Running title: Gpx1 in *S. cerevisiae* is an atypical 2-Cys peroxiredoxin

Kinetics and redox regulation of Gpx1, an atypical 2-Cys peroxiredoxin, in *Saccharomyces cerevisiae*

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Abbreviation

GPx, glutathione peroxidase; SeCys, selenocysteine; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; AMS, 4'-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid

Abstract

The budding yeast *Saccharomyces cerevisiae* has three homologues of glutathione peroxidase (*GPX1*, *GPX2*, and *GPX3*). Although structural homologues of the mammalian glutathione peroxidase, Gpx2 and Gpx3 have been proven to be atypical 2-Cys peroxiredoxins, which prefer to use thioredoxin as an electron donor. Here, we show that Gpx1 is also an atypical 2-Cys peroxiredoxin, but uses glutathione and thioredoxin almost equally. We determined the redox state of Gpx1 *in vivo*.

Keywords

Glutathione peroxidase, *S. cerevisiae*, peroxiredoxin, thioredoxin, glutathione

Glutathione peroxidase (GPx) is a key enzyme of the antioxidant system in eukaryotic organisms, which catalyzing the reduction of H_2O_2 and organic hydroperoxides to water and corresponding alcohols using reduced glutathione as an electron donor (for review see Margis *et al.*, 2008). Mammalian GPxs have selenocysteine (SeCys) at their active site, although a certain type of GPx contains cysteine instead of SeCys (Ghyselinck *et al.*, 1990). Non-SeCys-type GPxs have been found in plants (Criqui *et al.*, 1992; Holland *et al.*, 1993; Depège *et al.*, 1998; Roeckel-Drevet *et al.*, 1998), and yeasts (Inoue *et al.*, 1999). Non-SeCys-type GPxs often use thioredoxin as an electron donor to carry out the reaction (for review see Herbette *et al.*, 2007). The budding yeast *Saccharomyces cerevisiae* has three

homologues of glutathione peroxidase, *GPX1*, *GPX2*, and *GPX3* (Inoue *et al.*, 1999). Although structural homologues of mammalian GPxs, *S. cerevisiae* Gpx2 and Gpx3 have been found to be atypical 2-Cys peroxiredoxins (Delaunay *et al.*, 2002; Tanaka *et al.*, 2005). As a consequence of the peroxidase reaction of atypical 2-Cys peroxiredoxins, an intramolecular disulfide bond is formed, which is usually cleaved by thioredoxin (Hofmann *et al.,* 2002). The redox status of Gpx2 and Gpx3 *in vivo* is regulated by thioredoxin (Delaunay *et al.*, 2002; Tanaka *et al.*, 2005). In this study, we determined the enzymatic properties and the redox regulation of Gpx1.

To characterize Gpx1, we expressed it in *E. coli* cells basically the same method as our previous report (Tanaka *et al.,* 2005). The procedures for construction of plasmid are described in Supplementary Material. Since the recombinant Gpx1 is easily aggregated (Avery and Avery, 2001), purification and characterization of Gpx1 have not been succeeded. To avoid the aggregation of Gpx1, imidazole was removed from the enzyme fraction containing Gpx1 after Ni^{2+}-column chromatography by dialyzing stepwise against a buffer containing 100 mM, 50 mM, 10 mM, and 0 mM imidazole. GPx activity with the glutathione system (glutathione, glutathione reductase, and NADPH) and thioredoxin system (thioredoxin, thioredoxin reductase, and NADPH) was measured as described previously (Tanaka *et al.,* 2005). To determine the redox state of Gpx1 in yeast cells, we did the AMS (4’-acetamido-4’-maleimidylstilbene-2,2’-disulfonic acid) (Molecular Probes) assay and site-directed mutagenesis to change cysteine residues of Gpx1 to serine. Detail procedures are given in Supplementary Material. *S. cerevisiae* YPH250 (*MAT***a** *trp1*-*Δ1*

his3-Δ*200 leu2*-Δ*1 lys2-801 ade2-101 ura3-52*) and isogenic mutants (*gpx1*Δ::*KanMX4* and *gpx1*Δ::*KanMX4 trx1*Δ::*HIS3 trx2*Δ::*LEU2*) were used for the AMS assay. Cells were cultured in synthetic dextrose (SD) medium (2% glucose and 0.67% yeast nitrogen base without amino acids) with appropriate amino acids and bases until stationary phase of growth to induce the expression of *GPX1* (Ohdate *et al.,* 2010).

The purified Gpx1 exhibited peroxidase activity using both glutathione and thioredoxin as electron donors. The k_{cat}/K_m value of Gpx1 for reducing H_2O_2 was 4.10 x 10^6 $M^{-1}s^{-1}$ in the glutathione system, and 1.19 x 10^6 $M^{-1}s^{-1}$ in the thioredoxin system (Supplementary Table 1). So, the catalytic efficiency of Gpx1 was approximately four-fold higher in the glutathione system than thioredoxin system with H_2O_2 as a substrate. Meanwhile, the k_{cat}/K_m value of Gpx1 for reducing *tert*-butyl hydroperoxide (*t*-BHP) in the glutathione system (1.49 x 10^6 $M^{-1}s^{-1}$) was almost the same as that in the thioredoxin system (2.16 x 10^6 $M^{-1}s^{-1}$). We have previously reported that the catalytic efficiency of Gpx2 in the reduction of H_2O_2 was 100-times higher with the thioredoxin system than glutathione system (k_{cat}/K_m of 4.79 x 10^7 $M^{-1}s^{-1}$ *versus* 5.85 x 10^5 $M^{-1}s^{-1}$). Also, the k_{cat}/K_m value for *t*-BHP was approximately 10-times larger with the thioredoxin system (5.89 x 10^6 $M^{-1}s^{-1}$) than glutathione system (3.48 x 10^5 $M^{-1}s^{-1}$) (Tanaka *et al.,* 2005, also see Supplementary Table 1). These results indicate that Gpx2 prefers to use thioredoxin for the peroxidase reaction. Delaunay *et al.* (2002) reported that Gpx3 uses only thioredoxin as an electron donor for the peroxidase reaction. However, we have demonstrated in this study that Gpx1 uses glutathione and thioredoxin almost equally as electron donors.

In the peroxidase reactions of SeCys-type GPxs, selenol (Se-H) at the active site is oxidized to selenenic acid (Se-OH), and glutathione is bound to Se-OH to form Se-GS. Subsequently, Se-GS is reduced by another molecule of glutathione to revert Se-H, and consequently, glutathione disulfide (GS-SG) is formed. Meanwhile, for non-SeCys-type GPxs, two cysteine residues are involved in the peroxidase reaction, *i. e.* the first cysteine residue, referred to as the peroxidatic cysteine, is attacked by peroxide and oxidized giving sulfenic acid (Cys-SOH). Cys-SOH is reduced by another cysteine residue, referred to as the resolving cysteine, to form a disulfide bond between the peroxidatic cysteine and resolving cysteine. This disulfide bond is then reduced by thioredoxin. Since Gpx1 does not contain SeCys, cysteine residues are expected to be involved in the reducing reaction of peroxide using glutathione or thioredoxin. We then identified the active cysteine with the AMS assay. AMS is able to modify the sulfhydryl group (-SH) irreversibly, but not sulfenic acid (-SOH) and disulfide bonds (Hermanson, 1996). Gpx1 has six cysteines (Cys^{36}, Cys^{64}, Cys^{82}, Cys^{98}, Cys^{144}, and Cys^{152}) (Fig. 1A), and the molecular weight of AMS is approximately 500, therefore, the apparent molecular weight of Gpx1 theoretically increases 3000 if all cysteines are in the reduced form and modified by AMS. In addition, the oxidized protein having disulfide bonds migrates faster than the reduced protein in non-reducing SDS-PAGE (Tanaka *et al.,* 2005). So, Gpx1 in the reduced form and oxidized form is distinguishable by modification with AMS followed by non-reducing SDS-PAGE. To verify the validity of the assay, bacterially expressed Gpx1 was oxidized by treatment with H_2O_2 or reduced with dithiothreitol (DTT) followed by modification with AMS, and SDS-PAGE was

conducted. As shown in Fig. 1B, reduced Gpx1 (*i. e.* modified with AMS) migrated slowly, whereas, the oxidized Gpx1 migrated faster in SDS-PAGE. So, we introduced the plasmid GPX1-3HA into a *gpx1*Δ mutant, and determined the redox status of the Gpx1 protein. As shown in Fig. 1B, Gpx1 was basically in the reduced form under normal conditions, though half of the protein was oxidized when cells were treated with H_2O_2 or *t*-BHP. Next, we determined the redox status of Gpx1 in a mutant defective in thioredoxin (*gpx1*Δ*trx1*Δ*trx2*Δ) to evaluate the role of thioreoxin in the redox regulation of Gpx1. The oxidized form of Gpx1 was seen in cells lacking the cytosolic thioredoxin even though the cells were not treated with peroxides. This suggests that thioredoxin plays a role in the reduction of Gpx1 *in vivo*. This conclusion was supported by the result that Gpx1 was almost completely oxidized following treatment with H_2O_2 and *t*-BHP in the thioredoxin-deficient mutant (Fig. 1B). On the other hand, the GSH/GSSG balance may be decreased in a *trx1*Δ*trx2*Δ mutant. So, we treated yeast cells with diamide, which oxidizes glutathione (GSH) to form glutathione disulfide (GSSG). We determined the redox state of Gpx1 after treatment of yeast cells with 1.5 mM diamide for 60 min, under which conditions the GSH/GSSG balance was decreased as we have reported previously (Kuge *et al.*, 2001). As shown in Fig. 1C, Gpx1 was essentially in the reduced form. Next, we determined the redox state of Gpx1 in a *glr1*Δ mutant. The *GRL1* gene encodes glutathione reductase that catalyzes the reduction of GSSG to GSH, and we have previously reported that the disruption of *GLR1* enhanced the levels of GSSG *in vivo* (Izawa *et al.*, 1998). Under normal conditions (not treated with diamide), Gpx1 was in the reduced form. After treatment with diamide, Gpx1 was partially

oxidized (Fig. 1C). In *trx1*Δ*trx2*Δ cells, the redox state of Gpx1 was not substantially affected by treatment with diamide (Fig. 1C). Together, we concluded that thioredoxin plays crucial roles in regulating the redox state of Gpx1 *in vivo*.

In a typical 2-Cys peroxiredoxin, the disulfide bond is formed between two molecules of enzyme; whereas in an atypical 2-Cys peroxiredoxin, the disulfide bond is formed intramolecularly. Since no band whose molecular weight corresponds to a dimer of Gpx1 was seen in the AMS assay, Gpx1 seems to be an atypical 2-Cys peroxiredoxin like Gpx2 and Gpx3.

Next, to identify the cysteine residues involved in the redox regulation of Gpx1, we changed each cysteine residue of Gpx1 to serine, and introduced the mutants ($Gpx1^{C36S}$, $Gpx1^{C64S}$, $Gpx1^{C82S}$, $Gpx1^{C98S}$, $Gpx1^{C144S}$, and $Gpx1^{C152S}$) into *gpx1*Δ*trx1*Δ*trx2*Δ cells. Notably, no oxidized Gpx1 appeared in cells carrying $Gpx1^{C36S}$ protein even in the presence of *t*-BHP (Fig. 1C). This indicates that Cys^{36} plays a crucial role in the determination of the redox status of Gpx1 in yeast cells. Additionally, the position of this cysteine residue is well conserved among yeast GPxs, *i. e.* Cys^{37} and Cys^{36} are the peroxidatic cysteines in Gpx2 and Gpx3, respectively (Delaunay *et al.*, 2002; Tanaka *et al.,* 2005) (Fig. 1A). Together, Cys^{36} is thought to be the peroxidatic cysteine of Gpx1. Meanwhile, the pattern of reduced/oxidized protein of $Gpx1^{C144S}$ and $Gpx1^{C152S}$ was similar to that of $Gpx1^{WT}$, suggesting that Cys^{144} and Cys^{152} are not involved in the redox regulation of Gpx1.

Regarding the resolving cysteine, Cys^{83} and Cys^{82} are responsible in Gpx2 and Gpx3, respectively (Delaunay *et al.*, 2002; Tanaka *et al.,* 2005). Judging from an

alignment of primary structure among *S. cerevisiae* GPx homologues, Cys^{82} seems to be the resolving cysteine of Gpx1. Actually, in contrast to $Gpx1^{WT}$, no oxidized band of $Gpx1^{C82S}$ protein was seen in the AMS assay in thioredoxin-deficient cells under normal conditions (Fig. 1B), which suggests that Cys^{82} is involved in the redox regulation of Gpx1. However, since the oxidized band of $Gpx1^{C82S}$ protein merged following treatment of the cells with *t*-BHP, another cysteine residue may be involved in the formation of the intramolecular disulfide bond. A similar pattern was seen in the $Gpx1^{C64S}$ and $Gpx1^{C98S}$ mutants, so the possibility that these two cysteines are also responsible for the formation of a disulfide bond with Cys^{36} cannot be excluded, *i. e.* one of three disulfide bonds between either Cys^{36} and Cys^{64}, Cys^{36} and Cys^{82}, or Cys^{36} and Cys^{98}, might be formed if Cys^{64}, Cys^{82}, or Cys^{98} was substituted with serine.

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Figure legend

Fig. 1. Redox regulation of Gpx1. (A) Schematic diagram of the positions of cysteines in yeast GPxs. The numbers indicate the positions of cysteine residues. A line between cysteine residues represents a disulfide bond. In Gpx1, one of three disulfide bonds, between either Cys^{36} and Cys^{64}, Cys^{36} and Cys^{82}, or Cys^{36} and Cys^{98}, may be formed if Cys^{64}, Cys^{82}, or Cys^{98} is substituted with serine. (B) His-tagged Gpx1 expressed in *E. coli* cell (Gpx1-6His) was treated with 5 mM DTT or 1 mM H_2O_2 for 1 h, and precipitated with 20% trichloroacetic acid (TCA) solution. The samples were then subjected to the AMS assay. Gpx1 protein was detected using anti-His-tag antibody in Western blotting analysis. pRS416-$Gpx1^{WT}$-3HA (Gpx1-3HA) was introduced into *gpx1*Δ and *gpx1*Δ*trx1*Δ*trx2*Δ cells, and the cells cultured in SD medium at 28ºC. After 37 h, 0.4 mM H_2O_2 or 0.6 mM *t*-BHP was added, and cells were cultured for another 1 h at 28ºC. Cells of 40 A_{610} units of the culture were collected, washed twice with a 20% TCA solution and once with acetone, and then subjected to the AMS assay. Gpx1 protein was detected using anti-HA monoclonal antibody in Western blotting analysis. (D) Each cysteine-substituted mutant of *GPX1* harbored in pRS416 was expressed in *gpx1*Δ*trx1*Δ*trx2*Δ cells, and cells were treated with 0.6 mM *t*-BHP for 1 h. After treatment, the AMS assay was done as described in (B). We repeated each experiment three times, and we gained substantially the same results. Pictures represent the typical results. We repeated each experiment three times, and gained substantially the same results. Pictures represent the typical results.