Thiol-specific Antioxidant Protein (TSA)/Alkyl Hydroperoxide Peroxidase C (AhpC) Family

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Escherichia coli bacterioferritin comigratory protein (BCP), a putative bacterial member of the TSA/AhpC family, was characterized as a thiol peroxidase. BCP showed a thioredoxin-dependent thiol peroxidase activity. BCP preferentially reduced linoleic acid hydroperoxide rather than H₂O₂ and t-butyl hydroperoxide with the use of thioredoxin as an in vivo immediate electron donor. The value of Vₘₐₓ/Kₘₐₓ of BCP for linoleic acid hydroperoxide was calculated to be 5-fold higher than that for H₂O₂, implying that BCP has a selective capability to reduce linoleic acid hydroperoxide. Replacement of Cys-45 with serine resulted in the complete loss of thiol peroxidase activity, suggesting that BCP is a new bacterial member of TSA/AhpC family having a conserved cysteine as the primary site of catalysis. BCP exists as a monomer, and its functional Cys-45 appeared to exist as cysteine sulfenic acid. The expression level of BCP gradually elevated during exponential growth until mid-log phase growth, beyond which the expression level was decreased. BCP was induced 3-fold by the oxidation stress given by changing the growth conditions from the anaerobic to aerobic culture. Bcp null mutant grew more slowly than its wild type in aerobic culture including fatty acid hydroperoxide (1, 2). The alkyl hydroperoxide reductase (AhpC)/TSA family is a new type of peroxidase that has a conserved cysteine as the primary site of catalysis instead of the selenocysteine of glutathione peroxidase. The new type of peroxidase that has been discovered from prokaryotes to eukaryotes (3, 4) reduces hydroperoxides with the use of thioredoxin and other thiol-containing reducing agents (5–7).

Only two bacterial member of the new peroxidase family have been reported, although at least five types of thiol peroxidase exist in eukaryotic cell. In Escherichia coli. AhpC was reported to preferentially reduce alkyl hydroperoxide with electrons provided by either NADH or NADPH via the AhpF52 (8). Recently, we reported a novel type of peroxidase, p20, that acts as an antioxidant to remove peroxides such as H₂O₂ and alkyl hydroperoxide in the periplasmic space of E. coli (9).

The similarity of primary structure between bacterioferritin comigratory protein (BCP) and the TSA/AhpC family suggested that BCP could be another new member of the family. However, the function of BCP has not yet been clarified despite of the wide distribution of BCP in most pathogenic bacteria including Haemophilus influenzae, Helicobacter pylori, and Mycobacterium tuberculosis as p20. In this paper, we first showed that BCP is a new bacterial member of the TSA/AhpC family, acting as a general hydroperoxide peroxidase.

EXPERIMENTAL PROCEDURES

Cloning and Mutagenesis of E. coli BCP—The DNA sequence corresponding to BCP was obtained by the polymerase chain reaction (PCR) from E. coli genomic DNA using the forward primer (5'-G GAA TTC GGA TCC GGC GTG CTC) containing an NdeI (underlined) site and the initiation codon (boldface) and the reverse primer (5'-GGC GTG TTC TTT CAG CCA) containing a TCA GTT CAG-3' containing the BamHI site (underlined) and the stop codon (boldface). The amplified products were purified and digested with NdeI/BamHI. The digested fragments were subcloned into the T7 expression vector, pT7-7 digested with NdeI/BamHI, and the resulting plasmid was used to transform E. coli strain BL21 (DE3).

Three mutant proteins, C45S, C50S, and C99S, in which Cys-45, Cys-50, and Cys-99 were individually replace by serine, were generated by the standard PCR-mediated site-directed mutagenesis with complementary primers containing a 1-base pair mismatch, which converts the codon for cysteine to one for serine. The mutated PCR products were ligated into pT7-7 digested with NdeI/BamHI.

Expression and Purification of BCP and Its Mutant Proteins—Transformed cells were cultured at 37 °C overnight in LB medium supplemented with ampicillin (100 μg/ml) and then transferred to fresh medium to the ratio of 1 to 200. When the optical density of the culture at 600 nm reached 0.4, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.5 mM. After induction for 3 h, cells were harvested by centrifugation and stored at −70 °C until use.
Frozen cells were suspended in 50 mM Tris-HCl (pH 7.6) containing 2 mM phenylmethyl sulfonyl fluoride and 1 mM EDTA and disrupted by sonication. The supernatants clarified by centrifugation were loaded to Q-Sepharose column that had been equilibrated with 50 mM Tris-HCl (pH 7.6). Proteins were eluted with a linear gradient of NaCl from 0 to 1.0 M over 100 min at a flow rate of 2 ml/min. The fractions corresponding to the peak of BCP were pooled and dialyzed against 50 mM sodium acetate (pH 5.0). After dialysis, samples were applied to SP-Sepharose column that had been equilibrated with 50 mM sodium acetate (pH 5.0).

The samples eluted with a linear gradient of NaCl were dialyzed against 10 mM Tris-HCl (pH 7.4) and stored at 70 °C.

**Determination of Thiol-dependent Antioxidant Activity**—The antioxidant activity was determined by measuring the activity to protect the inactivation of *E. coli* glutamine synthetase (GS) by a thiol metal-catalyzed oxidation system (DTT/Fe3+/O2) (thiol MCO system) (10) as described previously (11). Instead of DTT, ascorbate was included as a non-thiol-reducing equivalent (non-thiol MCO system). The 30-μl reaction mixture containing 100 mM Hepes-NaOH (pH 7.0), 1.0 μg of GS, 3 μM FeCl3, various concentration of BCP and either 10 mM DTT or 10 mM ascorbate, was incubated at 37 °C, and then 0.5 ml of γ-glutamyltransferase assay mixture was added. After incubation at 37 °C for 10 min, the remaining activity of GS was determined by measuring the absorbance at 540 nm. To determine the thioredoxin (Trx)-linked antioxidant activity of BCP, the GS activity was measured in the reaction mixture containing 100 mM HEPS-NaOH (pH 7.0), 0.5 μg of GS, 3 μM FeCl3, various concentrations of BCP, 10 μM ascorbate, 10.8 μM Trx, 3.9 μM thioredoxin reductase (TR), and 1 mM NADPH.

**Chemical Modification of BCP with NEM, Iodoacetamide, and NBD-Cl**—BCP was preincubated in the absence or presence of 2.5 mM DTT at 30 °C for 30 min, and then chemical modification was carried out in a 100-μl reaction mixture containing 50 mM Tris-HCl (pH 8.0), 1.2 mg BCP, and 20 mM NEM or iodoacetamide at 30 °C for overnight as described previously (14). For the modification of BCP with NBD-Cl, 75 nmol of BCP was reacted with 450 nmol of NBD-Cl in 50 mM potassium phosphate with 0.1 mM EDTA at pH 7.0 at 30 °C for 2 h as described previously (15). The reaction mixtures were extensively dialyzed against 10 mM HEPS (pH 7.0) at 4 °C.

**HPLC Separation of Tryptic Peptides**—The NBD-treated BCP protein was denatured by 200 mM Tris buffer (pH 8.0) containing 3 M guanidine-HCl. The resulting protein was washed with acetonitrile, and the precipitate was suspended in 100 mM Tris-HCl buffer, pH 8.0, and the protein was digested with trypsin for 3 h at 30 °C. The additional digestion with fresh trypsin was carried out overnight. The tryptic peptides were separated by a reversed phase C18 column (Kromasil, 4.6 mm × 250 mm) with a linear gradient of 10–45% acetoniure in 0.1% trifluoroacetic acid over 45 min at a 1 ml/min flow rate. The peptides were simultaneously detected using a photodiode array detector (Shimadzu SPD-M10A,λ) and a fluorescence detector (Shimadzu RF-10AXL).

**Construction of BCP Promoter-lacZ Fusion**—To construct the BCP promoter-lacZ fusion, the upstream sequence of the initiation codon of the BCP gene was prepared by PCR. The forward primer (5'-CCG GAA TTC AAA AGC AAG CAG ACA GAA CCG-3') contains an EcoRI site (underlined), and the reverse primer (5'-CGG GGA TTC TAC TTA ACT...
or anaerobically in LB medium containing 50 mM Hepes-NaOH (pH 7.0), 3 mM FeCl₃, the indicated concentrations of BCP, 10 mM DTT (thiol MCO system; open circle), or 10 mM ascorbate (non-thiol MCO system; closed squares). In the Trx system (closed circle), 10.8 μM Trx, 3.9 μM TR, 1 mM NADPH, and 10 mM ascorbate instead of 10 mM DTT were added to the reaction mixture. After 14 min at 37 °C, the residual glutamine synthetase activity was measured as described under “Experimental Procedure.” B–D, removal of H₂O₂, t-BOOH, and linoleic acid hydroperoxide (LAOOH), respectively, by BCP in the presence of Trx, TR, and NADPH. Peroxidase reaction was carried out in 400 μl of reaction mixture containing 50 mM Hepes-NaOH (pH 7.0), 0.5 μM Trx, 0.3 μM TR, 0.26 mM NADPH, various concentrations of BCP (open squares, 0.7 μM; closed squares, 1.4 μM; open circle, 2.8 μM; closed circle, 5.7 μM), and 0.1 mM H₂O₂ (A), 0.1 mM t-BOOH (B), or 0.04 mM linoleic acid hydroperoxide (LAOOH, C) at room temperature. At the indicated times, the remaining peroxides were measured by using FOX1 reagent as described previously (11).

CCA TCC TGT TCA TC-3′ contains a BamHI site (underlined). The EcoRI/BamHI-digested PCR products were subcloned into EcoRI/BamHI-cut pRS415, and the resulting plasmid was used for transforming E. coli MC1061.

β-Galactosidase Assay—Transformed cells were cultured aerobically or anaerobically in LB medium containing 50 μg/ml ampicillin at 37 °C. At mid-log phase or indicated culture time, the optical density at 600 nm was measured, and cells were harvested. The cells were resuspended in 700 μl of Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 52 mM β-mercaptoethanol, adjusted to pH 7.0) and were mixed with 35 μl of 0.1% SDS and 35 μl of chloroform by vortexing for 10 s. The reactions were started by adding 140 μl of 13 μM o-nitrophenyl-β-D-galactoside. After incubation at 30 °C for 10 min, the reactions were stopped by the addition of 350 μl of 1 M Na₂CO₃. The cell debris was removed by centrifugation, and then β-galactosidase activity was measured in terms of the increase of absorbance at 420 nm because of the production of o-nitrophenol production. The activity was expressed as β-galactosidase units (16).

Construction of the bcp and ahpC Null Mutants—The null mutants were generated by the integrative disruption method. To construct the vector containing the bcp gene and its own promoter, PCR was carried out using the forward primer (5′-GGAA TTC CAT ATG CAG GCA GGC ACT GAA GAT ACC-3′), containing a NdeI site (underlined), and the reverse primer (5′-CCC AAG CTT AGA TTT TAC CAA CCA CTT GCA CAG 3′), containing an EcoRV site (underlined), and the reverse primer (5′-GA AGG CCT GAA CCG AGC ACC GGG TCG-3′), containing a StuI site (underlined). For the disruption of ahpC gene, the reverse primer (5′-CCC AAG CTT AGA TTT TAC CAA CCA CTT GCA CAG-3′), containing a PstI site (underlined), was used. Each PCR product was inserted into the EcoRV/StuI site of pBCP and Klenow-filled EcoRI/PstI site of pAhpC. The resulting plasmids were used as a template in PCR to produce 2.0- and 1.6-kilobase pair DNA fragments containing the bcp and ahpC genes disrupted by insertion of cat gene, respectively. These PCR products were used for transforming E. coli strain JC7623, and chloramphenicol-resistant cells were isolated.

RESULTS

Thioredoxin-linked Thiol Peroxidase Activity of BCP—The amino acid sequence identity among the BCP homologous proteins is >35%. The consensus sequence surrounding the unique conserved Cys among the BCP homologues (GCT) (Fig. 1) suggested that the BCP homologue would be a new member of the TSA/AhpC family. The BCP homologue is distributed in most
bacteria including H. influenzae, H. pylori, and M. tuberculosis (Fig. 1). To examine our speculation, E. coli BCP, a putative bacterial member of TSA/AhpC, was characterized. BCP was homogeneously purified from the E. coli recombinant highly overexpressing BCP (Fig. 2A). The homogeneous purity of the purified BCP was confirmed on SDS-PAGE gels (Fig. 2B). The BCP was detected at the molecular mass corresponding to that of monomer (18 kDa) regardless of the presence or absence of DTT. This result suggested that unlike E. coli AhpC, BCP exists as monomer even though it is in an oxidized state.

In the presence of a thiol-containing electron donor such as DTT, the TSA/AhpC family has the antioxidant activity of preventing the inactivation of GS by the MCO system, which is comprised of DTT, Fe$^{3+}$, and O$_2$ (i.e. thiol MCO system) (1). It has been well known that the replacement of a thiol-containing electron donor with a non-thiol electron donor such as ascorbate (i.e. non-thiol MCO system) resulted in the complete loss of the antioxidant activity, because of the lack of a thiol-containing electron donor to the TSA/AhpC protein (9). We therefore investigated such a thiol-dependent antioxidant activity exerted by BCP. Like TSA/AhpC protein, BCP prevented GS from the inactivation by a thiol MCO system, not by a non-thiol MCO system (Fig. 3A). The antioxidant activity of BCP in non-thiol MCO system was fully recovered by the addition of a Trx system comprised of NADPH, Trx, and Trx reductase (Fig. 3A). Taken together, these results suggested that BCP is a new member of TSA/AhpC family and also that the immediate electron donor to BCP is Trx.

To investigate the peroxidase activity of BCP, we directly measured the peroxidase activity of BCP toward H$_2$O$_2$, t-butyl hydroperoxide, and linoleic acid hydroperoxide in terms of the removal of peroxides in the presence of Trx system. The removal rates of all these peroxides increased as the concentration of BCP was increased (Fig. 3, B–D). It is worth noting that among the peroxides as the substrate, linoleic acid hydroperoxide was most rapidly removed at an equivalent concentration of BCP. To examine the substrate selectivity of BCP among various peroxides, the peroxide-dependent peroxidase activities were examined in the presence of the Trx system. The NADPH consumption rate increased as a function of BCP concentration (data not shown). This result indicated that the peroxidase activity of BCP should be supported by the Trx system. The initial rate of peroxide consumption by BCP was directly measured at the various concentrations of H$_2$O$_2$, t-butyl hydroperoxide, and linoleic acid hydroperoxide in the presence of the Trx system. The analysis of each Lineweaver-Burk plot (Fig. 4) showed that the $K_m$ values of BCP for H$_2$O$_2$, t-butyl hydroperoxide, and linoleic acid hydroperoxide were 47.8, 37.4, and 11.7 μM, respectively, and $V_{max}$ values for H$_2$O$_2$, t-butyl hydroperoxide, and linoleic acid hydroperoxide were 7.01, 1.93, and 8.23 μmol/min/μmol (i.e. 400, 110, 469 nmol/min/mg of protein), respectively. The value of $V_{max}/K_m$ of BCP for H$_2$O$_2$, t-butyl hydroperoxide, and linoleic acid hydroperoxide were calculated to be 0.147, 0.052 and 0.703 μmol/min/μmol, respectively. The higher value of $V_{max}/K_m$ of BCP for t-butyl hydroperoxide than those of for H$_2$O$_2$ and t-butyl hydroperoxide implied that BCP has a general hydroperoxidase activity. Thus, we suggest that BCP acts as a thioredoxin-dependent hydroperoxidase showing the substrate selectivity toward fatty acid hydroperoxide.

**Functional Cysteine Residues of BCP**—Although most of TSA/AhpC proteins exist in an intermolecular disulfide-linked homodimer, several TSA/AhpC enzymes such as mammalian ORF6 (17) and E. coli p20 proteins (9, 21) exist in a monomer. BCP contains three cysteine residues (Cys-45, Cys-50, and Cys-99) as shown in Fig. 1. To gain the information about the catalytic cysteine of BCP, each cysteine residue was replaced with serine. The resulting recombinant (C45S, C50S, and C99S) revealed that the Cys-45 is a catalytic cysteine residue for the antioxidant reaction. To determine the catalytic cysteine residue among the three putative cysteines, we examined the antioxidant activity of each recombinant protein to protect the inactivation of GS by thiol MCO system. Only C45S protein among three mutant proteins resulted in the complete loss of the antioxidant activity (Fig. 5A). In the presence of the Trx system, the peroxidase activities of mutant proteins were also determined directly by measuring the remaining amount of H$_2$O$_2$ (Fig. 5B) or indirectly by monitoring the decrease of absorbance at 340 nm owing to the H$_2$O$_2$-dependent oxidation of NADPH (Fig. 5C). As expected, only C45S protein did not show the Trx-dependent peroxidase activity. The activity analysis of three mutant proteins (C45S, C50S, and C99S) revealed that the Cys-45 is a primary catalytic site for the antioxidant reaction.

Members of the TSA/AhpC family can be divided into two subgroups such as 1-Cys and 2-Cys groups according to the number of the conserved cysteines within the protein (3). The 2-Cys protein exists as a homodimer. One mammalian member of the TSA/AhpC family, ORF6, as a 1-Cys protein contains one conserved Cys and thereby exists in monomer (17). To gain insight into the nature of a functional cysteine residue (Cys-45), we reacted BCP with a thiol-specific modification reagent, iodoacetate or NEM, in the absence or presence of DTT (Fig. 6). The modification of BCP, regardless of the presence or absence of DTT, resulted in the considerable loss of the antioxidant activity, which suggested that the SH group of the functional Cys-45 did not form the disulfide bond during the reduction reaction as did 2-Cys protein (6).
It was previously reported that the functional Cys of 1-Cys TSA/AhpC, as in ORF6, exists in the form of sulfenic acid (Cys-SOH) as a catalytic intermediate, which can be easily reduced to Cys-SH by DTT (17–18). The functional Cys of NADH peroxidase also exists as Cys-SOH (19). To investigate the possibility that the functional Cys-45 of BCP may exist in the form of sulfenic acid like those of ORF6 and NADH peroxidase, we reacted BCP with an electrophilic reagent, NBD-Cl, as a trapping agent for Cys-SOH. The reagent can react with Cys-S-OH and Cys-SH groups and form the corresponding thiol adducts, which have their own characteristic absorbance maxima (347 nm for Cys-S(O)-NBD and 422 nm for Cys-S-NBD) (15). The spectral analysis of NBD-Cl-treated BCP protein without DTT showed an absorbance maximum at 347 nm (data not shown), suggesting the existence of Cys-SOH in BCP. The NBD-Cl-treated BCP without DTT resulted in the complete loss of the antioxidant activity (Fig. 6). Taken together, these results could be taken as evidence supporting the existence of the functional Cys-45 as Cys-S-OH. In our attempt to identify the Cys-S(O)-NBD adducts within BCP, we analyzed the peptide(s) containing the Cys-S(O)-NBD adduct(s) by spectral analysis of tryptic digest derived from the NBD-Cl-treated BCP at the absorbance maximum (347 nm). The analysis of the tryptic peptide profiles (Fig. 7) suggested that at least two different Cys residues within BCP were reacted with NBD-Cl and resulted in the formation of the Cys-S(O)-NBD adduct. The peptide peaks (Fig. 7, a and b) were identified as the peptides containing Cys-45/Cys-50 and Cys-99, respectively, using the C99S mutant (data not shown). The fluorescence analysis of the tryptic digest at 527 nm (excitation at 422 nm) showed that unlike the peptide eluting at 37 min (peptide b), the peptide eluting at 24 min (peptide a) did not emit the fluorescence, which indicated that the peptide a contained only the Cys-S(O)-NBD adduct, not Cys-S-NBD adducts, which emit the fluorescence at 527 nm, a characteristic fluorescence of the Cys-S-NBD adduct (15). Therefore, these results collectively suggested that the functional Cys-45 of BCP (located within the peptide a) probably exists in the form of sulfenic acid (in its oxidation state).

**Inducibility of bcp Gene in Response to Oxidative Stress**—Most thiol peroxidase genes, including E. coli p20, are inducible by oxidative stress (20–23). To investigate the inducibility of bcp gene by oxygen stress, we fused the bcp promoter region to lacZ gene and measured the β-galactosidase activities expressed in the cells cultured aerobically and anaerobically. As shown in Fig. 8A, the fused lacZ activity was increased 3-fold in response to the oxygen stress. Also, the expression level of the

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**Fig. 6. Chemical modification of BCP protein.** After the protein was incubated without and with 2.5 mM DTT at 30 °C for 30 min, chemical modification was carried out in a 300-μl reaction mixture containing 50 mM Tris-Cl (pH 8.0), 1.2 mg of BCP with 20 mM iodoacetamide or NEM at 30 °C for overnight. In the case of the modification with NBD-Cl, the reaction was performed in a reaction mixture of 50 mM potassium phosphate (pH 7.0) and 1 mM EDTA at 30 °C for 2 h. The resulting protein mixtures were extensively dialyzed against 10 mM HEPES (pH 7.0), and then glutamine synthetase protection activities of modified BCP against the DTT oxidation system were determined. Curve 1, the antioxidant activity (GS protection activity) exerted by control BCP. Curve 2, the antioxidant activity exerted by iodoacetamide-treated BCP in the absence of DTT. Curve 3, the antioxidant activity exerted by iodoacetamide-treated BCP in the absence of DTT. Curves 4 and 5 indicate the antioxidant activities of iodoacetamide- and NEM-reacted BCPs in the presence of DTT. Curve 6, the antioxidant activity of NBD-Cl-treated BCP in the absence of DTT.

**Fig. 7. HPLC separation of the tryptic peptides derived from the NBD-Cl-treated BCP.** The reaction condition was the same as that of the corresponding experimental conditions described in the legend of Fig. 6. The tryptic peptide was simultaneously detected at 422 nm (profile 1) and 347 nm (profile 2). Also, the tryptic peptide (profile 3) was detected at the fluorescence emitted (527 nm) by excitation at 422 nm.
fused lacZ gene gradually elevated during exponential growth until mid-log phase growth, beyond which the expression level was decreased (Fig. 8D). The higher expression level of β-galactosidase in the exponentially growing cells could be explained in terms of rapid oxygen metabolism during the growth in which ROS would be relatively highly produced. Taken together, these results suggested that BCP acting as an in vivo antioxidant is an inducible protein in response to oxygen stress.

Physiology of the BCP Null Mutant—To investigate the in vivo antioxidant function of BCP, we made bcp null mutant (Δbcp) by an integrative disruption method. We also constructed ahpC null mutant (ΔahpC) with the same method as a control experiment. The disruption of each gene was confirmed by the colony PCR. As expected, the PCR products obtained from Δbcp and ΔahpC cells (2.0 and 1.6 kilobase pairs, respectively) were longer than those obtained from wild-type cells (1.15 and 0.78 kilobase pair, respectively) (data not shown), implying that the bcp and ahpC genes were disrupted by the insertion of cat gene. The Δbcp, ΔahpC, and their isogenic wild-type strain were cultured aerobically on LB media. The growth rates of null mutants were slower than that of the wild-type cells (Fig. 9). The viabilities of null mutant cells were also lower than that of wild-type cells (Fig. 10A). The sensitivities of each null mutation toward the various oxidants were examined. The null mutants showed hypersensitivity toward H2O2 and t-butyl hydroperoxide (Fig. 10, B and C, respectively). The low viability and the peroxide hypersensitivity of the Δbcp mutant could be complemented by the expression of bcp gene (Fig. 10, D–F). These results collectively suggest that BCP acts as an antioxidant in vivo.

DISCUSSION

The new type of thiol peroxidase, referred to TSA/AhpC protein, is the enzyme that defends against oxidative stress through decomposition of hydroperoxide with the use of a thiol equivalent such as thioredoxin. These data are the first demonstration that BCP is a new bacterial member of the TSA/AhpC family, which catalyzes the Trx-dependent reduction of hydroperoxide. Assay with H2O2, t-butyl hydroperoxide, and linoleic acid hydroperoxide as substrates indicated activity similar to that with selenium GSH peroxidase (i.e., several hundred nmol/min/mg of protein). In mammals, GSH peroxidase acts as a general hydroperoxide peroxidase to remove H2O2 and alkyl hydroperoxides, including fatty acid hydroperoxides (1, 2). The peroxidase activity of BCP toward linoleic acid hydroperoxide (469 nmol/min/mg) is comparable with that of mammalian liver GSH peroxidase toward linoleic acid hydroperoxide (23, 24).

On the basis of our results, we propose the reaction mechanism of BCP, which catalyzes the reduction of peroxides via cysteine sulfenic acid (Cys-SOH). The cysteiny1 sulfenic acid is reduced to Cys-SH by the catalyzing action of Trx. The reaction mechanism is similar to those of mammalian ORF6 (a 1-Cys

FIG. 8. Expression of bcp promoter-lacZ fusion dependent on culture condition (A) and growth phase (B). E. coli MC1061 carrying bcp-lacZ transcriptional fusion plasmid was cultured aerobically or anaerobically in LB medium containing 50 μg/ml ampicillin at 37 °C. A, the lacZ activity was measured with cells exponentially grown. B, at indicated culture times, the optical density at 600 nm (open circle) and β-galactosidase activity (closed circle) was measured. β-Galactosidase activity is expressed as Miller units and is representative of at least three experiments.

FIG. 9. The growth of Δbcp, ΔahpC, and their isogenic wild-type cells in aerobic culture. Wild-type E. coli JC7623 strain (closed squares), Δbcp (closed circle), and ΔahpC (open circle) cells were aerobically cultured in LB medium, and their optical densities at 600 nm were measured every hour.

FIG. 10. Sensitivity of E. coli BCP (Δbcp) and AhpC (ΔahpC) null mutants toward various oxidants (B and C) and the complementation by expression of their own genes (E and F). Wild-type strain (E. coli JC7623), Δbcp, and ΔahpC were compared for their ability to grow on LB plates without H2O2 (A), with H2O2 (B), with t-butyl hydroperoxide (t-BOOH) (C), Δbcp + pBCP, Δbcp carrying the plasmid expressing BCP under control of its own promoter (pBCP), ΔahpC + pAhpC, ΔahpC carrying the plasmid expressing AhpC under control of its own promoter (pAhpC). A and D, an overnight culture was diluted to 0.1 A600, then serially diluted (from 1/10 to 1 × 10^4); then 10 μl of each dilute culture was spotted on LB plates. B, C, E, and F, 10 μl of the culture diluted by 1 × 10^4 was spotted on LB plates containing H2O2 (B and E) or t-BOOH (C and F) of the indicated concentrations.
BCP as a Novel TSA/AhpC Member

TSA/AhpC and selenium (Se)-dependent GSH peroxidase, which catalyze the reduction of peroxides via Cys-SOH and Cys-SeOH, respectively (17, 25). The sulfinic acid form of GSH peroxidase is reduced to Cys-SeH by GSH. However, an immediate electron donor to ORF6 remains unsolved. There are several experimental evidence, supporting that our proposed reaction mechanism of BCP. We have suggested that the functional Cys-45 of BCP, which is involved in the reduction reaction as a primary catalyst with the use of Trx as its immediate electron donor, could exist in the form of sulfinic acid (Cys-SOH). C50S and C99S mutant proteins still exert strong activity, but C45S does not, excluding the possibility of the formation of an intradisulfide bond between Cys-45 and other Cys residues within BCP as a part of the catalytic cycle. BCP, one-Cys TSA/AhpC protein, exists in the form of a monomer in oxidation conditions, which also eliminates the involvement of an interdisulfide linkage in the catalytic cycle as does 2-Cys TSA/AhpC (6). The consensus sequence surrounding the unique conserved Cys among the bacterial BCP subfamily (GCT) (Fig. 1) lessened the possibility that the other Cys residues such as Cys-50 and Cys-99 might be involved in the catalysis. The proposed mechanism is summarized as follows.

\[
\text{Tpx-SH}^{45} + \text{H}_2\text{O}_2 \rightarrow \text{Tpx-Cys-S-OH} + \text{H}_2\text{O}
\]

\[
\text{Tpx-S-OH} + \text{HS-Trx-SH} \rightarrow \text{Tpx-S-S-Trx-SH} \rightarrow \text{Tpx-SH}^{45} + \text{oxidized Trx}
\]

where Tpx is thiol peroxidase.

In conclusion, bacterial BCP as a TSA/AhpC subfamily is a novel type of thioredoxin-dependent peroxidase exerting a general hydrogen peroxide peroxidase activity. It has been known that E. coli contains two members of the TSA/AhpC family (p20 and AhpC). One TSA/AhpC member, p20, exists in the periplasmic space, and the other member exists in the cytoplasm of E. coli (9, 26). Both types (p20 and AhpC) catalyze the reduction of peroxides supported by Trx and AhpF52, respectively (8, 9). Unlike AhpC, BCP and p20 utilizes the Trx system as an immediate electron donor to the reduction reaction. In addition to the peroxidase activity to remove H$_2$O$_2$ and 3-butyl hydroperoxide, BCP showed fatty acid hydroperoxide-selective peroxidase activity. These different kinetic properties among three TSA/AhpC members may imply their physiological significance in vivo.

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