) Spectra of cysteine-modified SOD1, oxidized using hydrogen peroxide, 2 h after oxidation. Most of the native SOD1 protein has been modified by two (sulfinic acid) (15 867.97 Da) or three (sulfonic acid) oxygen atoms (15 883.96 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 h after oxidation. All native SOD1 has been oxidized by two (15 867.97 Da) or three oxygen atoms (15 883.95 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 variants.[13] These experiments were repeated in triplicate.

ideal for cysteinylation. Control experiments revealed that, following endoproteinase digestion, rapid scrambling of SOD1 disulfides occurred, including scrambling of the native disulfide (Cys_{57} and Cys_{146}) with Cys_6 and Cys_{111}. This necessitated a "top-down" MS approach, whereby fragmentation occurs within the mass spectrometer such that disulfide scrambling cannot normally occur.[18] To localize the site of modification by cysteine, SOD1 was cysteinylated in vitro and analyzed via intact protein dissociation within the FTMS (Figure 4A). Cysteinylated SOD1 was fragmented in the FTMS using collisionally activated dissociation at the funnel−skimmer interface, yielding an abundant *b*-ion series (Figure 4B). The b_6-ion and larger *b*-ions (fragments containing the N-terminus and Cys_6) fit the theoretical mass of unmodified Cys_6 (Figure 4C), and no modified peaks were observed (on the basis of S/N, as little as 2% of modified Cys_6 could have been detected). In addition, we observed a y_{139}-ion at a mass of 14 454.09 Da. This mass is consistent with a cysteinylated 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 cysteinylated; we ruled out cysteinylation of cysteine 6 using MS/MS data and observed a large cysteinylated C-terminal SOD1 fragment, consistent with cysteinlyation of Cys_{111}. In a sister publication (DOI 10.1021/bi400613h), we present the 3-dimensional structure of cysteinylated SOD1, which is consistent with the results presented here, and also 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 organisms.[21]

More than half of all the SOD1 protein isolated here from postmortem 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 (Figures 1 and 2), indicating peroxide and cysteine are among the major modifiers of SOD1.

Putative cysteinylation of SOD1 was observed in vitro[7] and in preparations from blood[11,12] on the basis of 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 that disappears with DTT treatment and concluded that the modification was cysteine. However, this change in 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 MS^n 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, MS^n data of an LC−ESI-ion trap, and top-down MS, cysteinylation of SOD1 on residue 111 was confirmed. In addition, we suggest a possible role for SOD1 cysteinylation, 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 cysteinylation, which is consistent with some of the modifications we observed from human tissue occurring during the homogenization process. Note that cysteinylation from cystine occurs via thiol−disulfide exchange and is redox neutral, whereas cysteinylation 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 cysteinylation,[22] it did not do so in in vitro control experiments. Cysteinylation remained the most abundant PTM in the anaerobic (with no other chemicals) treatment.

An argument against nonenzymatic cysteinylation of SOD1 occurring during homogenization can be made on the basis of the cellular ratios of SOD1 to free cysteine/cystine (CSH/CSSC) and glutathione (GSH/GSSG). Although the amount of SOD1 is ~10 μM, the amount of cysteine inside the cell is approximately 2.5 μM,[23,24] and the amount of cystine is approximately 0.25−1.3 μM.[23,25−27] Given similarities in redox potential (E_0) for CSH/CSSC and GSH/GSSG (−0.22 and

dx.doi.org/10.1021/bi4006122 | *Biochemistry* 2013, 52, 6137−6144

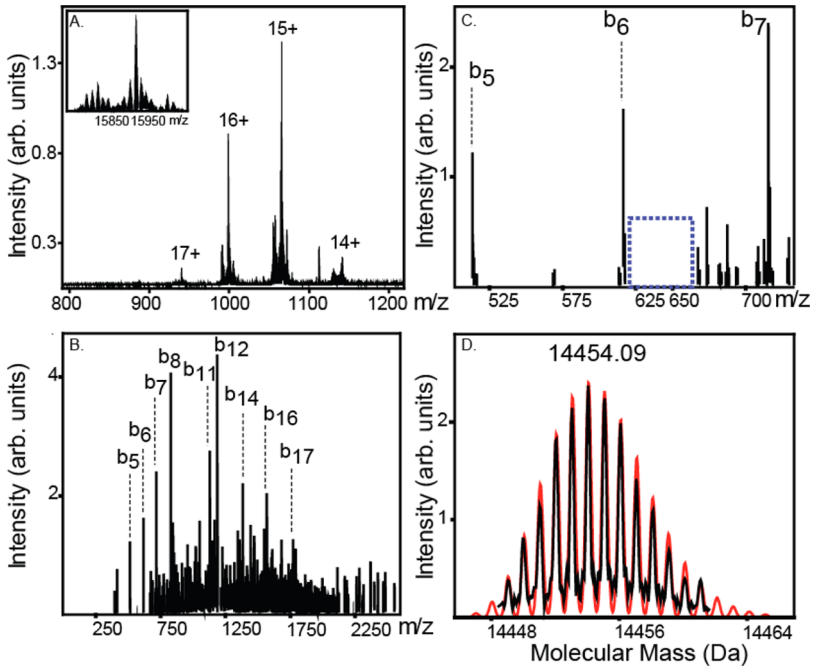


Figure 4. SOD1 Cys_{111}, the site of cysteinylation. (A) Mass spectrum of intact cysteinylated SOD1. (B) Fragmentation spectrum of cysteinlyated SOD1 using funnel−skimmer dissociation with the N-terminal *b*-ion series labeled. (C) Zoom in of the b_6-ion (contains cysteine 6). No peak corresponding to a cysteinlyated form of cysteine 6 is observed (approximately m/z 632; dotted blue box). (D) C-terminal fragment (y_{139}) of SOD1 (residues 16−153), which is consistent with the mass of a cysteinlyated SOD1 fragment. The red lines are the theoretical isotopic distribution modeled using the simulated isotopic pattern parameter in DataAnalysis (Bruker) of cysteinylated y_{139} (mass of y_{139} 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).

−0.24 V,[28] respectively) and the 1000-fold higher concentration of glutathione in the brain (~1−2 mM GSH and ~8 μM 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 plasma,[32] we do not observe cysteinylated SOD1 in blood preparations[14] (>50 purifications). On the other hand, cysteinylation was putatively assigned, though without MS/MS or protein digest confirmation, in other purifications.[11,12] Thus, on the basis of the protein concentrations in the cell, the cysteine concentration, and their redox potential, it is likely cysteinylation can occur in vivo.

Despite treatment with large excesses of cysteine, only one cysteine was detected to be bound per SOD1 dimer, consistent with cysteinylation of SOD1 Cys_{111} on one monomer blocking cysteinylation on the second monomer. This is probably due to the close proximity (~8−10 Å) of both cysteine residues in the dimer interface of SOD1;[7] we were unable to model two cysteine residues per dimer without them overlapping or being strained. A sister publication characterizes the 3-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 Cys_{111} (noncysteinylated Cys_{111}) 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 Cys_{111} are oxidized, there would be a coulombic 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 (three 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 Cys_{111}, cysteinylation 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 (average of 52 μM at age 26; average of 105 μM 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.

■ ASSOCIATED CONTENT

Supporting Information

MS, MALDI-TOF, and LC−MS/MS figures further characterizing cysteinylation. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*J. N. Agar: e-mail, j.agar@neu.edu; phone, (617) 373-5909.

Present Addresses

[⊥](J.R.A, J.P.S, J.N.A.) Department of Chemistry and Chemical Biology and Pharmaceutical Sciences and the Barnett Institute, Northeastern University, Boston, Massachusetts 02115, United States.

[¶](J.L.J.) Novartis Institute for Biomedical Research, Cambridge, Massachusetts 02139, United States.

Funding

This work was supported in part by grants from National Institutes of Health (1R01NS065263-01 to J.N.A. and

1R01NS067206-02 to D.A.B.), ALS Therapy Alliance/CVS Pharmacy to D.A.B., and Fidelity Biosciences Research Initiative to G.A.P. and D.R.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. P. John Hart for the generous gift of the YEP351-SOD1 plasmid and EGy118-(ΔSOD1) yeast cells used to express SOD1 in this study. We thank Dr. Nathalie Y. R. Agar for providing the postmortem human cortex used in the anaerobic control experiments. We also thank the patients who donated the tissue used in these experiments. We also thank members of the Agar and Petsko/Ringe Laboratories for thoughtful discussions, insights, and critically reviewing this manuscript.

■ REFERENCES

(1) Keithley, E. M., Canto, C., Zheng, Q. Y., Wang, X., Fischel-Ghodsian, N., and Johnson, K. R. (2005) Cu/Zn superoxide dismutase and age-related hearing loss. *Hear. Res. 209*, 76−85.

(2) Phillips, J. P., Tainer, J. A., Getzoff, E. D., Boulianne, G. L., Kirby, K., and Hilliker, A. J. (1995) Subunit-destabilizing mutations in Drosophila copper/zinc superoxide dismutase: neuropathology and a model of dimer dysequilibrium. *Proc. Natl. Acad. Sci. U. S. A. 92*, 8574−8578.

(3) Woodruff, R. C., Phillips, J. P., and Hilliker, A. J. (2004) Increased spontaneous DNA damage in Cu/Zn superoxide dismutase (SOD1) deficient Drosophila. *Genome 47*, 1029−1035.

(4) McCord, J. M., and Fridovich, I. (1968) The reduction of cytochrome c by milk xanthine oxidase. *J. Biol. Chem. 243*, 5753−5760.

(5) Fujiwara, N., Nakano, M., Kato, S., Yoshihara, D., Ookawara, T., Eguchi, H., Taniguchi, N., and Suzuki, K. (2007) Oxidative modification to cysteine sulfonic acid of Cys111 in human copper-zinc superoxide dismutase. *J. Biol. Chem. 282*, 35933−35944.

(6) Choi, J., Rees, H. D., Weintraub, S. T., Levey, A. I., Chin, L. S., and Li, L. (2005) Oxidative modifications and aggregation of Cu,Zn-superoxide dismutase associated with Alzheimer and Parkinson diseases. *J. Biol. Chem. 280*, 11648−11655.

(7) Liu, H., Zhu, H., Eggers, D. K., Nersissian, A. M., Faull, K. F., Goto, J. J., Ai, J., Sanders-Loehr, J., Gralla, E. B., and Valentine, J. S. (2000) Copper(2+) binding to the surface residue cysteine 111 of His46Arg human copper-zinc superoxide dismutase, a familial amyotrophic lateral sclerosis mutant. *Biochemistry 39*, 8125−8132.

(8) Redler, R. L., Wilcox, K. C., Proctor, E. A., Fee, L., Caplow, M., and Dokholyan, N. V. (2011) Glutathionylation at Cys-111 induces dissociation of wild type and FALS mutant SOD1 dimers. *Biochemistry 50*, 7057−7066.

(9) Schinina, M. E., Carlini, P., Polticelli, F., Zappacosta, F., Bossa, F., and Calabrese, L. (1996) Amino acid sequence of chicken Cu, Zn-containing superoxide dismutase and identification of glutathionyl adducts at exposed cysteine residues. *Eur. J. Biochem. 237*, 433−439.

(10) Wilcox, K. C., Zhou, L., Jordon, J. K., Huang, Y., Yu, Y., Redler, R. L., Chen, X., Caplow, M., and Dokholyan, N. V. (2009) Modifications of superoxide dismutase (SOD1) in human erythrocytes: a possible role in amyotrophic lateral sclerosis. *J. Biol. Chem. 284*, 13940−13947.

(11) Nakanishi, T., Kishikawa, M., Miyazaki, A., Shimizu, A., Ogawa, Y., Sakoda, S., Ohi, T., and Shoji, H. (1998) Simple and defined method to detect the SOD-1 mutants from patients with familial amyotrophic lateral sclerosis by mass spectrometry. *J. Neurosci. Methods 81*, 41−44.

(12) Shimizu, A., Nakanishi, T., and Miyazaki, A. (2006) Detection and characterization of variant and modified structures of proteins in blood and tissues by mass spectrometry. *Mass Spectrom. Rev. 25*, 686−712.

(13) Bosco, D. A., Morfini, G., Karabacak, N. M., Song, Y., Gros-Louis, F., Pasinelli, P., Goolsby, H., Fontaine, B. A., Lemay, N., McKenna-Yasek, D., Frosch, M. P., Agar, J. N., Julien, J. P., Brady, S. T., and Brown, R. H., Jr. (2010) Wild-type and mutant SOD1 share an aberrant conformation and a common pathogenic pathway in ALS. *Nat. Neurosci. 13*, 1396−1403.

(14) Taylor, D. M., Gibbs, B. F., Kabashi, E., Minotti, S., Durham, H. D., and Agar, J. N. (2007) Tryptophan 32 potentiates aggregation and cytotoxicity of a copper/zinc superoxide dismutase mutant associated with familial amyotrophic lateral sclerosis. *J. Biol. Chem. 282*, 16329−16335.

(15) Doucette, P. A., Whitson, L. J., Cao, X., Schirf, V., Demeler, B., Valentine, J. S., Hansen, J. C., and Hart, P. J. (2004) Dissociation of human copper-zinc superoxide dismutase dimers using chaotrope and reductant. Insights into the molecular basis for dimer stability. *J. Biol. Chem. 279*, 54558−54566.

(16) Hayward, L. J., Rodriguez, J. A., Kim, J. W., Tiwari, A., Goto, J. J., Cabelli, D. E., Valentine, J. S., and Brown, R. H., Jr. (2002) Decreased metallation and activity in subsets of mutant superoxide dismutases associated with familial amyotrophic lateral sclerosis. *J. Biol. Chem. 277*, 15923−15931.

(17) Cobb, J. S., Easterling, M. L., and Agar, J. N. (2010) Structural characterization of intact proteins is enhanced by prevalent fragmentation pathways rarely observed for peptides. *J. Am. Soc. Mass Spectrom. 21*, 949−959.

(18) Kellie, J. F., Tran, J. C., Lee, J. E., Ahlf, D. R., Thomas, H. M., Ntai, I., Catherman, A. D., Durbin, K. R., Zamdborg, L., Vellaichamy, A., Thomas, P. M., and Kelleher, N. L. (2010) The emerging process of Top Down mass spectrometry for protein analysis: biomarkers, protein-therapeutics, and achieving high throughput. *Mol. BioSyst. 6*, 1532−1539.

(19) Kleinova, M., Belgacem, O., Pock, K., Rizzi, A., Buchacher, A., and Allmaier, G. (2005) Characterization of cysteinylation of pharmaceutical-grade human serum albumin by electrospray ionization mass spectrometry and low-energy collision-induced dissociation tandem mass spectrometry. *RCM 19*, 2965−2973.

(20) Lim, A., Wally, J., Walsh, M. T., Skinner, M., and Costello, C. E. (2001) Identification and location of a cysteinyl posttranslational modification in an amyloidogenic kappa1 light chain protein by electrospray ionization and matrix-assisted laser desorption/ionization mass spectrometry. *Anal. Biochem. 295*, 45−56.

(21) Hochgrafe, F., Mostertz, J., Pother, D. C., Becher, D., Helmann, J. D., and Hecker, M. (2007) S-cysteinylation is a general mechanism for thiol protection of Bacillus subtilis proteins after oxidative stress. *J. Biol. Chem. 282*, 25981−25985.

(22) Johnson, J. M., Strobel, F. H., Reed, M., Pohl, J., and Jones, D. P. (2008) A rapid LC-FTMS method for the analysis of cysteine, cystine and cysteine/cystine steady-state redox potential in human plasma. *Clin. Chim. Acta 396*, 43−48.

(23) Sato, H., Tamba, M., Okuno, S., Sato, K., Keino-Masu, K., Masu, M., and Bannai, S. (2002) Distribution of cystine/glutamate exchange transporter, system x(c)-, in the mouse brain. *J. Neurosci. 22*, 8028−8033.

(24) Castagna, A., Le Grazie, C., Accordini, A., Giulidori, P., Cavalli, G., Bottiglieri, T., and Lazzarin, A. (1995) Cerebrospinal fluid S-adenosylmethionine (SAMe) and glutathione concentrations in HIV infection: effect of parenteral treatment with SAMe. *Neurology 45*, 1678−1683.

(25) Araki, K., Harada, M., Ueda, Y., Takino, T., and Kuriyama, K. (1988) Alteration of amino acid content of cerebrospinal fluid from patients with epilepsy. *Acta Neurol. Scand. 78*, 473−479.

(26) Arnesano, F., Banci, L., Bertini, I., Martinelli, M., Furukawa, Y., and O'Halloran, T. V. (2004) The unusually stable quaternary structure of human Cu,Zn-superoxide dismutase 1 is controlled by both metal occupancy and disulfide status. *J. Biol. Chem. 279*, 47998−48003.

(27) Lakke, J. P., and Teelken, A. W. (1976) Amino acid abnormalities in cerebrospinal fluid of patients with parkinsonism and extrapyramidal disorders. *Neurology 26*, 489−493.

(28) Jocelyn, P. C. (1967) The standard redox potential of cysteine-cystine from the thiol-disulphide exchange reaction with glutathione and lipoic acid. *Eur. J. Biochem. 2*, 327−331.
(29) Ratan, R. R., Murphy, T. H., and Baraban, J. M. (1994) Macromolecular synthesis inhibitors prevent oxidative stress-induced apoptosis in embryonic cortical neurons by shunting cysteine from protein synthesis to glutathione. *J. Neurosci. 14*, 4385−4392.
(30) Slivka, A., Mytilineou, C., and Cohen, G. (1987) Histochemical evaluation of glutathione in brain. *Brain Res. 409*, 275−284.
(31) Slivka, A., Spina, M. B., and Cohen, G. (1987) Reduced and oxidized glutathione in human and monkey brain. *Neurosci. Lett. 74*, 112−118.
(32) Brigham, M. P., Stein, W. H., and Moore, S. (1960) The Concentrations of Cysteine and Cystine in Human Blood Plasma. *J. Clin. Invest. 39*, 1633−1638.
(33) Auclair, J. R., Boggio, K. J., Petsko, G. A., Ringe, D., and Agar, J. N. (2010) Strategies for stabilizing superoxide dismutase (SOD1), the protein destabilized in the most common form of familial amyotrophic lateral sclerosis. *Proc. Natl. Acad. Sci. U. S. A. 107*, 21394−21399.
(34) Hornberg, A., Logan, D. T., Marklund, S. L., and Oliveberg, M. (2007) The coupling between disulphide status, metallation and dimer interface strength in Cu/Zn superoxide dismutase. *J. Mol. Biol. 365*, 333−342.
(35) Rakhit, R., Crow, J. P., Lepock, J. R., Kondejewski, L. H., Cashman, N. R., and Chakrabartty, A. (2004) Monomeric Cu,Zn-superoxide dismutase is a common misfolding intermediate in the oxidation models of sporadic and familial amyotrophic lateral sclerosis. *J. Biol. Chem. 279*, 15499−15504.
(36) Rakhit, R., Robertson, J., Vande Velde, C., Horne, P., Ruth, D. M., Griffin, J., Cleveland, D. W., Cashman, N. R., and Chakrabartty, A. (2007) An immunological epitope selective for pathological monomer-misfolded SOD1 in ALS. *Nat. Med. 13*, 754−759.
(37) Yarnell, A. (2009) Cysteine Oxidation New Chemical tools are poised to help scientists explore the roles of oxidized cysteine residues might play in biology. *Chem. Eng. News 87*, 38−40.