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Thioredoxin in the Periplasmic Space of *Escherichia coli* as a Physiological Electron Donor to Periplasmic Thiol Peroxidase, p20

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We previously reported that a novel thiol peroxidase (p20) from *Escherichia coli* is a distinct periplasmic peroxidase that detoxifies hydroperoxides together with glutathione or thioredoxin. Until now, there was no experimental evidence for the presence of thioredoxin (Trx) in the periplasmic space. In an attempt to confirm the physiological function of p20 as a thiol peroxidase supported by Trx in the periplasmic space, we have purified a Trx activity from the periplasmic space of *Escherichia coli* and identified the Trx as the same protein as the cytoplasmic Trx. The presence of Trx in the periplasmic space of *Escherichia coli* suggests that p20 is a unique extracellular Trx-linked thiol peroxidase.

Keywords: *Eschericia coli,* Periplasmic Space, Thiol Peroxidase, Thioredoxin, Translocation.

Introduction

We previously reported that p20, a periplasmic peroxidase, is a member of a new thiol peroxidase family whose constituents contain functional cysteine instead of selenocysteine (Cha *et al.*, 1995; Cha and Kim, 1996). Thiol peroxidase has been purified from a number of sources, such as *Escherichia coli* to mammals (Kim, *et al.*, 1988; 1989; Jacobson *et al.*, 1989; Lim *et al.*, 1994a; Cha *et al.*, 1995; 1996; Cha and Kim, 1996). Database searches have revealed about 20 proteins among all kingdoms that show similarity to thiol peroxidase (Chae *et al.*, 1993; Lim *et al.*, 1994b; Cha and Kim, 1998). The *E. coli* periplasmic p20 is a nonselenoperoxidase, structurally distinct from the known intracellular thiol peroxidase family (Cha and Kim, 1996). One prokaryotic member of the thiol peroxidase family, 21-kDa alkyl hydroperoxide reductase (AhpC), was purified from *E. coli* and *Salmonella typhimurium* and it was demonstrated that the peroxidase activity was supported by a 57-kDa AhpF coupled to NAD(P)H oxidation (Jacobson *et al.*, 1989). All members of the thiol peroxidase family have the capability to reduce peroxides in the presence of thiol-reducing equivalents such as glutathione (GSH), dithiothreitol (DTT), and thioredoxin (Trx) (Lim *et al.*, 1993; Chae *et al.*,1994; Kown *et al.*, 1994; Cha and Kim, 1995). Peroxidase activity of p20 is supported by both nonenzymatic and enzymatic thiol-reducing equivalents such as GSH and Trx, respectively.

Trx is a 12-kDa ubiquitous protein with a redox-active dithiol/disulfide in the active site (Holmgren, 1989). The redox-active dithiol is continuously generated by Trx reductase (TR) and NADPH (Holmgren, 1985). This Trx system has been known to serve as a disulfide-reducing system for general protein (Holmgren, 1985). Trx has been reported to be secreted through a leaderless secretory pathway by normal and transformed mammalian cells (Rubartelli *et al.*, 1992; 1995; Ericson *et al.*, 1992).

Recent results show that p20 is efficiently reduced by either *E. coli* cytoplasmic Trx system or GSH (Lim *et al.*, 1993). However, there are no reports concerning the presence of Trx in the periplasmic space of *E. coli.* The aim of the present study was to identify a p20 functional disulfide-reducing system in the periplasmic space of *E. coli.* The Trx system or chemicals such as GSH could serve as reducing equivalents to the periplasmic p20 and thereby reveal the major antioxidative role in an environment almost free from antioxidants such as catalase and peroxidases.

Materials and Methods

Strain and culture conditions *Escherichia coli* wild-type K12 was used for the preparation of Trx from both the cytoplasmic and periplasmic proteins. The *E. coli* temperature-sensitive SecY mutant, IQ85 (SecY24), and the SecA mutant, HB754 (SecA51),

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were used for the analysis of Trx in the periplasmic space. The mutants were generous gifts from Dr. C. K. Park, Department of Life Science, KAIST, Taejon, Korea. The mutants were cultured at 30°C and then the culture temperature was raised to 42°C for inactivation of SecY. A 447-bp *Trx* gene was amplified from *E. coli* K-12 chromosomal DNA using PCR techniques with an *Nde*1 forward primer and *Bam*H1 reverse primer. The *Trx* gene was inserted between the *Nde*I and *Bam*H1 sites of the pET vector, a T7 expression vector. The resulting *E. coli* recombinant BL21-pET/Trx, a recombinant for overproduction of *E. coli* Trx, was grown to OD_{600} of 0.9–1.0 with LB medium containing 100 μg/ml of ampicillin, and then overexpression of the cytoplasmic *Trx* gene was induced with 1 mM isopropyl-β-thiogalactopyranoside.

Preparation of periplasmic proteins and purification of thioredoxin from *E. coli* *E. coli* K-12 was grown in LB medium. Cells were washed three times with 10 mM Tris·HCl buffer, pH 7.4, containing 30 mM NaCl. The periplasmic fraction of the cells was isolated using an osmotic shock method (Heppel, 1967), where cells were incubated for 10 min with 20% sucrose, 1 mM EDTA, and 30 mM Tris·HCl buffer at pH 7.4, and then transferred to the same volume of cold deionized water. Proteins of the periplasmic space were released into the solution by such treatments, whereas the proteins of the cytoplasm were retained. After centrifugation, the supernatant was treated with ammonium sulfate to a concentraton of 80% to precipitate the periplasmic proteins. For the preparation of thioredoxin from the cytoplasm, the precipitated cells were resuspended in 50 mM Tris·HCl, pH 7.6, containing 2 mM phenylmethylsulfonyl fluoride. Following freezing-and-thawing and sonication (50% power) eight times for 3-min intervals interspersed with periods of cooling on ice, the cell debris was removed by centrifugation. The supernatant was brought to 80% ammonium sulfate by slow addition of solid ammonium sulfate. Precipitates from the periplasm and cytoplasm were each dissolved in 50 mM Tris·HCl, pH 7.6, and dialyzed against the same buffer. The dialyzed solution was applied to a DEAE-cellulose column (5 × 30 cm) equilibrated with 50 mM Tris·HCl buffer, pH 7.6. The thioredoxins were eluted by a linear gradient (0–400 mM KCl) in a total volume of 1 L of Tris·HCl buffer, pH 7.6. The active fractions were pooled, and the proteins were precipitated with 80% ammonium sulfate. The precipitate was dissolved in 50 mM Hepes-NaOH buffer, pH 7.4, and applied to a sephacryl S-200 column (2.5 × 75 cm). The ammonium sulfate precipitate of the fractions showing Trx activity was dissolved in 100 mM Hepes-NaOH buffer, pH 7.4, and applied to a sephacryl S-100 column (2.5 × 70 cm) equilibrated with 100 mM Hepes-NaOH buffer (pH 7.4) containing 200 mM NaCl. The active fractions were concentrated and applied to an additional sephadex G-50 column (1.5 × 100 cm). A 12-kDa protein showing Trx activity was obtained from each of the sephadex G-50 columns loaded with periplasmic and cytoplasmic proteins. The concentrated samples of the active fractions were each applied to an HPLC gel permeation column (G-300, 1.2 × 30 cm) for further purification.

Assays for thioredoxin Trx activity was determined by measurement of the increase of absorbance at 412 nm resulting from the reduction of DTNB in the presence of NADPH and *E. coli* TR.

Sequencing of tryptic peptides from the 20-kDa protein The purified thioredoxin (400 μg) was reductively denaturated in 6 M guanidine HCl solution containing 1 mM DTT and 50 mM Tris·HCl (pH 7.8). The sulfhydryl group(s) was labeled with TNB by treatment with 10 mM DTNB for 1 h at 37°C. The TNB-linked protein was precipitated with 5 vol of acetone. The resulting protein was suspended in 50 mM Tris·HCl (pH 7.8), and after digestion with 10 μg of trypsin for 3 h at 37°C, an additional digestion with another 10 μg of trypsin was carried out overnight at 30°C. The resulting peptides were applied to a preparative Vydac C_8 column (25 × 250 mm), and eluted with a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid over 40 min at a flow rate of 1 ml/min. Peptides containing cysteine residues were detected by monitoring at 328 nm.

Other methods Protein concentration was determined using the Bio-Rad Protein Assay kit. Detection of thioredoxin was carried out on 16% reducing SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R-250. Periplasmic proteins of BL21-pET/Trx were released by treating cells with chloroform (Ames *et al.*, 1984) for analysis of the Trx secretion on 16% SDS PAGE. Alkaline phosphatase activity was measured in terms of the increase in absorbance at 410 nm resulting from the production of *p*-nitrophenol from *p*-nitrophenyl phosphate.

Results and Discussion

Trx also exists in the periplasmic space of *Escherichia coli* The peroxidase activity of thiol peroxidase (p20) from *E. coli* periplasmic space was previously reported (Lim *et al.*, 1993). However, there is no report concerning the existence of Trx in the periplasmic space. Therefore, it is very important to identify a Trx or Trx-like protein in the periplasmic space as a physiological reducing equivalent to p20.

We found thioredoxin activity in the periplasmic crude proteins from *E. coli*. Figure 1 shows the gel filtration

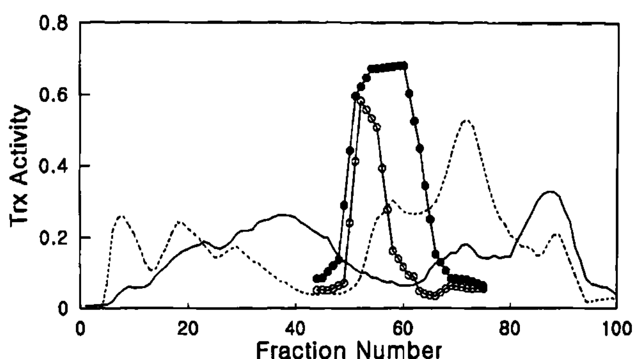

Fig. 1. Thioredoxin activity in the periplasmic proteins released from *E. coli* K-12. Each 100 mg of crude proteins from the periplasm and the cytoplasm was separated by Sephacryl S-200 gel permeation chromatography. The solid line without symbol represents the absorbance at 280 nm from the periplasmic proteins; dotted line, from the cytoplasmic proteins. Closed circle indicates the thiredoxin activity from the periplasmic proteins; open circle, the activity from the cytoplasmic proteins.

chromatography of periplasmic Trx activity on a Sephacryl S-200 column. The latter fractions contained Trx activity. We homogeneously purified the Trx activity from the periplasmic space and cytoplasm. Figure 2 shows the gel filtration chromatography of Trx activity on HPLC G-300 column as a final purification step. HPLC chromatographies of periplasmic Trx and cytoplasmic Trx yield single peaks showing Trx activities. The periplasmic Trx activity was eluted at the same volume as the cytoplasmic Trx indicating that it had the same molecular mass. An analysis of periplasmic Trx activity on a reducing SDS polyacrylamide gel electrophoresis (PAGE) (*inset* of Fig. 2) also displayed a single band on the SDS-PAGE with the apparent same molecular mass as that of the cytoplasmic Trx.

The amino-terminal sequences of periplasmic and cytoplasmic Trx were determined to be the same, as follows: SDKIIHLTDDSFDTDVLKAD. The absorbance pattern of the tryptic digest of reduced periplasmic Trx at 210 nm was exactly the same as that of cytoplasmic Trx (data not shown). The tryptic digests containing cysteines were modified with 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB) and the resulting TNB (5-thio-2-nitrobenzoic acid)-conjugated peptides were detected at 328 nm. The pattern of tryptic digest of the periplasmic Trx at 328 nm was also the same as that of cytoplasmic Trx (data not shown). Also, immunoblotting analysis with anti-cytoplasmic trx polyclonal antibodies (Fig. 3B) indicates that the periplasmic Trx has the same antigenicity towards the antibodies as the cytoplasmic Trx.

Taken together, these results indicate the presence of Trx in the periplasmic space of *E. coli*, suggesting the secretion of Trx from the cytoplasm to the periplasm.

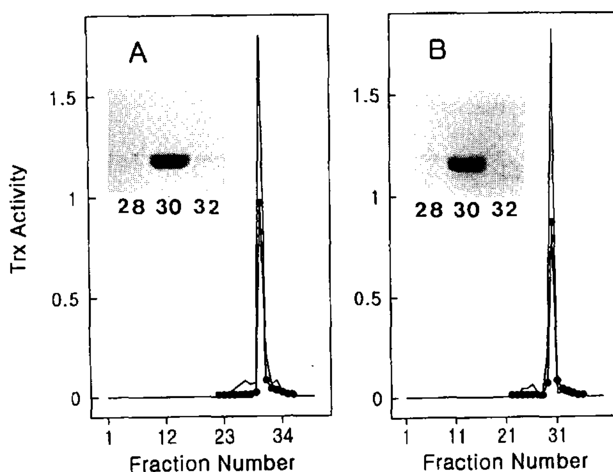

Fig. 2. Purification of thioredoxin from the periplasm and the cytoplasm of *E. coli*. Panel A represents the HPLC G-300 chromatogram of the periplasmic thioredoxin; panel B, that of the cytoplasmic thioredoxin. Each inset in the figures shows the corresponding thioredoxin visualized on 16% reducing SDS-PAGE.

Normal and mutant *E. coli* lacking SecY translocate thioredoxin to the periplasmic space In the *E. coli* periplasmic space, a protein showing Trx activity was purified and identified as Trx which has been known to

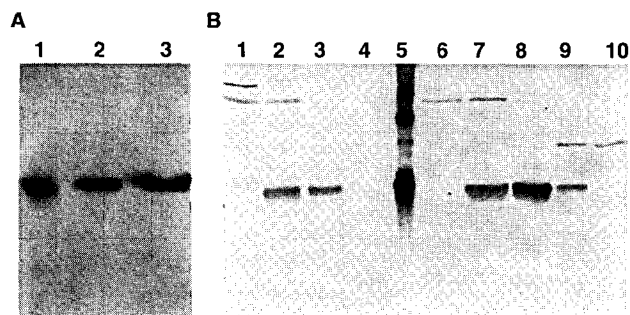

Fig. 3. Immunoblotting analysis of thioredoxin located in the periplasmic space of *E. coli*. Samples were separated by 16% SDS-PAGE, blotted onto a nitrocellulose membrane, and probed with anti-*E. coli* Trx polyclonal antibodies. Panel A: lane 1, 200 ng of *E. coli* Trx as a control; lane 2, 200 ng of Trx purified from the cytoplasm; lane 3, 200 ng of Trx purified from the periplasm. Panel B: immunoblot analysis of the Trx activities secreted to the periplasm of SecY mutants grown at permissive (lanes 1–4) and nonpermissive (lanes 6–10) temperatures (30 °C and 42°C, respectively). Details are described in the legend of Fig. 5. Lanes 1 to 4, 20 μl samples of fractions 20, 23, 26, and 28, respectively; lanes 6 to 10, samples of fractions 20, 23, 26, 28, and 30, respectively, indicated in Fig. 5B. Lane 5, *E. coli* Trx as a standard.

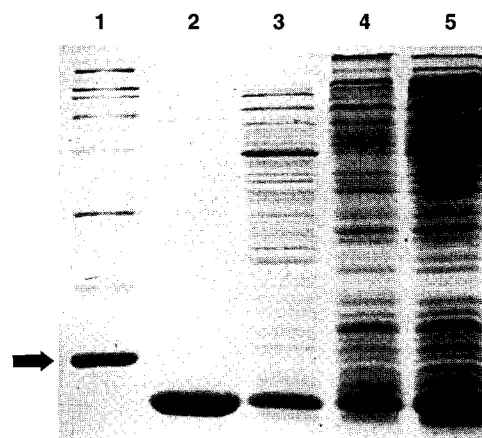

Fig. 4. SDS-PAGE analysis of translocation of overexpressed Trx to the periplasmic space of *E. coli*. The periplasmic proteins were released into 200 ml of Tris·HCl buffer (pH 7.4) by treatment of 3 ml culture of BL21-pET/Trx with chloroform. After washing two times with saline solution, the resulting cells without periplasmic proteins were suspended in the same volume of the same buffer to obtain the cytoplasmic proteins. The whole cells from the 1.5 ml culture were suspended in the 200 μl buffer to obtain whole proteins. Each 20 μl of the sample was loaded on to 16% reducing SDS-PAGE. Lane 1, size marker (arrow, 14.5 kDa); lane 2, 3 μg of Trx; lane 3, periplasmic proteins; lane 4, cytoplasmic proteins; lane 5, whole proteins.

function as an intracellular disulfide-reducing enzyme. Existence of Trx in the extracellular space, despite its lack of a signal sequence, suggests the secretion of Trx by *E. coli* through a leaderless secretory pathway.

In order to confirm the secretion of Trx to the periplasmic space of *E. coli*, an investigation on the expression of Trx in the periplasmic space was performed using *E. coli* recombinants, BL21-pET/Trx, which overproduce *E. coli* Trx. Evident distribution of Trx in the periplasmic space (Fig. 4) denotes the secretion of Trx to the periplasmic space.

A preprotein translocation system of *E. coli*, the Sec (secretion) protein complex, is involved in the secretion of periplasmic proteins such as alkaline phosphatase (Inouye *et al.*, 1982; Ames, 1986; Akiyama and Ito, 1989). The Sec complex consists of a soluble SecA protein and several membrane proteins including the SecY protein. Therefore, the Sec temperature-sensitive mutants grown at nonpermissive temperature were blocked in protein translocation through *E. coli* preprotein translocase (Wickner and Leonard, 1996). Figure 3B shows the immunoblot analysis of the secreted Trx in the periplasmic space of SecY mutants. The secretion of Trx in SecY mutants grown at nonpermissive temperature (42°C) was higher than that by the mutants grown at permissive temperature (30°C), suggesting that the secretion of Trx is not dependent on the Sec translocase. Also, we analyzed the extent of the secretion of Trx in terms of Trx activities (Fig. 5B). The activity of Trx in the periplasm of SecY mutants grown at 42°C was higher than that cultured at 30°C, supporting the result of the immunoblot experiment shown in Fig. 3B. However, the activity of alkaline phosphatase, which is exported to the periplasmic space through a Sec-dependent periplasmic protein translocation pathway (Akiyama and Ito, 1989), was significantly decreased (Fig. 5A). Taken together with these results and the fact that Trx does not have any signal sequences, we suggest that the secretion of Trx to the periplasmic space occurs through a leaderless secretory pathway. The secretion of Trx to the periplasmic space of *E. coli* is supported by other reports. Rubartelli *et al.* (1992) have shown that the secretion of Trx by normal and neoplastic cells occurs through a leaderless secretory pathway. In addition to Trx, proteins such as interleukin-1β, a cytokine of monocyte (Rubartelli, 1990), with a defined extracellular function but lacking a signal sequence, have been identified as secretory proteins.

In this paper, we have described the presence of Trx in the periplasmic space of *E. coli* as an electron donor to the periplasmic thiol peroxidase (p20) and confirmed that periplasmic Trx exists in the same form as cytoplasmic Trx. The presence of Trx in the periplasm, therefore, indicates that the secretion of Trx from cytoplasm to periplasmic space occurs through an unknown leaderless secretory pathway. Our result provides one explanation for how p20 can act as the major peroxidase in *E. coli* periplasm. Trx could be a major electron donor to electron-requiring periplasmic proteins of *E. coli*. Further studies will be required to provide explanation for how reducing equivalents are transferred to the oxidized Trx in the periplasmic space of *E. coli*.

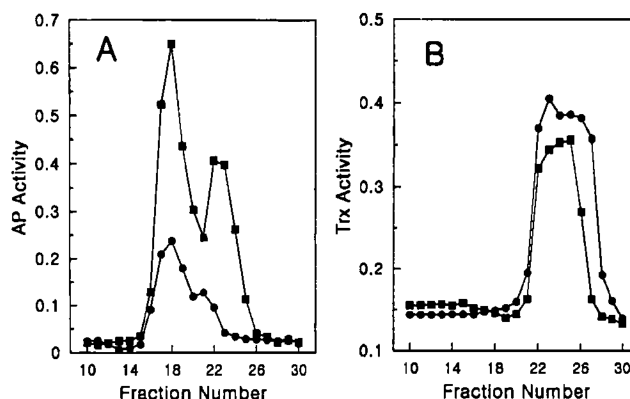

Fig. 5. Analysis of the secretion of thioredoxin to the periplasmic space of *E. coli* SecY mutant. The temperatuture-sensitive SecY mutant was cultured at 30°C for 4 h, and the culture was then shifted to 42°C for inactivation of the SecY protein. The periplasmic proteins released from the periplasm by osmotic shock were separated using Sephacyl S-200 gel permeation chromatography. Panel A shows the alkaline phosphatase activities of the periplasmic proteins from the 30°C culture (closed square) and the 42°C culture (closed circle). Panel B shows the thioredoxin activities of the periplasmic proteins from the 30°C culture (closed square) and the 42°C culture (closed circle).

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