Free Radical Biology and Medicine 94 (2016) 161–173

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Distinct oxidative cleavage and modification of bovine [Cu– Zn]-SOD by an ascorbic acid/Cu(II) system: Identification of novel copper binding site on SOD molecule

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ARTICLE INFO

Article history:
Received 25 November 2015
Received in revised form
21 January 2016
Accepted 26 January 2016
Available online 10 February 2016

Keywords:
SOD
Metal catalyzed oxidation
Copper
Ascorbic acid
Carbonylation
Mass spectrometry

ABSTRACT

We investigated the combined effect of ascorbate and copper [Asc/Cu(II)] on the integrity of bovine [Cu–Zn]-superoxide dismutase (bSOD1) as a model system to study the metal catalyzed oxidation (MCO) and fragmentation of proteins. We found Asc/Cu(II) mediates specific cleavage of bSOD1 and generates 12.5 and 3.2 kDa fragments in addition to oxidation/carbonylation of the protein. The effect of other tested transition metals, a metal chelator, and hydrogen peroxide on the cleavage and oxidation indicated that binding of copper to a previously unknown site on SOD1 is responsible for the Asc/Cu(II) specific cleavage and oxidation. We utilized tandem mass spectrometry to identify the specific cleavage sites of Asc/Cu(II)-treated bSOD1. Analyses of tryptic- and AspN-peptides have demonstrated the cleavage to occur at Gly31 with peptide bond breakage with Thr30 and Ser32 through diamide and α-amidation pathways, respectively. The three-dimensional structure of bSOD1 reveals the imidazole ring of His19 localized within 5 Å from the α-carbon of Gly31 providing a structural basis that copper ion, most likely coordinated by His19, catalyzes the specific cleavage reaction.

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1. Introduction

The metal catalyzed oxidation (MCO) of proteins introduces carbonyl moieties into protein side chains, and under certain conditions, causes fragmentation of the protein. MCOs have been proposed to play a role in oxidative stress, disease and aging [1,2]. *In vivo*, protein carbonylation has often been utilized as a hallmark of ROS-mediated protein oxidation as the carbonyl modifications are irreversible and are introduced directly by MCO or indirectly through lipid peroxidation or advanced glycation end product pathways [3–8]. *In vitro*, therapeutic proteins are prone to oxidative modification during manufacturing, processing, and storage that may in turn lead to degradation/fragmentation, aggregation, and immunogenicity [9–11]. While a number of studies have shown the effects of oxidative modifications such as methionine and cysteine oxidation on protein integrity, activity, and quality [12–15], little is known about the effect of oxidative carbonylation on therapeutic proteins. Previously, we had shown that the presence of trace amounts of transition metal Fe(II), H_2O_2, and ascorbic acid induced significant levels of carbonylation in proteins under moderate storage conditions [16]. In order to gain further insight and assess analytical methodologies characterizing MCO-mediated oxidative modification, carbonylation, and protein degradation, we investigated bovine [Cu–Zn]-superoxide dismutase (bSOD1) treated with ascorbic acid/Cu(II) as a model system. bSOD1 was chosen because it is a biochemically stable metalloenzyme, requiring the Cu(II) and Zn(II) ions for catalytic activity and protein stability, respectively [17–19]. The sites of protein oxidation and fragmentation by MCO have been proposed to be localized proximal to the metal binding sites [20,21]. In addition, mutations in SOD1 have been identified to be responsible for ~10–20% of the cases of the familial form of amyotrophic lateral sclerosis (fALS). Over 150 separate mutations scattered throughout the SOD1 molecules have been shown to induce ALS in an autosomal dominant fashion [22]. While the mechanism of pathology induced by mutant SOD1 is yet to be clarified, the misfolding and aggregation of mutant SOD1 proteins has been proposed as the primary biochemical basis underlying the pathogenesis of fALS

Abbreviations: bSOD1, bovine [Cu–Zn]-superoxide dismutase; MCO, metal catalyzed oxidation; ALS, amyotrophic lateral sclerosis; ROS, reactive oxygen species; Asc, sodium ascorbate; LC–ESI-Q-TOF-MS, liquid chromatography–electrospray ionization-quadrupole-time of flight-mass spectrometry; DTPA, diethylene-triamine-penta-acetic acid; CBB, Coomassie Brilliant Blue

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http://dx.doi.org/10.1016/j.freeradbiomed.2016.01.020
0891-5849/Published by Elsevier Inc.

[23–25]. Protein aggregation and inclusions are a common pathological hallmark of a number of neurodegenerative diseases such as Alzheimer's, Huntington's, and Parkinson's [26,27]. Furthermore, oxidation of free cysteine or histidine residues in wild type SOD1 or the apoenzyme lacking both metal ions were shown to form oligomers, implicating involvement of wild type SOD1 oxidation as a possible factor for sporadic ALS which constitutes the majority of ALS cases [28–31].

Ascorbic acid, Cu(II), and oxygen from air are widely used to model MCO systems (1). Protein oxidation and fragmentation by MCO takes place in close proximity to the metal binding site as a consequence of extreme reactivity and the short life of the hydroxyl radical generated. Thus, MCO has been utilized to identify the metal binding sites of a number of proteins [32–35].

The comparative analyses of bSOD1 oxidized by ascorbic acid/Cu(II) and H_2O_2 described here, demonstrate a distinct pattern of protein fragmentation and effects on protein activity that were not previously recognized. Identification and characterization of the cleavage sites and the nature of oxidative modification unique to each reactive oxygen species (ROS) provide further insight into how proteins are modified by different oxidants, how sites of modification are related to the protein structure, and how oxidative modifications impact protein integrity, quality, and activity.

2. Experimental procedures

2.1. Materials

Unless noted otherwise, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Sigma supplied recombinant bovine SOD1 (Cat No. S9697) and catalase (Cat No. C9322) were used for structure-function analyses. Sequencing grade trypsin and Asp-N protease were obtained from Promega (Madison, WI).

2.2. bSOD1 solution and activity

The lyophilyzed bSOD1 powder was dissolved in 20 mM Tris–HCl at pH 7.6. This was followed by buffer-exchange using a centrifuge filter, Amicon Ultra-0.5 MWCO 10K (Millipore, Billerica, MA) to remove trace amounts of free metal ions in the bSOD1 lyophilized powder. Using BSA as a standard, the protein concentration was determined by a modified Lowry assay (DC™ Protein Assay kit, Bio-Rad, Hercules, CA) and then adjusted to 1.6 mg/ml (50 μM) with the same buffer. It was then stored at −80 °C. Activity was determined using an assay kit from Cayman Chemical (Ann Arbor, MI) according to the manufacturer's instructions.

2.3. bSOD1 treatment

The bSOD1 protein (final concentration 8 – 10 μM in 10 mM Tris–HCl, pH 7.6) was treated with the indicated concentration of additive in a 10 – 15 μl final volume for 30 min at 37 °C. When the entire reaction mix was analyzed by SDS-PAGE, 4 × LDS sample buffer (Life Technology, Grand Island, NY) was added directly and analyzed by SDS-polyacrylamide gel. Alternatively, the reaction was halted by addition of DTPA and/or catalase to achieve a final concentration of 100 μM and 5 ng/μl, respectively.

2.4. SDS-PAGE and quantitation of protein fragments

The bSOD1 reaction mix in LDS sample buffer with or without DTT (final 50 mM) were heated at 75 °C for 10 min and analyzed on a 4 – 12% NuPAGE gel in Bis–Tris buffer using MES running buffer (Life Technologies, Grand Island, NY). After electrophoresis, the gel was fixed with 40% methanol/10% acetic acid, stained with SimplyBlue Safestain Coomassie Brilliant Blue (CBB) (Life Technologies), then de-stained with water. Protein in the bands was quantified utilizing the 700 nm channel of the Odyssey infrared imaging system (LI-COR, Lincoln, NE) and Image Studio software.

2.5. Carbonyl detection by western blot immunodetection

Western blot detection of the carbonyl moiety on bSOD1 was performed as described [36]. In brief, the carbonyl in 0.4 μg of bSOD1 reaction mix were derivatized with 5 mM dinitrophenylhydrazine in 1 N HCl and 3% SDS for 10 min at room temperature, and then neutralized by the addition of 1 M Tris-base. After separation on a 4 – 12% NuPAGE gel, the dinitrophenyl-derivatized bSOD1 was transferred to PVDF membrane, hybridized with goat anti-dinitrophenyl (1:2000 dilution) (Bethyl Laboratories, Montgomery, TX) and donkey anti-goat secondary antibody IRDYe800CW conjugated (LI-COR, Cat No. 926-32214) and visualized with the Odyssey on the 800 nm channel.

2.6. Carbonyl quantitation by ELISA

The 0.02 μg dinitrophenyl-derivatized bSOD1 reaction mix was used for quantification by ELISA as recently described [16].

2.7. Reduction and alkylation and enzymatic digestion

Control (untreated) or Asc/Cu(II) treated bSOD was reduced with 5 mM DTT in 5 M guanidine–HCl at 37 °C for 30 min, alkylated with 10 mM iodoacetamide (IAM) at 37 °C for 30 min, and excess IAM was quenched by addition of 20 mM DTT. The reaction mix was desalted by binding the bSOD1 to a C18-tip column (Thermo-Pierce) followed by elution with 0.1% formic acid/50% acetonitrile according to the instructions of the manufacturer. For analysis of non-digested bSOD1, the eluate was adjusted to 4 pmol/ml and used for LC–MS analysis. For enzyme digestion, the bSOD1 eluate was dried *in vacuo*, dissolved in 60 μl of 25 mM ammonium bicarbonate buffer at a protein concentration of 10 pmol/μl. 30 μl of the solution was used for trypsin or Asp-N digestion at a substrate/enzyme ratio of 1:40 or 1:30, respectively. After 24 h of digestion, formic acid was added to a final concentration of 0.1% and 5 μl of digests were analyzed.

2.8. Liquid chromatography–mass spectrometry (LC–MS) analysis

All LC–MS and tandem MS (MS/MS) analyses were conducted on an Agilent 1260 HPLC-Chip nano-electrospray-ionization 6520 Q-TOF MS system (LC–ESI-Q-TOF-MS). Solvent A was 0.1% formic acid in water and Solvent B was 0.1% formic acid in 95% acetonitrile. Mass correction was enabled during the run using internal reference ions with masses of 299.2945 and 1221.9906 Da. Intact protein mass measurement was performed using an Agilent 43 mm 300 Å C8 chip with a 40 nL trap column (G4240-63001 SPQ105). The bSOD1 samples were diluted to 130 ng/μl (4 μM) in 0.1% formic acid, and at least 4 pmol ($\geq$ 1 μl) was injected onto the trap column of the C8 chip at a flow rate of 2.5 μl/min in 100% Solvent A, then eluted at 0.5 μl/min with a linear gradient of 10–100% Solvent B for 18 min and held for an additional 4 min. The Q-TOF capillary voltage was 1890 V and that of the fragmentor was 225 V. The nitrogen gas temperature was 350 °C with a flow rate of 9 L/min. Data were analyzed with Agilent MassHunter and BioConfirm software (version B.05.00).