Thioredoxin-linked “Thiol Peroxidase” from Periplasmic Space of Escherichia coli*

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Three different molecular masses (24, 22, and 20 kDa) of antioxidant proteins were purified in Escherichia coli. These proteins exhibited the protective effects against the inactivation of glutamine synthetase activity and the cleavage of DNA by a metal-catalyzed oxidation system capable of generating reactive oxygen species. Their antioxidant activities were supported by a thiol-reducing equivalent such as dithiothreitol. Analysis of the amino-terminal amino acid sequences and the immunoblots between 24- and 22-kDa proteins indicates that the 24-kDa protein is an intact form of the 22-kDa protein that was previously identified 22-kDa subunit (AhpC) of E. coli alkyl hydroperoxide reductase (AhpC/AhpF). We isolated and sequenced an E. coli genomic DNA fragment that encodes 20-kDa protein. Comparison of the deduced amino acid sequence of the 20-kDa protein with that of AhpC revealed no sequence homology. A search of a data bank showed that the 20-kDa protein is a new type of antioxidant enzyme. The synthesis of this novel 20-kDa protein was increased in response to oxygen stress during growth. The 20-kDa protein resides mainly in the periplasmic space of E. coli, whereas the 24-kDa AhpC resides mainly in the matrix. The 20-kDa protein was functionally linked to the thioredoxin as an in vivo thiol-regenerating system and exerted a peroxidase activity. This 20-kDa protein is thus named “thiol peroxidase,” which could act as an antioxidant enzyme removing peroxides or H₂O₂ within the catalase- and peroxidase-deficient periplasmic space of E. coli.

In an aerobic environment, reactive oxygen species (O₂, H₂O₂, ROOH, and HO) are generated by many physiological processes such as incomplete reduction of molecular oxygen during respiration, NADPH oxidation linked to respiratory burst during phagocytosis, and redox cycling of xenobiotics (1). To prevent the deleterious effect of oxygen species, cells have equipped with a number of antioxidant enzymes, including catalases, peroxidases, and superoxide dismutase.

Recently, a 25-kDa antioxidant enzyme was purified from various eukaryotes, including yeast (2), human erythrocyte (3), and rat brain (4). These enzymes prevent the oxidative damage induced by oxidation system capable of generating reactive oxygen species in the presence of a thiol reducing equivalent such as DTT (2-4). However, such an antioxidant activity was abolished without a thiol-reducing equivalent. Thus, this enzyme has been named “TSA” (“thiol-specific antioxidant protein”). Previously, we have reported that the yeast TSA has a capability to destroy H₂O₂ in the presence of DTT (5), and such a peroxidase activity was greatly enhanced by the in vivo thiol-regenerating system (thioredoxin-thioredoxin reductase- NADPH) (6). However, its physiological significance as a peroxidase is still debatable because of the existence of conventional catalases and peroxidases in eukaryotic cytoplasm. Yeast and human genes that encode the 25-kDa TSA have been cloned and sequenced (7, 8). The deduced amino acid sequences showed no homology to known antioxidant enzymes (8, 9). An analysis of data bases revealed 27 additional protein sequences showing homology to the 25-kDa TSA. The biochemical functions of these homologous proteins (TSA family) are not yet clarified except for AhpC, one subunit of alkyl hydroperoxide reductase found in Salmonella typhimurium and Escherichia coli (10). The alignment of amino acid sequences of TSA family revealed two highly conserved cysteine residues. The yeast TSA whose conserved cysteines were replaced with serines completely lost antioxidant activity, which indicates that these cysteine residues are essential for the activity (11, 12). Thus, the TSA/AhpC family has been suggested to be a new type of peroxidase containing functional cysteines (6, 9, 12).

During aerobic growth, E. coli can be exposed to endogenous and exogenous reactive oxygen species from various oxidation reactions. These reactive oxygen species are known to damage cellular constituents. Alkyl hydroperoxides among many products of oxygen radical damage have a capability to initiate and propagate free radical chain reactions leading to DNA and membrane damages (13). In eukaryotes, glutathione peroxidases catalyze the reduction of alkyl hydroperoxides to the corresponding alcohols and H₂O. However, there has been no evidence that glutathione peroxidase exists in prokaryotes. A peroxidase was identified in both Salmonella typhimurium and E. coli (10). The purified activity required two separable subunits, 22- and 57-kDa proteins. The 57-kDa AhpF-linked and 22-kDa AhpC proteins converts alkyl hydroperoxides to the corresponding alcohols. This enzyme (AhpF/AhpC), hence, was suggested to serve as a prokaryotic equivalent to the glutathione reductase/glutathione peroxidase system in eukaryotes.

From E. coli, we purified three proteins (20, 22, and 24 kDa) showing thiol-dependent antioxidant activities. The 20-kDa antioxidant enzyme among the three proteins was shown to be a novel antioxidant enzyme, which resided in the periplasmic space of E. coli. In this paper we reported the purification and

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§ The abbreviations used are: DTT, dithiothreitol; DTNB, 5,5'-dithio- bis(2-nitrobenzoic acid); TNP, 2-nitro-5-thiobenzoate; PAGE, polyacrylamide gel electrophoresis; TSA or PRP, thiol-specific (or -dependent) antioxidant; AhpC, a 22-kDa component of alkyl hydroperoxide reductase; AhpF, a 57-kDa component of alkyl hydroperoxide reductase; Trx, thioredoxin; MCO, metal-catalyzed oxidation.
characterization of a novel E. coli 20-kDa antioxidant protein showing peroxidase activity and discussed its physiological function in the periplasmic space on the basis of thioredoxin (Trx)-linked "thiol peroxidase."

**EXPERIMENTAL PROCEDURES**

Materials—A stock solution of FeCl₃ was prepared in 0.1 n HCl. Glutamine synthetase was purified from the E. coli (Pgln/YMC10), as described (14). Two protein components, thioredoxin and thioredoxin reductase, also were purified from wild type E. coli K12 according to the methods reported previously (6).

**Purification of Bacterial Thiol Antioxidant Enzymes—** Wild type E. coli K12 were grown in 50 liters of LB medium. The harvested cells were centrifuged and resuspended in 100 ml of 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 1% of streptomycin sulfate by slow addition of 10% solution. After 30 min on ice, the nucleic acid precipitate was removed. The supernatant then was treated with ammonium sulfate to a concentration of 70% to precipitate proteins. The precipitate was dissolved in 10 ml of 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) previously equilibrated with 20 mM Tris buffer (pH 7.6). The thioldependent antioxidant activities of the enzymes were eluted by a linear gradient (0–400 mM KCl) in a total volume of 1 liter of Tris-HCl buffer (pH 7.6). The active fractions were pooled, and the proteins were precipitated with 70% ammonium sulfate. The precipitate was dissolved in 50 mM Hepes-NaOH buffer, pH 7.4, and was applied to a Sephadex G-75 column. All of the fractions revealed the antioxidant activity, but yielded two peaks, I and II (not shown). Each ammonium sulfate precipitate of the peaks I and II was dissolved in 100 ml Hepes-NaOH buffer (pH 7.4), containing 1.0 M ammonium sulfate, and applied to the respective phenyl-Sepharose CL-4B column (2.5 × 20 cm) previously equilibrated with 100 ml Hepes-NaOH buffer (pH 7.4) containing 1.0 M ammonium sulfate. Proteins were eluted from the columns by a linear gradient from 0.0 to 0.5 M ammonium sulfate to the buffer containing no salt and then eluted by cold deionized and distilled water. A strong thioldependent antioxidant activity was eluted by deionized and distilled water in the case of the phenyl column for Sephadex G-75 peak II fractions. A major thiol-dependent activity was eluted at the concentrations of 0.5–0.25 M ammonium sulfate in the case of the phenyl column for Sephadex G-75 peak I fractions. The active fractions from two phenyl columns were separately concentrated with Centricon 10 (Amicon). Each concentrated sample was applied to an additional Sephadex G-75 (for 20-kDa protein) or G-100 column (for 22-kDa protein). A 22-kDa protein showing the antioxidant activity was homogeneously obtained from the front part of protein peak of Sephadex G-100 column loaded by the phenyl fraction of Sephadex G-75 peak I. The thioldependent activity appeared in a shoulder just after the major protein peaks of Sephadex G-75 column for the phenyl fraction of Sephadex G-75 peak II. The concentrated sample of the active fractions was applied to a Sephadex G-50 column for the further purification. The active fractions were concentrated and washed with 50 ml Tris buffer (pH 7.6). The resulting sample was applied to an Accell-QMA column and eluted with a linear KCl gradient from 0.0 to 0.1 M KCl, which gave a 20-kDa of homogeneous thiol-dependent antioxidant protein at a concentration of 0.4–0.6 M KCl. The activities of E. coli antioxidant enzymes were assayed by monitoring their thioldependent antioxidant activity as described below.

**Determination of Thioldependent Thiol Antioxidant Activity—** Thioldependent antioxidant activities of E. coli thiol antioxidant enzymes were determined by monitoring their activities to inhibit the inactivation of E. coli glutamine synthetase by a metal-catalyzed thiol system (DTT, Fe²⁺, O₂) (thiol MCO system) (15) as described by Kim et al. (2). Instead of DTT, ascorbate was included as a non-thiol-reducing equivalent (non-thiol MCO system). Fifty µl of reaction mixture containing 5 µM of glutamine synthetase, 3 µM FeCl₃, 10 mM ascorbate, thiol antioxidant enzyme, and 100 mM Hepes-NaOH (pH 7.0) was incubated at 37°C. The remaining activity of glutamine synthetase was measured by addition of 10 µl of the reaction mixture to 2 ml of γ-glutamyltransferase assay mixture as described (2). To determine the antioxidant activity linked to Trx, the remaining glutamine synthetase activity was monitored in the reaction mixture containing 5 µM of glutamine synthetase, 3 µM FeCl₃, 10 µM DTT, 50 µM Trx, 50 µg/ml E. coli thioredoxin reductase, and 100 mM Hepes-NaOH buffer (pH 7.0).

Determination of Peroxidase Activity of the Antioxidant Enzyme—linked to Thioredoxin—To determine the peroxidase activity of the enzyme, the reaction was started by the addition of 1 mM H₂O₂ into the 50 µl of reaction mixture containing 2 mM NADPH, 12.5 µM E. coli Trx, 12.5 µg/ml E. coli thioredoxin reductase, 1 mM EDTA, and 50 mM Hepes-NaOH, pH 7.0, and then incubated at 37°C. At appropriate time intervals, 10 µl of the reaction mixture was added to 5 ml of trichloroacetic acid solution (12.5%, w/v) to stop the reaction, followed by the addition of 0.2 ml of 10 mM Fe(NH₄)₂(SO₄)₂ and 0.1 ml of 0.5 M KSCN to develop the complex, giving a purple color. The concentration of H₂O₂ was monitored by measurement of the decrease in absorbance at 480 nm, the absorbance maximum of the purple-colored complex. To determine the peroxidase activity of E. coli antioxidant enzyme linked to NADPH oxidation, the reaction was started by the addition of various amounts of the antioxidant enzyme to 50 mM Hepes-NaOH buffer (pH 7.0), containing 0.1 mM NADPH, varying concentrations of H₂O₂, 12.5 µM Trx, and 12.5 µM thioredoxin reductase. The resulting oxidation of NADPH was directly followed by the decrease in absorbance at 340 nm.

DNA Cleavage by Nonenzymatic MCO System—After reaction mixture (100 mM Hepes (pH 7.0)) containing the thiol-MFO system (3 µg FeCl₃, 10 mM DTT) was incubated with or without the enzyme for 40 min at 37°C, two µg of pUC19 plasmid was added for additional 4-h incubation (4). The resulting reaction mixture was subjected to phenol/chloroform extraction to obtain DNA, then applied to 1% agarose gel to examine its cleavage. Osmotic Shock to E. coli—Cells (10 g) were washed three times with 10 ml Tris buffer (pH 7.4), containing 30 mM NaCl, and were then osmotically shocked by incubation in 500 ml of 20% sucrose, 1 mM EDTA, 30 mM Tris-HCl buffer at pH 7.4 for 5 min and then transferred to 500 ml of cold deionized water. Proteins of the periplasmic space were released into solution by such treatments, whereas proteins of the cytoplasm were retained (16). Assays for Catalase, Superoxide Dismutase, Glutathione Peroxidase, and Thioredoxin—Catalase activity was determined by direct measurement of the decrease of absorbance at 250 nm caused by the decomposition of H₂O₂ by catalase (17). Superoxide dismutase activity to scavenge the hydroxyl radical by the method by Crapo (18). The rate of production of GSSG by glutathione peroxidase was measured in the presence of excess glutathione reductase by following the rate of NADPH oxidation (19). Trx activity was determined by measurement of the decrease of absorbance at 412 nm resulting from the reduction of DTNB.

**Sequence of Tryptic Peptides from the 20-kDa Protein—** The purified 20-kDa protein was reductively denatured by 6 M guanidine hydrochloride solution containing 1 mM DTT and 50 mM Tris-HCl (pH 7.8). The sulphydryl group(s) was labeled with TNB by 10 mM DTNB for 1 h at 37°C (20). The TNB-linked protein was precipitated with 10% trichloroacetic acid, and the precipitate was washed three times with acetone. The resulting protein was suspended in 50 mM Tris-HCl (pH 7.4) containing 10 mM EDTA, 0.2 M KCl, 0.1 M sucrose, and 10 mM Trx, and then transferred to 500 ml of cold deionized water. The resulting elution was applied to a preparative Vydac C₁₈ column (25 × 250 mm) and eluted with a linear gradient of 0–60% acetonitrile in 0.05% trifluoroacetic acid over 60 min at a 2 ml/min flow rate. Peptides containing cysteine residues were detected by monitoring at 328 nm.

**Cloning and Sequencing—** An E. coli K12 genomic DNA library in λgt11 (Clontech Laboratory, Inc.) was screened with rabbit polyclonal antibodies prepared against purified 20-kDa protein. The sequence determination was done by the dideoxy chain-termination method (21). Other Methods—Immunoblot analyses of E. coli thiol antioxidant enzymes were performed by using rabbit polyclonal antibodies against corresponding enzymes. Procedures for transfer of proteins from 12% SDS-polyacrylamide gels to nitrocellulose and for the processing of nitrocellulose blots have been described previously (4). Monospecific antibodies for the 20-kDa protein were prepared from the γ-globulin fraction using the 20-kDa protein immunized mice. Detection of polyclonal antibodies was carried out on 12% nonreducing SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R-250. The native molecular mass of protein was estimated from a graph of the logarithm of the molecular mass as a function of elution volume (Vₑ/Vₘ) made using the data from the protein standards such as egg albumin (45 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), soybean trypsin inhibitor.
Comparative Antioxidant Activities of p20 and AhpC—Fig. 3 shows the time-dependent inactivation of glutamine synthetase and thiol-dependent antioxidant activities of p20 and AhpC. Inactivation of yeast glutamine synthetase by DTT and Fe^3+ (2) was completely prevented by p20 (curve 2 in Fig. 3A) or AhpC (curve 2 in Fig. 3B). When thiol-reducing equivalent (DTT) is replaced with non-thiol-reducing equivalent (ascorbate), this thiol-dependent proteins no longer protect against the inactivation of glutamine synthetase by Fe^3+.-catalyzed oxidation (MCO) system (3) and AhpC. The extent of glutamine synthetase protection increased showing saturation kinetics (curve 3). The extent of glutamine synthetase protection increased showing saturation kinetics (curve 3). The extent of glutamine synthetase protection increased showing saturation kinetics.
DNA cleavage by thiol MCO system. Fifty nM p20 or AhpC was required to preserve completely glutamine synthetase activity. However, p20 showed the higher extent of protection degree than AhpC at the less than concentrations of 50 nM (not shown). The more effective protection by p20 against DNA cleavage confirms the superior capability of p20 to AhpC for antioxidant activity of p20 becomes restored, showing a saturation tendency as the concentration of DTT or GSH in the reaction mixture contained as follows: curve 1 A and B, glutamine synthetase plus 1 mM EDTA; curve 2 A and B, thiol MFO system plus 50 nM p20 or 50 nM p24, respectively; curves 3 A and B, non-thiol MCO system plus 50 nM p20 or p24, respectively. Curves 4 and 5 in A and B represent the inactivation of glutamine synthetase by non-thiol (ascorbate) MFO system (curve 4) or by thiol MCO system (curve 5) without p20 (A) and p24 (B).

Experiment Procedures: The inactivation mixture contained 10 μg of E. coli glutamine synthetase, 10 mM DTT for the thiol MCO system or 10 mM ascorbate for the non-thiol MCO system, 3 μM FeCl3, 50 mM Hepes, pH 7.0, in a total volume of 100 μL. All reactions were carried out at 37 °C. At indicated times, aliquots (10 μL) were removed and assayed for glutamine synthetase activity. Each inactivation reaction mixture contained as follows: curve 1 A and B, glutamine synthetase plus 1 mM EDTA; curve 2 A and B, thiol MFO system plus 50 nM p20 or 50 nM p24, respectively; curves 3 A and B, non-thiol MCO system plus 50 nM p20 or p24, respectively. Curves 4 and 5 in A and B represent the inactivation of glutamine synthetase by non-thiol (ascorbate) MFO system (curve 4) or by thiol MCO system (curve 5) without p20 (A) and p24 (B).

FIG. 3. Protection of glutamine synthetase by p20 and p24 against the DTT/Fe3+ (thiol MCO) system. The inactivation mixture contained 10 μg of E. coli glutamine synthetase, 10 mM DTT for the thiol MCO system or 10 mM ascorbate for the non-thiol MCO system, 3 μM FeCl3, 50 mM Hepes, pH 7.0, in a total volume of 100 μL. All reactions were carried out at 37 °C. At indicated times, aliquots (10 μL) were removed and assayed for glutamine synthetase activity. Each inactivation reaction mixture contained as follows: curve 1 A and B, glutamine synthetase plus 1 mM EDTA; curve 2 A and B, thiol MFO system plus 50 nM p20 or 50 nM p24, respectively; curves 3 A and B, non-thiol MCO system plus 50 nM p20 or p24, respectively. Curves 4 and 5 in A and B represent the inactivation of glutamine synthetase by non-thiol (ascorbate) MFO system (curve 4) or by thiol MCO system (curve 5) without p20 (A) and p24 (B).

FIG. 4. The concentration-dependent effects of DTT and glutathione (GSH) on the preventive activity of p20 against the inactivation of glutamine synthetase by ascorbate MFO system. A, various amounts of DTT were added into the ascorbate (i.e. non-thiol) MCO system containing 100 nM p20. After 40 min at 37 °C, the remaining glutamine synthetase activities were measured. B, various amounts of GSH were added into the non-thiol MFO system containing 100 nM p20. Lines 2 in A and B are the corresponding control experiments without p20.
not result in the inactivation of the antioxidant activity (not shown). These results indicate that a functional sulfhydryl group(s) (i.e. cysteine residue) of p20 is involved in the antioxidation reaction, and the resulting intramolecular disulfide bond can be regenerated by a thiol-reducing equivalent such as DTT.

The Antioxidant Activity of p20 Is Functionally Linked to Thioredoxin—We searched for an enzyme or an in vivo thiol-reducing equivalent capable of supporting the antioxidant activity of p20 against the ascorbate MFO system. Glutathione (GSH), known as an in vivo thiol-reducing equivalent, was monitored for its ability to give p20 potential for preventing the inactivation of glutamine synthetase by the ascorbate MFO system. Excess amount of GSH (>10 mM) was required to restore the antioxidant activity of p20 (Fig. 4B). Below 10 mM GSH, p20 did not show a superior antioxidant activity to GSH itself, reducing the possibility of glutathione as the in vivo thiol-reducing equivalent.

An enzymatic thiol regenerating system (Trx/Trx reductase/NADPH) was tested for its ability to regenerate the activity of p20. This system gave p20 capability for protecting the inactivation of glutamine synthetase by ascorbate MFO system. Fig. 5, A and B, show the NADPH dependence of the antioxidant activity of p20 against ascorbate MFO system in the presence of Trx and Trx reductase. As the concentration of NADPH was increased, the antioxidant activity of p20 became restored (Fig. 5, curve 2 in B, glutamine synthetase activity after 30 min at 37°C, in whole components containing various concentrations of NADPH ranging from 0.625 to 5 mM. Curve 2 in B, control glutamine synthetase activity in whole components containing varying concentrations of NADPH without p20.

Peroxidase Activity of p20—We examined the p20 for perox-

Fig. 5. Trx-linked antioxidant activity of p20. Glutamine synthetase was subjected to inactivation in 50 μl of a reaction mixture containing the non-thiol (10 mM ascorbate) MCO system, 100 μM p20, 25 μM Trx, 25 μM of Trx reductase, 50 mM Hepes-NaOH (pH 7.0), and various concentrations of NADPH. At various times, 8-μl aliquots were removed and assayed for glutamine synthetase. Curve 1 A, 5 mM NADPH; curve 2 in A, 1 mM NADPH; curve 3 in A, whole component without p20; curve 4 in A, whole component without Trx. Curve 1 in B, glutamine synthetase activity after 30 min at 37°C, in whole components containing varied concentrations of NADPH ranging from 0.625 to 5 mM. Curve 2 in B, control glutamine synthetase activity in whole components containing varying concentrations of NADPH without p20.

Fig. 6. Removal of H₂O₂ by p20 linked to the Trx system. Peroxidase reaction was carried out in a 0.5-ml reaction mixture containing 50 mM Hepes-NaOH (pH 7.0), 50 mM p20, 12.5 μM of Trx, 12.5 μM of Trx reductase, 0.12 mM H₂O₂, 0.25 mM NADPH (curve 2) at 37°C. Line 1, control experiment the on whole reaction component minus p20. At the indicated time, a 50-μl sample was removed, and the concentration of remaining H₂O₂ was measured with the use of ferrithiocyanate as described (5, 32). Peroxidase activity by measuring the decrease of H₂O₂ in the presence of the Trx system. The time course removal of H₂O₂ by p20 showed a characteristic first-order kinetics (Fig. 6). The velocity of the removal of H₂O₂ by p20 was increased showing a saturation pattern as the concentration of p20 increased (not shown). Peroxidase activity in the presence of Trx system can be measured indirectly by following the decrease in A₃₄₀ resulted from the oxidation of NADPH. The rate of the peroxidase-linked NADPH oxidation decreased with time and increased with the concentration of H₂O₂ (Fig. 7A) and t-butyl hydroperoxide (Fig. 7C). These results indicate the tight coupling between the peroxidase activity of p20 and the NADPH consumption rate. The Lineweaver-Burk plot constructed from the peroxides-dependent initial NADPH consumption rates (Fig. 7, B and D) shows that Kₘ values of p20 for H₂O₂ and t-butyl hydroperoxide are 60.6 and 15.6 μM, respectively, and Vmax values of p20 for H₂O₂ and t-butyl peroxide are 0.023 and 0.015 unit (μmol/min/μg), respectively. On the equivalent experimental conditions, the values of Vmax/Kₘ of p20 for t-butyl hydroperoxide and H₂O₂ were 9.62 × 10⁻⁴ and 3.80 × 10⁻⁴, respectively, indicating that t-butyl hydroperoxide is more specific substrate for p20. These results suggest that p20 linked to the in vivo thiol regenerating Trx system could be act as a peroxidase.

Induction of p20 under Aerobic Condition and Its Cellular Localization—Oxygenation of the growth medium increases the biosyntheses of p20 in E. coli. To confirm the oxygen stress on E. coli, the levels of AhpC (10) and manganese superoxide dismutase (25, 26), which have been known as inducible enzymes by an oxygen stress, were visualized by Western blot with polyclonal antibodies of AhpC and manganese superoxide dismutase, respectively. The immunoblot analysis (not shown) shows that both enzymes were induced by the oxygen stress.

The cells grown under anaerobic or aerobic conditions were subjected to osmotic shock. Proteins of periplasmic space were released into solution by the osmotic shocked treatment, whereas enzymes of the matrix space were retained (16). Catalase, which is known to be a matrix enzyme in E. coli, used as a marker enzyme. On the Western blot with polyclonal antibodies of catalase, the cytoplasmic catalase could not be detected in the periplasmic protein extract, indicating that the procedures used in preparing the shocked fluid caused little
Figure 7. Functional tight coupling between peroxidase activity of p20 and the Trx system. The decrease of NADPH was spectrophotometrically monitored in a 300-μl reaction mixture containing the Trx system (0.25 mM NADPH, 12.5 μg of Trx and 12.5 μg of Trx reductase), 50 mM Hepes-NaOH (pH 7.0), 50 mM p20, and various concentrations of H2O2 (A) and t-butyl hydroperoxide (C). After 2-min preincubation at 25°C, the reaction was started by the addition of substrate, H2O2, or t-butyl hydroperoxide to the reaction mixture. A. NADPH oxidation of Trx system coupled to the consumption of H2O2 by p20. A: traces 0–5, 0.04, 0.059, 0.088, 0.118, and 0.137 mM H2O2, respectively. B. Lineweaver-Burk plot of the initial rate of NADPH oxidation versus the concentration of H2O2 added. C. NADPH oxidation of the Trx system coupled to the consumption of t-butyl hydroperoxide by p20. C: traces 0–4, 0.06, 0.03, 0.023, and 0.035 mM t-butyl hydroperoxide, respectively. D. Lineweaver-Burk plot of the initial rate of NADPH oxidation versus the concentration of t-butyl hydroperoxide. One unit of peroxidase activity represents 1 μmol of peroxide which is converted to product/μg of p20/min.

release of matrix enzymes. Therefore, the evident p20 band in the shock fluid (not shown) indicates its existence in the periplasmic space. The comparisons of the band intensities on the Western blot of p20 with that of AhpC denote that p20 is much more abundant in the periplasmic space than AhpC.

In an attempt to clarify the existence of p20 in the periplasmic space, we purified the p20 from the osmotic shock fluid. p20 was purified to homogeneity by three sequential chromatographic steps on phenyl-Sepharose CL-4B and two rounds of G-50. However, the catalase activity was not detected in the periplasmic protein extract, indicating no contamination with cytoplasmic proteins. The immunoblot experiments revealed that the monospecific antibodies prepared against p20 are highly specific to the antigen. To determine the concentration of p20 in the periplasmic space of E. coli, the immunoreactivity was measured with from 10 to 40 μg of soluble proteins prepared from cytoplasm and periplasmic space of E. coli grown under aerobic condition. From the standard immunobLOTS in which the intensity of immunoblot increased with increasing concentrations of purified p20 from 12.5 to 400 ng (not shown), the amount of p20 in the periplasmic space was estimated to be between 0.5 and 1.0% of the total periplasmic proteins, whereas the amount of p20 in the cytoplasm was estimated to be between 0.05 and 0.1% of total cytoplasmic proteins. These results confirm the abundant existence of p20 in the periplasmic space of E. coli.

DISCUSSION

Recently, a family of TSA proteins, more recently referred to as thioredoxin-dependent peroxidases, has been rapidly growing (2–9). The similarity among these proteins, including E. coli AhpC, extended over the entire sequence, especially in the domains (VCP1 and VCP2 domains), which contain highly conserved cysteines (8). Therefore, AhpC has been suggested to be a prokaryotic counterpart of the eukaryotic TSA (12).

We purified a novel 20-kDa antioxidant protein (p20) from E. coli. p20 shares the same catalytic characteristics of TSA/AhpC proteins (i.e. thiol-dependent antioxidant properties) (2–9). p20 appeared to have a significant peroxidase activity to destroy H2O2 and alkyl peroxide such as t-butyl hydroperoxide. The predicted amino acid sequence of p20 does not show any significant homology to those of TSA/AhpC family (Fig. 2). A data bank search reveals that p20 is a novel E. coli protein. These results suggest p20 is a novel type of thiol-dependent peroxidase.

In order to understand a reason for the existence of two types of peroxidases such as p20 linked to the Trx system and AhpC linked to F52 (reductase component of alkyl hydroperoxide reductase) in E. coli, the differences of their physiological functions were examined. Their inducibilities of protein synthesis with response to oxidative stress were nearly same, but their different cellular compartmentalizations might give a clue to understand the reason. The distributions of p20 and p24 (AhpC) between the periplasmic space and cytoplasm of E. coli were different. p20 appears to be localized mainly in the periplasmic space, whereas AhpC resides mainly in the matrix of the cells. On the basis of these observations, it appears that p20, in the periplasmic space, could serve as a peroxidase to remove exogenous peroxides, while the AhpC, in the cytoplasm of the cell, acts as a peroxidase against endogenous peroxides.

Analogies to these cellular localizations were reported in the case of superoxide dismutase, a metalloenzyme found in all organisms (27). E. coli has two isoenzyme forms of superoxide dismutase: iron superoxide dismutase (28) and manganese superoxide dismutase (29). The cellular localizations of these isoenzymes are different. The periplasmic fluid contained 12 units of the magnesium form and 68 units of the iron form, whereas the shock-extracted cells (i.e. cytoplasm) contained 846 units of the manganese superoxide dismutase and 585 units of iron superoxide dismutase (26). Therefore, it appears that in the periplasmic space of the cells, iron superoxide dismutase converts exogenous O2- to H2O2. Without removing the periplasmic H2O2, very destructive hydroxyl radicals capable of damaging the cell membrane may be generated by Fe²⁺ via Fenton reaction. We tried to purify any peroxidase and catalase activities from the periplasmic fluids of E. coli, but these activities were not found in the periplasmic space. Thus, it is likely that p20, in the periplasmic space, might be a unique peroxidase to remove the periplasmic peroxides such as H2O2 and alkyl hydroperoxides.

This new enzyme shares the similar catalytic cycles of TSA protein (thioredoxin peroxidase), which involves the transfer of reducing equivalent by redox active disulfhydryls of Trx. However, this proposed mechanism is different from the previously reported catalytic cycles of TSA protein (9) in that the intramolecular disulfide linkage (not intermolecular disulfide bond of TSA proteins) of p20 was involved in the cycles, which is supported by the observations of the inactive monomer form of p20 in the absence of DTT (Fig. 1). p20 contains neither selenocysteine nor prosthetic group such as a heme or a flavin. Thus, it will be very interesting to investigate how p20 shows a peroxidase activity. The mechanism of p20 to destroy peroxides might be analogous to the mechanism proposed for selenocysteine glutathione peroxidases that have been not found in prokaryotes. The functional cysteine could be oxidized to an intermediate, –Cys–S–OH, on the assumption that redox-active cysteine of p20 gains abnormal strong nucleophilicity comparable with that of selenocysteine, –Cys–Se–OH, of glutathione peroxidase. It is likely that the thiol group in the active
cysteine residue of p20 would gain abnormal nucleophilicity by a microenvironmental effect. The analogy with the thio exhibited abnormal strong nucleophilicity was reported previously. The ovothiol, a mercaptimidazole, is more effective than catalase in destroying H₂O₂ (30). The capability of ovothiol was proven to be due to the strong nucleophilicity of the thiol group (31).

In conclusion, the novel 20-kDa antioxidant protein (p20), which is localized in the periplasmic space of E. coli, is a peroxidase linked to the Trx system, but its primary structure differs from that of the TSA/AhpC family of thioredoxin-dependent peroxidases. In order to discriminate this type of E. coli peroxidase having functional cysteine from the selenocysteine peroxidase such as glutathione peroxidase, we tentatively named p20 as “thiol peroxidase.” The identity of the normal physiological substrate(s) of thiol peroxidase (whether it is an alkyl hydroperoxide of lipid, hydrogen peroxide, hydroxyl radical, or some other cellular components containing oxygen radical) remains to be determined.

To investigate the mechanism of antioxidant action of thiol peroxidase and its physiological function, we are to make a thiol peroxidase deletion mutant and the point-mutated enzyme whose putative active cysteine(s) is changed to other amino acid.

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