OXIDATIVE MODIFICATION TO CYSTEINE SULFONIC ACID OF CYS111 IN HUMAN COPPER-ZINC SUPEROXIDE DISMUTASE

Noriko Fujiwara1,*, Miyako Nakano2,*, Shinsuke Kato1, Daisaku Yoshihara1, Tomomi Ookawara1, Hironobu Eguchi1, Naoyuki Taniguchi1 and Keiichiro Suzuki1

From the 1Department of Biochemistry, Hyogo College of Medicine, Nishinomiya, Hyogo, 663-8501, Japan, 2Department of Biochemistry, Osaka University Medical School & Graduate School of Medicine, Suita, Osaka, 565-0871, Japan, 3Department of Neuropathology, Institute of Neurological Sciences, Faculty of Medicine, Tottori University, Nishi-cho 36-1, Yonago 683-8504, Japan, 4Department of Disease Glycomics, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, 565-0871, Japan

Address correspondence to: Noriko Fujiwara, Department of Biochemistry, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan, Tel. 81-798-45-6537; Fax. 81-798-46-3164; E-mail: noriko-fi@hymed.ac.jp

*These authors contributed equally to this work.

Copper-zinc superoxide dismutase (SOD1) plays a protective role against oxidative stress. On the other hand, recent studies suggest that SOD1 itself is a major target of oxidative damage and has its own pathogenicity in various neurodegenerative diseases, including familial amyotrophic lateral sclerosis (FALS). Only human and great ape SOD1s among mammals have the highly reactive free cysteine residue, Cys111, at the surface of the SOD1 molecule. The purpose of this study was to investigate the role of Cys111 in the oxidative damage of the SOD1 protein, by comparing the oxidative susceptibility of recombinant human SOD1 modified with 2-mercaptoethanol at Cys111 (2-ME-SOD1) to wild-type SOD1. Wild-type SOD1 was more sensitive to oxidation by hydrogen peroxide generating fragments and oligomers compared with 2-ME-SOD1. Moreover, wild-type SOD1, but not 2-ME-SOD1, generated an upper shifted band in reducing SDS-PAGE even by air oxidation. Using mass spectrometry and limited proteolysis, this upper band was identified as an oxidized subunit of SOD1; the sulfhydryl group (Cys-SH) of Cys111 was selectively oxidized to cysteine sulfonic acid (Cys-SO2H) and to cysteine sulfonic acid (Cys-SO2H). The antibody raised against a synthesized peptide containing Cys111-SO2H reacted with only the Cys111-peroxidized SOD1 by Western blot analysis, and labeled Lewy-body-like hyaline inclusions and vacuole rings in the spinal cord of human SOD1-mutated ALS mice by immunohistochemical analysis. These results suggest that Cys111 is a primary target for oxidative modification and plays an important role in oxidative damage to human SOD1 including FALS mutants.

Cooper-zinc superoxide dismutase (Cu/Zn-SOD, SOD1) is a homodimer containing one copper ion and one zinc ion in each 16 kD subunit. SOD1 catalyzes the conversion of superoxide anion (O2−) into O2 and H2O2, thereby protecting cells against oxidative stress. On the other hand, SOD1 exhibits peroxidase activity and oxidizes various substrates in the presence of hydrogen peroxide, H2O2 (1). Although H2O2 is a substrate as well as a product of SOD1, incubation of bovine SOD1 with H2O2 caused oxidation of His118 (corresponding to His120 in human SOD1) to 2-oxo-histidines, inactivating the enzyme (2). Moreover, incubation with excess H2O2 caused oxidation of almost all histidine and cysteine residues (3), fragmentation (4, 5) and aggregation (6, 7) of SOD1 itself. Co-incubation with bicarbonate and H2O2 also induced bicarbonate radical anion formation, resulting in oligomerization of human SOD1 (8).

The familial form of amyotrophic lateral sclerosis (ALS) is associated with specific mutations in the SOD1 gene (SOD1) that encodes 153 amino acids (9, 10). To date, more than 110 familial ALS (FALS)-causing mutations in SOD1 have been identified (www.alsa.org/), however, the mechanism by which SOD1 mutants induce ALS remains unknown. The presence of intracellular aggregates that contain SOD1 in spinal cord motor neurons is thought to be a pathological hallmark of ALS. In particular, FALS-linked mutant SOD1s are prone to misfolding and aggregation (11, 12). Recently, Ezzi et al. reported that even wild-type SOD1 results in aggregation after oxidation, and the
peroxiredoxin (Prx) is oxidized to thiols under physiological conditions (21). In contrast, oxidation to sulfinic acid (SOH) is readily accomplished by thiols, such as DTT, 2-ME, glutathione or thioredoxin (Trx). In contrast, oxidation to sulfenic acid (SOH) or sulfenic acid (SO2H) is not reduced by these thiols under physiological conditions (21). For example, one cysteine in the active site of peroxiredoxin (Prx) is oxidized to sulfenic acid (SO2H) by incubation with an excess of substrate of this enzyme, H2O2, and re-reduced by a specific enzyme, sulfiredoxin, but not by general thiols (22, 23).

An upper shifted band of human wild-type or mutant SOD1s, with the exception of mutant Cys111Ser, on SDS-PAGE has been observed under a variety of conditions: during purification (24), or when hydrogen peroxide or copper ion is added (see Fig. 1). The SOD1 in the upper band is speculated to be irreversibly linked to another molecule via a covalent bond at Cys111, however, neither the molecule nor the modification site have been identified. Ube Industries Ltd. developed recombinant human SOD1 chemically modified with 2-mercaptoethanol at Cys111 (2-ME-SOD1, Cys111-S-S-CH2CH2OH). This 2-ME-SOD1 is stable for many years in aqueous solution, showing neither degradation nor a loss of activity. Thus, in this study, the role of Cys111 in oxidative damage of human SOD1 was investigated by comparing 2-ME-SOD1 and wild-type SOD1, and the identity of the molecule that is bound to human SOD1 in the upper band on SDS-PAGE was explored. Through mass spectrometry and limited proteolysis, it was determined that the mass size of the molecule is 32 and 48, and that the modification site in SOD1 is Cys111. We demonstrated that Cys111 in human SOD1 is selectively oxidized to cysteine sulfenic acid (Cys-SO2H) and to cysteine sulfonic acid (Cys-SO3H) even by air oxidation. Moreover, a polyclonal antibody was raised against a synthesized peptide containing Cys111SO3H. This antibody, denoted as anti-C111ox-SOD1, reacted with the upper band (oxidized SOD1) but not the original band by Western blot analysis. Using this anti-C111ox-SOD1, the role of Cys111 on the generation of SOD1 charge isomers and the presence of oxidized SOD1 in the spinal cord of ALS mice were investigated.

**EXPERIMENTAL PROCEDURES**

**Materials** - All chemicals used in this study were obtained either from Wako Pure Chemical Industries Ltd. (Osaka, Japan), Nacalai Tesque, Inc (Kyoto, Japan), or Sigma unless specified otherwise. Recombinant human SOD1, chemically modified with 2-mercaptoethanol (2-ME-SOD1), was kindly provided from Ube Industries Ltd. Horseradish peroxidase-conjugated goat anti-rabbit IgG and horseradish peroxidase-conjugated rabbit anti-goat IgG were used as secondary antibodies.

Human SOD1 has four cysteine residues, Cys6, Cys57, Cys111 and Cys146. An internal disulfide bond exists between Cys57 and Cys146 (14, 15), which contributes to the high stability of the SOD1 protein. This disulfide bond is highly conserved in SOD1s from various organisms, including yeast, plants, flies, fishes, and mammals. In contrast, two free cysteines, Cys6 and Cys111, are not conserved. Actually, yeast, fungi and spinach (plants) have no free cysteines, and residue 6 is Ala and residue 111 is Ser in these organisms (16). More evolved organisms, such as flies, fishes, and mammals, including the Japanese monkey have only one free cysteine, Cys6. Only humans and great apes (chimpanzee and orangutan) have two free cysteines, Cys6 and Cys111 (17). Notably, the amino acid sequence of chimpanzee SOD1 is identical to that of human SOD1. Although the evolutionary process may differ from humans and great apes, chicken SOD1 has three free cysteines including Cys6 and Cys111. The third free Cys residue is located at the C-terminus, Cys154 (18).

Because free cysteines generally are reactive and wild-type SOD1 is less thermo-stable than Ser111-SOD1 or Ala6-SOD1 (19), the Ala6Cys and Ser111Cys mutations during evolution are puzzling. In particular, Cys111 is located at the surface of the SOD1 molecule and is thought to be highly reactive. De Beus et al. reported that Cys111 was modified with persulfide (S-SH) in a human SOD1 isolated from erythrocytes that is commercially available (Sigma). The persulfide SOD1 was more resistant to copper-induced aggregation than wild-type SOD1 (20). The sulfur atom of cysteine is able to assume several different oxidation states. Reversible oxidation of cysteine to sulfide (-S-S) or sulfenic acid (-SOH) is readily accomplished by thiols, such as DTT, 2-ME, glutathione or thioredoxin (Trx). In contrast, oxidation to sulfenic acid (SOH) or sulfonic acid (SO2H) is not reduced by these thiols under physiological conditions (21). For example, one cysteine in the active site of peroxiredoxin (Prx) is oxidized to sulfenic acid (SOH) by incubation with an excess of substrate of this enzyme, H2O2, and re-reduced by a specific enzyme, sulfiredoxin, but not by general thiols (22, 23).
anti-goat IgG were purchased from Dako (Denmark). Lysylendopeptidase (Achromobacter Proteinase I) was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Sequencing grade modified-trypsin was purchased from Promega (Madison, WI). Sinapinic acid and α-cyano-4-hydroxycinnamic acid for matrix of MALDI-TOF MS were obtained from Bruker Daltonik GmbH (Bremen, Germany).

Conversion of 2-ME-SOD1 to wild-type SOD1 - 2-ME-SOD1 was incubated with 20 mM 2-ME for 1 h on ice and desalted with a PD-10 column. The resultant wild-type SOD1 and 2-ME-SOD1 were used in the experiments with the exception of the experiments in Fig. 1B and 1C.

Production and purification of wild-type and mutant SOD1 proteins - Overproduction of SOD1s by the baculovirus/insect cells system and purification of SOD1 proteins were carried out as described previously (25).

Oxidation of SOD1 and the analyses with MonoQ column - For strong oxidation, SOD1s diluted with miliQ water (1 mg/ml) were incubated with 5 mM H2O2 for 1 h at room temperature. For mild air oxidation, SOD1s diluted with miliQ water or appropriate buffer were filtered with 0.22 μm filter (MILLIPORE) and were slowly stirred at 30 rpm with a rotator (TAITEC, ROTATOR RT-50). The buffer or water containing oxidized SOD1s was changed with buffer A (2 mM potassium phosphate, pH 7.4) on a PD-10 column (Amersham Pharmacia). The SOD1s were applied to a HPLC (AKTA Explorer 10S) at a flow rate of 1 ml/min on a MonoQ column (MonoQ™ 4.6/100 PE, Amersham Biosciences). After washing with buffer A, the bound proteins were eluted with a linear gradient of KCl (0 - 100 mM) in buffer A. Adhesive proteins were washed with 0.5 M KCl in buffer A. Lysylendopeptidase treatment and the peptides analyses - SOD1 proteins were reduced with DTT and the free sulfhydryls were carbamidomethylated by adding iodoacetamide (IA) in the dark at room temperature for 30 min. After desalting on a PD-10 column with 50 mM Tris-HCl (pH 8.8), the proteins were digested with 0.25% (w/w) lysylendopeptidase (Wako Pure Chemicals) at 37 °C for 16 h. The resultant peptides were applied to a reverse-phase HPLC (AKTA Explorer 10S) at a flow rate of 1 ml/min on TSK-GEL ODS-80TM (4.6 mm x 250 mm, TOSOH). The peptides were separated by two linear gradients of 0 to 30% acetonitrile for 4 columns volume and 30 to 40% acetonitrile for 8 columns volume containing 0.05% trifluoroacetic acid (TFA). Peptides were detected by their absorbance at 215 nm. The peaks were subjected to the following MS analyses.

MALDI-TOF MS analysis - MALDI-TOF MS spectra of peptides and proteins were measured on an Ultraflex TOF/TOF mass spectrometer and analyzed by the Flexcontrol 1.2 software package (Bruker Daltonics GmbH, Bremen, Germany). For analyses of peptides, ions generated by a pulsed UV laser beam (nitrogen laser, λ=337 nm, 5 Hz) were accelerated to a kinetic energy of 20 kV in reflector mode using positive polarity. Metastable ions generated by laser-induced decomposition of the selected precursor ions were analyzed without any additional collision gas. Alpha-cyano-4-hydroxycinnamic acid (5 mg/ml in 50% acetonitrile containing 0.1% TFA) was used as a matrix for peptide analyses. For analyses of proteins, the determinations were performed in linear mode using positive polarity. Sinapinic acid (10 mg/ml in 50% acetonitrile containing 0.1% TFA) was used as matrix for protein analyses. Peptide or protein samples (1 μl each) were mixed with matrix solution (4 μl each), and an aliquot (1 μl each) was applied to a polished stainless steel target (Bruker Daltonics). The mixture was dried in air at room temperature for several minutes.

Infusion ESI MS analysis for peptide sequence - ESI mass spectra were measured on a Bruker Esquire HCT equipped with a quadrupole ion trap (Bruker Daltonics GmbH, Bremen, Germany). The solutions containing peptides digested with lysylendopeptidase were continuously introduced through the electrospray interface with a syringe infusion pump (Cole-Parmer, Vernon Hills, IL) at a flow rate of 5 μl/min. The MS-conditions were: Nebulizer gas (N2) 10 psi, dry gas (N2) 4 l/min, dry temperature 250 °C, capillary voltage 3500 V, HV end plate offset -500 V, capillary exit 190.6 V, skimmer 40 V, and trap drive 166.7. MS/MS spectra were sequenced using BioTool 2.2 software and Sequence editor 2.2 (algorithm provided by Bruker).

Trypsin digestion for MALDI-TOF MS - SOD1 proteins in 50 mM NH4HCO3 were digested with trypsin at 37 °C for 16 h. An aliquot of the digests (10 μl) was boiled, applied to ZipTip C18 P10 (Millipore, Bedford, MA; according to manufacture’s protocol) for desalting, and then subjected to MALDI-TOF MS analysis. When alkylation is needed, SOD1 proteins in 50 mM NH4HCO3 were treated with excess IA in the dark at room temperature for 30 min before trypsin.
digestion.

In-gel digestion of CBB-stained polyacrylamide gel for MALDI-TOF MS - The gel bands containing SOD1 protein after SDS-PAGE were clipped out and cut into small pieces in a 1.5-ml microtube. To remove CBB dye, the chopped gels were washed three times with 50 mM NH₄HCO₃ in 30% acetonitrile by shaking at room temperature for 20 min. The gels were further incubated with 500 µl of acetonitrile at room temperature for 10 min. After removing acetonitrile, the gels were incubated with an alkylating solution (500 µl) consisting of 40 mM IA, 10 mM EDTA and 50 mM NH₄HCO₃ in the dark at room temperature for 30 min. After washing twice with 50 mM NH₄HCO₃ (500 µl) for 10 min, the gels were incubated with 0.4 µg of trypsin in 50 mM NH₄HCO₃ at 37 °C overnight. After removing the pieces of gels, the remained solution was concentrated with a SpeedVac concentrator, and subjected to MALDI-TOF MS analyses.

Preparation of antibody for Cys111-sulfonylated SOD1 - Keyhole limpet hemocyanin-conjugated peptide (residues 103 – 114) containing sulfonlated Cys111 (Cys⁰¹⁻SO₃H) was obtained from Sigma genosys Japan. After the initial injection with the peptide-hemocyanin conjugate (200 µg peptide) mixed with complete Freund’s adjuvant, rabbits were subjected to five booster injections, each of 200 µg of peptide with incomplete Freund’s adjuvant, administered (at multiple subcutaneous sites) at 1 – 2 weeks intervals. Antisera were collected 1 week after the sixth booster injection, and the immunoglobulin G (IgG) fraction was precipitated with 50% (w/v) ammonium sulfate. The IgG fraction passed from the wild-type SOD1 coupled to NHS activated sepharose was bound to the oxidized SOD1 coupled to NHS activated sepharose. The bound IgG was eluted with 3 M MgCl₂ and collected. The IgG was desalted with PD-10 column and stored with 0.1 mg/ml BSA at deep freezer until use. This antibody was denoted as anti-C111ox-SOD1.

SDS-PAGE and Western blot analysis - Proteins were subjected to SDS-PAGE (14% gel) and then transferred to a PVDF membrane under semi-dry conditions by means of a Trans-blot (Bio-Rad). After blocking by incubation with 5% skim milk in Tris-buffered saline (20 mM Tris-HCl pH 8.0, 0.15 M NaCl, (TBS)) for 2 h at room temperature, the membrane was incubated with anti-C111ox-SOD1 (diluted 1:1000), or a goat polyclonal antibody against full length of human SOD1 (25), anti-SOD1 (diluted 1:1000), in TBS containing 0.05% Tween 20 (TBS-T) and 1% skim milk for 2 h at room temperature or for 18 h at 4 °C. After washing with TBS-T, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG (diluted 1:5000) for anti-C111ox-SOD1 or horseradish peroxidase-conjugated anti-goat IgG (diluted 1:5000) in TBS-T containing 1% skim milk for anti-SOD1, respectively, for 2 h at room temperature. After washing, the chemiluminescence method using an ECL or an ECL plus kit (GE Healthcare) was employed to detect peroxidase activity.

Two-dimensional Gel Electrophoresis (2-DE) - Sample proteins were dissolved in 8 M urea, 2% (w/v) CHAPS, 0.5% (v/v) immobilized pH gradient (IPG) buffer (GE Healthcare), and 12 µl/ml DeStreak™ Reagent (GE Healthcare), which forms stable disulfide bonds and prevent unspecific Cys residue oxidation during isoelectric focusing (50). The samples were applied to 11-cm IPG strips (pH 4 – 7), and the strips were then isoelectrically focused on an IPGphor (GE Healthcare) according to the following schedule: 500 Vhr at 500 V for Step and Hold, 800 Vhr at 1000 V for Gradient, 8800 Vhr at 6000 V for Gradient, and 4000 Vhr at 6000 V for Step and Hold. The strips were equilibrated for 20 min in 50 mM Tris-HCl (pH 8.8) containing 6 M urea, 2% (w/v) SDS, 30% (v/v) glycerol, and 1% (w/v) DTT. Second-dimension separation was run on 14% SDS-polyacrylamide gels and followed by Western blot analysis. Isoelectric points (pI) of spots were calculated according to the graph showing pH as function of distance at 20 °C and 8 M urea of IPG strips (pH 4 – 7) provided by GE Healthcare on line system.

ELISA - Wild-type SOD1 was air-oxidized, treated with and without 100 mM IA, and then diluted to 500 ng/ml with phosphate-buffered saline (PBS). 100 µl of the samples were added to each well of 96-well microplates (Maxisorp, Nunc), incubated overnight at 4 °C, washed three times with TBS-T and then blocked for 2 h at room temperature with 1% BSA in PBS. The plates were then washed three times with TBS-T and 100 µl of anti-C111ox-SOD1 and anti-SOD1 antibodies (diluted 1:1000 in TBS-T) was added, followed by incubation for 1 h at room temperature. The plates were washed three times with TBS-T and 100 µl of horseradish peroxidase-conjugated anti-rabbit IgG (diluted...
1:5000 in TBS-T) for anti-C111ox-SOD1 or horseradish peroxidase-conjugated anti-goat IgG (diluted 1:5000 in TBS-T) for anti-SOD1, respectively, was added and incubated for 1 h at room temperature. After washing five times with TBS-T, the plates were developed using 100 µl of o-phenylenediamine dihydrochloride solution and the reaction was stopped with 25 µl of 2 M HCl. The absorbance of each well was determined at 490 nm with a SPECTRAMax PLUS384 (Molecular Devices).

**Animals and Animal tissue preparation** - Four transgenic mice carrying the over-expressed human G94A mutant SOD1 at high copy G93A: [B6SIL-TgN(SOD1-G93A)1Gur; G1H-G93A] (Jackson Laboratory, Bar Harbor, ME, USA). Two age-matched littermates were used as controls. All animals were handled in accordance with Guideline for the Care and Use of Tottori University. All four G1H-G93A mice at 110 days of age neurologically exhibited hind limb paralysis, and two littermate mice at 110 days of age did not show any clinical symptoms. The G1H-G93A and littermate mice were euthanatized at 110 days of age. Animals were deeply anesthetized with sodium pentobarbital (0.1 ml/100 g body weight). After perfusion of three G1H-G93A and two littermate mice via the aorta with PBS at 37 °C, they were fixed by perfusion with 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.3). The spinal cords were removed and then post fixed in the same solution. The spinal cord of one mouse for Western blot analysis was removed after perfusion with PBS, quickly frozen in liquid nitrogen and stored at -80 °C until use.

**Protein extraction from G1H-G93A mouse spinal cord** - The G1H-G93A spinal cord tissues were spinal cord was homogenized in ice-cold homogenization buffer, 20 mM Tris-HCl (pH 6.8) containing Complete™ Mini Protease Inhibitor Mixture (Roche) and 100 mM 1A for preventing the secondary oxidation of SH groups. The sample was centrifuged at 17,000 x g at 4 °C for 30 min, and the pellet was homogenized in the ice-cold homogenization buffer containing 1% Triton X-100 (TX). The samples was centrifuged at 17,000 x g at 4 °C for 30 min, and the pellet was further homogenized in the ice-cold homogenization buffer containing 1% Triton X-100 and 2% SDS, and the sample was centrifuged at 17,000 x g at 4 °C for 30 min. The supernatants of each homogenization, buffer soluble, TX soluble, and SDS soluble fractions, were subjected to SDS-PAGE and Western blot analyses.

**Immunohistochemical analysis** - After fixation, the specimens were embedded in paraffin, cut into 5-µm-thick sections and examined for immunohistochemical analysis. Sections were deparaffinized, and then washed in PBS. Normal serum homologous with the secondary antibody diluted in 1% bovine serum albumin-containing PBS (BSA-PBS) was used as a blocking reagent. Tissue sections were incubated with anti-C111ox-SOD1 (diluted 1:1000 in BSA-PBS) at 4 °C for 18 h. Bound antibody was visualized by the avidin-biotin-immunoperoxidase complex (ABC) method using appropriate Vectastain Elite ABC Rabbit IgG kit (Vector Laboratories) and 3,3′-diaminobenzidine tetrahydrochloride (Wako, Osaka) as a chromogen. The endogenous peroxidase activity was quenched by incubation for 30 min with 3% H2O2 after the secondary antibody treatment to prevent nonspecific oxidation before anti-C111ox-SOD1 treatment.

**SOD1 activity** - SOD1 activity was assayed using the xanthine-xanthine oxidase/cytochrome c system as described previously (26).

**Protein assay** - SOD1 protein concentrations were estimated using a dimeric molar extinction at 280 nm of 10,800 M⁻¹cm⁻¹ (27). Protein concentrations of crude samples were determined using a BCA™ protein assay kit (PIERCE) with bovine serum albumin as a standard.

**RESULTS**

2-ME-SOD1 obtained from Ube Industries Ltd. was modified with 2-ME only at Cys111.

First, the chemical modification with 2-mercaptoethanol of recombinant human Cu/Zn-SOD (2-ME-SOD1) was confirmed. The molecular mass of 2-ME-SOD1 was determined to be 15865.5 (Supplementary Fig. S1A) - 15871 m/z (Supplementary Fig. S2A), which suggested the presence of 2-ME (76 Da) in apo-human SOD1 (monoisotopic mass: 15794.86, average mass: 15804.55). The metals, copper and zinc, of SOD1 were removed during MALDI-TOF MS analysis. Since this recombinant human SOD1 was expressed in E. coli, the N-terminus was not acetylated. To demonstrate that the 2-ME-SOD1 was in fact modified with 2-ME only at Cys111, the 2-ME-SOD1 was digested with trypsin and then analyzed by MALDI-TOF MS and MS/MS. The mass of the tryptic peptide (2533 m/z) indicating residues Asp92-Arg115 (2457 m/z)
plus the mass of 2-ME (76 Da) (Supplementary Fig. S1B) was analyzed by the collision-induced MS/MS. As shown in Supplementary Fig. S1C, the major fragment ions, y4 (457.1 m/z), y5 (635.9 m/z), y6 (772.9 m/z), y14 (1530.9 m/z), y19 (2074.4 m/z), and y23 (2418.7 m/z), indicated the presence of 2-ME at Cys111. The mass of the tryptic peptide (933 m/z, missed cleavages = 1), residues Ala1-Lys9, indicated that another free cysteine, Cys6, was not modified with 2-ME (Supplementary Fig. S1B). MS/MS analyses of the residue Ala1-Lys9 also indicated that 2-ME was not contained in this peptide (Supplementary Fig. S1D). These results demonstrated that Cys111, but not Cys6, was modified with 2-ME. The commercial human SOD1 from Sigma, modified with persulfide (S-SH) or with trisulfide (-S-S-S-) inter-subunit linkage at Cys111, exhibits an absorbance peak at 325 nm (20, 28). However, 2-ME-SOD1 did not exhibit the 325 nm peak (data not shown).

Additional 2-ME treatment recovered the 2-ME-SOD1 to wild-type SOD1.

Next, the ability to remove 2-ME from Cys111 was examined. 2-ME-SOD1 was incubated with 0 (H2O), 2, 20 and 200 mM 2-ME for 1 h on ice, removed the excess 2-ME on a PD-10 column with miliQ water, and then the mass of the proteins was analyzed. As shown in Supplementary Fig. S2A, the incubation with more than 20 mM 2-ME decreased the mass of the protein (15871 m/z to 15795 m/z). The difference in the mass was 76 m/z, indicating that incubation with 20 mM 2-ME removes 2-ME from Cys111. Thus, to confirm this finding, the 2-ME treated SOD1s were digested with trypsin after incubation with iodeacetamide (IA), and the mass of the resulting peptides was determined. Removal of 2-ME from Cys111, would allow the SH group of Cys111 to be carbamidemethylated by IA, yielding a mass of 2514 (2456 plus 58) m/z. As shown in Supplementary Fig. S2B, the mass of the tryptic peptide containing Cys111 (residues Asp92 - Arg115) treated with more than 20 mM 2-ME was 2514 m/z. In contrast, the mass of the 2-ME-SOD1 treated with H2O was 2533 m/z, indicating that the SH group of Cys111 remained bound to 2-ME. These results show that incubation of 2-ME-SOD1 with 2-ME in excess of 20 mM removed 2-ME from Cys111, converting 2-ME-SOD1 to wild-type SOD1. The SOD activities of 2-ME-SOD1 and the wild-type SOD1 were 4181 units/mg and 4056 units/mg, respectively, indicating that both SOD1s have similar activities.

Upper band of oxidized human SOD1 on SDS-PAGE involves Cys111.

2-ME-SOD1 and wild-type SOD1 were incubated with various concentrations of H2O2 for 20 min, and then subjected to reducing SDS-PAGE. Although 2-ME-SOD1 was slightly affected by H2O2 treatment, wild-type SOD1 showed an additional upper band when incubated with more than 1 mM H2O2 (Fig. 1A). When commercial gradient gels (5 - 20%, e-PAGEL, ATTO) were used for the SDS-PAGE, the upper band was not observed (data not shown). It is thought that the two bands are unable to separate on the gradient gels. Next, various purified wild-type and mutant human SOD1 proteins expressed in baculovirus/insect cells system (25) were oxidized with 1 mM H2O2, followed by reducing SDS-PAGE and Western blotting. As shown in Fig. 1B, the additional upper band appeared in all SOD1s, except C111S (Cys111Ser mutant), after oxidation. Furthermore, the effects of various metal ions on the generation of the upper band were investigated. Only Cu2+ ion, among all metal ions examined, formed an upper band similar to that observed after oxidation with H2O2 (Fig. 1C). Although Fe2+ and Fe3+ are thought to be oxidants, neither Fe3+ (data not shown) nor Fe2+ treatment generated the upper band. Wild-type SOD1 diluted with various pH buffers was slowly stirred (30 rpm with a rotator) for 24 h at room temperature. As shown in Fig. 1D, the upper band was generated when the pH of the incubation buffer was higher than pH 7. These results indicated that Cys111 was readily oxidized by oxygen in ambient air and that the sulphydryl group (SH) of Cys111 was needed to provide a thiolate anion (S-) for the oxidative modification.

Role of Cys111 in the generation of negatively charged molecules after oxidation

To examine the role of Cys111 in the generation of negatively charged molecules after oxidation, 2-ME-SOD1 and wild-type SOD1 were incubated with 5 mM H2O2 for 1 h and were applied to a MonoQ column. Some of the fractions were then subjected to reducing SDS-PAGE and Western Blotting. Since incubation with 5 mM H2O2 caused oxidation of almost all histidine and cysteine residues in bovine SOD1 (3), it is thought that negatively charged molecules were generated in both SOD1s. As shown in the upper panels of Fig. 2A and 2B,
several peaks containing oxidized SOD1 proteins were eluted with similar patterns in both SOD1s. However, the results of Western Blotting were quite different (lower panels in Fig. 2A and 2B). In oxidized 2-ME-SOD1, only one fragment (labeled with asterisk) from the first peak and slight polymer bands from the last fractions, which were obtained by washing the column with 0.5 M KCl, were observed (Fig. 2A). The single fragment resulting from oxidation of 2-ME-SOD1 has been identified by Ookawara et al., as a large fragment cleaved between Pro62 and His63 (5). Because Ookawara et al. also used recombinant human SOD1 (2-ME-SOD1) obtained from Ube Industries Ltd., it can be concluded that the identity of the single fragment in the present study and that of Ookawara et al. are the same. In contrast, oxidation of the wild-type SOD1 resulted in, not only the upper band, but also in several additional fragments and polymer bands (Fig. 2B). Oxidation of Cys111 may become a trigger of fragmentation and polymerization. Zhang et al. reported that a covalently cross-linked dimer (polymer) of human SOD1 was induced by bicarbonate and H2O2 (8). So, the effects of bicarbonate on the oxidation of 2-ME-SOD1 and wild-type SOD1 were investigated. However, no difference in dimer formation between the two SOD1 variants was observed (data not shown), suggesting that the cross-linkage between monomers was not mediated by Cys111.

Identification of the molecule in the upper band

Next, the identity of the molecule in the upper band was explored. Slow stirring in miliQ water did not cause fragmentation and polymerization of SOD1, but generated the upper band. Thus, in order to exclude effects of the buffer system, 2-ME-SOD1 and wild-type SOD1 were oxidized by stirring in miliQ water. Then, the molecular masses were measured by MALDI-TOF MS. The air-oxidized wild-type SOD1 showed two masses, 15792 m/z and 15838 m/z, but the mass of 2-ME-SOD1 did not change (Fig. 3A). In contrast, the upper and original bands from reducing SDS-PAGE (Fig. 3D) of peak (b) from the MonoQ column were clipped out, alkylated with IA and digested with trypsin. The resultant peptides were subjected to MALDI-TOF MS analyses. In the upper band, a major mass, 2489 m/z, corresponding to tryptic peptide 92-115 plus 32 was also observed (Fig. 3E). In contrast, in the original band, a mass of 2514 m/z resulting from carbamidemethylation (plus 58) of triptic peptide 92-115 was detected (Fig. 3F). These results indicate that amino acids in the residues 92-115, probably Cys111, in the upper band, were oxidized with two or three molecules of oxygen (Cys111-SO2H or Cys111-SO3H). However, the amounts of these peptides were too small for MS/MS analyses to determine the amino acid sequence.

To obtain greater quantities of oxidized peptides, SOD1 proteins in peaks (a) and (b) separated with the MonoQ column (Fig. 3B) were reduced by DTT, alkylated with IA and digested with lysylendopeptidase, but not with trypsin. The resultant peptides were applied to a reverse-phase HPLC (ODS column). As shown in Fig. 4A, the HPLC elution profiles were nearly identical, but two additional peaks (d) and (e) were observed after the last peak (c) only in digests from peak (b) of the Mono Q column (i.e., the lower panel). Peak (c) has already been identified as residues 92-122 containing carbamidemethylated Cys111 in previous work (25). When fractions containing the additional peaks (d) and (e) were re-applied to the ODS column, four fractions containing three distinct peaks were obtained (Fig. 4B). Each fraction was subjected to MALDI-TOF MS analyses. As a result, peptide (c) in fractions 1 and 2, corresponded to residues 92-122 containing carbamidemethylated Cys111 (3320.5 m/z) as expected. Peptide (d), in fractions 2 and 3, and
The resultant IgG, which was denoted as columns as described in Experiment reduced purified to exclude the reactivity with antibody against the peptide containing SOD1 (Cys111). Anti-SOD1 at Cys111 resulted in the upper band shift oxidation by air, and that the peroxidation of SOD1 (Cys111) was readily oxidized to Cys111-oxo (Fig. 5G). Analyses based on the assumption indicating that Cys111 was oxidized to Cys111-oxo resulted in the mass of peptide (e) corresponding to residues 92-122 plus 32 (3295.5 m/z) gave the major fragment ions, y5 (611.3 m/z), y10 (1152.0 m/z), y12 (1424.6 m/z), y13 (1561.7 m/z) and y21* ([M+2H]2+ = 1610.0 m/z), indicating that Cys111 was carbamidemethylated, as expected (Fig. 5E). The mass of peptide (d) (3295.5 m/z) ([M+2H]2+ = 1648.3 m/z) gave the major fragment ions, y11 (1264.6 m/z), y12 (1399.7 m/z), y13 (1536.7 m/z) and b20 (2031.6 m/z), which was identified to be residues 92-122 containing Cys111(ox)-SO2H (Fig. 5F). Furthermore, the mass of peptide (e) (3311.6 m/z) ([M+2H]2+ = 1657.2 m/z) gave the major fragment ions, y9 (1038.6 m/z), y12 (1415.6 m/z), y13 (1552.7 m/z), y18 (2012.0 m/z), and y21 (2310.8 m/z), indicating that Cys111 was oxidized to Cys-SO2H (Fig. 5G). Analyses based on the assumption that His110 and/or His120 were oxidized to 2-oxo-histidine showed that the corresponding y ions and b ions were absent (data not shown). These results clearly indicated that Cys111 was readily oxidized to Cys-SO2H, which underwent further oxidation to Cys-SO2H without His oxidation by air, and that the peroxidation of SOD1 at Cys111 resulted in the upper band shift in reducing SDS-PAGE.

**Anti-C111ox-SOD1 recognized only Cys111-oxidized SOD1.**

To explore the possibility of immunological detection of Cys111-oxidized SOD1 (Cys111-SO2H-SOD1), a rabbit polyclonal antibody against the peptide containing Cys111-SO2H was prepared. The antisemum was purified to exclude the reactivity with reduced-form SOD1 (Cys111-SH) by affinity columns as described in Experimental procedure. The resultant IgG, which was denoted as anti-C111ox-SOD1, reacted with only the upper band of Cys111-peroxidized SOD1 but not the original band of wild-type SOD1 (Fig. 6A). The anti-C111ox-SOD1 did not recognize 2-ME-SOD1 both with and without oxidation, and mouse SOD1, either. These data further demonstrated that the upper band is oxidized form of SOD1 containing sulfonylated Cys111. Also, in ELISA experiments, the anti-C111ox-SOD1 specifically reacted with IA-treated air-oxidized wild-type SOD1, but neither with 2-ME-SOD1 nor with IA-treated wild-type SOD1 (Fig. 6B). However, when wild-type SOD1 was not treated with IA before ELISA, the wild-type SOD1 was also reacted with anti-C111ox-SOD1, indicating that SH of Cys111 of the wild-type SOD1 was oxidized during coating on the 96-well plate.

**2-DE characterization of oxidized SOD1.**

It is well known that human, bovine and recombinant human SOD1 proteins have several charge isomers detected by HPLC, an isoelectric gel focusing (IEF) or 2-DE (49 - 51). The reason of the heterogeneity is still unknown although some hypotheses, such as different metallation, different conformation and different oxidation of Cys residues, were presented (13, 50). Thus, 2-DE of oxidized SOD1 was performed to examine the effects of Cys111 oxidation on the generation of charge isomers. As shown in Fig. 7A, wild-type SOD1 presented the main spot 3 (pI 5.15) and three tiny spots, I (pI 4.92), 2 (pI 5.02), and spot 4 (pI 5.8). 2-ME-SOD1 also presented the similar four spots (Fig. 7B). Three of them, the spots, I, 2, and 3, were thought to correspond to three isomers of recombinant human SOD1 with pI of 4.99, 5.06 and 5.14, which was previously determined by isoelectric gel electrophoresis (49). When 2-ME-SOD1 was oxidized by H2O2, three major spots, 5 (pI 5.32), 6 (pI 5.52) and 7 (pI 5.65), were newly generated between spot 3 and spot 4 (Fig. 7C). In contrast, when wild-type SOD1 was oxidized by H2O2, further new spots, 5’ (pI 5.3), 6’ (pI 5.46), and 7’ (pI 5.6), which were probably their upper and acidic shifted spots of the spots, 5 (pI 5.32), 6 (pI 5.52) and 7 (pI 5.65), respectively, were generated (Fig. 7D and 7E). Furthermore, two major spots, I’, 2’ and weak spot 3’, just above the spots I, 2, and 3, respectively, were also detected by anti-C111ox-SOD1 (Fig. 7E). Air oxidation of wild-type SOD1 generated only the two major spots, I’ and 2’, and one tiny spot 7’ (Fig. 7F and 7G). Therefore, these results indicated that spots, I’, 2’ and 7’, were generated by the oxidation of Cys111, and that the
generation of the spots, 5, 6, and 7 (Fig. 7C), was caused by the oxidation of another amino acid residues.

**Cys111-peroxidized SOD1 was detected in spinal cord of G1H-G93A mice.**

Because this new antibody, anti-C111ox-SOD1, is a good tool for detection of Cys111-peroxidized SOD1, G1H-G93A mouse spinal cord extract was subjected to Western blot analysis to examine the involvement of oxidized SOD1 in ALS. The anti-C111ox-SOD1 clearly reacted with approximately 25 kD band in TX-soluble fraction (Fig. 8A, left panel). Although some weak bands were detected, the upper band of oxidized SOD1 was not detected in all fractions. Immunostaining with anti-SOD1 detected a large amount of human SOD1 over-expressed in the G1H-G93A mouse and mouse SOD1 (Fig. 8A, right panel). In contrast, anti-C111ox-SOD1 detected the upper band of oxidized SOD1 (positive control), but neither the reduced form of G93A-SOD1 nor mouse SOD1 (Fig. 8A, left panel). Therefore, the 25 kD band, selectively recognized by anti-C111ox-SOD1, was thought to be some molecule-bound oxidized SOD1. Basso et al detected mono- and poly-ubiquitinated SOD1 (24, 32, 40, 48 kD spots in 2-DE) in TX-insoluble fraction of G93A tg mice spinal cords (50). Thus, the 25 kD band was speculated to be mono-ubiquitinated SOD1, and then the immunostaining with anti-ubiquitin on the same membrane was performed. Although the 25 kD band appeared to be one of the ubiquitinated proteins (data not shown), the evidence of mono-ubiquitination has not been obtained. We are currently exploring the identity of the molecule bound to Cys111-peroxidized SOD1. Finally, immunohistochemical study of paraffin-embedded spinal cord sections of G1H-G93A mice was performed. The G1H-G93A mice examined at 110 days of age revealed severe loss of anterior horn cells with gliosis, and both Lewy-body-like hyaline inclusions (LBHIs)- and vacuolation-pathologies (52). The anti-C111ox-SOD1 selectively labeled the LBHIs in the neuropil and in the cytoplasm of the neurons (Fig. 8B) and the rim of the vacuoles in the neuropil (Fig. 8C). When the paraffin sections were incubated with BSA-PBS alone, or anti-C111ox-SOD1 pretreated with an excess amount of air-oxidized SOD1 or peptide containing sulfenylation Cys111, no staining was detected. The spinal cords of the two littermates exhibit neither distinct histopathological changes nor staining with anti-C111ox-SOD1. These results suggested that the Cys111-peroxidized SOD1 was involved in the formation of the LBHIs and the vacuoles in ALS spinal cords.

**DISCUSSION**

Because SOD1 catalyzes the conversion of superoxide radicals into molecular oxygen and hydrogen peroxide, SOD1 is thought to be a major target of oxidative stress. The results of this study show that the Cys111 residue plays an important role in oxidative-fragmentation and aggregation of human SOD1 (Fig. 2). Moreover, the upper-shifted band on reducing SDS-PAGE generated after oxidation (Fig. 1) was determined to be an oxidized SOD1 subunit containing sulfenic acid (Cys\(^{111}\)SO\(_3\)H) and sulfonic acid (Cys\(^{111}\)SO\(_4\)H) (Fig. 3 - 5). The newly developed specific antibody against a peptide containing Cys\(^{111}\)SO\(_3\)H, anti-C111ox-SOD1, recognized the upper band (Cys\(^{111}\)SO\(_3\)H form) but not original band (Cys\(^{111}\)SH form) by Western blot analyses (Fig. 6 - 8). These results further demonstrated that the upper band is oxidized form of SOD1 containing Cys\(^{111}\)SO\(_3\)H. Although the increment in the mass, 32 or 48, is small, oxidized SOD1 has slower mobility on the SDS-PAGE. This phenomenon is, however, frequently observed in SOD1. For example, mutant G85R has faster mobility in SDS-PAGE although the difference in the mass is 99 (53). Mouse SOD1 also has faster mobility than human SOD1 in SDS-PAGE although both SOD1s have similar molecular weight (Fig. 8A).

Some cysteine residues are sensitive to oxidation because their environment promotes ionization of the thiol (Cys-SH) group, even at a neutral pH, to the thiolate anion (Cys-S\(^{-}\)), which is more readily oxidized to sulfenic acid (Cys-SOH) than is Cys-SH (29, 30). The sulfenic acid group generally is unstable and reacts with any accessible thiol to form a disulfide (S-S) bond. Or, sulfenic acid may undergo further oxidation to sulfonic acid (Cys-SO\(_3\)H) and to sulfonic acid (Cys-SO\(_4\)H) in the presence of strong oxidants (29). In the active site of some proteins, such as Prx and Trx, one cysteine is in the thiolate form, and, as a result, can react with \(\text{H}_2\text{O}_2\) (31, 32). In the case of PrxI, Cys51 is selectively oxidized to Cys-SO\(_3\)H, but not to Cys-SO\(_4\)H, as evidenced by the difference of 32 mass units between reduced...
and oxidized PrxI proteins. Additional oxidation
with \( \text{H}_2\text{O}_2 \) did not increase Cys-SO\(_2\)H even in vitro (22, 29). Cys\(^{51}\)-SO\(_2\)H is re-reduced to Cys\(^{51}\)-SH by sulfiredoxin, but not DTT or Trx (23, 33). In contrast, in intact human SOD1, Cys111 appears to be oxidized to Cys\(^{111}\)-SO\(_2\)H, even by mild oxidation in air. As shown in this study, air oxidation of SOD1 resulted in two proteins with a mass unit difference of about 48, i.e., three oxygen atoms (Fig. 3). Oxidation of Cys111 occurred above pH 7, suggesting that Cys111 is in thiolate form at physiological pH, and can therefore react not only with \( \text{H}_2\text{O}_2 \) but also with oxygen in ambient air (Fig. 1). Cys111 also is readily modified with \( N \)-ethylmaleimide or 4-vinylpyridine (28, 34), or bound to another sulfhydryl, such as \( 2\text{ME} \) (Supplementary Fig. S1) or cysteine (34). In contrast, the free cysteine at residue 6, Cys6, is less reactive with oxygen, \( 2\text{ME} \) or \( \text{N} \)-ethylmaleimide (present study, 28), probably because it exists in a \( \beta \)-sheet 1a and is buried within the SOD1 molecule (35).

In other studies, exposure of bovine SOD1 to excess of \( \text{H}_2\text{O}_2 \) resulted in selective oxidation of His118 (corresponding to His120 in human SOD1), inactivating the enzyme (2). Rakhit et al. showed that four amino acids (His48, 80, 120 and Phe20) in human SOD1 were prone to oxidation by ascorbic acid/CuCl\(_2\) (6). However, neither oxidation of His120 in wild-type SOD1 (Fig. 5) nor change in mass of 2-ME-SOD1 (Fig. 3) by air oxidation was observed. These results indicate that most amino acids in human SOD1 are not oxidized by air, the exception being Cys111. Therefore, these findings demonstrate that, in human SOD1, Cys111 is the most reactive and sensitive amino acid to oxygen and other oxidizing agents.

The oxidation of Cys\(^{51}\)-SH to Cys\(^{51}\)-SO\(_2\)H causes acidic shifting of PrxI on 2-DE (22). In contrast, human, bovine and recombinant human SOD1 originally have some charge isomers before oxidation (49, 51). Fig. 7A and 7B also showed that both wild-type SOD1 and 2-ME-SOD1 have similar four spots although they have their own mass. Cys111 oxidation by air oxidation generated two major spots, \( l \) and \( l' \); just above the two minor isomers, \( l \) and \( 2 \) (pl 4.92 and 5.02) (Fig. 7F and 7G). However, these spots appear like acidic shifted spots of original main spot 3 (pl 5.15) because no spot above the main spot was generated by air oxidation. These results suggest that peroxidation of Cys111 also causes acidic shifting of SOD1. Further oxidation by \( \text{H}_2\text{O}_2 \) further generated several spots, and totally 13 spots were observed on 2-DE (Fig. 7D). Therefore, isomers of SOD1 observed in the previous reports (13, 50) may be due to both oxidative modification and charge isomers of SOD1 itself.

Although more than 110 FALS mutations in the SOD1 have been identified, the mechanism by which the FALS-linked mutant SOD1s cause motor neuron degeneration is not completely understood. Two hypotheses have been proposed explaining the toxic gain of function that is associated with these mutations (12, 36). The “copper hypothesis” proposes that copper, either bound to or released from FALS-linked mutant SOD1s generates reactive oxygen species (ROS) harmful to motor neurons (37 - 39). The “aggregation hypothesis” supposes that FALS-linked mutant SOD1s are structurally unstable and tend to aggregate, resulting in degeneration of neuronal cells analogous to that observed in other neurodegenerative disorders such as Alzheimer’s, Parkinson’s and Huntington’s diseases (40 - 42). However, recent reports suggest that these two hypotheses are inter-related. Copper ion oxidized Cys111 in human SOD1 (Fig. 1). Oxidation may result in misfolding and aggregation even in wild-type SOD1 (6, 43). Oxidized wild-type SOD1 exhibits characteristics of FALS-linked mutant SOD1s: conjugation with poly-ubiquitin, interaction with Hsp70 or chromogranin B, and toxic effects on motor neurons (7). Although this study demonstrated that the SH of Cys111 underwent irreversible peroxidation to Cys-SO\(_2\)H and to Cys-SO\(_3\)H, Cys111 may also participate in disulfide bond linkage with other cysteine residues and oligomerization (47). Furukawa et al. presented that incorrect inter-molecule disulfide cross-linking of immature, miss-folded FALS-linked mutant SOD1s leads to formation of insoluble aggregates (44 - 46). On the other hand, immunohistochemical study using the anti-C111ox-SOD1 revealed that Cys111-peroxidized mutant SOD1 accumulated in the vacuole structures and LBHIs (Fig. 8B and 8C). Because the most characteristic neuropathological findings in ALS model mice are LBHIs and vacuoles (52, 53), the peroxidation of Cys111 may contribute to the pathology of the degeneration/death of FALS motor neurons. However, the amount of the Cys111-peroxidized SOD1 appears to be quite limited (Fig 8A). Large quantities of thiol compounds, such as glutathione and cysteine, probably protect the SH
of Cys111 in healthy cells. The survived neuron cells were not immunostained by anti-C111ox-SOD (Fig. 8B and 8C). Ferri et al. proposed that Cys111 was a key mediator of mitochondrial association of SOD1 and subsequent mitochondrial dysfunction, because the Cys111Ser mutant was less associated with mitochondria (47). Recently, it was also reported that A4V-C111S protein was more stable than A4V protein in cells (54). However, it is still an open question whether Cys111 is essential for the etiology of ALS, because mice that express mouse SOD1-G85R developed ALS-like symptoms even though Ser, not Cys, is at residue 111 of mouse SOD1 (48). It is thought that Cys111 may enhance human ALS development. A comparative study of symptoms between mice expressing ALS mutation with C111S (G93A-C111S etc.) and traditional ALS model mice (G93A etc.) would answer the question.

In summary, we demonstrated that Cys111 in human SOD1 is a primary target for oxidation, and is readily oxidized to Cys\textsuperscript{111}-SO\textsubscript{2}H. The specific antibody against the Cys\textsuperscript{111}-SO\textsubscript{2}H would be a useful tool for detecting oxidized human SOD1. Precise studies of the role of oxidized SOD1 on ALS are currently underway.

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FOOTNOTES

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The abbreviations used are: SOD, superoxide dismutase; ALS, amyotrophic lateral sclerosis; FALS, familial amyotrophic lateral sclerosis; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; ESI, electrospray ionization; MS, mass spectrometry; MS/MS, tandem MS; TBS, Tris-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; DTT, dithiothreitol; 2-ME, 2-mercaptoethanol; IA, iodoacetamide; HPLC, high-performance liquid chromatography; CBB, Coomassie brilliant blue; 2-DE, two-dimensional gel electrophoresis; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; TX, Triton X-100

**FIGURE LEGENDS**

**Fig. 1.** Generation of upper shifted band of SOD1 on SDS-PAGE under reducing conditions. A, 2-ME-SOD1 and wild-type SOD1 treated with various concentrations of H2O2 for 20 min, diluted with miliQ water and boiled with SDS-PAGE loading buffer containing 5% 2-ME. Five μg of protein per lane was subjected to SDS-PAGE (14% gel). Rainbow™ colored protein molecular weight markers purchased from GE Healthcare were used for the molecular weight marker (left side). B, Western blot analyses of wild-type and mutant SOD1s treated with 1 mM H2O2 for 20 min. C, Western blot analyses of wild-type SOD1 treated with 1 mM CuCl2, ZnCl2, MnCl2, FeCl3, CoCl2, NiSO4, MgCl2 and H2O2 for 1 h. Wild-type and mutant SOD1s used in (B) and (C) were produced by the baculovirus/insect cells system. SOD1 proteins in (B) and (C) were immunostained with anti-SOD1. D, SDS-PAGE of wild-type SOD1 stirred for 24 h in various pH buffers (50 mM), citric acid-NaOH (pH 3.0), sodium citrate-disodium phosphate-NaOH (pH 5.0), sodium phosphate (pH 7.0), glycine-NaOH (pH 9.0 and pH 11.0). Arrowheads with solid lines indicate SOD1 subunits and arrowheads with broken lines indicate modified SOD1 subunits.

**Fig. 2.** Separation of oxidized SOD1s with MonoQ column. 2-ME-SOD1 and wild-type SOD1 were incubated with 5 mM H2O2 for 1 h and were applied to a MonoQ column; some fractions were subjected to reducing SDS-PAGE and Western blot analyses. A, Chromatogram profiles of oxidized and intact 2-ME-SOD1 separated with MonoQ column (upper panel), and Western blot analysis of some fractions indicated (lower panel). B, Chromatogram profiles of oxidized and intact wild-type SOD1 separated with MonoQ column (upper panel), and Western blot analysis of some fractions indicated (lower panel). SOD1 proteins in (A) and (B) were immunostained with anti-SOD1. Arrowheads with solid lines indicate SOD1 subunits and arrowheads with broken lines indicate modified SOD1 subunits.

**Fig. 3.** Analyses of upper and original bands on SDS-PAGE. A, MALDI-TOF MS spectra of intact and stirred 2-ME-SOD1 and wild-type SOD1, respectively. B, Chromatogram profiles of stirred wild-type SOD1 and 2-ME-SOD1 on MonoQ column. C, MALDI-TOF MS spectra of SOD1 in the peaks, (a) and (b), separated with MonoQ column (B). D, SDS-PAGE of intact (i) and stirred (s) 2-ME-SOD1, and SOD1s in the peaks (a) and (b). Arrowheads with solid lines indicate SOD1 subunits and arrowheads with broken lines indicate modified SOD1 subunits. Precision plus protein standards purchased from Bio Rad were used for the molecular weight marker (left side). E and F, MALDI-TOF MS spectra of tryptic peptides (residues Asp92-Arg115) from the upper band (E) and the original band (F), respectively, in right side lane of (D).

**Fig. 4.** Purification and analyses of lysylendopeptidase-digested peptides. SOD1s in peaks (a) and (b) in Fig. 3B were digested with lysylendopeptidase after treatments with DTT and iodoacetamide, and the digests were fractionated by HPLC using a TSK-GEL ODS-80TM column. A, Chromatogram profiles of the digests from peaks, (a) (upper panel) and (b) (lower panel), separated with MonoQ column (Fig. 3B). B, Re-chromatogram profile of fractions containing peaks, (d) and (e), in lower panel of (A). C, MALDI-TOF MS spectra of peptides in Fr. 1 to 4 in (B).
Fig. 5. Sequence analysis of modified peptide 92-122 by ESI MS. A, Schematic representation of peptides obtained from fragmentations by MS/MS analysis. B - D, Cys modification observed in this experiment. Carbamidemethyl Cys (C-CAM) (B), sulfynil Cys (C-SO₂H) (C), and sulfonyl Cys (C-SO₃H) (D). E - G, MS/MS sequence analysis of the peptide 92-122 digested with lysylendopeptidase in Fr. 1, 3 and 4 (Fig. 4B). E, MS/MS analysis of the peptide modified with C-CAM ([M+2H]⁺ = 1661.2 m/z) in Fr. 1. F, MS/MS analysis of the peptide modified with C-SO₂H ([M+2H]⁺ = 1648.3 m/z) in Fr. 3. G, MS/MS analysis of the peptide modified with C-SO₃H ([M+2H]⁺ = 1657.2 m/z) in Fr. 4. The y-ions and b-ions labeled with asterisks are the doubly charged ions. The mass number labeled with diamond means that of the precursor ion in (E), (F) and (G).

Fig. 6. Validation of anti-C111ox-SOD1 that selectively recognizes Cys111-peroxidized SOD1. A, Western blot analyses for 2-ME-SOD1 and air-oxidized SOD1 detected by anti-SOD1 (upper panel) and anti-C111ox-SOD1 (lower panel). An arrowhead with solid lines indicates wild-type SOD1 subunits and arrowheads with broken lines indicate Cys111-peroxidized SOD1 subunits. The PVDF membrane after reacting anti-C111ox-SOD1 was de-proved by treatment with stripping buffer and re-incubated with anti-SOD1. B, ELISA for 2-ME-SOD1 and air-oxidized SOD1 treated with and without iodoacetamide (IA) detected by anti-C111ox-SOD1 (left panel) and anti-SOD1 (right panel). The labels, Ox, WT and 2-ME, indicate air-oxidized SOD1, wild-type SOD1 and 2-ME-SOD1, respectively. Data are presented as the means ± S.D. of triplicate experiments.

Fig. 7. Two-dimensional Western blot analyses for wild-type and 2-ME SOD1s with and without oxidation detected by anti-SOD1 (A, B, C, D and F) and anti-C111ox-SOD1 (E and G). Five µg of SOD1s treated with and without 1 mM H₂O₂ for 1 h or air oxidation for 24 h were subjected to 2-D and Western blot analysis. The PVDF membranes after reacting anti-C111ox-SOD1 were de-proved by treatment with stripping buffer and re-incubated with anti-SOD1. Data are presented as the means ± S.D. of triplicate experiments.

Fig. 8. Cys111-peroxidized SOD1 in spinal cord of G1H-G93A mice. A, Western blot analyses for G1H-G93A spinal cord extracts detected by anti-C111ox-SOD1 (left panel) and anti-SOD1 (right panel). Sixty µg proteins of buffer soluble (B), TX soluble (T), and SDS soluble (S) fractions, respectively, were applied. Arrowheads with solid lines indicate human and mouse SOD1 subunits and a double arrowhead with broken line indicates Cys111-peroxidized SOD1 subunits. Arrowheads indicate 25 kD band reacted with anti-C111ox-SOD1. Twenty ng of air-oxidized SOD1 (Ox) and wild-type SOD1 (WT) were applied as controls. The PVDF membranes after reacting anti-C111ox-SOD1 were de-proved by treatment with stripping buffer and re-incubated with anti-SOD1. Precision plus protein standards purchased from Bio Rad were used for the molecular weight marker. B and C, Immunohistochemical analyses of paraffin-embedded G1H-G93A spinal cord sections detected by anti-C111ox-SOD1. B, Double arrows indicate LBHIs in the neuropil and in the cytoplasm of the neurons immunostained with anti-C111ox-SOD1. A single arrow indicates a rim of the vacuoles in the neuropil immunostained with anti-C111ox-SOD1. An arrowhead indicates the survived motor neuron, which is not stained with anti-C111ox-SOD1. Scale bar = 25 µm. C, Single arrows indicate rims of vacuoles detected by anti-C111ox-SOD1. An arrowhead indicates the survived motor neuron, which is not stained with anti-C111ox-SOD1. Scale bar = 50 µm.
Fig. 1

A

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(Mr, kD)

B

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SOD1

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SOD1

D

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Fig. 2

A

Oxidized 2-ME-SOD1

Intact 2-ME-SOD1

Absorbance at 280 nm (mAU)

Fr. No. 8 10 12 14 16 22 24 28 37 38

B

Oxidized wild-type SOD1

Intact wild-type SOD1

Absorbance at 280 nm (mAU)

Fr. No. 9 11 14 16 18 22 26 30 37 38
Fig. 3

A

2-ME-SOD1

2-ME-SOD1 + stirring

wild-type SOD1

wild-type SOD1 + stirring

B

Absorbance at 280 nm (mAU)

C

Peak a

Peak b

D

2-ME-SOD1

Wild-type SOD1 MonoQ

i

s

a

b

E

Upper band

F

Original band

(Mr, kD)
Fig. 4

A

Peak a

Absorbance at 215 nm (mAU)

Elution (ml)

Acetonitrile (%)

B

Absorbance at 215 nm (mAU)

Elution (ml)

Acetonitrile (%)

C

Fr.1

Fr.2

Fr.3

Fr.4
Fig. 6

A

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<tr>
<th>Wild-type SOD1</th>
<th>Ox-SOD1</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 40 60 80 100 (ng/lane)</td>
<td>20 40 60 80 100</td>
</tr>
</tbody>
</table>

Anti-SOD1

Anti-C111ox-SOD1

B

Absorbance at 490 nm

<table>
<thead>
<tr>
<th>Anti-C111ox-SOD1</th>
<th>Anti-SOD1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ox+IA WT+IA 2-ME WT</td>
<td>Ox+IA WT+IA 2-ME WT</td>
</tr>
</tbody>
</table>

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Fig. 7

Wild-type SOD1

2-ME-SOD1 + H₂O₂

Wild-type SOD1 + H₂O₂

Wild-type SOD1 + air-oxidation

A

Anti-SOD1

1 2 3 4

B

Anti-SOD1

1 2 3 4

C

Anti-SOD1

1 2 3 5 6 7 4

D

Anti-SOD1

1' 2' (3') 5' 6' 7'

E

Anti-C111ox-SOD1

1' 2' (3') 5' 6' 7'

F

Anti-SOD1

1 2' 3 4

G

Anti-C111ox-SOD1

1' 2' 7'
Fig. 8

A

Anti-C111ox-SOD1

(Mr. kD)

25
20
15

Ox WT B T S

G1H-G93A spinal cord

Anti-SOD1

h SOD1

m SOD1

B

C

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J. Biol. Chem. published online October 3, 2007

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