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Oxidation, inactivation and aggregation of protein disulfide isomerase promoted by the bicarbonate-dependent peroxidase activity of human superoxide dismutase

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ABSTRACT

Protein disulfide isomerase (PDI) is a dithiol–disulfide oxidoreductase that has essential roles in redox protein folding. PDI has been associated with protective roles against protein aggregation, a hallmark of neurodegenerative diseases. Intriguingly, PDI has been detected in the protein inclusions found in the central nervous system of patients of neurodegenerative diseases. Oxidized proteins are also consistently detected in such patients, but the agents that promote these oxidations remain undefined. A potential trigger of protein oxidation is the bicarbonate-dependent peroxidase activity of the human enzyme superoxide dismutase 1 (hSOD1). Therefore, we examined the effects of this activity on PDI structure and activity. The results showed that PDI was oxidized to radicals that lead to PDI inactivation and aggregation. The aggregates are huge and apparently produced by covalent cross-links. Spin trapping experiments coupled with MS analysis indicated that at least 3 residues of PDI are oxidized to tyrosyl radicals (Y^{63}, Y^{116} and Y^{327}). Parallel experiments showed that PDI is also oxidized to radicals, inactivated and aggregated by the action of photolytically generated carbonate radical and by UV light. PDI is prone to inactivation and aggregation by one-electron oxidants and UV light probably because of its high content of aromatic amino acids.

Introduction

Protein disulfide isomerase (PDI)[1] is a ubiquitous dithiol–disulfide oxidoreductase chaperone that is located mainly in the endoplasmic reticulum (ER), although it has also been reported at the cell surface and other locations, including the mitochondria, nucleus, and cytosol. PDI contains 508 amino acid residues organized as an U-shaped structure bearing sequential domains named a-b-b′-a′-c, the latter containing the C-terminal ER-retention sequence KDEL. The a and a′ domains at the arms of the "U" contain one CGHC redox active motif each, while the b and b′ domains at the bottom of the "U" are rich in hydrophobic residues involved in substrate binding [1–4]. PDI (other gene names are PDIA1 and P4HB) is the founding member of a family containing more than 20 members, most of which have between one and three dithiol redox motifs (CXXC) and all of which display thioredoxin fold structure (for reviews, see [1–4]). PDI is actively involved in protein folding by oxidizing, reducing and isomerizing disulfide bonds [5–7]. In addition, PDI has a chaperone-like activity that is not directly related to its dithiol–disulfide oxidoreductase activity, thereby permitting PDI to fold denatured proteins that do not contain disulfide bonds [8–10]. Not surprisingly, PDI has been associated with protective roles against protein misfolding and aggregation, both of which are hallmarks of neurodegenerative diseases [11–19].

In the case of amyotrophic lateral sclerosis (ALS), PDI has been shown to be up-regulated in the spinal cords of a mouse model of the disease before symptom onset and in post-mortem tissues of human patients [15]. Additionally, PDI has been shown to co-localize with protein inclusions in the motor neurons of ALS patients [15] and in a mouse model of the disease [16]. Similarly, PDI has been detected in the protein inclusions of Alzheimer's [17] and Parkinson's patients [18]. These findings suggest the recruitment of PDI to the central nervous system to protect against

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[1] *Abbreviations used:* ALS, amyotrophic lateral sclerosis; bSOD1, bovine Cu,Zn-superoxide dismutase 1; cobalt complex, carbonato-tetrammine cobalt(III) complex ($[Co(NH_3)_4CO_3]^+$); DBNBS, 3,5-dibromo-4-nitrosobenzenesulfonic acid; DMPO, 5,5-dimethylpyrroline-*N*-oxide; DTPA, diethylene triamine pentaacetic acid; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; hSOD1, human Cu,Zn-superoxide dismutase 1; PDI, human protein disulfide isomerase (type P4HB).

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protein aggregation [19]. Conversely, post-translational modifications of recruited PDI may critically affect its activity and its anti-aggregation effects. Along this line, inactivation of PDI by S-nitrosation of one of its critical cysteine residues has been reported in Parkinson's [11], Alzheimer's [11,13] and ALS disease [14]. S-nitrosated PDI has also been detected in the detergent-insoluble fraction of spinal cord homogenates from a mouse model of ALS [20].

S-nitrosation is one of the many post-translational modifications of proteins that occur under oxidative conditions, including those associated with neurodegenerative diseases. Indeed, protein oxidation, carbonylation, nitration and oligomerization are consistently detected within the pathologically affected areas of the central nervous system of patients and animal models of neurodegenerative diseases (see, for instance, [21–25]). The triggers and targets of these protein modifications, however, remain poorly understood. A possible candidate to trigger protein oxidation in ALS and other neurodegenerative diseases is the bicarbonate-dependent peroxidase activity of the abundant enzyme superoxide dismutase 1 (SOD1) [23,26–33]. During this activity, SOD1 consumes considerable amounts of hydrogen peroxide to produce the carbonate radical. This potent oxidant diffuses away from the active site and oxidizes exogenous targets, including nearby proteins [26,30,32,33].

In addition to oxidizing nearby targets, the bicarbonate-dependent peroxidase activity of human SOD1 (hSOD1) oxidizes its own tryptophan and histidine residues [29]. Interestingly, the unique solvent-exposed tryptophan^{32} residue is only present in human and other simian SOD1s and it is oxidized to hSOD1-tryptophanyl radical (hSOD1-W$^{\cdot 32}$). This radical either decays to hSOD1-*N*-formyl-kynurenine and hSOD1-kynurenine [31] or reacts with another hSOD1-W$^{\cdot 32}$ to produce a covalent dimer cross-linked by a ditryptophan bond (hSOD1-W-W-hSOD1) [27]. Since PDI has been detected in the protein inclusions found in the central nervous system of patients of neurodegenerative diseases [15,17,18], the focus of this work was to examine the effects of the bicarbonate-dependent peroxidase activity of hSOD1 on PDI structure and activity.

Materials and methods

Materials

Unless stated otherwise, all chemicals were purchased from Sigma–Aldrich, Merck or Fisher and were analytical grade or better. The hydrogen peroxide solutions were prepared immediately before use, and the concentrations were determined spectrophotometrically by reaction with horseradish peroxidase to produce compound I ($\Delta\varepsilon_{403} = 5.4 \times 10^4\ M^{-1}\ cm^{-1}$) [34]. Mass spectrometry grade trypsin (Gold) was purchased from Promega (Madison, WI, USA). Bovine superoxide dismutase 1 (bSOD1) was purchased from Alexis Biochemicals. Bovine liver catalase was purchased from Boehringer. The perchlorate salt of the carbonato-tetrammine cobalt(III) complex ($[Co(NH_3)_4CO_3]ClO_4$) [31] was a generous gift from Dr. V.F. Ferreira (Universidade Federal Fluminense). All solutions and buffers were prepared with distilled water purified in a Millipore Milli-Q system and treated with Chelex-100® resin (Sigma–Aldrich).

Expression and purification of hSOD1

Plasmids (pET-3d) encoding the enzymes hSOD1^{WT} and hSOD1^{G93A} were kindly provided by Dr. J. S. Beckman from the Linus Pauling Institute. The plasmids were expressed in *Escherichia coli* strain BL21 (DE3) pLysS, and the enzymes were purified and analyzed as previously described [28]. Typically, recombinant hSOD1^{WT} and mutant hSOD1^{G93A} contained approximately 0.7 copper and 0.7 zinc ions per monomer. SOD1 dismutase activity was determined by the cytochrome c method, as previously described [35]. Typically, recombinant hSOD1^{WT} and mutant hSOD1^{G93A} exhibited specific dismutase activities of 3900 ± 400 U/mg (mg of protein normalized by the copper content). Here, the concentrations of hSOD1 are always expressed as the dimer; hSOD1 is a non-covalent homodimer containing 1 copper and 1 zinc ion per monomer.

Expression and purification of PDI

A histidine-tagged human *PDI* gene (P4HB or PDIA1) cloned in pET28a vector (Novagen) was expressed in *E. coli* strain BL21 (DE3) pLysS grown in medium broth containing doubled amounts of yeast extract and tryptone at 37 °C. Protein expression was induced with 0.1 mM isopropyl β-D-thioglucopyranoside. When the apparent absorbance of the culture at 600 nm reached 0.6, protein expression was induced with 0.1 mM IPTG for 6 h. Protein was extracted from the bacterial lysate by adding lysozyme (200 μg/ml) to the resin equilibration/washing buffer (50 mM sodium phosphate and 300 mM NaCl, pH 7.0). The lysate was incubated on ice for 2 h under orbital agitation followed by centrifugation at 12,000 rpm to obtain the supernatant. PDI was finally purified from the lysate by immobilized metal affinity resin (TALON affinity resin) according to the manufacturer's instructions. After protein purification, samples were dialyzed against equilibration buffer to remove imidazole. Reduced PDI was obtained by treating the protein with excess DTT, and after 2 h, excess DTT and its products were removed with a FPLC desalting column. Most of the experiments were performed with reduced PDI but oxidized PDI was used in some experiments. To obtain oxidized PDI the enzyme was treated with a 10 times molar excess of hydrogen peroxide, and after 2 h, excess hydrogen peroxide was removed with a FPLC desalting column. When required, the protein was concentrated by ultrafiltration (Amicon Ultra, cut-off 50 kDa, Millipore). The PDI used in all experiments was fully active with respect to the reductase activity [36]. The residue numbering employed was for human PDI with the signal sequence.

Enzymatic incubations

Unless otherwise stated, the reaction mixtures (50 μl) contained hSOD1 (10 μM per dimer units), PDI (10 μM per monomer units), hydrogen peroxide (1 mM), sodium bicarbonate (25 mM) and DTPA (0.1 mM) in phosphate buffer (25 mM) adjusted to a final pH of 7.4, and the mixtures were incubated at 37 °C for 1 h.

Photolysis experiments

PDI (10 μM) in phosphate buffer (25 mM) containing DTPA (0.1 mM), pH 7.4, was mixed with carbonatotetrammine cobalt(III) complex ($[Co(NH_3)_4CO_3]ClO_4$) (4 mM). The mixtures were transferred to EPR flat cells and irradiated for 30 s at 254 nm in a Photoreactor ICH-2 (Luzchem, Ottawa Ontario, Canada) and the energy incident in the sample was approximately, 6.3 mW × cm^{-2}.

SDS–PAGE analysis

The spent reaction mixtures were diluted with Laemmli buffer, heated at 95 °C for 10 min and submitted to electrophoresis (12% SDS–PAGE). The samples were run on the gel at 80 V for 15 min and then at 120–150 V for approximately 2–3 h. The staining was performed by dipping the gels in 50 mL colloidal Coomassie solution overnight. The gels were destained overnight either with