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Relation	

Oxidative Modification to Cysteine Sulfonic Acid of Cys¹¹¹ in Human Copper-Zinc Superoxide Dismutase*[§]

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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. 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, oligomers, and charge isomers 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 sulfenic acid (Cys-SO₂H) and to cysteine sulfonic acid (Cys-SO₃H). The antibody raised against a synthesized peptide containing Cys¹¹¹-SO₃H reacted with only the Cys¹¹¹-peroxidized SOD1 by Western blot analysis and labeled Lewy body-like hyaline inclusions and vacuole rims in the spinal cord of human SOD1-mutated amyotrophic lateral sclerosis 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 familial amyotrophic lateral sclerosis mutants.

Copper-zinc superoxide dismutase (Cu/Zn-SOD)³ (SOD1) is a homodimer containing one copper ion and one zinc ion in each 16-kDa 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-oxohistidines, 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 (available on the World Wide Web); 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.* (7) reported that even wild-type SOD1 results in aggregation after oxidation, and the oxidized wild-type SOD1 gains properties like FALS mutant SOD1s. In addition to ALS, oxidative damaged SOD1 proteins were detected in the brains of patients with Alzheimer and Parkinson diseases (13). These findings suggest that oxidized SOD1 plays a role in the pathophysiology of various neurodegenerative diseases.

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³ The abbreviations used are: SOD, superoxide dismutase; ALS, amyotrophic lateral sclerosis; FALS, familial amyotrophic lateral sclerosis; MALDI, matrix-assisted laser desorption/ionization; TOF, time of flight; ESI, electrospray ionization; MS, mass spectrometry; MS/MS, tandem MS; TBS, Tris-buffered saline; PVDF, polyvinylidene fluoride; DTT, dithiothreitol; 2-ME, 2-mercaptoethanol; IA, iodoacetamide; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; Prx, peroxiredoxin; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; IPG, immobilized pH gradient; LBHI, Lewy body-like hyaline inclusion.

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However, the role of oxidized wild-type and FALS-linked mutant SOD1s on these diseases remains unclear.

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 are generally reactive, and wild-type SOD1 is less thermo-stable than Ser¹¹¹-SOD1 or Ala⁶-SOD1 (19), the A6C and S111C 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.* (20) 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 disulfide (-S-S-) or sulfinic acid (-SOH) is readily accomplished by thiols, such as DTT, 2-ME, glutathione, or thioredoxin. In contrast, oxidation to sulfinic acid (-SO₂H) or sulfonic acid (-SO₃H) is not reduced by these thiols under physiological conditions (21). For example, one cysteine in the active site of peroxiredoxin (Prx) is oxidized to sulfinic acid (-SO₂H) by incubation with an excess of substrate of this enzyme, H₂O₂, and rereduced 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 C111S, 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 sulfinic 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 Cys¹¹¹-SO₃H. 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 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. 1, B and C.

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 milliQ water (1 mg/ml) were incubated with 5 mM H₂O₂ for 1 h at room temperature. For mild air oxidation, SOD1s diluted with milliQ water or appropriate buffer were filtered with a 0.22- μ m filter (Millipore) and were slowly stirred at 30 rpm with a rotator (rotator RT-50; Taitec). 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 Biosciences). The SOD1s were applied to a high performance liquid chromatograph (AKTA Explorer 10S) at a flow rate of 1 ml/min on a MonoQ column (MonoQTM 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 Peptide Analyses—SOD1 proteins were reduced with DTT, and the free sulfhydryls were carbamidemethylated 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 high performance liquid chromatography.

graph (AKTA Explorer 10S) at a flow rate of 1 ml/min on TSK-GEL ODS-80TM (4.6 × 250 mm; Tosoh). The peptides were separated by two linear gradients of 0–30% acetonitrile for 4 column volumes and 30–40% acetonitrile for 8 column volumes containing 0.05% trifluoroacetic acid. 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; $\lambda = 337$ 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. α -Cyano-4-hydroxycinnamic acid (5 mg/ml in 50% acetonitrile containing 0.1% trifluoroacetic acid) 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% trifluoroacetic acid) was used as matrix for protein analyses. Peptide or protein samples (1 μ l each) were mixed with matrix solution (4 μ l each), and an aliquot (1 μ l 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 μ l/min. The MS conditions were as follows: nebulizer gas (N_2), 10 p.s.i.; dry gas (N_2), 4 liters/min; dry temperature, 250 °C; capillary voltage, 3500 V; high voltage end plate offset, -500 V; capillary exit, 190.6 V; skimmer, 40 V; 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 NH_4HCO_3 were digested with trypsin at 37 °C for 16 h. An aliquot of the digests (10 μ l) was boiled, applied to ZipTip C18 P10 (Millipore, Bedford, MA; according to the manufacturer's protocol) for desalting, and then subjected to MALDI-TOF MS analysis. When alkylation is needed, SOD1 proteins in 50 mM NH_4HCO_3 were treated with excess IA in the dark at room temperature for 30 min before trypsin digestion.

In-gel Digestion of Coomassie Brilliant Blue-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 Coomassie Brilliant Blue dye, the chopped gels were washed three times with 50 mM NH_4HCO_3 in 30% acetonitrile by shaking at room temperature for 20 min. The gels were further incubated with 500 μ l of acetonitrile at room temperature for 10 min. After removing acetonitrile, the gels were incubated with an alkylating solution (500 μ l) consisting of 40 mM IA, 10 mM EDTA, and

50 mM NH_4HCO_3 in the dark at room temperature for 30 min. After washing twice with 50 mM NH_4HCO_3 (500 μ l) for 10 min, the gels were incubated with 0.4 μ g of trypsin in 50 mM NH_4HCO_3 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-sulfonated SOD1—Key-hole limpet hemocyanin-coupled peptide (residues 103–114) containing sulfonated Cys¹¹¹ (Cys¹¹¹-SO₃H) was obtained from Sigma. After the initial injection with the peptide-hemocyanin conjugate (200 μ g of peptide) mixed with complete Freund's adjuvant, rabbits were subjected to five booster injections, each of 200 μ g of peptide with incomplete Freund's adjuvant, administered (at multiple subcutaneous sites) at 1–2-week intervals. Antisera were collected 1 week after the sixth booster injection, and the IgG fraction was precipitated with 50% (w/v) ammonium sulfate. The IgG fraction passed from the wild-type SOD1 coupled to *N*-hydroxysuccinimide-activated Sepharose was bound to the oxidized SOD1 coupled to *N*-hydroxysuccinimide-activated Sepharose. The bound IgG was eluted with 3 M MgCl₂ and collected. The IgG was desalted with a PD-10 column and stored with 0.1 mg/ml bovine serum albumin (BSA) at deep freeze 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 semidry conditions by means of a Trans-blot (Bio-Rad). After blocking by incubation with 5% skim milk in Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 8.0, 0.15 M NaCl) 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 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—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 μ l/ml DeStreakTM reagent (GE Healthcare), which forms stable disulfide bonds and prevents nonspecific Cys residue oxidation during isoelectric focusing (26). The samples were applied to 11-cm IPG strips (pH 4–7), and the strips were then isoelectrically focused on an IPGphor isoelectric focusing system (GE Healthcare) according to the following schedule: 500 V-h at 500 V for step and hold, 800 V-h at 1000 V for gradient, 8800 V-h at 6000 V for gradient, and 4000 V-h 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. pI values of spots were calculated according to the graph

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showing pH as function of distance at 20 °C and 8 M urea of IPG strips (pH 4–7) provided by the 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 μ l 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 μ l 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 μ l 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 μ l of *o*-phenylenediamine dihydrochloride solution, and the reaction was stopped with 25 μ l 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 a high copy number of the human G93A SOD1 gene (B6SJL-TgN[SOD1-G93A]1Gur, G1H-G93A) mice, were purchased from the Jackson Laboratory (Bar Harbor, ME). Two age-matched littermates were used as controls. All animals were handled in accordance with the guidelines for care and use (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 euthanized at 110 days of age. Animals were deeply anesthetized with sodium pentobarbital (0.1 ml/100 g of 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 postfixed 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 was homogenized in ice-cold homogenization buffer, 20 mM Tris-HCl (pH 6.8) containing Complete™ miniprotease inhibitor mixture (Roche Applied Science), and 100 mM IA for preventing the secondary oxidation of SH groups. The sample was centrifuged at 17,000 \times *g* at 4 °C for 30 min, and the pellet was homogenized in the ice-cold homogenization buffer containing 1% Triton X-100. The sample was centrifuged at 17,000 \times *g* at 4 °C for 30 min, 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 \times *g* at 4 °C for 30 min. The supernatants of each homogenization, buffer-soluble, Triton X-100-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- μ m-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% BSA-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 the appropriate Vectastain Elite ABC rabbit IgG kit (Vector Laboratories) and 3,3'-diaminobenzidine tetrahydrochloride (Wako, Osaka, Japan) 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 (27).

Protein Assay—SOD1 protein concentrations were estimated using a dimeric molar extinction at 280 nm of 10,800 M⁻¹ cm⁻¹ (28). Protein concentrations of crude samples were determined using a BCA™ protein assay kit (Pierce) with BSA 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 15,865.5 (supplemental Fig. S1A) to 15,871 *m/z* (supplemental Fig. S2A), which suggested the presence of 2-ME (76 Da) in apo-human SOD1 (monoisotopic mass, 15,794.86; average mass, 15,804.55). The metals, copper and zinc, of SOD1 were removed during MALDI-TOF MS analysis. Since this recombinant human SOD1 was expressed in *Escherichia 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) (supplemental Fig. S1B) was analyzed by the collision-induced MS/MS. As shown in supplemental Fig. S1C, the major fragment ions, γ 4 (457.1 *m/z*), γ 5 (635.9 *m/z*), γ 6 (772.9 *m/z*), γ 14 (1530.9 *m/z*), γ 19 (2074.4 *m/z*), and γ 23 (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 (supplemental Fig. S1B). MS/MS analyses of the residues Ala¹–Lys⁹ also indicated that 2-ME was not contained in this peptide (supplemental 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-) intersubunit linkage at Cys¹¹¹, exhibits an absorbance peak at 325 nm (20, 29). 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; the excess 2-ME was removed on a PD-10 column with milliQ water, and then the mass of the proteins was analyzed. As shown in supplemental Fig. S2A, the incubation with more than 20 mM 2-ME decreased the mass of the protein (from 15,871 to 15,795 *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 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 carbamidomethylated by IA, yielding a mass of 2514 (2456 plus 58) *m/z*. As shown in supplemental 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 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 the baculovirus/insect cell 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, after oxidation. Furthermore, the effects of various metal ions on the generation of the upper band were investigated. Only the Cu²⁺ ion, among all metal ions examined, formed an upper band similar to that observed after oxidation with H₂O₂ (Fig. 1C). Although Fe³⁺ and Fe²⁺ are thought to be oxidants, neither Fe³⁺ (data not shown) nor Fe²⁺ treatment generated the upper band. Next, 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 sulfhydryl 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

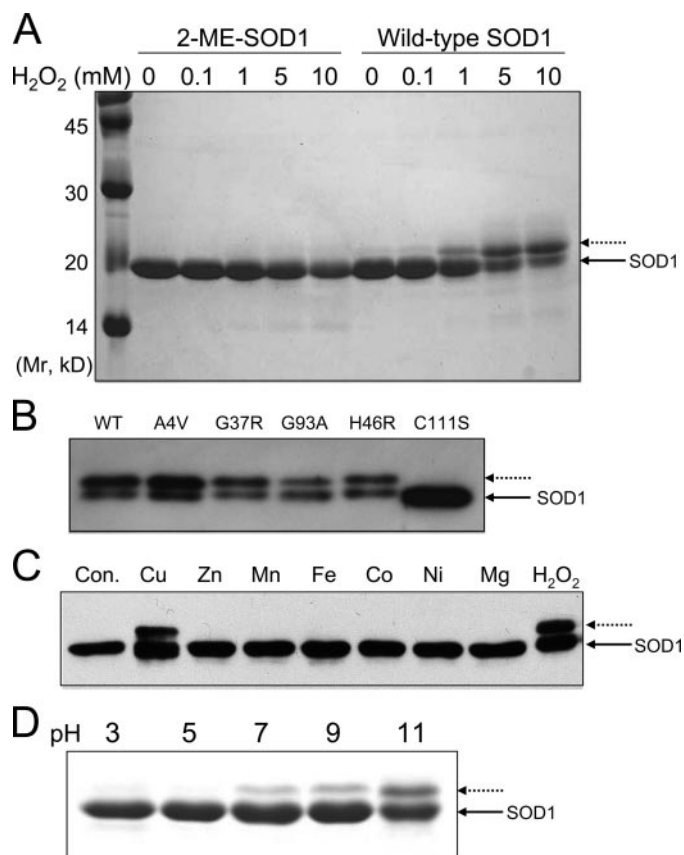


FIGURE 1. Generation of upper shifted band of SOD1 on SDS-PAGE under reducing conditions. A, 2-ME-SOD1 and wild-type SOD1 treated with various concentrations of H₂O₂ for 20 min, diluted with milliQ water, and boiled with SDS-PAGE loading buffer containing 5% 2-ME. 5 μ g of protein/lane was subjected to SDS-PAGE (14% gel). RainbowTM colored protein molecular weight markers purchased from GE Healthcare were used as molecular weight markers (left). 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, FeCl₂, 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), and glycine-NaOH (pH 9.0 and 11.0). The arrowheads with solid lines indicate SOD1 subunits, and arrowheads with broken lines indicate modified SOD1 subunits.

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. 2, A and B, 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. 2, A and B). In oxidized 2-ME-SOD1, only one fragment (labeled with an 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.* (5) as a large fragment cleaved between Pro⁶² and His⁶³. Because Ookawara *et al.* (5) 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.* (5)

Peroxidation of Cys¹¹¹ in Human SOD1

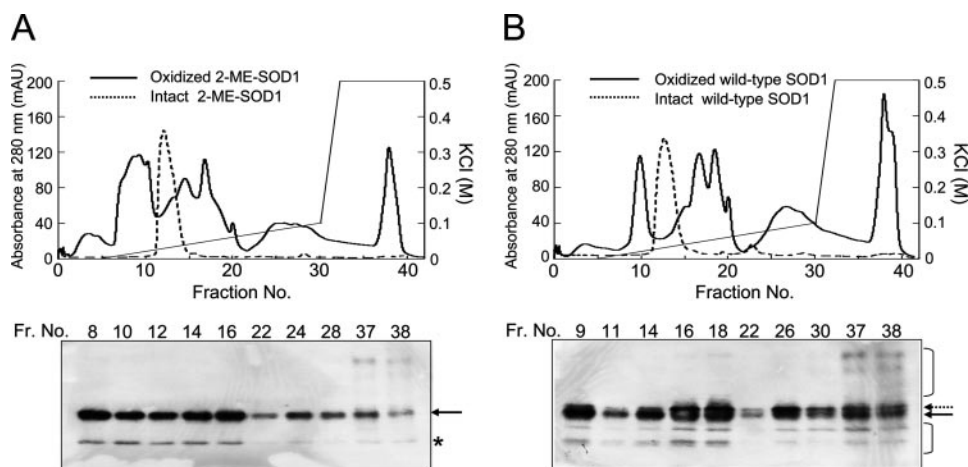


FIGURE 2. Separation of oxidized SOD1s with a MonoQ column. 2-ME-SOD1 and wild-type SOD1 were incubated with 5 mM H₂O₂ for 1 h and were applied to a MonoQ column; some fractions were subjected to reducing SDS-PAGE and Western blot analyses. *A*, chromatogram profiles of oxidized and intact 2-ME-SOD1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

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. 2*B*). Oxidation of Cys¹¹¹ may become a trigger of fragmentation and polymerization. Zhang *et al.* (8) reported that a covalently cross-linked dimer (polymer) of human SOD1 was induced by bicarbonate and H₂O₂. Therefore, 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 milliQ 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 milliQ water. Then the molecular masses were measured by MALDI-TOF MS. The air-oxidized wild-type SOD1 showed two masses, 15,792 and 15,838 *m/z*, but the mass of 2-ME-SOD1 did not change (Fig. 3*A*). Fig. 3*B* shows the elution patterns of air-oxidized 2-ME-SOD1 and wild-type SOD1 on the MonoQ column. The stirred wild-type SOD1 (*solid line*) was separated into two peaks (*a* and *b*), whereas the stirred 2-ME-SOD1 (*dotted line*) was not separated. MALDI-TOF MS also showed that the SOD1 protein in peak *b* also gave two masses, 15,793 and 15,841 *m/z* (Fig. 3*C*), and gave two bands on reducing SDS-PAGE (Fig. 3*D*). It is noteworthy that the SOD activity in peak *b* (3716 units/mg) was similar to the activity in peak *a* (3753 units/mg) and that SOD1 proteins in both peaks retained more than 90% of SOD activity compared with the original wild-type SOD1. These results suggested that oxidative modification at Cys¹¹¹ did not affect on SOD activity and that His residues in the active site were still intact. The difference in mass units between the SOD1 subunit in the upper band and the SOD1 subunit in the original band

appeared to be about 48, suggesting the presence of three oxygens at Cys¹¹¹. Next, the upper and original bands from reducing SDS-PAGE (Fig. 3*D*) 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, 2505 *m/z*, corresponding to tryptic peptide 92–115 (2457 *m/z*) plus 48 was detected. A minor mass, 2489 *m/z*, corresponding to tryptic peptide 92–115 plus 32 was also observed (Fig. 3*E*). In contrast, in the original band, a mass of 2514 *m/z* resulting from carbamidemethylation (plus 58) of tryptic peptide 92–115 was detected (Fig. 3*F*). These results indicate that amino acids in 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. 3*B*) were reduced by DTT, alkylated with IA, and digested with lysylendopeptidase, but not with trypsin. The resultant peptides were applied to a reverse-phase high performance liquid chromatograph (ODS column). As shown in Fig. 4*A*, 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 MonoQ 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 reapplied to the ODS column, four fractions containing three distinct peaks were obtained (Fig. 4*B*). 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 peptide *e*, in fractions 3 and 4, gave masses corresponding to residues 92–122 plus 32 (3295.5 *m/z*) and residues 92–122 plus 48 (3311.6 *m/z*), respectively (Fig. 4*C*).

To directly demonstrate the formation of Cys¹¹¹-SO₂H and Cys¹¹¹-SO₃H, these peptides (*c*–*e*), were further analyzed by infusion ESI MS/MS. This method was used to ascertain the site of oxidative modification of SOD1 by determination of the amino acid sequence of the peptides. The amino acid sequence of the peptide 92–122 was determined based on the assumption that Cys¹¹¹ was modified with carbamidemethyl (Fig. 5*B*), sulfonic acid (SO₂H) (Fig. 5*C*), and sulfonic acid (SO₃H) (Fig. 5*D*), respectively. The mass of peptide *c* (3320.5 *m/z*), Cys-carbamidemethyl ([*M* + 2*H*]²⁺ = 1661.2 *m/z*) gave the major fragment ions, *y*₅ (611.3 *m/z*), *y*₁₀ (1152.0 *m/z*), *y*₁₂ (1424.6 *m/z*), *y*₁₃ (1561.7 *m/z*), and *y*₂₁* ([*M* + 2*H*]²⁺ = 1161.0 *m/z*), indi-

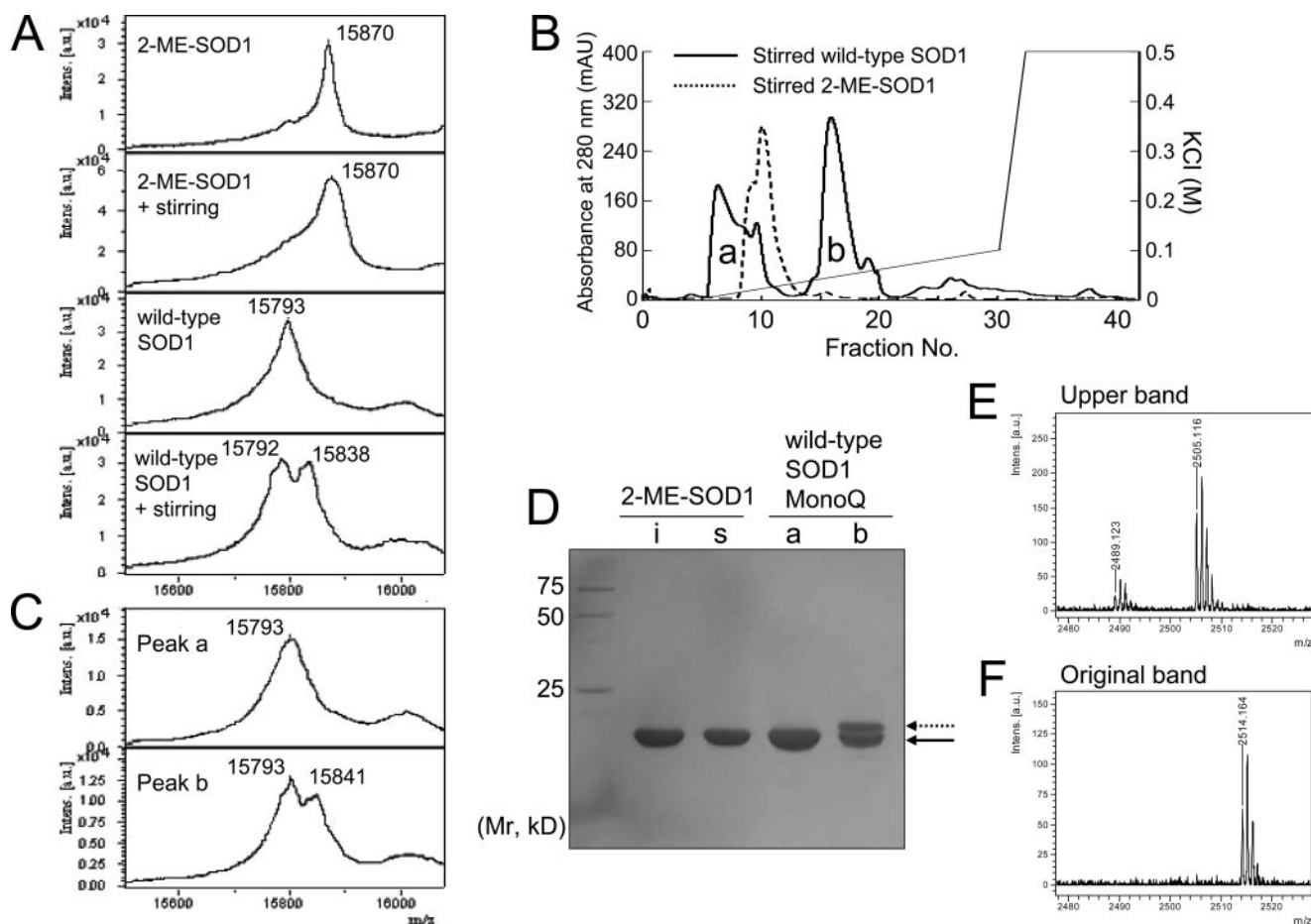


FIGURE 3. Analyses of upper and original bands on SDS-PAGE. *A*, MALDI-TOF MS spectra of intact and stirred 2-ME-SOD1 and wild-type SOD1, respectively. *B*, chromatogram profiles of stirred wild-type SOD1 and 2-ME-SOD1 on a MonoQ column. *C*, MALDI-TOF MS spectra of SOD1 in peaks *a* and *b*, separated with a MonoQ column (*B*). *D*, SDS-PAGE of intact (*i*) and stirred (*s*) 2-ME-SOD1 and SOD1s in peaks *a* and *b*. The 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 the right lane of *D*. MAU, milliabsorbance units.

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, γ_{11} (1264.6, *m/z*), γ_{12} (1399.7 *m/z*), γ_{13} (1536.7 *m/z*), and β_{20} (2031.6 *m/z*), which was identified to be residues 92–122 containing Cys¹¹¹-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, γ_9 (1038.6 *m/z*), γ_{12} (1415.6 *m/z*), γ_{13} (1552.7 *m/z*), γ_{18} (2012.0 *m/z*), and γ_{21} (2310.8 *m/z*), indicating that Cys¹¹¹ was oxidized to Cys-SO₃H (Fig. 5G). Analyses based on the assumption that His¹¹⁰ and/or His¹²⁰ were oxidized to 2-oxo-histidine showed that the corresponding γ ions and β ions were absent (data not shown). These results clearly indicated that Cys¹¹¹ was readily oxidized to Cys-SO₂H, which underwent further oxidation to Cys-SO₃H 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-peroxidized SOD1—To explore the possibility of immunological detection of Cys¹¹¹-peroxidized SOD1 (Cys¹¹¹-SO₃H-SOD1), a rabbit polyclonal antibody against the peptide containing Cys¹¹¹-SO₃H was prepared. The antiserum was purified to exclude the reactivity with reduced form SOD1 (Cys¹¹¹-SH) by affinity col-

umns as described under “Experimental Procedures.” The resultant IgG, which was denoted as anti-C111ox-SOD1, reacted with only the upper band of Cys¹¹¹-peroxidized SOD1 but neither the original band of wild-type SOD1 nor 2-ME-SOD1 (Fig. 6A). These data further demonstrated that the upper band is the oxidized form of SOD1 containing sulfonated 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.

Two-dimensional Gel Electrophoresis Characterization of Oxidized SOD1—It is well known that human, bovine, and recombinant human SOD1 proteins have several charge isomers detected by HPLC, isoelectric gel focusing, or two-dimensional gel electrophoresis (26, 30, 31). The reason for the heterogeneity is still unknown, although some hypotheses, such as different metallation, different conformation, and different oxidation of Cys residues, were presented (13, 26). Thus, two-dimensional gel electrophoresis of oxidized SOD1 was performed

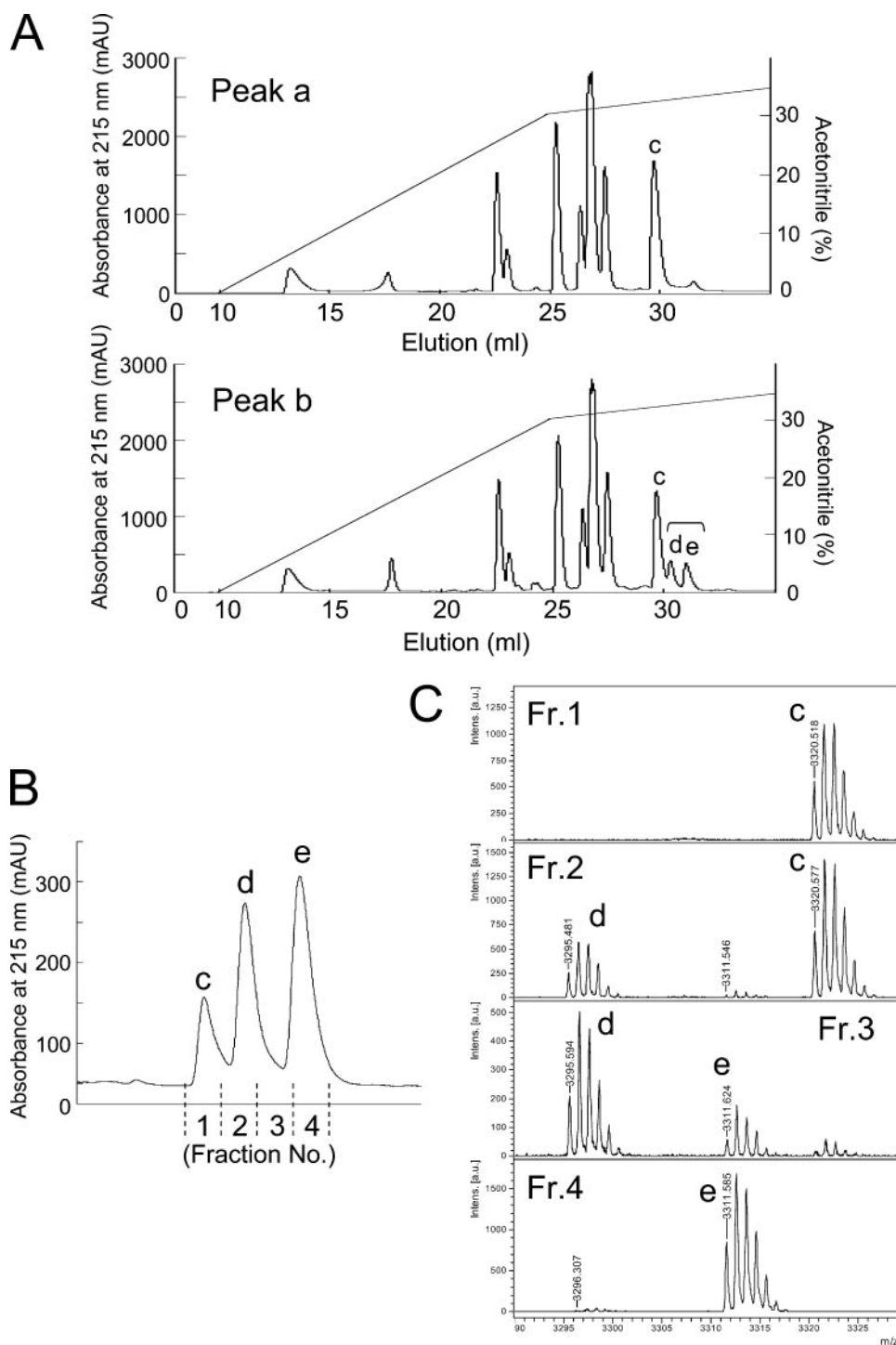


FIGURE 4. Purification and analyses of lysylendopeptidase-digested peptides. SOD1s in peaks *a* and *b* in Fig. 3B were digested with lysylendopeptidase after treatments with DTT and iodoacetamide, and the digests were fractionated by HPLC using a TSK-GEL ODS-80TM column. *A*, chromatogram profiles of the digests from peaks *a* (top) and *b* (bottom), separated with a MonoQ column (Fig. 3B). *B*, rechromatogram profile of fractions containing peaks *d* and *e*, at the bottom of *A*. *C*, MALDI-TOF MS spectra of peptides in fractions 1–4 in *B*. mAU, milliabsorbance units.

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, 1 (pI 4.92), 2 (pI 5.02), and 4 (pI 5.8). 2-ME-SOD1 also presented four similar spots (Fig. 7B). Three of them, spots 1–3, 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 (30). 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 spots 3 and 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. 7, D and E). Furthermore, two major spots, 1' and 2', and weak spot 3', just above spots 1, 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, 1' and 2', and one tiny spot, 7' (Fig. 7, F and G). Therefore, these results indicated that spots 1', 2', and 7' were generated by the oxidation of Cys¹¹¹ and that the generation of spots 5, 6, and 7 (Fig. 7C) was caused by the oxidation of other 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 the ~25 kDa band in the Triton X-100-soluble fraction (Fig. 8A, left). 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 overexpressed in the G1H-G93A mouse and mouse SOD1 (Fig. 8A, right). 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). Therefore, the 25 kDa band, selectively recognized by anti-C111ox-SOD1, was thought to be some molecule-bound oxidized SOD1. Basso *et al.* (26) detected mono- and polyubiquitinated SOD1 (24, 32, 40, 48 kDa spots in two-dimensional gel electrophoresis) in the Triton X-100-insoluble

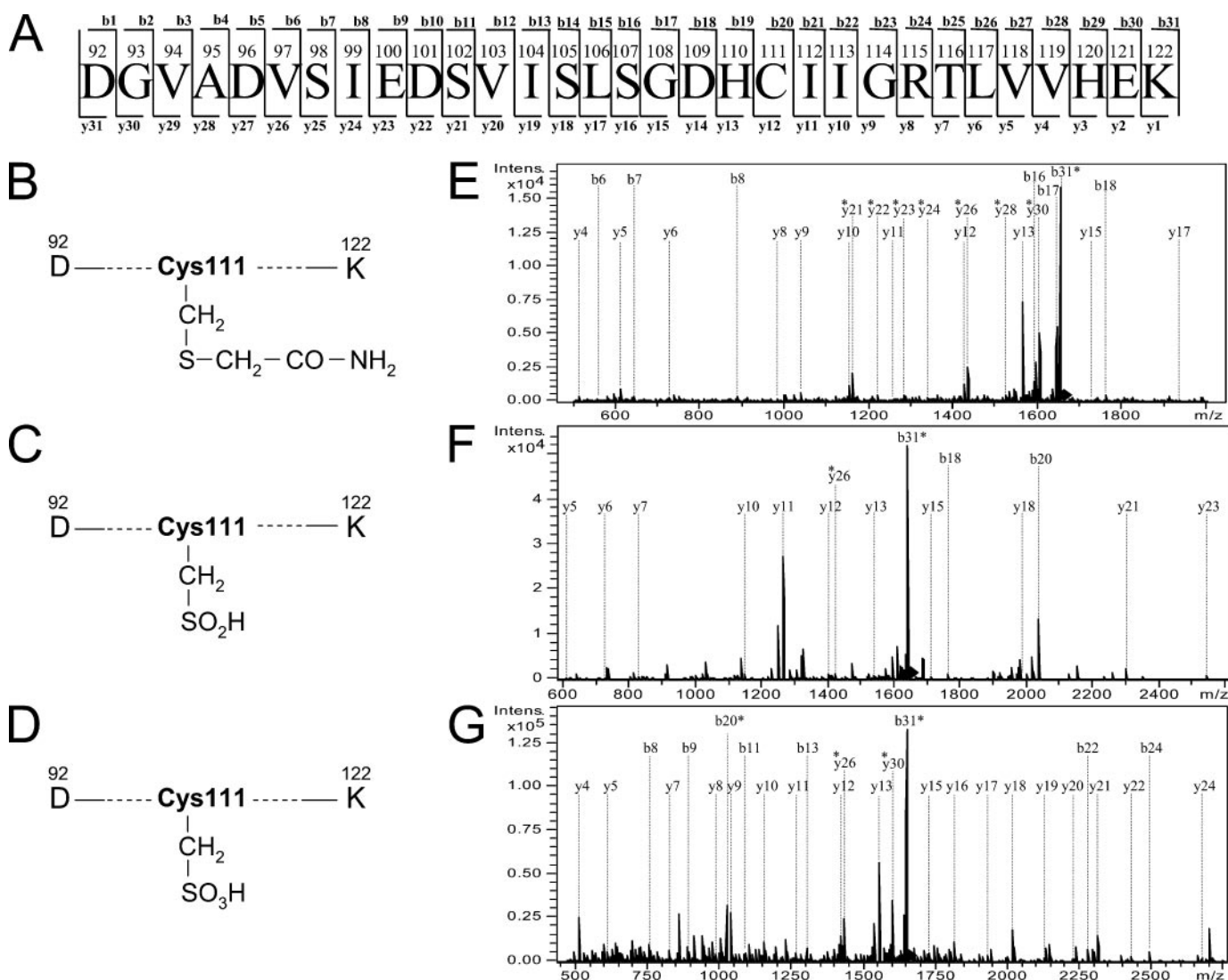


FIGURE 5. Sequence analysis of modified peptide 92–122 by ESI MS. A, schematic representation of peptides obtained from fragmentations by MS/MS analysis. B–D, Cys modification observed in this experiment. Carbamidemethyl-Cys (B), sulfanyl Cys (C-SO₂H) (C), and sulfonyl Cys (C-SO₃H) (D). E–G, MS/MS sequence analysis of the peptide 92–122 digested with lysylendopeptidase in fractions 1, 3, and 4 (B). E, MS/MS analysis of the peptide modified with carbamidemethyl-Cys ($[M + 2H]^{2+} = 1661.2$ m/z) in fraction 1. F, MS/MS analysis of the peptide modified with C-SO₂H ($[M + 2H]^{2+} = 1648.3$ m/z) in fraction 3. G, MS/MS analysis of the peptide modified with C-SO₃H ($[M + 2H]^{2+} = 1657.2$ m/z) in fraction 4. The y-ions and b-ions labeled with asterisks are the doubly charged ions. The mass number labeled with diamond is that of the precursor ion in E–G.

fraction of G93A transgenic mice spinal cords. Thus, the 25 kDa band was speculated to be monoubiquitinated SOD1, and then the immunostaining with anti-ubiquitin on the same membrane was performed. Although the 25 kDa band appeared to be one of the ubiquitinated proteins (data not shown), evidence of monoubiquitination has not been obtained. We are currently exploring the identity of the molecule bound to Cys¹¹¹-peroxidized SOD1. Finally, an 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 (32). 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 with anti-C111ox-SOD1 pretreated with an excess

amount of air-oxidized SOD1 or peptide containing sulfonated 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 sulfinic acid (Cys¹¹¹-

Peroxidation of Cys¹¹¹ in Human SOD1

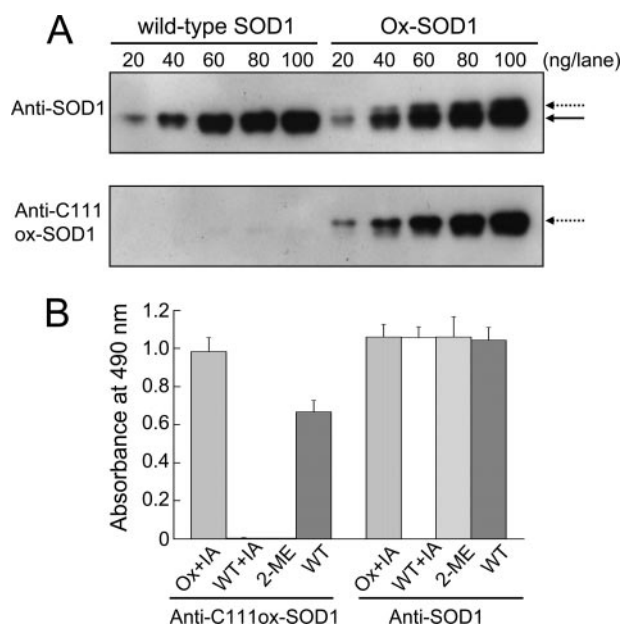


FIGURE 6. Validation of anti-C111ox-SOD1 that selectively recognizes Cys¹¹¹-peroxidized SOD1. A, Western blot analyses for 2-ME-SOD1 and air-oxidized SOD1 detected by anti-SOD1 (top) and anti-C111ox-SOD1 (bottom). An arrowhead with solid lines indicates wild-type SOD1 subunits, and arrowheads with broken lines indicate Cys¹¹¹-peroxidized SOD1 subunits. The PVDF membrane was first reacted with anti-C111ox-SOD1. The antibody was stripped from the membrane, which was reincubated with anti-SOD1. B, ELISA for 2-ME-SOD1 and air-oxidized SOD1 treated with and without IA detected by anti-C111ox-SOD1 (left) and anti-SOD1 (right). Ox, WT, and 2-ME, air-oxidized SOD1, wild-type SOD1, and 2-ME-SOD1, respectively. Data are presented as the means \pm S.D. of triplicate experiments.

SO₂H) and sulfonic acid (Cys¹¹¹-SO₃H) (Figs. 3–5). The newly developed specific antibody against a peptide containing Cys¹¹¹-SO₃H, anti-C111ox-SOD1, recognized the upper band (Cys¹¹¹-SO₃H form) but not the original band (Cys¹¹¹-SH form) by Western blot analyses (Figs. 6–8). These results further demonstrated that the upper band is the oxidized form of SOD1 containing Cys¹¹¹-SO₃H. 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 (33). 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 (34, 35). 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 sulfinic acid (Cys-SO₂H) and to sulfonic acid (Cys-SO₃H) in the presence of strong oxidants (34). In the active site of some proteins, such as Prx and thioredoxin, one cysteine is in the thiolate form and, as a result, can react with H₂O₂ (36, 37). In the case of PrxI, Cys⁵¹ is selectively oxidized to Cys-SO₂H but not to Cys-SO₃H, as evidenced by the difference of 32 mass units between reduced and oxidized PrxI proteins. Additional oxidation with H₂O₂ did not increase Cys-SO₃H even *in vitro* (22, 34). Cys⁵¹-

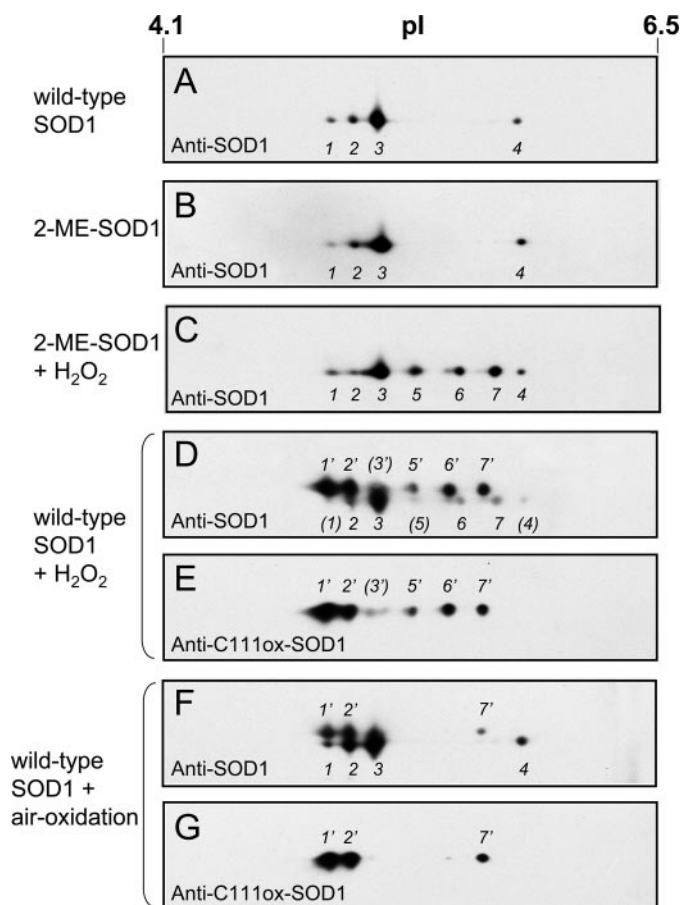


FIGURE 7. Two-dimensional Western blot analyses for wild-type and 2-ME SOD1s with and without oxidation detected by anti-SOD1 (A–D and F) and anti-C111ox-SOD1 (E and G). 5 μ g of SOD1s treated with and without 1 mM H₂O₂ for 1 h or with air oxidation for 24 h were subjected to two-dimensional gel electrophoresis and Western blot analysis. The PVDF membrane was first reacted with anti-C111ox-SOD1. The antibody was stripped from the membrane, which was reincubated with anti-SOD1.

SO₂H is rereduced to Cys⁵¹-SH by sulfiredoxin but not by DTT or thioredoxin (23, 38). In contrast, in intact human SOD1, Cys¹¹¹ appears to be oxidized to Cys¹¹¹-SO₃H, 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 the thiolate form at physiological pH and can therefore react not only with H₂O₂ but also with oxygen in ambient air (Fig. 1). Cys¹¹¹ also is readily modified with *N*-ethylmaleimide or 4-vinylpyridine (29, 39) or bound to another sulfhydryl, such as 2-ME (supplemental Fig. S1) or cysteine (39). In contrast, the free cysteine at residue 6, Cys⁶, is less reactive with oxygen, 2-ME, or *N*-ethylmaleimide (present study) (29), probably because it exists in β -sheet 1a and is buried within the SOD1 molecule (40). In other studies, exposure of bovine SOD1 to an excess of H₂O₂ resulted in selective oxidation of His¹¹⁸ (corresponding to His¹²⁰ in human SOD1), inactivating the enzyme (2). Rakhit *et al.* (6) showed that four amino acids (His⁴⁸, His⁸⁰, His¹²⁰, and Phe²⁰) in human SOD1 were prone to oxidation by ascorbic acid/CuCl₂. 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

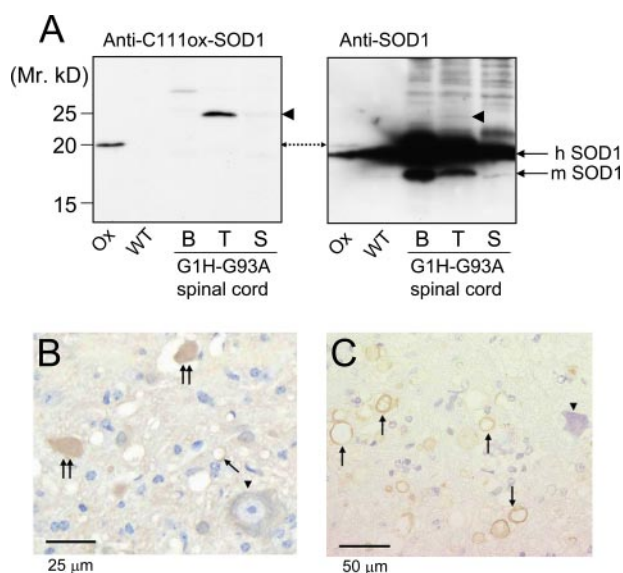


FIGURE 8. Cys¹¹¹-peroxidized SOD1 in spinal cord of G1H-G93A mice. A, Western blot analyses for G1H-G93A spinal cord extracts detected by anti-C111ox-SOD1 (left) and anti-SOD1 (right). 60 μ g of proteins of buffer-soluble (B), Triton X-100-soluble (T), and SDS-soluble (S) fractions, respectively, were applied. The arrowheads with solid lines indicate human and mouse SOD1 subunits, and a double arrowhead with a broken line indicates Cys¹¹¹-peroxidized SOD1 subunits. The arrowheads indicate a 25 kDa band reacted with anti-C111ox-SOD1. 20 ng of air-oxidized SOD1 (Ox) and wild-type SOD1 (WT) were applied as controls. The PVDF membrane was first reacted with anti-C111ox-SOD1. The antibody was stripped from the membrane, which was reincubated with anti-SOD1. Precision Plus protein standards purchased from Bio-Rad were used as molecular weight markers. B and C, immunohistochemical analyses of paraffin-embedded G1H-G93A spinal cord sections detected by anti-C111ox-SOD1. B, double arrows indicate LBHs 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 surviving motor neuron, which is not stained with anti-C111ox-SOD1. Scale bar, 25 μ m. C, single arrows indicate rims of vacuoles detected by anti-C111ox-SOD1. An arrowhead indicates the surviving motor neuron, which is not stained with anti-C111ox-SOD1. Scale bar, 50 μ m.

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 Cys⁵¹-SH to Cys⁵¹-SO₂H causes acidic shifting of PrxI upon two-dimensional gel electrophoresis (22). In contrast, human, bovine, and recombinant human SOD1 originally have some charge isomers before oxidation (30, 31). Fig. 7, A and B, also showed that both wild-type SOD1 and 2-ME-SOD1 have four similar spots, although they have their own mass. Cys¹¹¹ oxidation by air oxidation generated two major spots, 1' and 2', just above the two minor isomers, 1 and 2 (pI 4.92 and 5.02) (Fig. 7, F and G). However, these spots appear like acidic shifted spots of original main spot 3 (pI 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 H₂O₂ further generated several spots, and a total of 13 spots were observed on two-dimensional gel electrophoresis (Fig. 7D). Therefore, isomers of SOD1 observed in the previous reports (13, 26) 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, 41). The "copper hypothesis" proposes that copper, either bound to or released from FALS-linked mutant SOD1s generates reactive oxygen species harmful to motor neurons (42–44). 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, Parkinson, and Huntington diseases (33, 45, 46). However, recent reports suggest that these two hypotheses are interrelated. Copper ion oxidized Cys¹¹¹ in human SOD1 (Fig. 1). Oxidation may result in misfolding and aggregation even in wild-type SOD1 (6, 47). Oxidized wild-type SOD1 exhibits characteristics of FALS-linked mutant SOD1s: conjugation with polyubiquitin, 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-SO₂H and to Cys-SO₃H, Cys¹¹¹ may also participate in disulfide bond linkage with other cysteine residues and oligomerization (48). Furukawa *et al.* (49–51) showed that incorrect intermolecule disulfide cross-linking of immature, misfolded FALS-linked mutant SOD1s leads to formation of insoluble aggregates. On the other hand, an immunohistochemical study using the anti-C111ox-SOD1 revealed that Cys¹¹¹-peroxidized mutant SOD1 accumulated in the vacuole structures and LBHs (Fig. 8, B and C). Because the most characteristic neuropathological findings in ALS model mice are LBHs and vacuoles (32, 33), 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 surviving neuron cells were not immunostained by anti-C111ox-SOD (Fig. 8, B and C). Ferri *et al.* (48) proposed that Cys¹¹¹ was a key mediator of mitochondrial association of SOD1 and subsequent mitochondrial dysfunction, because the C111S mutant was less associated with mitochondria. Recently, it was also reported that A4V/C111S protein was more stable than A4V protein in cells (52). 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, although Ser, not Cys, is at residue 111 of mouse SOD1 (53). It is thought that Cys¹¹¹ may enhance human ALS development. A comparative study of symptoms between mice expressing the 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 Cys¹¹¹-SO₃H. The specific antibody against the Cys¹¹¹-SO₃H will be a useful tool for detecting oxidized human SOD1. Precise studies of the role of oxidized SOD1 in ALS are currently under way.

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**Oxidative Modification to Cysteine Sulfonic Acid of Cys¹¹¹ in Human
Copper-Zinc Superoxide Dismutase**

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