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Short communication

Hydrogen peroxide induce modifications of human extracellular superoxide dismutase that results in enzyme inhibition

Randi H. Gottfredsen [a], Ulrike G. Larsen [a], Jan J. Enghild [b], Steen V. Petersen [a,*]

[a] Department of Biomedicine, Aarhus University, DK-8000 Aarhus, Denmark
[b] Department of Molecular Biology and Genetics, Aarhus University, DK-8000 Aarhus, Denmark

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ABSTRACT

Superoxide dismutase (EC-SOD) controls the level of superoxide in the extracellular space by catalyzing the dismutation of superoxide into hydrogen peroxide and molecular oxygen. In addition, the enzyme reacts with hydrogen peroxide in a peroxidase reaction which is known to disrupt enzymatic activity. Here, we show that the peroxidase reaction supports a site-specific bond cleavage. Analyses by peptide mapping and mass spectrometry shows that oxidation of Pro112 supports the cleavage of the Pro112–His113 peptide bond. Substitution of Ala for Pro112 did not inhibit fragmentation, indicating that the oxidative fragmentation at this position is dictated by spatial organization and not by side-chain specificity. The major part of EC-SOD inhibited by the peroxidase reaction was not fragmented but found to encompass oxidations of histidine residues involved in the coordination of copper (His98 and His163). These oxidations are likely to support the dissociation of copper from the active site and thus loss of enzymatic activity. Homologous modifications have also been described for the intracellular isozyme, Cu/Zn-SOD, reflecting the almost identical structures of the active site within these enzymes. We speculate that the inactivation of EC-SOD by peroxidase activity plays a role in regulating SOD activity *in vivo*, as even low levels of superoxide will allow for the peroxidase reaction to occur.

Introduction

Extracellular superoxide dismutase (EC-SOD) is an extracellular antioxidant that catalyzes the dismutation of superoxide ($O_2^{\bullet -}$) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). The protein is highly expressed in the lung and the vasculature where it is immobilized by the binding to a number of ligands in the extracellular space [1–4]. Hence, EC-SOD plays an important role in diseases where reactive oxygen species are known to be involved, including hypertension, atherosclerosis, and pulmonary fibrosis [5]. Recently, EC-SOD has also been shown to be involved in bacterial clearance [6,7], suggesting a role not only in protection against superoxide-mediated macromolecular damage [3,4,8,9] but also as a component involved in modulating the immune response to bacterial infection.

The amino acid sequence representing the central part of EC-SOD is homologous to the sequence of the intercellular counterpart, Cu/Zn-SOD [10]. Structural analyses show that Cu/Zn-SOD and the central part of EC-SOD shares the same fold albeit with some minor differences in loop structures [11,12]. The amino acid residues involved in coordination of the catalytic active copper atom and the zinc atom are conserved and share nearly identical spatial orientations, supporting the finding that the enzymatic properties of these enzymes are comparable. Apart from the ability to dismutate the superoxide radical, Hodgson and Fridovich showed that Cu/Zn-SOD also displayed peroxidase activity and found that this reaction inhibited the enzyme [13]. A large body of literature has described this activity in great detail suggesting the formation of a copper-bound hydroxyl radical (OH•) that mediates the loss of SOD activity by subsequent oxidations [14]. By using HPLC and electrochemical detection, Uchida and Kawakishi showed that His118 (numbering according to bovine protein) was oxidized to 2-oxo-histidine in Cu/Zn-SOD inactivated by H_2O_2 [15]. A more recent study using LC–MS/MS revealed that His44, His46, as well as His118 were oxidized after exposure to H_2O_2 [16]. These histidine residues are all known to coordinate copper [11] and oxidation is thus likely to destroy enzymatic activity by disrupting the coordination sphere. In addition, studies have shown that Pro60 can be oxidized leading to fragmentation of the Pro60–His61 peptide bond [16,17]. However, although several mechanisms for oxidative fragmentation at

Abbreviations: DDC, diethyldithiocarbamate; DMPO, 5,5-dimethyl-pyrroline N-oxide; EC-SOD, extracellular superoxide dismutase; FA, formic acid; MALDI, matrix assisted laser desorption/ionization; TFA, trifluoroacetic acid

* Correspondence to: Department of Biomedicine, Aarhus University, Bartholins Allé 6, Building 1245, DK-8000 Aarhus C, Denmark. Tel.: +45 87167825; fax: +45 86196128.
E-mail address: svp@biokemi.au.dk (S.V. Petersen).

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the prolyl bond have been proposed [18–20], it is not clear how oxidation at Pro60 mediates fragmentation of Cu/Zn-SOD.

The peroxidase activity and subsequent inhibition of enzymatic activity has also been shown for EC-SOD [21]. Interestingly, these authors showed that the presence of uric acid at physiological relevant concentrations could prevent inhibition *in vitro*. Moreover, when using an *in vivo* model of atherosclerosis, they were able to show that EC-SOD was partially inhibited by H_2O_2 during oxidative stress and that the activity was restored after increasing the level of urate in the circulation [21]. In line with these results, inhibition of EC-SOD activity has also been suggested to play a role in high-volume hypertension [22] and in persistent pulmonary hypertension of the newborn [23]. Collectively, these studies show that the inhibition of EC-SOD by H_2O_2 is relevant to disease, and that inhibition may allow for the development and progression of diseases involving reactive oxygen species.

Here we describe the mechanism of H_2O_2-induced EC-SOD inhibition and show that the modifications are similar to those described for Cu/Zn-SOD including histidine oxidation and site-specific fragmentation. Our data underscore that EC-SOD is subject to product inhibition and that the presence of EC-SOD subunits in tissue extracts and extracellular fluids may not necessarily correlate with protein activity.

Materials and methods

Proteins and reagents

Human EC-SOD was purified from aorta tissue or from cell culture supernatants by heparin-affinity chromatography and anion exchange chromatography as previously described [24]. Standard chemicals including diethylene triamine pentaacetic acid (DTPA) and α-cyano-4-hydroxysinnamic acid were obtained from Sigma. The MALDI matrix 2,5-dihyroxyacetophenone (DHAP) and standards for calibration of the mass spectrometer were obtained from Bruker Daltonics. The spin trap 5,5-dimethyl-pyrroline N-oxide (DMPO) was purchased from Enzo Life Sciences and diethyldithiocarbamate (DDC) and hydrogen peroxide (30%) were from Merck. Sequence grade porcine trypsin and bovine chymotrypsin were obtained from Promega and xanthine oxidase and PNGaseF were from Roche.

Expression of recombinant P112A human EC-SOD

The sequence encoding full-length EC-SOD with an optimized Kozak sequence was previously established in the pIRES vector [25]. The Pro112Ala substitution was introduced by PCR using the Quick change site-directed mutagenesis kit provided by Stratagene and the sequence of the obtained expression plasmid was verified by sequencing. HEK293 cells were stably transfected as previously described and protein expression conducted in serum-free medium [25]. The expressed P112A EC-SOD was active as evaluated by using the cytochrome C assay (see below) and activity staining [24] indicating that the protein was folded correctly.

Exposure of purified EC-SOD to hydrogen peroxide

Samples containing purified EC-SOD were prepared in PBS containing 0.1 mM DTPA and increasing amounts of H_2O_2 as indicated. In order to evaluate the role of the copper ion within the EC-SOD subunit, DDC was added to the samples and allowed to incubate 5 min prior to the addition of H_2O_2. When indicated, 1 mM DMPO was added to the reaction mixture to allow for detection of any protein-centered radicals generated. The reaction mixtures were incubated for 1 h at 37 °C and processed for further analysis (see below).

SDS-PAGE and protein visualization

Prior to electrophoresis, H_2O_2 was removed by reverse-phase chromatography using Poros50 R1 micro-columns as previously described [26]. Proteins were separated by polyacrylamide gel electrophoresis using uniform 10% polyacrylamide gels and the glycine/2-amino-2-methyl-1,3-propanediol-HCl buffer system [27]. Samples were analyzed under reducing conditions by boiling in the presence of 0.5% (w/v) SDS and 50 mM dithiothreitol prior to electrophoresis. Separated proteins were subsequently visualized by silver staining.

SOD activity assay

The activity of EC-SOD exposed to H_2O_2 was evaluated using the cytochrome C assay [28] modified for use in a 96-well plate format. In brief, samples containing EC-SOD and H_2O_2 were diluted in 50 mM $NaHCO_3$, 0.1 mM EDTA, pH 10 containing 0.1 mM xanthine and cytochrome C and 100 μl added to wells of a microtiter plate. As negative control, wells received 100 μl reaction mixtures without EC-SOD. To initiate the reaction, 100 μl of 50 mM $NaHCO_3$, 0.1 mM EDTA, pH 10 containing cytochrome C and xanthine oxidase was added and the absorbance at 550 nm measured between 0 and 2 min with intervals of 20 s using an EnSpire 2300 multimode plate reader (Perkin Elmer). The SOD activity was evaluated as ΔAbs/min and the relative activity determined by defining the activity of the sample containing no H_2O_2 as 100%.

Peptide maps of EC-SOD exposed to H_2O_2

EC-SOD exposed to H_2O_2 was desalted into 50 mM Tris–HCl, pH 8.0 containing 6 M guanidinium hydrochloride, 10 mM 8-hydroxyquinoline and 30 mM DTT by using Zeba spin desalting columns (Thermo Scientific). The material was incubated at 37 °C for 30 min and subsequently added 60 mM iodoacetamide to block free cysteine residues and incubated for another 30 min at 22 °C in the dark. The material was acidified and recovered by desalting using a Poros50 R1 reverse-phase micro-column, lyophilized, and subsequently added porcine trypsin in 50 mM ammonium bicarbonate. The digestion was allowed to proceed at 37 °C over night. The generated peptides were separated by UPLC reverse-phase chromatography by using a BEH300 C18 column (2.1 mm × 15 cm; 1.7 μm) operated by an Aquity UPLC system (Waters). The column was developed at a flow rate of 300 μl min^{-1} using a 1% B min^{-1} linear gradient of solvent B (90% acetonitrile, 0.08% (v/v) trifluoroacetic (TFA)) in solvent A (0.1% (v/v) TFA). Fractions were collected manually and analyzed by MALDI mass spectrometry.

Mass spectrometric analyses

Samples containing EC-SOD were acidified by the addition of TFA and H_2O_2 was removed by desalting using Poros50 R1 reverse phase micro-columns. The recovered protein was lyophilized and resuspended in 0.1% TFA and mixed thoroughly with DHAP matrix solution prepared in 20 mM diammonium hydrogen citrate, 75% (v/v) ethanol. The mass spectra were acquired using a Bruker Autoflex III instrument operated in linear mode and calibrated in the mass range from 5000 Da to 17,500 Da using Protein calibration standard I (Bruker Daltronics). The analysis of tryptic peptides recovered by off-line reverse phase chromatography was