Oxidation of the Tryptophan 32 Residue of Human Superoxide Dismutase 1 Caused by Its Bicarbonate-dependent Peroxidase Activity Triggers the Non-amyloid Aggregation of the Enzyme*

Background: Protein aggregation is a hallmark of neurodegenerative diseases.

Results: Oxidation of the hSOD1-Trp32 residue promotes enzyme covalent dimerization, oligomerization, and aggregation.

Conclusion: A novel pathway for hSOD1 aggregation is revealed.

Significance: The uniqueness of the Trp32 residue makes its oxidation potentially relevant to ALS pathogenesis.

The role of oxidative post-translational modifications of human superoxide dismutase 1 (hSOD1) in the amyotrophic lateral sclerosis (ALS) pathology is an attractive hypothesis to explore based on several lines of evidence. Among them, the remarkable stability of hSOD1WT and several of its ALS-associated mutants suggests that hSOD1 oxidation may precede its conversion to the unfolded and aggregated forms found in ALS patients. The bicarbonate-dependent peroxidase activity of hSOD1 causes oxidation of its own solvent-exposed Trp32 residue. The resulting products are apparently different from those produced in the absence of bicarbonate and are most likely specific for simian SOD1s, which contain the Trp32 residue. The aims of this work were to examine whether the bicarbonate-dependent peroxidase activity of hSOD1 (hSOD1WT and hSOD1G93A mutant) triggers aggregation of the enzyme and to comprehend the role of the Trp32 residue in the process. The results showed that Trp32 residues of both enzymes are oxidized to a similar extent to hSOD1-derived tryptophanyl radicals. These radicals decayed to hSOD1-N-formylkynurenine and hSOD1-kynurenine or to a hSOD1 covalent cross-linked by a dityrphanyl bond, causing hSOD1 unfolding, oligomerization, and non-amyloid aggregation. The latter process was inhibited by tempol, which recombines with the hSOD1-derived tryptophanyl radical, and did not occur in the absence of bicarbonate or with enzymes that lack the Trp32 residue (bovine SOD1 and hSOD1W32F mutant). The results support a role for the oxidation products of the hSOD1-Trp32 residue, particularly the covalent dimer, in triggering the non-amyloid aggregation of hSOD1.

The cytosolic enzyme Cu,Zn-superoxide dismutase (SOD1)2 is an intriguing enzyme. It is one of the most important antioxidant defenses (1) but can also present secondary pro-oxidant activities, such as the bicarbonate-dependent peroxidase activity (2–8). In addition, hSOD1 is a small protein (153 amino acids and 1 copper and 1 zinc ion per monomer), which in the native state is a remarkably stable non-covalent homodimer highly resistant to detergents and proteolysis. Nevertheless, mutations in hSOD1 (>170 have been described) are associated with the fatal neurodegenerative disease amyotrophic lateral sclerosis (ALS). As is the case with other neurodegenerative diseases, protein misfolding and aggregation are hallmarks of ALS. A distinctive feature of ALS is that it displays amorphous protein aggregates instead of the amyloid aggregates characteristic of most protein aggregation diseases (9, 10).

SOD1 mutations are responsible for a small percentage of the familial cases of ALS (20%). Nevertheless, SOD1-linked ALS patients exhibit a pathology and symptoms similar to those of sporadic ALS patients, arguing for a common pathogenic mechanism for both forms of the disease (11). Consequently, ALS and ALS-SOD1 mutants have been extensively studied in recent years. Despite the many advances made, the pathogenic mechanism of ALS remains elusive (reviewed in Refs. 12–17). It is agreed that ALS-SOD1 mutants cause motor neuron death via a toxic gain-of-function that is independent of the normal antioxidant function of the enzyme. Over the years, many theories about the nature of this toxic gain-of-function have been proposed, such as an ability to generate oxidants, a higher sensitivity to oxidants and to denaturing conditions, and a higher propensity for aggregation (11–16). The latter characteristic is currently the most emphasized, although it may be related to the other properties.

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2 The abbreviations used are: SOD, superoxide dismutase; ALS, amyotrophic lateral sclerosis; hSOD1, bovine Cu,Zn-superoxide dismutase 1; DBNB, 3,5-dibromo-4-nitrosobenzenesulfonic acid; DLS, dynamic light scattering; DTPA, diethylene triamine pentaacetic acid; CR, congo red; hSOD1, human superoxide dismutase 1; FTC, fluorescein-5-thiosemicarbazide; MILS, mean intensity of light scattering.
In fact, the so-called non-native hSOD1 forms, which are detected in the affected areas of the central nervous systems of patients and animal models of the disease and are considered crucial to ALS pathogenesis, include unfolded/misfolded hSOD1 forms (18–20), over oxidized/carbonylated hSOD1 (18–24), and dimer-sized hSOD1 species (reviewed in Ref. 24). These non-native hSOD1 forms have been characterized primarily by immuno-based techniques, which provide limited structural information. Therefore, it cannot be excluded that some of the non-native hSOD1 forms are oxidized to some extent.

The possibility of oxidative modifications of hSOD1 triggering hSOD1 unfolding and aggregation was first proposed in 2002 by Chakrabartty and co-workers (25). Subsequent studies by these and other investigators have shown that hSOD1 oxidized by long incubations with hydrogen peroxide, added in bolus or generated by other agents, undergoes major structural changes and non-amyloid aggregation resembling that observed in ALS (10, 18, 19, 26). These structural modifications have been shown to occur downstream to the oxidation of His80 and His32 residues in the active site of hSOD1 (10). These oxidations are caused by Fenton chemistry, including that associated with the peroxidase activity of SOD1, in which the enzyme consumes modest amounts of hydrogen peroxide to become oxidized and inactivated (27).

However, the physiologically ubiquitous bicarbonate buffer greatly modifies the peroxidase activity of SOD1 and its ensuing consequences (2–8). In the presence of bicarbonate, SOD1 consumes considerable amounts of hydrogen peroxide to generate the carbonate radical before becoming inactivated. This highly oxidizing radical can diffuse away from the active site and oxidize distant targets, such as other proteins (28, 29) and, in the case of hSOD1, its own solvent-exposed Trp32 residue (3). The produced hSOD1-trypthrophanyl radical (hSOD1-Trp32) decays to hSOD1-N-formylkynurenine and hSOD1-kynurenine (3) or reacts with another hSOD1-Trp32 to produce the covalent dimer cross-linked by a ditryptophan bond (hSOD1-Trp-Trp-hSOD1) (30). Apparently, the latter hSOD1 oxidation products are different from those produced in the absence of the bicarbonate buffer (10) and are likely specific for human and other simian SOD1s, which are always expressed as the dimer.

Oxidation, Unfolding, and Aggregation of hSOD1

Expression, Purification and Analysis of Human Recombinant SOD1WT and Mutants SOD1G93A and SOD1W32F—Plasmids (pET-3d) encoding the enzymes, hSOD1WT and hSOD1G93A, were kindly provided by Dr. J. S. Beckman from the Linus Pauling Institute. To obtain the mutant hSOD1W32F, we employed a QuikChange site-directed mutagenesis kit (Agilent Biotechnology). Two pairs of reverse complementary primers harboring the appropriate single mutation were designed (forward primer: GGACCAGTGAGGTTCGG-AAGCATTAAAGGAC and reverse primer: GTCTCTTAATGCTTCCGAACACCTCTA1CCTGG) and purchased from Extend Inc. The PCR was performed according to the standard procedure of the site-directed mutagenesis kit. The pET3D plasmid containing the new SOD1W32F mutant gene was transformed into Escherichia coli and the mutation was confirmed by DNA sequencing (GenBank™ number KJ179904). The plasmids were expressed in E. coli strain BL21(DE3) pLySS, and the enzymes were purified and analyzed as previously described (6). Protein concentration was determined by the Bradford method using a commercial reagent (Bio-Rad) or by spectrophotometry at 280 nm (ε280 = 10.8 × 10³ M⁻¹ cm⁻¹) for the native dimeric enzyme (9). SOD1 metal contents were determined spectrophotometrically at 500 nm under denaturing conditions by the 4-(2-pyridylazo)resorcinol assay (6). The concentration of nonspecifically bound metals under non-denaturing conditions was below the detection limit of the method (0.5 μM). Typically, recombinant hSOD1WT and mutants hSOD1G93A and hSOD1W32F contained ~0.7 copper and 0.7 zinc ions per monomer. The commercial bovine SOD1 contained ~0.35 copper and zinc ions per monomer. The commercial bovine SOD1 contained ~0.35 copper and zinc ions per monomer. SOD1 activity was monitored spectrophotometrically at 550 nm using the cytochrome c method (9). Typically, recombinant hSOD1WT and mutants hSOD1G93A and hSOD1W32F exhibited specific dismutase activities of 3,900 ± 400 units/mg (mg of protein normalized by the copper content). Here, the concentrations of hSOD1 are always expressed as the dimer.

Bicarbonate-dependent Peroxidase Activity of SOD1—The activity was monitored spectrophotometrically by the oxidation of dihydroxynadimine 123 to rhodamine (ε500 nm = 78.8 × 10³ M⁻¹ cm⁻¹) (6). The incubations contained SOD1 (1 μM in terms of dimer units), DHR (100 μM), bicarbonate (25 mM), DTPA (100 μM), and H2O2 (1 mM) in phosphate buffer (25 mM) adjusted to a final pH of 7.4 and performed at 37 °C. One unit was defined as the amount of enzyme that produces 1 μmol of rhodamine/min. Recombinant hSOD1 expressed in our laboratories exhibited specific peroxidase activity of 29 ± 8 units/mg.
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Incubation Mixtures—Unless otherwise stated, the reaction mixtures contained hSOD1WT, hSOD1G93A, or hSOD1W32F (25 µM in terms of dimer), hydrogen peroxide (1 mM), bicarbonate (50 mM), and DTPA (0.1 mM) in phosphate buffer (50 mM) adjusted to a final pH 7.4; the mixtures were kept tightly closed and incubated for 1 h at 37 °C ± 2 °C before being subjected to different analyses. The reactions were started by the addition of hydrogen peroxide. In the case of controls, the mixtures did not contain bicarbonate or hydrogen peroxide or both.

Hydrogen Peroxide Consumption—Hydrogen peroxide consumption after 1 h incubation at 37 °C was monitored by measuring the remaining oxidant via the ortho-phenidinomide method as previously described (6).

Quantification of Released Copper—In the incubations used to quantify the copper released from hSOD1 after a 1-h incubation, DTPA (0.1 mM) was replaced by bathocuproine disulfonic acid (0.5 mM). Released copper(I) ion was quantitated by the absorbance at 485 nm (ε485 = 1.24 × 10^4 M⁻¹ cm⁻¹) of its bathocuproine disulfonate complex (34).

EPR-Spin Trapping Experiments—The incubations contained hSOD1 (WT or mutants) (30 µM in terms of dimer units), H₂O₂ (1 mM), HCO₃⁻ (25 mM), DTPA (0.1 mM), and DBNBS (10 mM) in phosphate buffer (50 mM) adjusted to a final pH of 7.4, and incubations were performed at 37 °C. Aliquots taken after 10 min of incubation were transferred to a flat cell, and the EPR spectra were recorded at room temperature (25 ± 2 °C) on a Bruker EMX instrument equipped with a Super High Q cavity.

Analysis by Reducing and Non-reducing SDS-PAGE—After a 1-h incubation at 37 °C, sample aliquots (corresponding to 10 µg of protein) were removed, resuspended in Laemml buffer (Tris (62 mM, pH 6.8), glycerol (10%), bromphenol blue (0.05%), SDS (2%) and β-mercaptoethanol (4%), heated at 95 °C for 5 min, and submitted to electrophoresis (15% SDS-PAGE) with the running buffer composed of Tris (25 mM), glycine (192 mM), and SDS (10%). Alternatively, the samples were submitted to non-reducing and partially denaturing electrophoresis (35), in which case aliquots were transferred to the loading buffer (Tris buffer (62 mM, pH 6.8), glycerol (10%), bromphenol blue (0.05%), and SDS (0.4%)) and were not heated; the running buffer was composed of Tris (25 mM) and glycine (192 mM). The gels were stained with Coomassie Blue.

Analysis of hSOD1 Carbonylation—Protein carbonyl contents were determined as previously described (36). After 1 h of incubation, the reactions were stopped by the addition of catalase (2 units/ml). Aliquots (corresponding to 50 µg of protein) were removed and added (1:0.5, v/v) to a solution of fluorescein-5-thiosemicarbocarbo (FTC; 1 mM) in phosphate buffer (50 mM), pH 6.0. The mixture was incubated at 37 °C for 2 h. The protein was then precipitated by the addition of a 10-fold excess of cold acetone (−20 °C), and the mixture was kept overnight at −20 °C. After centrifugation (16,000 × g) for 10 min at 4 °C, the protein pellets were broken up and washed twice with cold acetone. The pellets were then resuspended in water, and the protein concentration was determined using a Bradford assay (Bio-Rad). Protein aliquots (10 µg) were diluted in Laemml buffer and subjected to 15% SDS-PAGE electrophoresis. Fluorescent protein images from the gel were captured with a Typhoon 9400 imager using an excitation wavelength of 488 nm and an emission filter at 520 nm (40 nm bandpass). A relative quantification of the bands was performed with ImageJ 1.44p software (NIH). The gels were then fixed with methanol (50%) and acetic acid (10%) for 10 min, followed by an overnight staining with Coomassie Blue.

Circular Dichroism Measurements—The incubations contained hSOD1 (15 µM in dimer), H₂O₂ (1 mM), HCO₃⁻ (50 mM), and DTPA (0.1 mM) in phosphate buffer (50 mM) adjusted to a final pH of 7.4. The incubations were performed at 37 °C with the samples tightly closed. After a 1-h incubation, the samples were transferred to 0.1-cm quartz cuvettes, and the CD spectra were recorded in a Jasco J-715 spectropolarimeter (Jasco Corporation, Tokyo, Japan) at 25 °C. The averaged spectra of 15 scans were corrected for the buffer blank. Estimations of the secondary structure contents were made through the DicroWeb site (37) using the CDSSTR method (38).

Analysis of Unfolded hSOD1 Forms—Unfolded hSOD1 forms were detected with a conformation-specific antibody (USOD) targeted against hSOD1 residues 42–48 that recognizes hSOD1 with an unfolded β barrel (18, 39). The unfolded hSOD1 standard was prepared by dissolving hSOD1 (15 µM) in guanidine hydrochloride (6 M) containing DTT (2 mM) and EDTA (1 mM); these mixtures were incubated at room temperature for at least 2 h. In parallel, samples of hSOD1WT or hSOD1G93A (20 µM) were incubated with hydrogen peroxide (1 mM), bicarbonate (25 mM), and DTPA (0.1 mM) in phosphate buffer (50 mM), pH 7.4, for 2 h at 37 °C ± 2 °C. The reactions were stopped by the addition of catalase (5 units/ml). Some reactions were incubated in the presence of tempol (10 µM). The control mixtures did not contain bicarbonate or hydrogen peroxide. Next, 96-well plates were coated with either a typical reaction mixtures or unfolded hSOD1 (150 ng/well) overnight. After blocking with BSA (1%) in PBS, the plates were incubated with 100 µl/well of affinity-purified USOD (1 µg/ml) for 2 h followed by secondary antibody (anti-rabbit IgG peroxidase conjugate, KPL, 100 µl/well of a 1:750 dilution) for 2 h. TMB and hydrogen peroxide were used as substrates for peroxidase. The oxidation of TMB was determined spectrophotometrically at 450 nm. Anti-SOD was used as a loading control.

LC-MS and MS/MS Analysis of hSOD1 Hydrolsates—After a 1-h incubation at 37 °C, aliquots corresponding to 50 µg of protein were removed, resuspended in Laemml buffer, heated at 95 °C for 5 min, and submitted to electrophoresis (15% SDS-PAGE). The proteins spots corresponding to the monomer (~20 kDa) and dimer (~40 kDa) were excised from the gels and destained with ammonium bicarbonate (50 mM) in acetonitrile (50%). The destained gels were treated with dithiotreitol (10 mM) in ammonium bicarbonate (50 mM) for 1 h at 37 °C and then incubated with iodoacetamide (50 mM) in ammonium bicarbonate (50 mM) for 1 h at 37 °C in the dark. The gel pieces were washed 3 times with 100 µl of ammonium bicarbonate (50 mM) for 10 min, dehydrated by the addition of acetonitrile, rehydrated with ammonium bicarbonate (50 mM), and shrunk again by the addition of acetonitrile. The gel pieces were then completely dried in a vacuum microcentrifuge. Proteolysis with trypsin was performed in ammonium bicarbonate (50 mM) containing calcium chloride (2 mM), pH 8.0, for 17 h at 37 °C. A
substrate/trypsin ratio of 30 was employed. The peptides were extracted with formic acid (5%) in acetonitrile (50%) at a constant sonication (10 min for each change) at room temperature and dried in a SpeedVac (40). The samples were resuspended in water containing 1% TFA. The tryptic hydrolysates were applied in a ZipTip® (Millipore) before analysis. The hydrolysates were analyzed by LC-MS on a UHR-ESI-Q-TOF Bruker Daltonics MaXis 3G system with Captive Spray source in the positive mode coupled on line to a nano-UPLC system. The hydrolysates were subjected to reverse-phase chromatography using a ZORBAX 300SB-C18 (150 mm × 75 μm, particle size 3.5 μm) (Agilent Technologies) (36). The interface conditions for the nano captive source were as follows: capillary, 1.8 kV; dry heater, 150 °C; dry gas, 6 liters/min. Nitrogen was used as the collision gas and the CID (collision-induced dissociation) energy was optimized by the equipment. The instrument was externally calibrated using an ESI low concentration tuning mixture over a m/z range of 100 to 2000 and an internal standard for calibration (lock mass m/z 1221.9906). The Mascot (Matrix Science) and Bruker Data Analysis software (version 4.0) were employed for data acquisition and processing. Under the experimental conditions, mass errors were lower than 10 ppm for MS and 0.5 Da for MS/MS. Peaks with scores lower than 4.0) were employed for data acquisition and processing. Under “Results” are followed by the S.E. from the fitting. The values for the parameters in Equation 1 presented among the experimental groups was determined by analysis of variance followed by a Bonferroni post-test. All tests were performed with GraphPad Prism 4.0 (GraphPad Software). All data are presented as mean ± S.D. Values of p < 0.05 were considered significant.

RESULTS

hSOD1<sup>WT</sup> and hSOD1<sup>G93A</sup> Display Similar Bicarbonate-dependent Peroxidase Activities and Are Similarly Oxidized—The ALS-associated mutant hSOD1<sup>G93A</sup> is the most studied hSOD1 mutant and is expressed in rodents to provide animal models of ALS. This mutant is classified as a hSOD1<sup>WT</sup>-like mutant because the enzymes are structurally and functionally similar (12). Accordingly, the recombinant hSOD1<sup>WT</sup> and hSOD1<sup>G93A</sup> expressed and purified in our laboratories presented similar superoxide dismutase (3,900 ± 400 units/mg) and bicarbonate-dependent peroxidase (29 ± 8 units/mg) activities (see “Experimental Procedures”). To compare the oxidative and structural modifications undergone by the enzymes as a result of their bicarbonate-dependent peroxidase activities, the enzymes (25 μM) were incubated with hydrogen peroxide (1 mM), bicarbonate (50 mM), and DTPA (0.1 mM) in phosphate buffer (50 mM), pH 7.4, as described under “Experimental Procedures.” After a 1-h incubation, the overall consumption of hydrogen peroxide by the enzymes in the absence of bicarbonate was ~45% of that in its presence (0.40 ± 0.08 and 0.92 ± 0.07 mM, respectively). In parallel, similar amounts of hSOD1 were inactivated (~50%) but the amount of copper(I) ion released was slightly higher for the enzyme incubated in the absence than in the presence of bicarbonate (~30 and 20%, respectively, of the initial concentration of copper ions in hSOD1 (50 μM)). All of these results are in line with previously reported data for hSOD1<sup>WT</sup> (6, 36, 43).

Previous studies have also shown that hSOD1<sup>WT</sup> residues, in particular Trp<sup>32</sup> and His residues, are targets of the carbonate radical produced during the bicarbonate-dependent peroxidase activity, being oxidized to the corresponding hSOD1-Trp<sup>32</sup>- and hSOD1-His<sup>red</sup> radicals. These short-lived species decay to carbonylated products (3, 36, 44) and to the hSOD1-Trp-hSOD1 covalent dimer (30). Here, we show by spin-trapping experiments with DBNBS that hSOD1<sup>G93A</sup> is oxidized to hSOD1-Trp<sup>32</sup> similarly to the hSOD1<sup>WT</sup> enzyme in a process that was dependent on the presence of both, bicarbonate and hydrogen peroxide (Fig. 1A) (3, 43). Part of the produced hSOD1-Trp<sup>32</sup> recombined to produce a covalent hSOD1 dimer as monitored by reducing SDS-PAGE (Fig. 1B) (30). To confirm that the produced hSOD1<sup>G93A</sup> dimer was also constituted by 2 hSOD1 monomers cross-linked through a dityrosine bond, we isolated the covalent dimer band (~40 kDa) from gels similar to those shown in Fig. 1B and subjected it to tryptophan hydrolysis and LC-MS and MS/MS analysis (see “Experimental Procedures”). The tryptic digests of the hSOD1<sup>G93A</sup> dimer presented the peaks at m/z values of 933.48, 700.36, and
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FIGURE 1. Oxidation, dimerization, and carbonylation of hSOD1WT and hSOD1G93A resulting from their bicarbonate-dependent peroxidase activity. A, representative EPR spectra of DBNBS radical adducts produced from hSOD1 incubated under the specified conditions. The mixtures contained hSOD1 (30 μmol), bicarbonate (25 mM), DTPA (0.1 mM), and H2O2 (1 mM) in phosphate buffer (50 mM) adjusted to pH 7.4. After 10 min of incubation at 37 °C, the spectra were run at room temperature. The instrumental conditions were as follows: microwave power, 20 milliwatt; modulation amplitude, 2.0 G; sweep time, 41 s; time constant, 41 ms. B, representative image of SDS-PAGE gel of the specified reaction mixtures after 1 h of incubation at 37 °C stained with Coomassie Blue (B) or FTC (C). The mixtures containing hSOD1 (25 μmol), bicarbonate (50 mM), DTPA (0.1 mM), and H2O2 (1 mM) in phosphate buffer (50 mM) were adjusted to pH 7.4. All of the experimental details are described under "Experimental Procedures." The protein carbonyl index values are shown. The bands such as those shown in Fig. 1 were also subjected to non-reducing SDS-PAGE (Fig. 3). The dimeric peptide exhibited the missed trypsin cleavage and the ditryptophan cross-link is cleaved to produce an apparently monomeric peptide monoisotopic mass of 2797.47 Da, corresponding to the characteristic dimeric peptide (24ESNGPVKVGSKI36)2, with 3, 4, and 5 charges, respectively. The MS/MS sequencing of the peak at m/z 700.36 is shown in Fig. 2. This dimeric peptide exhibited the missed trypsin cleavage and the ditryptophan cross-link, which cleaved under MS/MS conditions to produce y fragments and the related fragments with 2 Da from their masses along fragments y5 to y13 of the y-series (Fig. 2) as previously reported for hSOD1WT (30, 36). Additionally, stated, during the MS/MS fragmentation of the dimeric peptide most of the ditryptophan cross-link is cleaved to produce an apparently non-modified Trp residue and a Trp residue missing 2 H atoms.

Next, we compared the overall production of carbonylated hSOD1, which can result from the decay of the hSOD1-Trp32-carboxy radical to o-formylkynurenine and kynurenine (3, 36, 44), and the decay of the hSOD1-His110 radicals to oxo-histidine (Table 1). Carbonylation of both hSOD1WT and hSOD1G93A were higher in the presence of bicarbonate than in its absence (Fig. 1C). Apparently, bicarbonate alone leads to some enzyme carbonylation (Fig. 1C). However, the background noted in the lanes corresponding to the enzymes incubated in phosphate buffer containing bicarbonate is an artifact of the method, probably arising from reversible addition of carbon dioxide, which equilibrates with bicarbonate to the protein.3 Indeed, LC-MS and MS/MS analyses of the trypsin hydrolysates of the enzymes treated with phosphate buffer plus bicarbonate showed that the enzymes were not oxidized (see Table 1). Additionally, hSOD1 incubated with phosphate buffer alone did not show staining with the FTC reagent (see Ref. 36 and also, below). Despite the background, it is clear that the higher consumption of hydrogen peroxide by hSOD1 in the presence of bicarbonate (6, 36) leads to higher oxidation of its residues in quantitative terms (Fig. 1C).

In qualitative terms, the only observed difference was the oxidation of hSOD1-Trp32 in the presence but not absence of bicarbonate. Indeed, isolation of the bands of oxidized monomers such as those shown in Fig. 1C, followed by trypsin hydrolysis and LC-MS and MS/MS analysis, showed that the oxidized monomer bands of both hSOD1WT and hSOD1G93A obtained in the presence of bicarbonate were composed of a mixture of non-oxidized and oxidized monomers. The latter presented tryptic peptides containing o xo-histidine at His53, His80, and His110, hydroxylated phenylalanine at Phe20 and Phe64, and N-formylkynurenine at Trp32; in the case of hSOD1WT, kynurenine32 was also present (Table 1). In the case of the enzymes incubated with hydrogen peroxide in the absence of bicarbonate, the modified monomers contained the same His and Phe residues oxidized but no oxidized Trp (Table 1). This result is in line with previous reports of hSOD1WT oxidation by hydrogen peroxide under different experimental conditions (10, 26, 43).

Taken together, the above results confirm that the bicarbonate-dependent peroxidase activities of hSOD1WT and hSOD1G93A are similar and cause oxidation of the enzymes to a greater extent than the bicarbonate-independent activity (Fig. 1) (3, 6, 36, 43). In qualitative terms, a major difference is the oxidation of the Trp32 residue of both enzymes, leading to covalently dimerized hSOD1 and hSOD1-N-formylkynurenine and hSOD1-kynurenine (Fig. 2; Table 1), which should contribute to the overall detection of carbonylated hSOD1 monomers (Fig. 1C). Next, the structural modifications of the enzymes caused by their bicarbonate-dependent peroxidase activity were examined.

Enzyme Unfolding—To examine structural changes in hSOD1WT and hSOD1G93A turned over in the presence of bicarbonate, the 1-h incubation samples and their controls were also subjected to non-reducing SDS-PAGE (Fig. 3A). The enzymes incubated with bicarbonate alone migrated primarily as the native dimer, whereas the enzymes incubated with hydrogen peroxide alone presented less defined native dimer bands accompanied by ill-defined bands of lower molecular weight, indicating unfolding of the enzymes to some extent. The enzymes that consumed hydrogen peroxide in the presence of bicarbonate presented even less well defined bands at the native dimer region, accompanied by ill-defined bands of lower and higher molecular weights than the native dimer (Fig. 3B).
FIGURE 2. ESI-Q-TOF MS/MS analysis of the dimeric tryptic peptide \((24\text{ESNGPVKVWGSIK}_{36})_2\) obtained from hSOD1\(^{G93A}\). The MS/MS sequencing of the peak at \(m/z\) 700.36, which corresponds to the characteristic peptide \((24\text{ESNGPVKVWGSIK}_{36})_2\) (monoisotopic mass 2797.47 Da) with 4 charges is shown. The inset displays the MS of the peptide with 4 charges (right side) and the expected peptide fragments of the \(y\)-series (left side). As previously demonstrated, the ditryptophan cross-link cleaves under ESI-Q-TOF MS/MS conditions to produce \(y\) fragments and the related fragments with minus 2 Da from their masses along fragments \(y_5\) to \(y_{13}\) of the \(y\)-series (30). The hSOD1\(^{G93A}\) spot corresponding to the covalent dimer produced under similar conditions as those of Fig. 1 was excised from the gel, digested with trypsin, and submitted to ESI-Q-TOF-MS and MS/MS analysis as described under “Experimental Procedures.”

TABLE 1

| MS and MS/MS characterization of the oxidative modifications of hSOD1 (WT, G93A, and W32F) resulting from its bicarbonate-dependent and -independent peroxidase activities |
|---|---|---|---|---|
| Each enzyme (25 μM) was incubated in phosphate buffer containing bicarbonate (50 mM) or hydrogen peroxide (1 mM) or bicarbonate (50 mM) plus hydrogen peroxide (1 mM) and for 1 h at 37 °C. The samples were submitted to SDS-PAGE and the protein spots corresponding to the hSOD1 monomer (approximately 20 kDa) and, when present, to the hSOD1 dimer (approximately 40 kDa) were excised from the gels, reduced, alkylated, digested with trypsin, and submitted to LC-MS and MS/MS analysis as described under “Experimental Procedures.” The enzymes extracted from all the spots were covered from residue 4 to 153 but only the peptides found oxidized were included in the table. The enzymes incubated with bicarbonate alone were also analyzed but their peptides did not show oxidation, presenting the expected theoretical mass value. |

| Modified peptides | Theoretical (MH)\(^{a}\) | H\(_2\)O\(_2\) monomer (MH)\(^{b}\) | Dimer (MH)\(^{b}\) | Modification | Modified hSOD\(^{a}\) |
|---|---|---|---|---|---|
| 24CDDGPVQKINIFRKDK\(^{e}\) | 1501.7 | 1517.7 | 1517.7 | Phe + 16 | WT, G93A, W32F |
| 24ESNGPVKVWGSIK\(^{b}\) | 1400.7 | 1432.7 | 2798.3 | Trp\(^{b}\) + 32 (NFK)\(^{d}\) | WT, G93A |
| 31WKSIZK\(^{c}\) | 3519.6 | 3535.6 | 3535.6 | His\(^{b}\) + 16; Phe\(^{b}\) + 16 | WT, G93A, W32F |
| 31WKSIZK\(^{d}\) | 3225.6 | 3251.6 | 3251.6 | His\(^{b}\) + 16 | WT, G93A, W32F |
| 31WKSIZK\(^{e}\) | 3720.8 | 3746.8 | 3746.8 | His\(^{b}\) + 16 | WT, G93A, W32F |
| 31WKSIZK\(^{f}\) | 2471.2 | 2497.2 | 2497.2 | His\(^{b}\) + 16 | WT, G93A, W32F |

\(^{a}\) Theoretical mass value of the non-modified peptide.

\(^{b}\) Experimental mass value obtained for the corresponding modified peptide found in hSOD1 extracted from the spot at 20 kDa (monomer) and 40 kDa (dimer), respectively, and characterized by MS/MS analysis.

\(^{c}\) The enzymes that showed the specified modification are listed.

\(^{d}\) NFK, Kyn, and W-W refers to \(N\)-formylkynurenine, kynurenine and the ditryptophan cross-linked dimer (ESNGPVKVWGSIK), respectively.

\(^{e}\) Peptides oxidized either at His\(^{b}\) or at Phe\(^{b}\) were characterized.
A. This result indicated the presence of low amounts of partially unfolded forms and considerable amounts of low-order oligomers of both hSOD1WT and hSOD1G93A. Parallel analysis by CD spectroscopy showed that the overall secondary structure of hSOD1 was not much affected after a 1-h incubation (Fig. 3B). Indeed, estimations of the secondary structure contents showed that no more than 20% of /H9252-sheet was lost and 10% of random coil was gained. Unfolding was also indicated by competitive ELISA experiments with a conformation-specific antibody (USOD) targeted against hSOD1 residues 42–48 (Fig. 3C) (10, 18, 39). Surprisingly, the enzymes assayed after the bicarbonate-dependent peroxidase activity had a higher affinity for the USOD antibody than the chemically unfolded hSOD1. These results were in apparent contrast with those obtained by CD spectroscopy (Fig. 3B). However, the CD spectra of the enzymes were run immediately after the 1-h incubation and should reflect a short run unfolding that is apparently modest (Fig. 3B). In the ELISA experiments, binding to the USOD antibody is preceded by an overnight incubation of the enzymes to coat the plates. This long incubation and interactions with the plate are likely responsible for the increased unfolding of the enzymes after the bicarbonate-dependent peroxidase activity.

**Enzyme Oligomerization and Aggregation**—Next, we examined the structural alterations of the enzymes by DLS after 1 h and thereafter for several days. Extensive DLS analysis showed that incubation of hSOD1WT and hSOD1G93A with bicarbonate for 1 h at 37 °C presented a nearly single exponential autocorrelation function, demonstrating that the system is monomodal. An average hydrodynamic diameter of ~6 nm was obtained from the exponential fit of the autocorrelation function, a value that is in the range of the native hSOD1 dimer (Fig. 4) (9, 45). After 1 h, the sample containing the enzyme and 1 mM hydrogen peroxide also showed a single exponential autocorrelation function but with a slightly increased hydrodynamic diameter of ~7 nm. Although it is small, this 1-nm difference was observed in several experiments and suggests unfolding into some extent of the native hSOD1 dimer, in agreement with the non-reducing SDS-PAGE, CD, and USOD antibody data (Fig. 3). Interestingly, the analysis of light scattering data of hSOD1 in the presence of hydrogen peroxide and bicarbonate showed a monomodal size distribution with an average hydrodynamic diameter of ~6 nm.
dynamic diameter of 10 nm (Fig. 4). This value represents an increase of ~2.8 times the surface area compared with the native dimer. Such an increase in the surface area is suggestive of a loose tetramer, which is consistent with the ill-defined bands of molecular weight higher than that of native hSOD1 dimer observed in the non-reducing SDS-PAGE gel (Fig. 3A). These structural modifications after 1 h of incubation were similar for both hSOD1WT and hSOD1G93A (Fig. 4).

Thereafter, all of the samples were maintained tightly closed in the dark at 25 ± 2 °C and re-analyzed in 24-h intervals (Fig. 5). In the case of the enzymes treated with hydrogen peroxide and bicarbonate, broad monomodal size distributions were seen in up to 48 h (Fig. 5, A and B). However, at 72 h, the fit of the autocorrelation function of samples provided bimodal size distributions, with the original mode preserved and a new mode of particles with higher sizes, centered near to 450 nm. From then on, the population of the higher aggregates started to increase, at the expense of the population of smaller aggregates. Additionally, a systematic increase in the mode center with the incubation time was observed, from 2.8 ± 0.11 (Fig. 5, A and B) to 5 ± 1.01 nm (Fig. 5, C and D) (41). The $t_{1/2}$ values obtained for hSOD1WT and hSOD1G93A were similar (80 ± 5 and 73 ± 1 h, respectively), demonstrating that both enzymes have 50% of aggregation completed at nearly the same time. Although the $t_{1/2}$ values for hSOD1WT and hSOD1G93A are very similar, a difference in the $\Delta t$ values between the two enzymes was observed (9.3 ± 2.9 and 10.5 ± 2.7 h, respectively), reflecting a tendency of the mutant enzyme to have a slightly higher rate constant of aggregation ($k = 1/\Delta t$) ($k = 0.16 ± 0.03 h^{-1}$) than the wild type enzyme ($k = 0.11 ± 0.03 h^{-1}$). The higher $\Delta t$ value of hSOD1WT in comparison to that observed for hSOD1G93A compensates for the small difference in $t_{1/2}$ between the enzymes and leads the aggregation to start at the same time, as attested by the similar lag time ($t_{lag} = t_{1/2} - 2\Delta t$) values for hSOD1WT and hSOD1G93A (61 ± 10 and 61 ± 5 h, respectively).

We then examined the nature of hSOD1 aggregates by the Congo Red (CR) assay and transmission electronic microscopy (Fig. 6). In the case of the CR assay, the enzymes in the plateau phase of aggregation (96 h incubation) were added to a 20 μM solution of CR, and the visible spectra were scanned. None of the samples promoted the red shift of the CR absorbance peak that is characteristic of CR binding to amyloid (Fig. 6A) (9, 10). In agreement, transmission electronic microscopy analysis of both hSOD1WT and hSOD1G93A showed that enzymes in the plateau phase of aggregation (96 h) presented irregular particles with a high degree of aggregation but no clear amyloid fibers (Fig. 6B). Accordingly to the DLS experiments (Fig. 5, B and D), the enzymes incubated with bicarbonate alone did not show aggregates (Fig. 6B). Taken together, these results show that the hSOD1 aggregates resulting from its bicarbonate-dependent peroxidase activity are non-amyloid similarly to the protein aggregates found in ALS patients and animal models (9, 10).

**Trp32 Oxidation Is Critical for Triggering hSOD1 Aggregation**—To determine which oxidative modification of hSOD1 caused by its bicarbonate-dependent activity was critical for triggering the observed aggregation, we performed a series of
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control experiments. First, we compared the behavior of hSOD1WT with that of bovine SOD1 (hSOD1). The latter has been the most SOD1 studied with regard to peroxidase activity, which is similar to that of hSOD1WT (36, 43). The enzymes have 83% homology, but hSOD1 does not possess Trp32, which is unique for simian SOD1s. In addition to Phe20 and Phe64 that are present in both enzymes, hSOD1 has another aromatic residue, Tyr108, whereas Thr32 substitutes for Trp32 in hSOD1. The bicarbonate-dependent peroxidase activity of hSOD1 was comparable with those of the human enzymes (when normalized to copper ion contents) (data not shown) but did not produce radical adducts detectable with DBNBS or a covalent dimer as previously reported (3). Additionally, hSOD1 did not aggregate up to 120 h after the bicarbonate-dependent peroxidase activity ended as monitored by DLS experiments (data not shown). These results argue against a major role for the oxidation of the free thiols of SOD1 (hSOD1 and bSOD1 have 2 (Cys6 and Cys111) and 1 (Cys6) free thiols, respectively) in triggering hSOD1 aggregation. This hypothesis was confirmed by the fact that hSOD1 exposed to hydrogen peroxide alone does not aggregate after up to 174 h under our experimental conditions (Fig. 5, C and D). Additionally, control experiments showed that previous alkylation of hSOD1WT with 2-iodoacetamide does not prevent hSOD1 dimerization and aggregation (data not shown). These results suggest a critical role for the oxidation of the hSOD1-Trp32 residue in hSOD1 aggregation and were also supported by experiments in the presence of tempol. This cyclic nitroxide marginally affects the bicarbonate-dependent peroxidase activity of hSOD1WT but inhibits its ensuing oxidation to N-formylkynurenine, kynurenine, and covalent hSOD1 dimer by recombining with the hSOD1-Trp32 radical (36). Total inhibition required a tempol concentration equal to that of hSOD1 monomers. Here, we showed that this concentration of tempol inhibited hSOD1WT aggregation for up to 120 h (data not shown). Additionally, we showed that tempol concentrations lower than those of hSOD1 monomers partially inhibited enzyme unfolding as followed by reaction with the USOD antibody (Fig. 2C).

Last, to confirm the role of the oxidation of the Trp32 residue in triggering hSOD1 aggregation, we obtained the hSOD1W32F mutant as described under “Experimental Procedures.” This mutant displayed similar dismutase and bicarbonate-dependent peroxidase activities of hSOD1WT and hSOD1G93A but was not oxidized to radicals detectable with the spin trap DBNBS (Fig. 7A) (3). In addition, the mutant was not oxidized to the covalent dimer (Fig. 7B) and did not show aggregation for up to 120 h (Fig. 7C) despite being carbonylated, most likely due to the oxidation of His residues (Table 1).

DISCUSSION

Here we compared the oxidative and structural modifications undergone by hSOD1WT and its ALS-associated mutant G93A as a result of their bicarbonate-dependent and -independent peroxidase activities (Figs. 1–6, Table 1). Only in the presence of the biologically ubiquitous bicarbonate buffer are the Trp32 residues of both enzymes oxidized in a considerable and similar extent to hSOD1-derived tryptophan radicals (hSOD-Trp32) (Fig. 1) (3, 30, 36, 43). These radicals decay to hSOD1-N-formylkynurenine and hSOD1-kynurenine or to a hSOD1 covalent dimer cross-linked by a ditryptophan bond (hSOD1-Trp-Trp-hSOD1) (Figs. 1 and 2, Table 1) (3, 30, 36) causing hSOD1 unfolding, oligomerization, and non-amyloid aggregation (Figs. 3–6). The latter processes were inhibited by tempol (Fig. 3), which recombines with the hSOD1-Trp32 radical (36), and did not occur in the absence of bicarbonate (Figs. 3–6), or in the presence of bicarbonate with enzymes that lack the Trp32 residue, such as hSOD1 (see “Results”) and the hSOD1W32F mutant (Fig. 7, Table 1). These results argue for a role of the oxidation products of the hSOD-Trp32 residue in triggering the non-amyloid aggregation of hSOD1 under our experimental conditions.
The aggregation process was slow as expected from a complex process that requires specific intermolecular interactions (9, 45–47). The $t_{1/2}$ values of aggregation obtained for hSOD1WT and hSOD1G93A were similar (80 $\pm$ 5 and 73 $\pm$ 1 h, respectively) (Fig. 5). The mutant showed a tendency to have a higher rate constant of aggregation ($k = 0.16 \pm 0.03$ h$^{-1}$) than the wild-type enzyme ($k = 0.11 \pm 0.03$ h$^{-1}$) but the difference was not statistically significant.

Although our results cannot establish which of the oxidative modifications of hSOD1-Trp32 triggers the aggregation process (Table 1), the covalent hSOD1 dimer is likely to play the major role. The roles of SOD1-N-formylkynurenine and hSOD1-kynurenine are probably less important because other hSOD1-carbonylated products did not cause detectable hSOD1 aggregation under our experimental conditions (compare Figs. 1C, 5, and 7, B and C). Interestingly, after a 1-h incubation, a time in which most hydrogen peroxide is consumed and most of the covalent dimer is produced (6, 30, 36, 43), the DLS data showed that hSOD1 is primarily present as 10-nm diameter hydrodynamic particles compared with the 6 nm of the native enzyme (Fig. 4) (9, 10, 45). This result is suggestive of a loose tetramer, which is expected to result from the formation of the ditryptophan cross-link between 2 native non-covalent hSOD1 dimers (Fig. 8). Therefore, it is plausible to consider that the ditryptophan cross-link weakens the non-covalent bonds between the hSOD1 monomers, facilitating unfolding, and provides hydrophobic surfaces acting as seeds for hSOD1 aggregation. The inset shows a stereo view of the (5)-ditryptophan cross-link in a dipeptide.

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Other oxidative post-translational modifications of hSOD1 are receiving increasing attention in the ALS literature, such as oxidation of His residues (see, for instance Refs. 10, 18, 21, and 26) glutathionylation at Cys111 (48–50), and disulfide scrambling producing disulfide-linked oligomers (51). These oxidative modifications lead to hSOD1 aggregation, as is the case of the oxidation products of hSOD1-Trp32 (this work). The particular relevance of the latter oxidation products arises from the fact that Trp32 residues are unique to simian SOD1s. In this respect, it is worth recalling early studies attempting to prove a viral origin for ALS (52). These investigators injected homogenates of brain and spinal cord tissues from patients who died of the disease intracranially into mice and guinea pigs, but failed to transmit the disease. By injecting the homogenates into monkeys, they were able to reproduce ALS-like symptoms in the animals after a few years. The hypothetical virus-like agent was transferred to other monkeys without attenuation but remained non-pathogenic for mice and other laboratory animals (52). It is feasible that the virus-like agents were non-native hSOD1 forms present in the patient interacting with mon-
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key SOD1 (17, 53, 54), which also possesses the Trp^{32} residues that may become oxidized as the animal ages.

A role for oxidative post-translational modifications of hSOD1 in ALS pathology is an attractive hypothesis to explore based on several lines of evidence. The late-onset of ALS suggests a connection to the aging process rendering the organism less able to cope with an ever present threat that was previously tightly regulated, such as the homeostatic level of reactive oxygen species (55–57). The deregulation of these species during ALS, and hence, oxidative stress, is supported by the consistent detection of oxidative damage in proteins, lipids, and DNA within the pathologically affected areas of the central nervous system of ALS patients and animal models (13). Conversely, hSOD1^{WT} and several of its ALS-associated mutants are remarkably stable in the native state, in contrast to other proteins implicated in neurodegenerative diseases, such as the A\beta peptide in Alzheimer disease and PrP in the prion diseases (10).

Therefore, hSOD1 oxidation may be crucial for triggering its conversion to non-native hSOD1 forms, such as those that have been detected in affected areas of the central nervous system of ALS patients (18–24). Such a view is supported by recent reports showing that over-expression of hSOD1^{WT} in mice causes ALS (58) and that over-oxidized/carbonylated hSOD1^{WT} is present in lymphocytes of sporadic ALS patients (23).

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