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Original Contribution

A ditryptophan cross-link is responsible for the covalent dimerization of human superoxide dismutase 1 during its bicarbonate-dependent peroxidase activity

Danilo B. Medinas [a,*], Fabio C. Gozzo [b], Luiz F.A. Santos [b], Amadeu H. Iglesias [b], Ohara Augusto [a,*]

[a] Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, 05513-970 São Paulo, SP, Brazil
[b] Departamento de Química Orgânica, Instituto de Química, Universidade Estadual de Campinas, Campinas, SP, Brazil

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ABSTRACT

Unlike intermolecular disulfide bonds, other protein cross-links arising from oxidative modifications cannot be reversed and are presumably more toxic to cells because they may accumulate and induce protein aggregation. However, most of these irreversible protein cross-links remain poorly characterized. For instance, the antioxidant enzyme human superoxide dismutase 1 (hSod1) has been reported to undergo non-disulfide covalent dimerization and further oligomerization during its bicarbonate-dependent peroxidase activity. The dimerization was shown to be dependent on the oxidation of the single, solvent-exposed Trp^{32} residue of hSod1, but the covalent dimer was not isolated nor was its structure determined. In this work, the hSod1 covalent dimer was isolated, digested with trypsin in H_2O and $H_2^{18}O$, and analyzed by UV–Vis spectroscopy and mass spectrometry (MS). The results demonstrate that the covalent dimer consists of two hSod1 subunits cross-linked by a ditryptophan, which contains a bond between C3 and N1 of the respective Trp^{32} residues. We further demonstrate that the cross-link cleaves under usual MS/MS conditions leading to apparently unmodified Trp^{32}, partially hinders proteolysis, and provides a mechanism to explain the formation of hSod1 covalent trimers and tetramers. This characterization of the covalent hSod1 dimer identifies a novel oxidative modification of protein Trp residues and provides clues for studying its occurrence in vivo.

The abundance of proteins in cells and extracellular fluids argues for their importance as biological targets of reactive oxygen- and nitrogen-derived species. Indeed, it has been estimated that proteins are likely to scavenge 50–75% of the reactive species produced in vivo [1]. As a result, the proteins are reversibly or irreversibly oxidized. Reversible protein oxidation usually involves Cys residues and modulates a variety of protein functions, including their roles in cell signaling [2,3]. Irreversible protein oxidation involves covalent modifications other than the formation of disulfide bonds, such as protein cleavage, carbonylation, nitration, hydroxylation, halogenation, and protein cross-linking with other proteins, lipids, and nucleic acids [1,4–11]. To guarantee cell homeostasis, oxidized proteins are targeted to degradation by the proteasome [12,13]. Under pathological conditions involving oxidative stress, however, the levels of overoxidized and cross-linked proteins build up because these species are poor substrates for the proteasome. This may lead to protein aggregation, a hallmark of neurodegenerative diseases [1,4–13]. Recent studies indicate that small oligomeric intermediates in the aggregation process may be the most neurotoxic to the central nervous system (reviewed in [14]). Despite the potential of irreversible protein–protein cross-links constituting the intermediates of protein aggregation, the chemical nature of these bonds remains largely uncharacterized except for a few of them [4–7,15–18].

Recently, the covalent dimerization and further oligomerization of the antioxidant enzyme human superoxide dismutase 1 (hSod1) have been reported during its bicarbonate-dependent peroxidase activity [19–21]. These processes are specific for the human enzyme and could intermediate the buildup of the toxic protein deposits found in human neurodegenerative diseases. Based on spin trapping and site-directed mutagenesis experiments, Kalyanaraman and co-workers demonstrated that the covalent dimerization of hSod1 completely depends on the oxidation of its single, solvent-exposed tryptophan residue (Trp^{32}) by the enzymatically produced carbonate radical [19–21]. The well-known tryptophan oxidation products *N*-formylkynurenine and kynurenine [22] were detected in oxidized hSod1 and suggested to be precursors of the covalent cross-link [19–21]. Nonetheless, the covalent hSod1 dimer was not isolated nor was its structure determined.

Abbreviations: hSod1, human superoxide dismutase 1; DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*′-pentaacetic acid.

* Corresponding authors. D.B. Medinas is to be contacted at Department of Chemical and Systems Biology, Stanford University School of Medicine, Stanford, CA 94305, USA. Augusto, Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, Caixa Postal 26077, CEP 05513-970 São Paulo, SP, Brazil. Fax: +55 11 3091 2186.
E-mail addresses: medinas@stanford.edu (D.B. Medinas), oaugusto@iq.usp.br (O. Augusto).

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We reasoned that the full characterization of the covalent hSod1 dimer produced during its bicarbonate-dependent peroxidase activity was likely to provide new information about the oxidative modifications allowed for Trp residues in proteins [9–11]. Therefore, the covalent hSod1 dimer was isolated, hydrolyzed, and analyzed by UV–Vis and mass spectrometry. The results demonstrated that the hSod1 covalent dimer is constituted by two hSod1 subunits bound by a novel ditryptophan cross-link (hSod1–Trp–Trp–hSod1). Properties of the cross-link are described, and the possibilities of its occurrence in vivo are addressed.

Experimental procedures

Materials

Mass spectrometric grade trypsin (Gold) was purchased from Promega (Madison, WI, USA). Isotope-labeled water ($H_2^{18}O$, 97%) was obtained from ISOTEC (Miamisburg, OH, USA). All other reagents were from Sigma, Merck, Fluka, Invitrogen, or USB and were of analytical grade or better. All solutions and buffers were prepared with distilled water purified in a Millipore Milli-Q system.

Human recombinant Sod1 expression and purification

The enzyme was expressed in *Escherichia coli* and purified as previously described [23].

Preparation and purification of the hSod1 oxidized monomer and covalent dimer

Human Sod1 (20 μM in monomers) was incubated with 1 mM H_2O_2 in 200 mM $NaHCO_3/CO_2$ (equilibrated with 50% CO_2 balanced with air) buffer containing 100 μM DTPA (pH 7.4) with stirring for 2 h at room temperature (25 ± 2 °C) to produce the hSod1 covalent dimer [15–17]. As control, the enzyme was incubated under the same experimental conditions without the addition of H_2O_2 and referred to here as native hSod1. After the incubation, the sample was dried in a vacuum centrifuge and solubilized in a denaturing buffer consisting of 10 mM Tris–HCl with 6 M guanidine hydrochloride, 10 mM DTPA, and 30 mM dithiothreitol (pH 8.0) under reduced oxygen tension. The sample was incubated for 4 h at 37 °C to reduce the disulfide bonds. Next, 150 mM sodium iodoacetate was added and the sample incubated for 3 h in the dark at 37 °C to alkylate the free thiols. After the alkylation step, the buffer was changed to 50 mM Hepes containing 150 mM NaCl (pH 7.0) by ultrafiltration, and then the protein was concentrated. The concentrated protein was subjected to size-exclusion chromatography (Tosohaas TSK-Gel, G-3000 SWXL, 300 × 7.8 mm i.d., particle size 5 μm), which produced two major peaks that consisted of the hSod1 covalent dimer and oxidized monomer. These fractions were collected and concentrated by ultrafiltration in 50 mM NH_4HCO_3 buffer (pH 8.0).

Digestion of hSod1 with trypsin and peptide analysis by HPLC/UV–Vis and HPLC/mass spectrometry with electrospray ionization (MS–ESI)

Proteolysis with trypsin was carried out in 50 mM NH_4HCO_3 containing 2 mM $CaCl_2$ (pH 8.0) at 37 °C for 20 h. A substrate/trypsin ratio of 50 was employed for all samples (native hSod1, oxidized monomer, and covalent dimer). For the digestion in $H_2^{18}O$ medium, the protein sample was divided into two aliquots of 50 μg each, dried in a vacuum centrifuge, and solubilized in 50 μl of H_2O or 50 μl of $H_2^{18}O$. Then, 1 μl of 2.65 M NH_4HCO_3, 1 μl of 106 mM $CaCl_2$, and 1 μl of trypsin stock solution (1 μg/μl) were added to the protein samples. The $H_2^{18}O$ medium had 91% enrichment after the addition of the NH_4HCO_3, $CaCl_2$, and trypsin solutions. The hydrolysates of hSod1 samples were subjected to reverse-phase chromatography in an HPLC system (Shimadzu, Tokyo, Japan) with a C-18 μBondapack Waters column (300 × 3.9 mm i.d., particle size 10 μm) employing a binary gradient with 0.1% trifluoroacetic acid as eluent A and 0.1% trifluoroacetic acid in 90% acetonitrile as eluent B. The gradient used consisted in 0% B over 15 min, 0 to 26% B over 52 min, 26% B over 10 min, 26 to 40% B over 28 min, 40 to 80% B over 10 min, 80% B over 5 min, 80 to 0% B over 5 min, and 0% B over 5 min and a flow rate of 1 ml/min. The hydrolysates were analyzed by UV–Vis spectroscopy using a diode-array detector (SPD-M20A, Shimadzu) and by mass spectrometry using a triple-quadrupole mass spectrometer (Micromass, Manchester, UK) with the electrospray ionization source in the positive ion mode. The instrumental conditions were set to sample cone voltage at 20, 50, and 90 V and extractor cone voltage at 5 V. The capillary voltage was set at 4.50 kV, the source temperature was 100 °C, and the desolvation temperature was 200 °C.

Mass spectrometry analysis of hSod1 hydrolysates by ESI-Q-TOF MS/MS

The digestion products of the hSod1 covalent dimer were analyzed by LC–MS/MS in a Synapt HDMS (Waters, Milford, MA, USA) coupled online to a nanoAcquity UPLC system. The digests were loaded and desalted using a C-18 Waters Symmetry column (20 mm × 180 μm, particle size 5 μm). After the desalting step, the sample was subjected to reverse-phase chromatography using a C-18 Waters BEH130 column (100 mm × 100 μm, particle size 1.7 μm) employing a binary gradient with 0.1% formic acid as eluent A and 0.1% formic acid in acetonitrile as eluent B. The gradient used consisted in 3 to 30% B over 20 min, 30 to 70% B over 20 min, 70% B over 10 min, 70 to 3% B over 1 min, and 3% B over 9 min and a flow rate of 1.0 μl/min. Typical operating conditions of

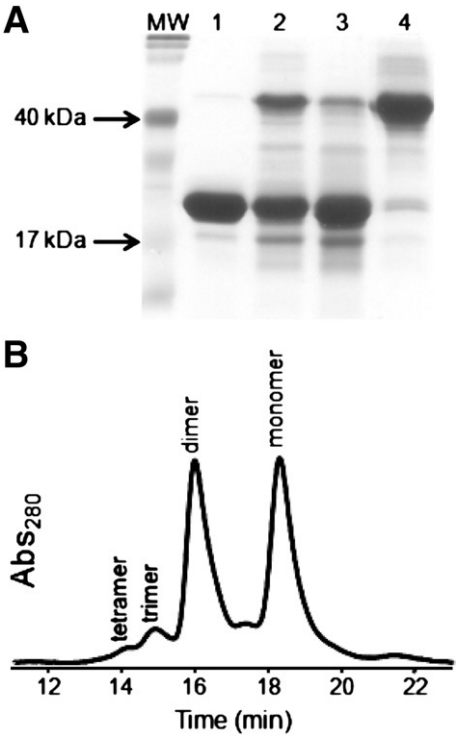

Fig. 1. Covalent oligomerization of hSod1 during its bicarbonate-dependent peroxidase activity. (A) SDS–PAGE analysis of native hSod1 (lane 1), hSod1 after incubation with H_2O_2 in $NaCO_3/CO_2$ buffer (lane 2), size-exclusion chromatography fractions of hSod1 oxidized monomer (lane 3), and covalent dimer (lane 4). MW refers to the molecular weight marker. (B) Size-exclusion chromatogram of turned-over hSod1 after treatment with denaturing, reducing, and alkylating agents. Human Sod1 (20 μM in monomers) was incubated with 1 mM H_2O_2 in 200 mM $NaHCO_3$ (50% CO_2/50% air) buffer containing 100 μM DTPA at pH 7.4 for 2 h at 25 °C. Then, the protein was subjected to a denaturing/reducing/alkylating treatment as described under Experimental procedures.