Journal of Biochemistry and Molecular Biology, Vol. 34, No. 2, March 2001, pp. 139-143

JOURNAL OF
Biochemistry and Molecular Biology

Essential Cysteine Residues of Yeast Thioredoxin 2 for an electron donor to Thioredoxin Peroxidases

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Received 6 November 2000, Accepted 11 January 2001

Thioredoxin (Trx) is a redox protein possessing conserved sequence Cys-Gly-Pro-Cys in all organisms. Trx acts as an electron donor of many proteins including thioredoxin peroxidase (TPx). Yeast Trx 2 has two redox active cysteine residues at positions 31 and 34. To investigate the redox activity of each cysteine, we generated mutants C31S, C34S, and C31S/C34S using site directed mutagenesis and examined the redox activity of Trx variants as an electron donor for yeast TPx enzymes. None of the three Cys-mutated Trx proteins was active as a redox protein in the 5', 5'-dithiobis-(2-dinitrobenzoic acid) reduction under the condition of the presence of NADPH and thioredoxin reductase, and in the thioredoxin dependent peroxidase activity of yeast TPx II. C34S enhanced the glutamine synthetase protection activity of yeast TPx I, even though 100 times more protein was needed to exhibit the same activity to WT. The formation of a mixed disulfide intermediate between Trx and TPx II subunits was analyzed by SDS-PAGE. The mixed dimer form of TPx II was found only for C34S. These results suggest that Cys-31 more effectively acts as an electron donor for TPx enzymes.

Keywords: Thioredoxin, Thioredoxin peroxidase, Cysteine

Introduction

A thioredoxin (Trx) is a small soluble protein composed of 105-110 amino acid residues in a single polypeptide chain in which two cysteine residues are arranged in a characteristic -Cys-Gly-Pro-Cys- form. Trx is exhibited either in a reduced form with dithiol, or a oxidized form with a disulfide bond between two cysteine residues. The oxidized Trx is reduced by the action of thioredoxin reductase (TR) with NADPH, and the reduced Trx is a reductant that reduces oxidized target proteins (Holmgren, 1985; Follmann *et al.*, 1995-1996). A function of Trx as an electron donor is consistent with the reduction of an active center disulfide in target proteins. The pKa value of the front cysteine residue, Cys-32, in the active site of *E. coli* Trx (-Cys^{32}-Gly-Pro-Cys^{35}-) has been calculated to be 6.7. It was suggested that the thiolate anion of the acidic cysteine of Trx reacts to disulfide of a target protein, and then formed a mixed disulfide intermediate with the protein in the reduction of the target protein by Trx (Holmgren, 1985).

Since Trx was discovered in *E. coli* as the hydrogen donor of ribonucleotide reductase for the first time, the protein is known to present in most living cells from prokaryotes to eukaryotes as an oxidoredox protein (Follmann *et al.*, 1995-1996). Trx is induced by stress, such as viral infection and oxidative stress, and acts as a regulatory factor of the transcription factors such as NF-B, AP-1, Ref-1, p53, heat shock factor and IRP1, regulator of iron metabolism (Oblong *et al.*, 1994; Hirota *et al.*, 1997; Leppa *et al.*, 1997; Ueno *et al.*, 1999; Lee *et al.*, 2000). Recently, it was discovered that Trx is an immediate electron donor for peroxiredoxin (Prx) that catalyzes the reduction of peroxides (Chae *et al.*, 1994; Kwon *et al.*, 1994; Kang *et al.*, 1998; Cha and Kim, 1999; Jeong *et al.*, 1999; Jeong *et al.*, 2000).

In yeast *Saccharomyces cerevisiae,* three types of Trx are known: Trx 1 and Trx 2 are in cytosol and Trx 3 is in mitochondria (Oliveira *et al.*, 1999). These three Trx proteins are electron donors for yeast thioredoxin peroxidase I (TPx I) and II (TPx II), members of the Prx family (Chae *et al.*, 1994; Kwon *et al.*, 1994; Jeong *et al.*, 1999). It was suggested that the disulfide bond of yeast TPx I is reduced by Trx in the mechanism of peroxide reduction catalyzed by the enzyme (Chae *et al.*, 1994). In this paper, in order to investigate the essential cysteine residue of yeast Trx 2 for redox protein as an electron donor for TPx, we characterized the redox properties of Trx variants generated by site directed mutagenesis of cysteine residues.

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Materials and Methods

Materials TR (Kwon *et al.*, 1994), TPx I (Kim *et al.*, 1988), and TPx II (Jeong *et al.*, 1999) were purified from *S. cerevisiae* BJ986. Glutamine synthetase was purified from *E. coli* pgln6 (Kim *et al.*, 1985).

Cloning and mutagenesis of the yeast Trx 2 gene The DNA fragment corresponding to Trx 2 was obtained by a polymerase chain reaction (PCR) from the yeast *S. cerevisiae* cDNA library (Clontech, Palo Alto, USA) using the forward primer (5'-AAA CAT ATG GTC ACT CAA TTA AAA TCC GCT TCT-3') containing an *Nde*I (underlined) site, the initiation codon (boldface), and the reverse primer (5'-AAA GAA TTC **CTA** GTT GGA AGC AAT AGC TTG-3') containing the *EcoR* I site (underlined) and the stop codon (boldface). The amplified product was purified and digested with *Nde I/EcoR* I. The digested fragment was subcloned into the pET17b expression vector and the resulting recombinant plasmid was transformed into the *E. coli* strain BL21(DE3)pLysS. Three Trx mutant proteins, C31S, C34S, and C31S/C34S, in which Cys-31, Cys-34, and Cys-31, 34 were replaced by serine, were generated by the PCR-mediated site directed mutagenesis with complementary primers containing a 1-base pair mismatch, which converts the cordon for cysteine residue to one for serine. The mutated PCR products were ligated into pET17b and then transformed into the *E. coli* strain BL21(DE3)pLysS.

Expression and purification of recombinant Trx variants *E. coli* BL21(DE3)pLysS cells containing the Trx mutant plasmid were grown to the log phase and induced with 4 mM Isopropyl-1-thio-β-D-galactopyranoside for an additional 4 hrs. Harvested cells were resuspended in 20 mM Tris-HCl (pH 7.6) and sonicated. Cell debris was removed by centrifugation at 10,000 rpm. The supernatant was applied to a DEAE-Sephacel ion exchange column, and bound proteins were eluted with a 900 ml 0-500 mM NaCl gradient in 20 mM Tris-HCl (pH 7.6). Fractions containing the recombinant Trx protein were pooled, precipitated with 95% ammonium sulfate and applied to the Sephadex G-75 gel filtration column preequilibrated with a 200 mM sodium phosphate buffer (pH 7.0). Analysis of the recombinant Trx proteins by immunoblots with anti-yeast Trx antisera was performed.

5', 5'-dithiobis-(2-dinitrobenzoic acid) assay The 5', 5'-dithiobis-(2-dinitrobenzoic acid) (DTNB) assay was monitored for the increase of A_{412} in a 0.5 ml reaction mixture containing a 200 mM phosphate buffer (pH 7.0), 1 mM DTNB, 0.5 mM NADPH, 5 μg of yeast TR, and 10 μg of purified Trx variants. The reaction was started by the addition of 10 μl of Trx variants solutions (Riddles *et al.*, 1983; Miranda-Vizuete *et al.*, 1997).

Thioredoxin dependent peroxidase assay NADPH oxidation was monitored for the decrease of A_{340} in a 0.5 ml reaction mixture containing 50 mM Hepes (pH 7.0), 0.2 mM NADPH, 5 μg of yeast TR, 1 mM H_2O_2, 10 μg yeast TPx II, and 50 μg of recombinant Trx variants. The reaction was started by addition of 10 μl of peroxide solution, and the mixture was incubated at 30°C (Chae *et al.*, 1994; Jeong *et al.*, 1999).

Glutamine synthetase protection assay Glutamine synthetase inactivation was performed in a 25 μl reaction mixture containing 50 mM Hepes (pH 7.0), 1 μg of glutamine synthetase, 10 mM dithiothreitol (DTT), 3 μM $FeCl_2$, 1.0 μg of yeast TPx I, and various concentrations of Trx 2 variants. After 10 min at 37°C, the remaining glutamine synthetase activity was measured by the γ-glutamyl transferase method (Kim *et al.*, 1988; Kwon *et al.*, 1994).

Formation of a mixed disulfide intermediate between Trx variants and yeast TPx II WT and Cys-mutant Trx proteins were preincubated in the absence or presence of yeast TPx II for 10 min then a 15% SDS-PAGE analysis was performed under the non-reducing condition. In addition to making a larger amount of an intermediate mixed disulfide of Trx C34S and TPx II, preincubation was performed with 1 mM DTT and 10 mM H_2O_2 for 10 min and analyzed on SDS-PAGE under non-reducing and reducing conditions. The intermediate between the C34S and TPx II subunits was eluted from the polyacrylamide gel and analyzed on SDS-PAGE under non-reducing and reducing conditions to conform to the intermediate composed of Trx C34S and TPx II subunit.

Results

Purification of Trx variants WT and Cys-mutant Trx proteins, C31S, C34S, and C31S/C34S, were purified from the *E. coli* that was highly overexpressed with the proteins using DEAE-Sephacel ion exchange and gel filtration chromatographic separations. The purity of the WT and Cys-mutant Trx proteins were confirmed on SDS-PAGE. Under reducing conditions, WT, C31S, C34S, and C31S/C34S were all detected at a monomeric form (Fig. 1). Under non-reducing conditions, however, the mobility patterns were complex. The major band of WT, C31S and C31S/C34S proteins corresponded to the molecular size of monomers, whereas the major band of C34S corresponded to the molecular size of a dimer, and the portion of dimer increased during storage (Fig. 4 lanes 1-4).

Activity of WT and Cys-mutants in DTNB assay DTNB is an artificial disulfide substrate and a fast oxidant of Trx-$(SH)_2$, which keeps the concentration of Trx-S_2 constant. DTNB is often used in the assay of Trx, where one molecule of DTNB is split into two 5'-thionitrobenzoic acid molecules by reduced Trx (Holmgren, 1985). To investigate the catalytic cysteine residue, we examined the ability of Trx variants as a substrate for DTNB reducing activity of TR. When the DTNB reducing activity was measured in the presence of NADPH and yeast TR, only WT supported the DTNB reducing activity (Fig. 2).

WT and Cys-mutants Trx as an electron donor for peroxidase activity of TPx Trx is known as an immediate electron donor of TPx, which is the major member of the peroxiredoxin family (Chae *et al*, 1994; Kwon *et al*, 1994; Jeong *et al.*, 1999). The Trx system is composed of NADPH,

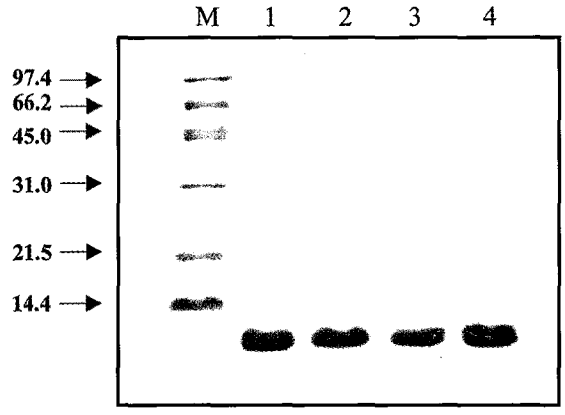


Fig. 1. SDS-PAGE of purified WT and Cys-mutant Trx proteins. Purified Trx variants (5 μg of protein) were separated on the 15% gel. Lane M, low molecular weight standard marker; lane 1, WT; lane 2, C31S; lane 3, C34S; lane 4, C31S/C34S.

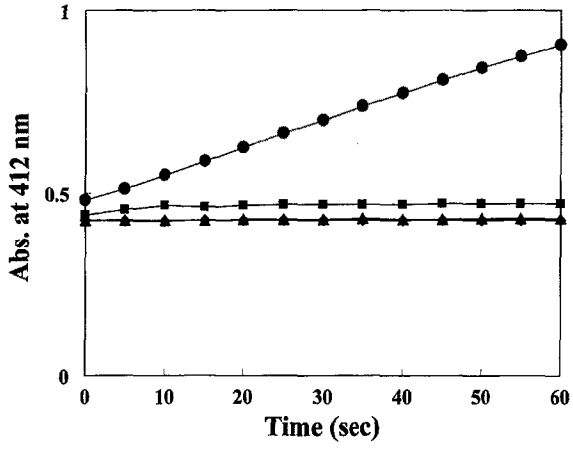


Fig. 2. Activity of WT and Cys-mutant Trx proteins in DTNB Assay. The DTNB assay was monitored as the increase in A_{412} in a 0.5 ml reaction mixture containing 200 mM phosphate buffer (pH 7.0), 1 mM DTNB, 0.5 mM NADPH, 5 μg of yeast TR, and 10 μg of recombinant Trx variants. The reaction was started by addition of 10 μl of a Trx protein solution. -●-, WT; -■-, C31S; -▲-, C34S; -◆-, C31S/C34S.

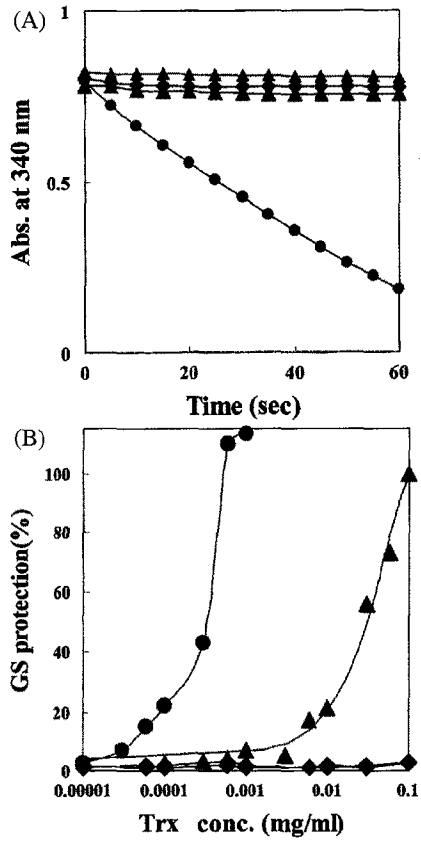


Fig. 3. Effects of WT and Cys-mutant Trx proteins on the thioredoxin dependent peroxidase activity of yeast TPx II and the glutamine synthetase protection activity of TPx I. (A) NADPH oxidation was monitored as the decrease in A_{340} in a 0.5 ml reaction mixture containing 50 mM Hepes (pH 7.0), 0.5 mM NADPH, 5 μg of yeast TR, 1 mM H_2O_2, 10 μg yeast TPx II, and 50 μg of Trx variants. The reaction was started by addition of 10 μl of a peroxide solution, then the mixture was incubated at 30°C. (B) Glutamine synthetase inactivation was performed in a 25 μl reaction mixture containing 50 mM Hepes (pH 7.0), 1 μg of glutamine synthetase, 10 mM DTT, 3 μM $FeCl_2$, 1.0 μg of yeast TPx I, and various concentrations of Trx variants. After 10 min at 37°C, the remaining glutamine synthetase activity was measured. -●-, WT; -■-, C31S; -▲-, C34S; -◆-, C31S/C34S.

TR, and Trx. Yeast TPx II is known to be a peroxidase that reduces H_2O_2 and alkyl hydroperoxide (Jeong *et al.*, 1999). To investigate the effect as an electron donor for TPx II, we examined whether the Cys-mutated Trx proteins could support the thioredoxin-dependent peroxidase activity of TPx II. Yeast TPx II showed no peroxidase activity with Trx systems comprised of the mutated Trx proteins. Only WT supported the peroxidase activity of the enzyme (Fig. 3A). All three mutant Trx proteins supported no peroxidase activity of TPx I (data not shown).

Effect of WT and Cys-mutants on the glutamine synthetase protection activity of TPx I It is known that yeast TPx I is able to protect glutamine synthetase against damage by the thiol oxidation system comprised of Fe^{3+}, O_2, and DTT (Kim *et al.*, 1988). In the presence of Trx, the glutamine synthetase protection activity of yeast TPx I against the MCO system containing DTT was increased 10 fold (Chae *et al.*, 1994; Kwon *et al.*, 1994). We tested whether or not the Cys-mutated Trx proteins could also enhance the glutamine synthetase protection activity of yeast TPx I. Under the concentration of 25 μg/ml of yeast TPx I, the protection of glutamine synthetase against the MCO system with DTT was not observed. WT Trx showed the increased activity of glutamine synthetase protection activity that was previously reported. Complete protection was observed in the presence of 1.0 μg/ml of WT in the MCO system with DTT, as shown in Fig. 3B. Neither C31S nor C31S/C34S showed any effect on

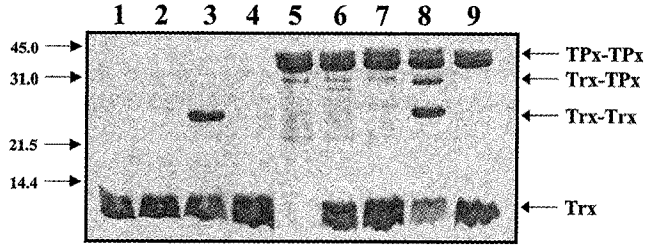


Fig. 4. SDS-PAGE analysis of an intermediate formation by mixed disulfide between Trx and TPx II subunits. WT and Cys-mutant Trx proteins were preincubated in the absence (lanes 1-4) or presence (lanes 6-9) of yeast TPx II for 10 min, then a SDS-PAGE (15%) analysis was performed under the non-reducing condition. Lanes 1 and 6, WT; lanes 2 and 7, C31S; lanes 3 and 8, C34S; lanes 4 and 9, C31S/C34S; lane 5, TPx II only. The arrows indicate a TPx dimer, a hetero dimer of Trx and TPx II, Trx dimer and Trx monomer.

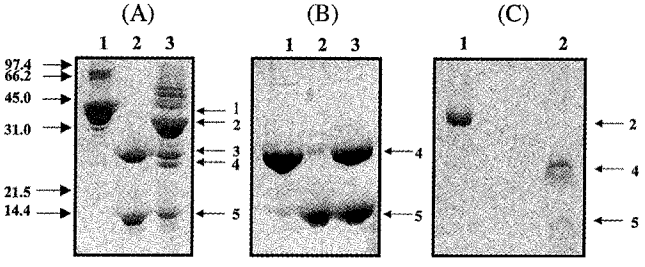


Fig. 5. SDS-PAGE analysis of an intermediate formation by mixed disulfide between C34S and TPx II subunits with 1 mM DTT and 10 mM H_2O_2. A and B, C34S, TPx II, and mixtures of C34S and TPx II were preincubated with 1 mM DTT and 10 mM H_2O_2 for 10 min, then a SDS-PAGE (15%) analysis was performed under the non-reducing condition (A) and reducing condition (B). Lane 1, TPx II only; lane 2, C34S only; lane 3, C34S and TPx II. C, Band 2 of lane 3 in A was eluted from the polyacrylamide gel and analyzed on SDS-PAGE under the non-reducing condition (lane 1) and the reducing condition (lane 2). The arrows indicate each protein band; 1, a TPx dimer; 2, a hetero dimer of Trx and TPx II; 3, Trx dimer; 4, TPx II monomer; 5, Trx monomer.

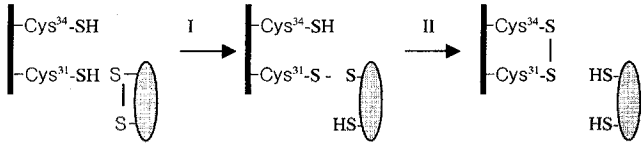
the protection of glutamine synthetase by TPx I. C34S, however, showed the enhancing effect at the concentration of 0.03 mg/ml as IC_{50}, which was about 100 fold higher than that of WT, 0.3 μg/ml (Fig. 3B).

Formation of a mixed disulfide intermediate between Trx variants and yeast TPx II The NFκB DNA binding activity is enhanced by reduction with Trx. A kinetically stable mixed disulfide intermediate between NFκB and a peptide of human Trx was obtained (Qin . *et al.,* 1995). Trx is an electron donor of TPx II (Jeong *et al.,* 1999). We tested whether thioredoxin binds to TPx II, and which cysteine residue is important to the binding. WT and Cys-mutant Trx proteins were preincubated with TPx II for 10 min, then a SDS-PAGE analysis was performed. A new band on SDS-PAGE was detected only for TPx II and C34S, and the band was estimated as 30 kDa (Fig. 4). The new band formed from Trx C34S and TPx II did not cross-react well with antibodies against either yeast Trx or TPx II. The intensity of the band was increased under the presence of 10 mM H_2O_2 and 1 mM DTT in the reaction mixture for preincubation of C34S and TPx II (Fig. 5A). There was no remarkable change in the pattern of SDS-PAGE for the other Trx proteins (data not shown). Under reducing conditions, only two protein bands, TPx II and Trx 2, were detected (Fig. 5B). This means that the newly formed band is caused by a disulfide bond rather than other covalent bonds. To confirm that the new band comprised TPx II and Trx 2, we eluted the band from gel, then analyzed it on SDS-PAGE. Under non-reducing conditions, one band was detected that was the size of the mixed disulfide proteins of TPx II and Trx 2. Under reducing condition, two bands corresponding to Trx 2 and TPx II were detected (Fig. 5C).

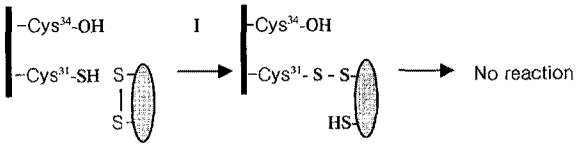
Discussion

In order to investigate the essential cysteine residue in Trx that is needed to interact with Prx as an electron donor, we studied three mutants, C31S, C34S and C31S/C34S. These replaced the cysteine residue to serine, respectively, by site directed mutagenesis. Under non-reducing conditions, WT, C31S and C31S/C34S were detected as monomer. C34S, however, was detected as a dimer on SDS-PAGE, and the portion of the dimer was increased during storage. Since the pKa value of the front cysteine (Cys^{31}) is very low, the reactivity is higher than Cys^{34} and in C34S, the more reactive cysteine residue, Cys^{31}, formed a intermolecular disulfide bond with other C34S because there was no Cys^{34} in that protein.

The formation of a mixed disulfide intermediate was found only for C34S with TPx II. With 10 mM H_2O_2 and 1 mM DTT, the intensity of the mixed disulfide band increased. The new band that was comprised of TPx II and Trx was confirmed by protein elution and SDS-PAGE analysis. Under non-reducing conditions, one band was detected the size of the mixed disulfide proteins of TPx II and Trx 2. Under reducing conditions, the two bands were detected as Trx 2 and TPx II (Fig. 5C). A mixed disulfide intermediate from TPx II could not be formed with C31S and C31S/C34S mutant Trx proteins because of the absence of the Cys^{31} residue. The mixed disulfide bond was formed from Cys^{31} of WT Trx and TPx II was reduced by the thiol group of Cys34, as suggested by Holmgren. (1985) (Scheme I). The mixed disulfide between Cys^{31} of Trx C34S and TPx II, however, can be trapped because of the absence of Cys^{34} (shown in Scheme II).



Scheme I



Scheme II

Neither the DTNB reduction activity, nor the thioredoxin-dependent peroxidase activity was supported by the Cys-mutated Trx proteins. It is well known that both cysteine residues in the active site of Trx are required for the substrate of TR. In the glutamine synthetase protection activity of TPx I, however, C34S showed the enhancing effect even though there is 100 times more protein needed compared to WT. The mixed disulfide bond in the trapped intermediate could not be reduced by TR, but reduced by DTT (Fig. 2, 3). Thus, in the MCO system containing DTT, Trx C34S can be active in the enhancement of the enzyme protection activity of TPx II.

These results suggested that cysteine-31 of yeast Trx 2 is more reactive in the reduction of disulfide bonds of yeast TPx enzymes and first reacts with TPx II.

Acknowledgments This study was supported by a grant from the Academic Foundation of the Chonnam National University in 1997.

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