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Dual functions of Arabidopsis sulfiredoxin: Acting as a redox-dependent sulfinic acid reductase and as a redox-independent nuclease enzyme

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ABSTRACT

Based on the fact that the amino acid sequence of sulfiredoxin (Srx), already known as a redox-dependent sulfinic acid reductase, showed a high sequence homology with that of ParB, a nuclease enzyme, we examined the nucleic acid binding and hydrolyzing activity of the recombinant Srx in Arabidopsis (AtSrx). We found that AtSrx functions as a nuclease enzyme that can use single-stranded and double-stranded DNAs as substrates. The nuclease activity was enhanced by divalent cations. Particularly, by point-mutating the active site of sulfinate reductase, Cys (72) to Ser (AtSrx-C72S), we demonstrate that the active site of the reductase function of AtSrx is not involved in its nuclease function.

1. Introduction

Since reactive oxygen species (ROS) generated not only from the environmental stresses but also from diverse metabolic processes are highly reactive, they can cause oxidative damages to many biological macromolecules including nucleic acids, lipids, and proteins, etc. [1,2]. Responding to changes of intracellular ROS levels, the susceptible molecules in cells perform their specific reversible or irreversible modification, which results in an alteration of their functions and activities [3,4]. Among the processes, the sulphydryl group of proteins is particularly sensitive to oxidative modification. Thus, the status of thiol groups in certain protein reflects the redox changes of cells [5,6].

Even though, the overoxidized form of sulfinic- and sulfonic-acid proteins has been viewed as an irreversible protein modification, at recent it was shown that the sulfinic form of some types of peroxiredoxins (Prxs) were reversibly reduced by sulfiredoxin (Srx) with the help of thioredoxin (Trx)- or glutaredoxin (Grx) system [7]. However, up to date, the sulfonic form of Prxs remains as an irreversible overoxidation. The Srx, a small redox protein, was firstly identified from yeast as a reducer of the sulfinic form of 2-Cys peroxiredoxin (2-Cys Prx). In this reaction, the highly conserved cysteine residue in Srx plays a critical role as its active site [8–10] and the presence of ATP, Mg^{2+} and a thiol-reductants are necessitated [8,11,12]. Through this antioxidative function of Srx, it has also been shown that the Srx participates diverse cellular regulatory functions, such as peroxide-mediated signaling cascades, H_2O_2 signaling fluxes in Pap1 pathway [13], cell proliferation [14], tumor promotion [15,16], and regulation of post-translational modification against oxidative and/or nitrosative stresses [17,18]. And the transcript level of *Srx* is significantly induced by H_2O_2 and its deletion in yeast cells led to a reduced tolerance to oxidative stress [8,13].

Importantly, during the searches of the Arabidopsis Information Resource database (TAIR ID: At1g31170), we retrieved an interesting result that the amino acid sequence of Srx shared a high sequence homology with part of the prokaryotic ParB protein [19]. The ParB containing a helix-turn-helix (HTH) motif in its amino acid sequence was well characterized to play a role in bacterial chromosome partitioning and plasmid inheritance with the help of two other components, such as *cis*-acting site (*parS*) and an ATPase (parA). In the reaction, the ParB protein played a critical role in DNA binding and exhibited a Ca^{2+}-dependent nuclease function [20,21]. Even though the molecular mechanism remains unclear [22], it has been known that the ParB binds to *parS* site and spreads laterally along the DNA forming a nucleoprotein complex. Then the ParA, Walker-box ATPase, separates the ParB-*parS* complex into the opposite poles of cells. The process of chromosome segregation can be enhanced by recruitment of structural maintenance chromosomes (SMC) into the replication origin by ParB-*parS* [23].

Despite the overall sequence homology of Srx with ParB, the Srx does not have HTH motif known as an important domain for DNA binding of ParB. And the highly conserved amino acid, Glu of ParB, was replaced with Cys residue in Srx [19]. Even though it was

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demonstrated the two proteins of ParB and Srx have high sequence and structural homologies [19], there has been no report on the examination of nuclease function of Srx. Therefore, in this study, we examined whether the Srx truly has nuclease function using the bacterially expressed recombinant protein of Arabidopsis Srx, designated AtSrx. From the experiment, we clearly demonstrate that the AtSrx plays dual functions, acting not only as a redox-dependent sulfinic acid reductase but also as a redox-independent nuclease function.

2. Materials and methods

2.1. Materials

M13mp8 (single-stranded DNA) and M13mp8RFI (double-stranded DNA) were purchased from New England Biolabs (NEB). $CaCl_2$, $MgCl_2$, $CoCl_2$, $ZnCl_2$ and EDTA were obtained from Sigma and pUC19 DNA was from Takara Co.

2.2. Preparation of recombinant AtSrx and AtSrx-C72S proteins and their purification

A cDNA (312 bp) encoding the AtSrx was cloned from an Arabidopsis cDNA library by polymerase chain reaction (PCR) with the use of following primers (Forward primer, 5′-TATAGGATCCATGAACGGTTCGCCGCCGGT-3′ and Reverse primer, 5′-CTCGAGTCAGCGAAGATGATGCCTTAATGTTT-3′), and the PCR product was ligated into the pGEM-Teasy vector (Promega). Using the *AtSrx* DNA in pGEM-Teasy vector as a template, the DNA encoding AtSrx-C72S in which the Cys72 was replaced by Ser, was prepared by PCR-mediated mutagenesis technique as described [24] using the following specific primers (Forward primer, 5′-ATGGGTTCTCGGGATCCCAC-3′; Reverse primer, 5′-ATCTGTGGGATCCCGAGAAC-3′). For the protein expression of AtSrx and AtSrx-C72S in *Escherichia coli* BL21 (DE3), the *Bam*HI/*Xho*I-digested *AtSrx* and *AtSrx-C72S* DNA inserts were ligated with the pET28a expression vector (Novagen), respectively. Sequence of the constructs was verified by nucleotide sequencing. After transforming the *AtSrx and AtSrx-C72S* genes into *E. coli* BL21 (DE3) and 0.2 mM IPTG induction, the histidine (His)-tagged recombinant AtSrx and AtSrx-C72S proteins were expressed and purified by an Ni-NTA affinity agarose gel chromatography, as described [25]. The His-tag was cleaved by thrombin treatment and the native forms of AtSrx and AtSrx-C72S proteins were obtained by dialyzing them into 20 mM Hepes-KOH (pH 8.0), which were used for biochemical analyses.

2.3. Preparation of Arabidopsis 2-Cys Prx protein and its reduction analysis

Arabidopsis 2-Cys Prx was obtained with the same procedures as used for the preparation of AtSrx protein in *E. coli* and the protein was hyper-oxidized by H_2O_2 (5 mM) treatment as described [9]. Reduction of sulfinic form of 2-Cys Prx by AtSrx was examined in the 100 μl reaction mixture containing 50 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM ATP, 10 mM GSH, 6 μg sulfinic acid form of 2-Cys Prx, 2 μg AtSrx or AtSrx-C72S at 30 °C for 120 min. The oxidized status of the 2-Cys Prx was investigated by western blot analysis with the use of antibodies specifically reacted either with the hyperoxidized 2-Cys Prx (purchased from the LabFrontier company) or the whole protein of 2-Cys Prx (prepared from our laboratory).

2.4. Analyses of nucleic acid binding and hydrolyzing activities

Nucleic acid binding activity of AtSrx was analyzed by gel retardation technique using various kinds of DNA as substrates as described [26]. 200 ng of pUC19, M13mp8 or M13mp8RFI was incubated with varying concentrations (0, 1, 2, 5 μg) of the purified AtSrx protein in 15 μl binding buffer (20 mM Tris–HCl, pH 7.5 containing 2 mM EDTA, 4 mM KCl, 5% glycerol, and 50 μg/ml bovine serum albumin). The reaction mixture was maintained on ice for 30 min and their DNA binding activity was measured by gel shift on agarose gel which was visualized by ethidium bromide staining (EtBr). And the nuclease activity was assayed as described [21] with a slight modification. Various concentrations (0.1, 0.5, 1 and 5 μg) of AtSrx were incubated with 200 ng substrates of pUC19, single-stranded or double-stranded DNA in the 30 μl reaction buffer (20 mM Tris–HCl, pH 7.5) containing 3 mM β-mercaptoethanol, 5 mM $CaCl_2$ at 37 °C for 20 min. After the reaction was stopped by adding 2 μl of 500 mM EDTA, the mixture was electrophoresed in a 0.8% agarose gel. The nuclease activity was visualized by EtBr staining. Instead of AtSrx, glutathione S-transferase (GST: 5 μg) was used as a control.

3. Results

3.1. Identification of a novel function of AtSrx acting as a nuclease enzyme

Based on the fact that the deduced amino acid sequences of AtSrx showed a high sequence similarity to those of prokaryotic ParB proteins, already known as nuclease enzymes (Supplemental Fig. 1) [19], we tried to investigate the nucleic acid hydrolyzing activity of AtSrx using the various kinds of substrates. For the experiment, the cDNA clone (312 bp) of AtSrx was isolated from an Arabidopsis cDNA library, subcloned it into the pET28a expression vector and expressed the protein in *E. coli*. Treatment of 1 mM IPTG to the transformed *E. coli* significantly induced the expression levels of AtSrx (Fig. 1A). After the His-tagged recombinant AtSrx was homogeneously purified using a Ni-NTA agarose affinity column chromatography, the His-tag was removed by thrombin treatment. Then, it was possible to get a single protein band of AtSrx on a SDS–PAGE gel (Fig. 1A). To investigate the role of active Cys residue in AtSrx, we also prepared the Cys mutant form of AtSrx-C72S, in which the active site Cys residue (positioned at 72) was replaced by Ser. Using the proteins, we confirmed reductase function of AtSrx by Western blotting, which efficiently reduced the sulfinic form of Arabidopsis 2-Cys Prx (Fig. 1B). In this experiment, we used two antibodies that specifically recognized either the hyperoxidized group of 2-Cys Prx or whole protein of 2-Cys Prx, respectively. As expected, it was verified again that the Cys72 in AtSrx was essential to reduce the sulfinic acid form of Arabidopsis 2-Cys Prx.

In addition, we examined the DNA binding activity of AtSrx with the use of many kinds of DNA substrates including pUC19, single-stranded (ss) M13mp8 phage DNA, and double-stranded (ds) DNA molecules. We found AtSrx bound with all the DNA molecules tested, suggesting that AtSrx interacted with DNAs without showing substrate specificity. Thus, in the presence of more than 1 μg of AtSrx in the reaction mixture, AtSrx resulted in a supershift of its electro-mobility on the gels (Fig. 2A). In contrast, no interaction was detected from the incubation of DNA substrates with the excessive amount (5 μg) of GST used as a control. To determine the effect of salt on the DNA binding of AtSrx, we assessed its binding affinity to substrate DNAs with increasing the concentrations of KCl and analyzed it on agarose gels. In the presence of KCl up to 200 mM concentrations, AtSrx bound with its diverse target DNAs (Fig. 2B). Interestingly, it can be concluded from the results that the AtSrx previously identified as a sulfinic acid reductase [27,28] can also have binding activity with diverse forms of DNA molecules.