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Original Contribution

Cloning and characterization of *Arenicola marina* peroxiredoxin 6, an annelid two-cysteine peroxiredoxin highly homologous to mammalian one-cysteine peroxiredoxins ☆

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ABSTRACT

Peroxiredoxins (PRDXs) are a superfamily of thiol-dependent peroxidases found in all phyla. PRDXs are mechanistically divided into three subfamilies, namely typical 2-Cys, atypical 2-Cys, and 1-Cys PRDXs. To reduce peroxides, the N-terminal peroxidatic Cys of PRDXs is first oxidized into sulfenic acid. This intermediate is reduced by forming a disulfide bond either with a resolving Cys of another monomeric entity (typical 2-Cys) or of the same molecule (atypical 2-Cys). In 1-Cys PRDXs, the resolving Cys is missing and the sulfenic acid of the peroxidatic Cys is reduced by a heterologous thiol-containing reductant. In search of a homolog of human 1-Cys PRDX6 in *Arenicola marina*, an annelid worm living in intertidal sediments, we have cloned and characterized a PRDX exhibiting high sequence homology with its mammalian counterpart. However, *A. marina* PRDX6 possesses five Cys among which two Cys function as peroxidatic and resolving Cys of typical 2-Cys PRDXs. Thus, *A. marina* PRDX6 belongs to a transient group exhibiting sequence homologies with mammalian 1-Cys PRDX6 but must be mechanistically classified into typical 2-Cys PRDXs. Moreover, PRDX6 is highly expressed in tissues directly exposed to the external environment, suggesting that this PRDX may be of particular importance for protection against exogenous oxidative attacks.

Introduction

Peroxiredoxins (PRDXs) are an ubiquitous family of peroxidases able to reduce H_2O_2, alkyl hydroperoxides (ROOH), and peroxynitrite ($ONOO^-$) [1–3]. These enzymes are highly conserved throughout evolution and widely distributed among bacteria, archaea, and eukaryotes [3,4]. Unlike most peroxidases that exhibit high catalytic efficiency with a selenocysteine, a flavin, or a heme ring in their active site [3], PRDXs lack cofactors or prosthetic groups. Their activity is depending on cysteines (Cys). PRDXs were initially classified into two subfamilies (2-Cys and 1-Cys PRDXs) according to the number of catalytically active Cys residues in their sequence [2]. Then, 2-Cys PRDXs were yet divided into two different subfamilies, namely typical and atypical 2-Cys PRDXs based on their catalytic mechanism [5]. All PRDXs share a peroxidatic Cys in their N-terminal domain which is oxidized into sulfenic acid (-SOH) in a first catalytic step during reaction with peroxides [1]. The second step of the reaction distinguishes the three PRDX subfamilies and consists in the reduction of sulfenic acid. For all 2-Cys PRDXs, this reactivation step requires an additional Cys called resolving Cys [1]. Typical 2-Cys PRDXs form homodimers with two identical active sites through intersubunit disulfide bonds reduced most frequently by thioredoxins (TXNs) [1]. In atypical 2-Cys PRDXs, the resolving Cys directly attacks the peroxidatic Cys by forming an intramolecular disulfide bond. Their classical physiological reductants are also TXNs [5]. The last subfamily, 1-Cys PRDXs, only contains the single peroxidatic Cys [6,7]. In this case, the sulfenic acid is reduced by an external thiol. Recently, it has been shown that one physiological

Abbreviations: Bt, *Bos taurus*; CHP, cumene hydroperoxide; C_p, peroxidatic cysteine; C_r, resolving cysteine; DAPI, 4′,6-diamidino-2-phenylindole; Dox, oxidized dimer; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FITC, fluoresceine isothiocyanate; GPX, glutathione peroxidase; GR, glutathione reductase; GRX, glutaredoxin; GSH, glutathione; HMW, high molecular weight; H_2O_2, hydrogen peroxide; H_2S, hydrogen sulfide; *Hs*, *Homo sapiens*; HSP, heat shock protein; M, monomer; MALDI, matrix-assisted laser desorption/ionization; *Mm*, *Mus musculus*; Mox, oxidized monomer; Mred, reduced monomer; NADPH, nicotinamide adenine dinucleotide phosphate; $ONOO^-$, peroxynitrite; ORF, open reading frame; PBS, phosphate buffer saline; PCR, polymerase chain reaction; πGST, glutathione S-transferase π; PLOOH, phospholipid hydroperoxide; RACE, rapid amplification of cDNA ends; *Rn*, *Rattus novergicus*; ROOH, organic hydroperoxide; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; -SOH, sulfenic acid; TBHP, *tert*-butyl hydroperoxide; TOF, time of flight; TXN, thioredoxin; TXNRD, thioredoxin reductase.

☆ The nucleotide sequence reported in this paper has been submitted to GenBank with Accession Number DQ059567.

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reactivation process in mammals consists in a glutathione S-transferase π (πGST)-mediated reduction with glutathione (GSH). πGST facilitates the access toward the active site by establishing a transient hydrogen bond with sulfenic acid [8]. After a reaction of condensation, peroxidatic Cys can form a heterodimer with GSH which is finally reduced by another molecule of GSH.

PRDXs have been historically classified using different criteria. First, as described before, PRDXs have been classified according to their catalytic mechanisms. Nevertheless, this classification is possible only when the catalytic mechanism is fully characterized. Another classification is based on sequence homology [3,4]. Interestingly, sequence alignment of PRDXs from all kingdoms forms clusters including typical and atypical 2-Cys PRDXs [3,4]. A significant example is illustrated by human PRDX5, the prototype of an atypical 2-Cys PRDX. Human PRDX5 belongs to the same cluster as *Saccharomyces cerevisiae* Ahp1p which is mechanistically classified as a typical 2-Cys PRDX [9].

PRDXs are expressed at high levels in organisms. They are among the 10 most abundant proteins in *E. coli* and constitute 0.1–0.8% of soluble proteins in mammalian cells [10]. However, some mammalian PRDXs have been shown to display a marked vulnerability to being inactivated during their catalytic process [11]. This suggested another role for mammalian PRDXs. Besides their cytoprotective antioxidant function, PRDXs appeared to play a role in cell proliferation, differentiation, immune response, and control of apoptosis, processes involving a redox signaling [2]. Also, more recently, several PRDXs were shown to present a chaperone function [12–14]. This function has been reported for 2-Cys PRDXs from *S. cerevisiae* [12] and also for 2-Cys PRDXs from *Homo sapiens* [13] and *Helicobacter pylori* [14] which can form high molecular weight (HMW) structures induced by overoxidation. With regard to these characteristics, PRDXs resemble heat shock proteins. It casts doubt on the notion that PRDXs are only peroxidases.

Previous studies on bovine 1-Cys PRDX6 showed that, besides GSH, the toxic H_2S could also reactivate the enzyme by reducing the sulfenic acid [15]. The homologs of bovine PRDX6 could then be important for species living in a sulfide-rich environment like the marine annelid *Arenicola marina* [16]. Moreover, H_2S is also a potent inhibitor of catalase, which together with peroxidases detoxify H_2O_2. Apart from this high sulfide exposure, *A. marina* is also exposed to oxidative attacks caused by photochemical reaction in which oxygen reduction is induced by solar radiation via excitation of dissolved organic matter [17]. Another cause of oxidative stress originates from the hypoxic-reoxygenation cycles of animal burrows associated with low and high tides [18]. Thus, PRDXs could be major antioxidant enzymes for species adaptation in such environments.

In the present study, we report the cloning and characterization of an annelid PRDX. This novel PRDX which was cloned in *A. marina* is homologous to mammalian PRDX6. Although mammalian PRDX6s exhibit a unique peroxidatic Cys, it appeared that *A. marina* PRDX6 has also a resolving Cys involved in the formation of intermolecular disulfide bonds and therefore must be mechanistically classified among typical 2-Cys PRDXs.

Materials and methods

cDNA cloning and sequence analysis

Specimens of the lugworm *A. marina* were collected on the Aber of Roscoff (France). Entire specimens were crushed in liquid nitrogen and total RNA was extracted using RNAble buffer (Eurobio) and poly(A) RNA was isolated using a mRNA purification kit (Amersham). First-strand cDNA was obtained with Moloney leukemia virus reverse transcriptase (Superscript II, Invitrogen) using oligo$(dT)_{25}$ as primer according to the manufacturer's instructions. Degenerate primers were designed to amplify by PCR the cDNA sequence coding for *A. marina* PRDX6. Indeed, using a *Drosophila melanogaster* PRDX6 sequence (GenBank Accession Number AF311878), we searched in public databases (http://www.ncbi.nlm.nih.gov/) for homologous sequences from different species in order to identify conserved domains. The amino acid sequence alignment of *D. melanogaster* (GenBank Accession Number AF311878), *Anopheles gambiae* (GenBank Accession Number AAAB01008807, and *Aplysia californica* (GenBank Accession Number AF510851) PRDX6s revealed the presence of two strictly conserved blocks separated by a less conserved sequence coded by 279 nucleotides. Using these two domains, we perfomed the design of degenerate primers using the CODEHOP software (consensus degenerate hybrid oligonucleotide primer, http://www.bioinformatics.weizmann.ac.il/blocks/codehop.html). Degenerate nucleotide sequences were proposed according to codon usage of the annelid *Hirudo medicinalis*. Then, a partial cDNA fragment was successfully PCR-amplified using the Goldstar DNA polymerase (Eurogentec) during 35 cycles of PCR with 5′-GCC GAT TWC ACC CCA GTC TGY ACN ACN GA-3′ and 3′-TAR CCN GCN ACC ACC GGA AGA AAC TTC-5′ degenerate primers. PCR cycles were as follows: denaturing for 30 s at 94°C, annealing for 45 s at 50°C, extension for 1 min at 72°C, and after 35 cycles, a final extension step for 5 min at 72°C. The PCR product of 349 bp was gel-purified with nucleospin extract 2.1 (Macherey Nagel) and cloned into pCR2.1 vector with TA TOPO cloning kit (Invitrogen) The cloned PCR product was sequenced. Subsequently, the full-length *A. marina PRDX6* cDNA from whole animals was obtained by rapid amplification of cDNA ends (RACE) with Expand High Fidelity DNA polymerase (Roche Molecular Biochemicals) using a Marathon cDNA kit (Clontech) and *A. marina* specific primers 3′-CTG CCG GGC TGT GTT CAT CAT TGG TCC-5′and 5′-GCC GAC CAC AAA GAG TGG TCT GAG GAC G-3′ for 5′-amplification and 3′-amplification, respectively. The PCR product was cloned and sequenced as described before. Based on the new cDNA sequence obtained by RACE-PCR, a 716-bp sequence of cDNA from whole animals was amplified using 5′-TGG CAG TCG GTG ACA GTA TAG TTC-3′ and 3′-CGG TCG CTC CCG GGG ATC AGC G-5′ primers. The corresponding sequence of the PCR product including the coding sequence was proven to be identical to the sequence obtained by RACE-PCR. The deduced amino acid sequence of *A. marina* PRDX6 was used to perform a BLAST search at the NCBI web site (http://www.ncbi.nlm.nih.gov/). The homology with mammalian PRDX6 was confirmed but, equally, the important conservation between amino acid sequences of PRDX6s of all phyla. Multiple alignments of peptidic sequences and the phylogenetic analysis were performed with CLUSTAL W (version 1.8) [19] according to neighbor joining method. Afterward, a phylogenetic tree was visualized with treeview [20].

Protein expression and purification

PCR amplification of *A. marina PRDX6* cDNA was performed as described above with Expand High Fidelity DNA polymerase, using primers 5′-TTC GAC **GGT ACC** GGT ATC ACC CTT GGA GAA GTC-3′ (forward) and 3′-GCT ATA CGC CCC AGC CCA AGT AG**A AGC TT**G CTC CC-5′ (reverse) that allowed the cloning into pQE30 (Qiagen) expression vector thanks to restriction sites *Kpn*I and *Hind*III, respectively (in bold type and underlined in primer sequence). Another PCR amplification with primers 5′-TCG ACA **CCA TGG** GTA TCA CCC TTG GAG-3′ (forward) and 3′-CTA TAC GCC CCA GCC CAA GT**C CAT GG**T CGC T-3′ (reverse) allowed the cloning and the ligation in the expression vector pQE60 (Qiagen) with restriction sites *Nco*I (in bold type and underlined in primer sequence). In pQE30, a 6xHis tag is fused at the N-terminal extremity of the recombinant protein while in pQE60, 6xHis tag is fused at the C-terminal extremity. To determine the role of the different Cys in the catalytic mechanism, mutants C45S, C71S, C86S, C127S, and C183S were generated by PCR-mediated site-directed mutagenesis using complementary primers containing base mismatch that converted the codon for Cys to a codon for Ser. The mutants were cloned into pQE60. Mismatched primers used were: