Escherichia coli Periplasmic Thiol Peroxidase Acts as Lipid Hydroperoxide Peroxidase and the Principal Antioxidative Function during Anaerobic Growth*

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To clarify the enzymatic property of Escherichia coli periplasmic thiol peroxidase (p20), the specific peroxidase activity toward peroxides was compared with other bacterial thiol peroxidases. p20 has the most substrate preference and peroxidase activity toward organic hydroperoxide. Furthermore, p20 exerted the most potent lipid peroxidase activity. Despite that the mutation of p20 caused the highest susceptibility toward organic hydroperoxide and heat stress, the cellular level of p20 did not respond to the exposure of oxidative stress. Expression level of p20 during anaerobic growth was sustained at the −50% level compared with that of the aerobic growth. Viability of aerobic p20∆ without glucose was reduced to the −65% level of isogenic strains, whereas viability of aerobic p20∆ with 0.5% glucose supplement was sustained. The deletion of p20 resulted in a gradual loss of the cell viability during anaerobic growth. At the stationary phase, the viability of p20∆ was down to −10% level of parent strains. An analysis of the protein carbonyl contents of p20∆ as a marker for cellular oxidation indicates that severe reduction of viability of anaerobic p20∆ was caused by cumulative oxidative stress. P20∆ showed hypersensitivity toward membrane-soluble organic hydroperoxides. An analysis of protein carbonyl and lipid hydroperoxide contents in the membrane of the stress-imposed p20∆ demonstrates that the severe reduction of viability was caused by cumulative oxidative stress on the membrane. Taken together, present data uncover in vitro function for p20 as a lipid hydroperoxide peroxidase and demonstrate that, as the result, p20 acts as the principal antioxidant in the anaerobic habitats.

Physiologically, Escherichia coli is versatile and well adapted to its characteristic habitats. The bacteria can grow in the presence or absence of O2. Under anaerobic conditions, it will grow by means of fermentation. However, it can also grow by means of anaerobic respiration because it is able to utilize NO3, NO2, or fumarate as final electron acceptors for respira-

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§ The abbreviations used are: ROS, reactive oxygen species; AhpC, alkyl hydroperoxide reductase; BCP, bacterioferritin-comigratory protein; TPx, thiol-dependent antioxidant; TSA, thiol-dependent antioxidant; DTT, dithiothreitol; GS, glutamine synthetase; DNP, 2,4-dinitrophenyl; LAOOH, linoleic acid hydroperoxide.
Antioxidative Function of E. coli p20

Cloning of Three Types of E. coli Thiol Peroxidases—Basic cloning protocols were described in Sambrook and Russell (19). The DNA sequences corresponding to AhpC, BCP, and p20 were obtained by PCR from E. coli genomic DNA using the forward primers (5’-GGGA CTTC ATG TCA CAA ACC CTT CAT TTC-3’ for p20, 5’-GGA ATTC CAT ATG TCA CAA ACC CTT CAT TTC-3’ for AhpC, and 5’-GGA ATTC CAT ATG AAT CCA CTG AAA GCC GGT-3’ for BCP) containing a NdeI (underlined) site and the initiation codon (boldface) and the reverse primers (5’-GGG GAA TCC TTA TGG TTC CAG AAC CCA-3’ for p20, 5’-GCC GGA TCC TTA GAT TTC ATT AAC CAC ACC GTC-3’ for AhpC, and 5’-GCC GGA TCC TTA GCC GGT TTC TTT CAG CCA CCA-3’ for BCP) containing the BamHI site (underlined) and the stop codon (boldface). The amplified products were purified and digested with NdeI/BamHI. The digested fragments were cloned into the pBS15 (a low copy number plasmid) (22), and the resulting plasmid was used for transformation of E. coli XLI-Blue.

Expression and Purification of E. coli Thiol Peroxidase—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 250. When the optical density of the culture at 600 nm reached 0.4, isopropyl-1-thio-β-galactopyranoside was added to a final concentration of 1.0 mM. After induction for 4 h, cells were harvested by centrifugation and stored at −70 °C until use. Frozen cells were suspended in 50 mM Tris-HCl (pH 7.4) containing 2 mM phenylmethylsulfonyl fluoride and 1 mM EDTA and disrupted by sonication. The supernatants clarified by centrifugation were used for purification of p20, AhpC, and BCP proteins. The TPx proteins were purified according to the methods reported previously (8, 9, 20).

Construction of AhpC and p20 Promoter-lacZ Fusion—To construct the AhpC or p20 promoter-lacZ fusion, the upstream sequence of the initiation codon of the BCP gene was prepared by PCR. The forward primers (5’-GGG GAA TCC ACT GAA GGC CGG GTT CCAC-3’ for p20 and 5’-GGG GGG ACC AGG TAA GAG CTT AGA TCA GGTG-3’ for AhpC) contain a KpnI site (underlined), and the reverse primers (5’-GGG GGA TCC ATT ATC TTC CCT GATTAC-3’ for p20 and 5’-GGG GGG ACC AGG TAA GAG CTT AGA TCA GGTG-3’ for AhpC) contain a BamHI site (underlined). The KpnI/BamHI-digested PCR products were cloned into the pBS15 vector, and the resulting plasmid was used for transformation of E. coli XLI-Blue.

β-Galactosidase Assay—Relative transcriptional activities of p20 and AhpC promoters were determined in terms of the expression level of lacZ reporter gene. Cells were suspended in Z buffer (60 mM Na2HPO4, 40 mM NaCl, 1 mM MgSO4, and 5 mM KCl) containing 2-mercaptoethanol and disrupted by sonication, and the expressed β-galactosidase activity was assayed using o-nitrophenyl-β-galactoside as a artificial substrate according to the method described previously (23). The β-galactosidase activity was expressed as unit (increase of optical density at 412 nm that resulted from o-nitrophenyl-β-galactoside hydrolysis by β-galactosidase per 10 min) for each sample.

Preparation of Cytosolic and Membrane Protein Extracts—10-ml samples were taken from the cultures at the indicated times, centrifuged at 13,000 × g for 5 min, washed with phosphate-buffered saline, and resuspended 1 ml of phosphate-buffered saline. Cells were disrupted by sonication. After centrifugation, the supernatant was saved for the cytosolic fraction. The pellet was resuspended with 50 mM Tris-HCl buffer (pH 7.4) and washed three times with the same buffer. The pellet was resuspended the Tris-HCl buffer containing 5% SDS and heated at 100 °C for 3 min. After centrifugation, 20,000 × g for 30 min, the soluble membrane protein content was measured using the Bio-Rad DC protein assay for SD5-containing samples.

Preparation of Membrane Lipid Peroxide—15 μg of E. coli membrane pellet was suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 1% SDS. After sonication and two times of washing with a cold deionized water to remove SDS, the pellet was dried under vacuum at −5 °C and dissolved in 1 ml of a methanol/chloroform (2:1 v/v) solution (24). The suspension was vortexed at room temperature for 1 h, and then FOX II reagent (methanol/acidified FOX reagent) was added to the suspension (21). After further vortex at room temperature for 1 h, the sample was centrifuged at 13,000 × g for 10 min at room temperature to obtain clear supernatant. The amount of membrane lipid peroxidase was measured by monitoring the absorbance at 560 nm.

Immunodetection of Protein Carbonyls—The protein carbonyl content in cytoplasmic or membrane fraction was determined according to the dinitrophenylhydrazine derivatization method described by Levine et al. (25). Samples were mixed with Laemmli sample buffers and separated electrophoretically on 8% acrylamide gel. The gels were then equilibrated in electrophoresis buffer (25 mM Tris, 191 mM glycine, 8770

thermore, as a reaction intermediate, a sulfenic acid intermediate (Cys-SOH) at Cys-61 was produced as the reaction product during the peroxidation of AhpC family in that the corresponding alcohol (akyl alcohol) was produced as the reaction product during the peroxidation reaction of p20 with alkyl hydroperoxide (17). E. coli proteomic studies by Link et al. (14) showed the relatively high expression of p20 during the growth phase without induction by H2O2, which indicates a sustained requirement for peroxide detoxification in the periplasmic space during growth. Periplasmic localization of TPx during the growth in relatively high level would give an advantage to a periplasmic thiol peroxidase (p20) in detoxification of peroxides prior to cytosolic entry (17), which may indicate an in vivo function of p20 as a periplasmic thiol peroxidase of E. coli. However, the in vivo function still remains to be defined.

On the basis of the substrate preference of alkyl hydroperoxides over H2O2, p20 has been suggested to act as an alkyl hydroperoxide reductase. Here, for the first time, in vivo evidence is presented, indicating that p20 acts as lipid peroxidase to inhibit bacterial membrane oxidation. In addition, we demonstrate that p20 acts as principal antioxidant for E. coli during anaerobic growth.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth of Cells—AhpC, p20, or BCP deletion mutants strains used in this experiment are our laboratory stocks described previously (8, 16). The wild type strain is JC7623 (AB1157 recB21 recC22 sbcB15 sbcC201). Cells were grown in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) (18, 19). LB medium was supplemented with 0.5% glucose or 0.05% glucose (or without glucose) for carbon-starvation culture. Aerobic growth of cells was carried out in 200-ml Erlenmeyer flasks, one-tenth filled, and aerated in a rotary shaker at 250 rpm. Anaerobic cultures were shaken in sealed anaerobic culture bottles filled to top with medium and stirred with 10 glass beads (1 mm diameter). Turbidity was measured with SPECTRONIC 20 D+ spectrophotometer at 600 nm. To calculate cell viability, appropriate dilutions of the cultures were placed on plates containing 1.5% agar and incubated at 37 °C, and colony-forming units were determined with cultures taken at the same absorbance. Various oxidative stresses or heat shock were subjected to the exponential phase cells and harvested at indicated times.

Determination of TSA 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 (DIT, Fe3+, and O2) (thiol metal-catalyzed oxidation system) as described previously (20). The 30-μl reaction mixture containing 100 mM Hepes-NaOH (pH 7.0), 1.0 μg of GS, 3 μM FeCl3, various concentrations of E. coli TPx, and 10 mM DTT 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.

Determination of Peroxidase Activity of TPx—Peroxidase reaction was performed in 50 μl of a reaction mixture containing 50 mM Hepes-NaOH (pH 7.0), 0.5 mM DTT, varying concentrations of TPx, and 50–700 μM of peroxides at 37 °C. The residual amount of peroxide was determined by FOX (ferrous oxidation in the presence of glyoxal orange) assay (20). Peroxidase reaction was started by the addition of 50 μM DTT. The reaction mixture was added to 1 ml of FOX I reagent and then incubated at room temperature for 30 min. The remaining amount of peroxide was measured by monitoring the absorbance at 560 nm. Linoleic acid hydroperoxide (LAOOH) was generated by incubating 100 μM linoleic acid with 10 μg/ml soybean lipoxidase in 100 mM Tris-HCl (pH 7.4) at room temperature for 30 min. The concentration of LAOOH was determined spectrophotometrically using an extinction coefficient at 234 nm of 25,000 M−1 cm−1.
Periplasmic TPx (p20) Shows the Most Potent Lipid Peroxidase Activity among E. coli TPx Family—Each E. coli Tsa/ AhpC family (p20, BCP, and AhpC) was fractionated in a 15% formaldehyde-agarose gel and transferred to a nylon membrane, and the resultant blot was hybridized with 32P-labeled p20 or AhpC structural gene

RESULTS

**Periplasmic TPx (p20) Shows the Most Potent Lipid Peroxidase Activity among E. coli TPx Family**—Each E. coli Tsa/AhpC family (p20, BCP, and AhpC) was fractionated in a 15% formaldehyde-agarose gel and transferred to a nylon membrane, and the resultant blot was hybridized with 32P-labeled p20 or AhpC structural gene.

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15% methanol) and electrophoresed to nitrocellulose membranes. Following the electrophoretic procedure, the nitrocellulose membranes were removed from the blotting apparatus and dried completely at room temperature. Prior to derivatization, membrane was equilibrated in 20% (v/v) methanol, 80% (v/v) Tris-buffered saline for 5 min. Continuous shaking was used during all incubation and washing steps. Membranes were incubated in 2×HCl for 5 min. The membranes were next incubated in a solution of 2,4-dinitrophenylhydrazine (0.5 mM) in 2× HCl for exactly 5 min each as described by Talent et al. (26). The membranes were washed three times in 2×HCl (5 min each) and five times in 50% methanol for 5 min each wash. The immunodetection for protein carbonyls using anti-dinitrophenyl (DNP) antibody was carried out according to the method described previously (26). The anti-DNP antibody was supplied by Sigma (D9656) and used at a 1/1,000 dilution. The secondary antibody was a goat anti-rabbit antibody conjugated with alkaline phosphatase.

**Other Methods**—Patch assays were done as described below. Aliquots (10 μL) containing varying cell numbers of an overnight culture were spotted on LB plates containing various oxidants. The plates were monitored after 1 day. In contrast to the case of BCP Δ, AhpC Δ, or Gram-positive, but there is no data indicating that Gram-negative bacteria have p20. The unique presence of p20 in Gram-negative bacteria could be taken as the evidence supporting the presence of p20 in the periplasmic space. In addition, this observation suggests in vivo function of p20 as an important peroxidase to detoxify peroxides prior to cytosolic entry. However, the in vivo function still remains to be defined.

As a first step toward understanding the physiological function of p20, we tried to investigate comparatively the specific activity of p20, BCP, and AhpC toward various peroxides. To our knowledge, this was the first comparative study on the peroxide substrate preference of the three types of E. coli TPx.

We measured the thiol peroxidase activity toward H2O2, t-butyl hydroperoxide, and LAOOH. Fig. 1A showed that among E. coli TPx family in addition to GS protecting activity against the inactivation by metal-catalyzed oxidation system, p20 has the most potent peroxidase activity regardless kinds of peroxide. Furthermore, p20 has a high preference toward alkyl hydroperoxide as a substrate (LAOOH = t-butyl-hydroperoxide > H2O2). The over 5-fold higher LAOOH peroxidase activity of p20 to the other TPx family suggests the in vivo function of p20 as an alkyl hydroperoxidase. The −2× higher preference for a LAOOH over H2O2 suggests that p20 may be designed to remove the hydroperoxide linked to long chain alkyl groups such as the alkyl groups of membrane lipids. Recent crystal structure of the oxidized form of E. coli p20 supports our finding (27). Based on a modeled structure of the reduced p20 in complex with 15-hydroperoxyeicosatetraenoic acid, it was suggested that the size and shape of the binding site are particularly suited for long chain fatty acid hydroperoxide.

To investigate the in vivo antioxidant function of p20 as an alkyl hydroperoxide peroxidase, we imposed various hydroperoxides on each of E. coli TPx mutants (p20Δ, BCPΔ, and AhpCΔ) growing aerobically on LB plate. As shown in Fig. 1B, in contrast to the case of BCPΔ and AhpCΔ, p20Δ exerted the
highest sensitivity toward alkyl hydroperoxides but not toward \( \text{H}_2\text{O}_2 \), indicating the possibility that p20 acts as an alkyl hydroperoxide-specific peroxidase. Taken together with the outstanding lipid hydroperoxide peroxidase activity of p20 when compared with AhpCΔ and p20Δ, this result suggests that p20 acts as a lipid hydroperoxide peroxidase in the periplasmic space of \( E. \text{coli} \).

**P20 Is Not Oxidative Stress-inducible Protein**—We have described above that exposure of p20Δ to oxidative stress remarkably reduced the cell viability. This observation suggests the possibility that p20 as an antioxidant might be induced if the cell was imposed to oxidative stress. However, to our knowledge, there is no study on transcriptional response of p20 to oxidative stress. Therefore, we investigated the response of p20 to oxidative stress.

To investigate p20 transcriptional response to oxidative stress, exponentially growing cells were exposed to various oxidants (paraquat, \( \text{H}_2\text{O}_2 \), \( \text{t} \)-butyl hydroperoxide, cumene hydroperoxide, and diamide) for 30 min and transcriptional activities of p20 and AhpC in terms of the \( \beta \)-galactosidase activity expressed under control of the p20 promoter were analyzed. As a positive control, transcriptional activity of AhpC under control of OxyR transcription factor was also examined because it is well known that the transcriptional level of AhpC is sharply induced in response to oxidative stress (28). Fig. 2A shows that transcriptional activity of p20 was insensitive to the exposure of various oxidative stresses, but as expected, that of AhpC was remarkably induced, exerting the concentration dependence. To confirm this result in vivo, we also directly determined the mRNA levels by Northern blot. The level of AhpC mRNA was greatly increased in response to diamide, whereas the level of p20 mRNA did not significantly respond to the oxidant throughout the concentrations tested (Fig. 2B). The immunoblot analysis for p20 and AhpC in the same experimental condition described above indicates that, in contrast to AhpC, p20 protein levels did not changed upon exposure to various oxidants such as \( \text{t} \)-butyl hydroperoxide, paraquat, and diamide (Fig. 2C). Taken together, these results demonstrate that p20 is not a oxidative stress-inducible protein.

**P20 Is Necessary for Survival of E. coli against Heat Stress**—As an another approach to investigate in vivo function of p20 in \( E. \text{coli} \), heat shock as an oxidative stress-induced process was subjected to three log-phased \( E. \text{coli} \) TPx mutants (p20Δ, BCPΔ, and AhpCΔ) by elevating culture temperature from 30 to 51 °C and the cell viabilities were comparatively investigated. Heat-stressed cells were plated on LB plate, and the cell viability was measured in terms of the number of the survival colonies. Fig. 3A showed viabilities of the three types of mutant as a function of the exposed time at 51 °C. An analysis of the viability data indicates that deletion of p20 in \( E. \text{coli} \) gave the most harmful effect on the survival against the heat stress. An analysis of protein carbonyl contents of the p20Δ using DNP antibody as a marker for cellular oxidation shown in Fig. 3B suggests that the severe reduction of the viability of heat-stressed p20Δ is caused by cumulative oxidative stress. Deletion of BCP (data not shown) and AhpC did not result in significant increases of protein carbonyl contents when compared with their isogenic strains, which is consistent with the viability data. To see more clearly the protein oxidation pattern, two-dimensional electrophoresis was used for the separation of the protein mixture. Fig. 3C showed clearly the significant increase of the protein oxidation in the heat-
stressed p20Δ compared with that of parent strains. Taken together with result showing that mutation of p20 renders cells more sensitive to organic peroxide killing than AhpC and BCP deletion (Fig. 1B), these data showing the lowest viability of p20Δ against heat shock lead us to speculate that this probably occurs because p20 has much higher specific activity toward organic peroxide as a lipid peroxidase than other E. coli thiol peroxidases (Fig. 1A). This speculation could be supported by previous reports that p20 is localized in periplasmic space of E. coli (9) and that heat shock causes oxidation of bacterial membrane lipid (29).

P20 Is Necessary for the Survival of E. coli during Respiration-dependent Growth—E. coli proteomics studies by Link et al. (14) showed that during the growth phase in minimal media and without induction by H₂O₂, p20 (~1.6 μM) is 3.5-fold less abundant than AhpC. Moreover, p20 is a housekeeping-type antioxidant but AhpC is a highly inducible protein by oxidative stress (Fig. 2). Despite the suggestions that p20 is the most effective antioxidant for the survival against oxidative stress and heat shock (Figs. 1B and 3) because of the highest catalytic activity shown in Fig. 1A, it was not possible to rule out the possibility that p20 is involved in a unique defense mechanism different from AhpC and BCP or in a yet undefined function.

To see the superior antioxidative capability of p20, we examined comparative viability of each E. coli TPx mutant (p20Δ, AhpCΔ, and BCPΔ). The stationary-growth-phased cells in LB media were plated on LB plate, and the cell viability was measured in terms of the number of the survival colonies. Both viabilities of AhpCΔ and BCPΔ appeared to be 13–15% higher than that of parent strains, whereas the viability of p20Δ was ~35% lower than that of parent strains (data not shown here). Taken together with previous observations of the most sensitivity of p20Δ to organic peroxide killing, heat shock killings, and the outstanding fatty acid hydroperoxide (LAOOH) peroxidase activity of p20, this result suggests the possibility that p20 is involved in a unique defense mechanism different from AhpC and BCP. As one possibility, we propose that p20 probably plays a crucial role in preventing bacterial membrane from the oxidation during respiration. To figure out the p20 function, we explored more precisely the effect of p20 mutation on the growth phenomenon and survival during aerobic growth in rich media (LB) without glucose or with glucose (0.5%). P20 mutant and its parent strains as a control were grown to the stationary phase. In aerobic condition without glucose, growth of p20Δ was significantly retarded, especially in the stationary phase when compared with the wild type strain (Fig. 4A). To investigate why the growth is retarded, the viabilities of the exponential phase and stationary phase cells were determined (Fig. 4B). Exponential and stationary growth-phased cells were plated on LB plate, and the cell viability was measured in terms of the number of the survival colonies. Both p20Δ and its isogenic strain, each viability of stationary phase cells increased more by ~10% than the corresponding viability of exponential phase cells. However, the cell viability of p20Δ was significantly lower by 30–35% than the wild strains in both growth phases, which taken together with more severe growth retardation of p20Δ than the wild strains in case of stationary-phased cells suggests the possibility that p20 is required for the aerobic (i.e., respiratory) growth. To investigate this possibility, we compared growth of p20Δ with those of the wild strains in LB media containing glucose. In sharp contrast to the case of the aerobic growth in carbon starvation condition with 0.05% glucose, the growth of p20Δ in aerobic condition supplemented with 0.5% glucose was not retarded when compared with the growth of its parent strain (Fig. 4C). Also, the viabilities of the cells were determined after the cells were plated on LB plate. An analysis of the viability data indicated that deletion of p20 did not affect the viability of cells growing aerobically in glucose-rich LB medium (i.e., growing on fermentation of glucose), but the mutation resulted in ~50% decrease of cell viability growing aerobically in the glucose starvation medium (i.e., growing on respiration) when compared with that of the wild strains (Fig. 4D). Collectively, these data demonstrate that p20 is necessary for survival of E. coli during respiration-dependent growth.

P20 Is Principal Antioxidant for Survival of E. coli during the Anaerobic Growth—Physiologically, E. coli is versatile and well adapted to its characteristic habitats. In the absence of O₂,
bacteria can grow by means of anaerobic respiration. In this study, we have demonstrated that p20 is a housekeeping antioxidant and that p20 is required for the survival for aerobic respiration. Thus, it is worthwhile to investigate whether p20 is required for survival of *E. coli* during anaerobic respiration or not. To answer the question, we examined the effect of p20 mutation on the growth phenomenon and survival during anaerobic growth in rich media (LB). P20 mutant and its parent strains as a control were grown to the stationary phase. Fig. 5, A and B, showed that the growth of p2oΔ was dramatically retarded when compared with the wild type strain regardless of presence or absence of glucose. To figure out the reason why the growth is so retarded, the viabilities of the exponential and stationary-phased parent strains. These results suggest that p20 is almost essentially required for survival of *E. coli* during anaerobic respiration. This observation leads us to suggest that p20 probably becomes an essential antioxidant for *E. coli* in anaerobic habitats.

A strain devoid of p20 exhibited defect in anaerobic respiration-dependant growth, which probably suffered from acute oxidative stress. To demonstrate the antioxidative function of p20 in anaerobic *E. coli*, we determined the transcriptional activity of p20 and the acute oxidative stress subjected to p20Δ in anaerobic culture. Fig. 6A showed that the transcriptional activity increased as a function of anaerobic culture time, although the activity was lower by 50% than that in aerobic culture, which is consistent with p20 level determined by the immunoblot against p20 antibody (Fig. 6B). Also, to see whether deletion of p20 results in the cellular oxidative stress, the soluble and membrane protein extracts from anaerobic and aerobic cultures in LB media were analyzed by Western blotting using DNP antibodies (Fig. 6, C and D). The protein carbonyls were used for an *in vivo* marker for cellular oxidative stress. In addition to the case of aerobic cultures, anaerobic cultures of p20Δ, which showed a severe growth arrest and loss of cell viability, exerted considerable increases in DNP band intensity in both cytosolic and membrane protein extracts when compared with those of the wild strains. The carbonyl level of p20Δ grown in anaerobic or aerobic condition was much higher than the respective carbonyl levels of the wild strains. Also, the much higher membrane carbonyl level of anaerobic p20Δ than that of aerobic p20Δ (Fig. 6D) gave the rationale for the severe growth arrest and the loss of cell viability in anaerobic growth. Taken together, these results demonstrate that p20 is necessary for survival of *E. coli*, especially during anaerobic growth.

**In Vivo Function of P20 Is to Prevent Bacterial Membrane Oxidation**—We have observed several lines of the evidence supporting that the *in vivo* function of p20 probably prevents bacterial membrane oxidation. (i) P20 has the most potent lipid peroxidase activity among the *E. coli* Tpx family. (ii) P20 mutant exerted the highest susceptibility toward alkyl hydroperoxides and heat shock among *E. coli* Tpx mutants. (iii) P20 is required for survival during aerobic or anaerobic respiration-dependant growth.

To investigate the *in vivo* function of p20, we comparatively studied the relative levels of the oxidative damage on cellular proteins caused by deletion of p20, AhpC, and BCP in *E. coli*. The soluble protein extracts from aerobic and anaerobic cultures of *E. coli* in the presence or absence of various oxidative stresses were analyzed by Western blotting against DNP antibodies. An analysis of the intensities of DNP-reacted bands gave us the information that BCP and AhpC were not so crucial as antioxidants as much as p20 (Fig. 7) because the deletions did not affect the protein oxidation when compared with their parent strains (data for BCP not shown). It is worthwhile to note that in anaerobic culture of p20Δ, almost all of oxidative protein damage was already processed before imposing oxidative stresses. The preoxidative damage in anaerobic p20Δ also supported the severe growth arrest caused by the reduction of cell viability shown in Fig. 5. Fig. 8 shows that, in aerobic growth of p20Δ, oxidative stresses such as heat shock, cumene hydroperoxide, and t-butyl hydroperoxide capable of damaging the bacterial membrane caused the profound oxidative damage on the membrane proteins of p20 mutant cells but oxidative stress that cannot damage the membrane (*i.e.* H2O2, diamide, and paraquat) did not so much. Taken together, the selective and profound effect of the membrane-damaging agents on the oxidation of soluble and membrane proteins in p20Δ could be taken as the evidence, supporting that p20 is an essential...
antioxidant to prevent bacterial membrane oxidation.

To demonstrate further the in vivo function of p20 as a lipid peroxidase to prevent membrane oxidation, we tried to find out the correlation between growth retardation of p20/H9004 caused by membrane-soluble organic peroxides and their membrane damages (Fig. 9). As a marker for oxidative damage on bacterial membrane, protein carbonyls in the membrane fractions (Fig. 9B) and membrane lipid hydroperoxide (Fig. 9C) were determined as a function of the concentration of t-butyli hydroperoxide or cumene hydroperoxide. Considering that cumene hydroperoxide acts as a significantly more potent oxidant for membrane than t-butyli hydroperoxide, the higher susceptibility of p20Δ to cumene hydroperoxide could be expected on the basis of the in vivo function of p20 as a lipid hydroperoxide peroxidase. Analysis of the data demonstrates the close relationship between the degrees of growth retardation of p20Δ (Fig. 9A) and its oxidative damage on the membrane shown by in terms of protein carbonyl contents (Fig. 9B) and lipid hydroperoxide (Fig. 9C).

Taken together, all of the data present here uncovered possible function for E. coli p20 as a lipid hydroperoxide peroxidase. P20 is more powerful in blocking the oxidation process occurring in bacterial membrane destructive of the cell than either of the other thiol peroxidases, AhpC, and BCP because p20 has potent lipid peroxidase activity.

**DISCUSSION**

The high structural diversity among three members of bacterial TSA/AhpC family (p20, BCP, and AhpC) (10, 11, 27) indicates their distinct physiological functions. To explain the
multiple existences, the differential cellular localization of p20, AhpC, and BCP has been cited. P20 has been characterized as a periplasmic protein (9), whereas AhpC and BCP have been localized to the cytoplasm (7, 14). However, the in vivo function still remains to be defined although they have been suggested to be alkyl hydroperoxide reductase serving as additional defenses in E. coli against alkyl hydroperoxide (TB), 0.5 mM and 1 mM; paraquat (PQ), 0.5 mM and 1 mM; cumene hydroperoxide (CH), 0.5 mM and 1 mM. Heat stress was subjected to log-phased cells by elevating culture temperature from 30 to 42, 45, 48, and 51 °C. Each 30 μg of soluble protein extract was used for the separation on 8% SDS-PAGE.

Fig. 7. SDS-PAGE quantification of oxidized cytosolic proteins. Panel A represents the oxidized proteins of aerobic Wild, AhpCΔ, and p20Δ imposed on various oxidative stresses during 2 h. Panel B shows the oxidized proteins of anaerobic Wild, AhpCΔ, and p20Δ imposed on various oxidative stresses during 2 h. The oxidized protein was immunostained with DNP antibody. Various oxidative stresses were subjected to the corresponding strain at the log-phased cells at 30 °C as follows: H2O2, 1 mM and 2 mM; diamide (DA), 1 mM and 2 mM; t-butyl hydroperoxide (TB), 0.5 mM and 1 mM; paraquat (PQ), 0.5 mM and 1 mM; cumene hydroperoxide (CH), 0.5 mM and 1 mM. Heat stress was subjected to log-phased cells by elevating culture temperature from 30 to 42, 45, 48, and 51 °C. Each 30 μg of soluble protein extract was used for the separation on 8% SDS-PAGE.

Fig. 8. Capability of organic hydroperoxide and heat stress to oxidize membrane proteins. Various oxidative stresses were imposed on exponentially growing cells in aerobic condition for 2 h, and the cells were harvested for analysis of the oxidation of cytosolic and membrane proteins. Panel A shows the oxidized proteins of wild and p20Δ, which were heat-stressed at 51 °C for 2 h. C and M represent cytosolic and membrane proteins, respectively. + and + represent without and with heat stress, respectively. Panel B represents the oxidized proteins of wild and p20Δ exposed to various oxidative stresses during 2 h. Data for the wild strains were not shown for simplicity as follows: H2O2, 1 mM; DA, 1 mM; TB, 0.5 mM; PQ, 1 mM; CH, 0.5 mM; and HS, from 30 to 51 °C.
physiology of p20Δ with that of AhpCΔ suggests that p20 may be involved in a unique defense mechanism against oxidative stress different from other cytosolic TPxs. The goal of this study was to figure out the possible distinct physiological functions.

Comparative analysis of the specific activities of p20, BCP, and AhpC toward various peroxides demonstrates that p20 has the most potent organic hydroperoxide peroxidase activity. The much higher LAOOH peroxidase activity of p20 compared with the other TPx family suggests the in vivo function of p20 serving as a lipid peroxidase. The higher preference for a LAOOH suggests that p20 may be designed to remove the hydroperoxide linked to membrane lipid. Our suggestion can be supported by a recent report on the crystal structure of the oxidized form of p20 (27). It was suggested that the size and shape of the binding site are particularly suited for long chain fatty acid hydroperoxide (27). Present comparative analyses of bacterial TPxs kinetics and response of each mutant to various oxidants have allowed us to clarify the role of p20 in E. coli and demonstrate that p20 as a periplasmic lipid peroxidase is essential for the prevention of oxidative damage on bacterial membrane during aerobic or anaerobic respiration-dependent growth, although it was not completely possible to rule out the possibility that the in vivo function resulted from the advantage given by both potent catalytic power of p20 toward various peroxides regardless of the types of peroxides and its periplasmic localization. Periplasmic localization of p20 could be taken as one of the benefits needed to combat exogenous or/endogenous reactive oxygen species prior to the entry to cell membrane and cytoplasm.

To provide a detailed description of the oxidative stress and the site specificity of hydroperoxide-induced oxidative stress in p20Δ, we have characterized the action of various antioxidants. As described previously, membrane-soluble hydroperoxides (cumene and t-butyl hydroperoxides) and heat stress (Fig. 3), capable of lipid peroxidation of cell membrane, specifically caused significantly more cumulative oxidative stress on the membrane and soluble proteins of p20Δ when compared with AhpCΔ, BCPΔ, and the wild strain (Figs. 3, 7, and 8). Also, the content of membrane lipid hydroperoxide of cumene hydroperoxide-subjected p20Δ was much higher when compared with that of the parent strain, exerting concentration dependence (Fig. 9). These findings support that p20 can protect bacterial membrane from lipid peroxidation, which could result in higher survival of the bacterial cells against membrane-damaging agents such as heat stress and organic hydroperoxide.

To explain the physiological function of p20Δ in E. coli, the response of p20Δ to various growth conditions has been comparatively investigated. Despite that mutation of p20 caused the highest susceptibility toward oxidative stress (Fig. 1B), in contrast to AhpC, the cellular level of p20 did not respond to the

**Fig. 9.** The correlation between growth retardation of p20Δ and the membrane-oxidative damages by organic peroxides. Exponentially growing cultures of p20Δ and isogenic strain were challenged with the indicated concentrations of t-butyl hydroperoxide (TBOOH) and cumene hydroperoxide (COOH). After 2 h, the cell density was measured at 600 nm. Growth arrest was represented as percent value of the growth during 2 h after addition of organic hydroperoxide compared with that of the growth during same time in the absence of organic peroxide (A). The membrane protein extracts from aerobic cultures of E. coli in the presence or absence of TBOOH and COOH were analyzed by Western blotting against DNP antibodies. As a marker for oxidative damage on bacterial membrane, protein carbonyls in the membrane fractions (B) and membrane lipid hydroperoxide (C) were determined as function of the concentration of t-butyl hydroperoxide or cumene hydroperoxide indicated in the corresponding figure. The amount of membrane lipid peroxide was monitored by measuring the absorbance at 560 nm as described in under "Experimental Procedures." Closed circle represents the value of the detected absorbance derived from the membrane of p20Δ in the presence of cumene hydroperoxide ranging from 0.1 to 0.5 mM; open circle represents the value from the p20Δ with t-butyl hydroperoxide; and closed and open squares represent the values with cumene hydroperoxide and t-butyl hydroperoxide, respectively.
exposure of the stress (Fig. 2). This observation indicates a sustained p20 requirement for detoxification of oxidative stress during normal growth. The viability of p20Δ grown aerobically in the absence of glucose was considerably reduced, whereas in the case of the glucose-supplemented growth, the viability was not changed when compared with that of the isogenic strain (Fig. 4), which suggests the requirement of p20 in the respiration-dependent growth. To investigate the function of p20 in anaerobic culture, E. coli was subjected to anaerobic respiration-dependent growth in LB medium without supplement of glucose. In that environment, the stationary-phased p20Δ almost died (Fig. 5C). Analysis of the carbonyl contents of p20Δ indicates that severe reduction of the viability is caused by oxidative stress on the membrane (Fig. 6, C and D), which is taken as the evidence supporting that p20 acts as lipid peroxide to prevent membrane oxidation. Taken together, these results demonstrate that p20 is almost essential antioxidant during anaerobic respiration-dependent growth. In contrast to the case of aerobic growth with glucose, the growth retardation of anaerobic p20Δ was not recovered by the presence of glucose (Fig. 5B). This is probably because of not only the sustained anaerobic respiration in the presence of glucose to meet the demand for redox neutrality but also the lack of protein synthesis of other aerobic-inducible antioxidant proteins in the anaerobic culture of E. coli (5, 28, 30).

The enteric bacterium E. coli thrives in the gastrointestinal tract of humans and other warm-blooded animals. In this environment, oxygen required for respiration and energy generation is in limited supply. Thus, the cell must derive energy from anaerobic respiration with alternative electron acceptors (1, 2). The demand of E. coli for redox neutrality is met by electron transfer from reducing equivalents to an internal electron acceptor (i.e. anaerobic respiration). When growing on glucose, the anaerobic respiration results in a mixed acid fermentation (1, 2). The antioxidative function of p20 in anaerobic growth probably adapts E. coli to its intestinal (anaerobic) habitats. Taken together, present data uncover in vivo function for p20 as a lipid hydroperoxide peroxidase to prevent membrane oxidation by ROS generated during respiration and demonstrate that, as the result, p20 acts as the principal antioxidant in its anaerobic habitats.

REFERENCES
1. Clark, D. P. (1989) FEMS Microbiol. Rev. 5, 223–234
2. Holmes, H. (1996) FEMS Microbiol. Rev. 18, 85–116
3. Tenhaken, R., Levine, A., Bruson, L. F., Dixon, R. A., and Lamb, C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4158–4163
4. Ferri, A., and Calza, R. (1995) Biochem. Mol. Biol. Int. 35, 691–697
5. Lynch, A. S., and Lin, E. C. C. (1996) in E. coli and Salmonella: Cellular and Molecular Biology VI (Neidhardt, F. C., ed) 2nd Ed., pp. 1526–1538, American Society for Microbiology Press, Washington, D.C.
6. Carlussi, A., and Touati, D. (1986) EMBO J. 5, 623–630
7. Costa Seaver, L., and Inlay, J. A. (2001) J. Bacteriol. 183, 7173–7181
8. Jeong, W., Cha, M. K., and Kim, I. H. (2000) J. Biol. Chem. 275, 2924–2930
9. Cha, M. K., Kim, H. K., and Kim, I. H. (1995) J. Biol. Chem. 270, 28625–28641
10. Poole, L. B. (2003) in Signal Transduction by Reactive Oxygen and Nitrogen Species: Pathways and Chemical Principles (Torres, M., Fukuto, J. M., and Forman, H. J., eds) pp. 80–101, Kluwer Academic Publishers, Norwell, MA
11. Hofmann, B., Hecht, H. J., and Flohe, L. (2002) Biol. Chem. 383, 347–364
12. Poole, L. B., Reynolds, C. M., Wood, Z. A., Ellis, H. R., and Li Calzi, M. (2000) Eur. J. Biochem. 267, 6126–6133
13. Wood, Z. A., Schrader, E., Harris, J. R., and Poole, L. B. (2003) Trends Biochem. Sci. 28, 32–40
14. Link, A. J., Robison, K., and Church, G. M. (1997) Electrophoresis 18, 1259–1313
15. Wan, X.-Y., Zhou, Y., Yan, Z.-Y., Wang, H.-L., Hou, Y.-D., and Jin, D.-Y. (1997) FEMS Lett. 407, 32–36
16. Cha, M. K., Kim, H. K., and Kim, I. H. (1996) J. Bacteriol. 178, 5610–5614
17. Baker, L. M. S., and Poole, L. B. (2003) J. Biol. Chem. 278, 9203–9211
18. Neidhardt, F. C., Bloch, P. L., and Smith, D. F. (1974) J. Bacteriol. 119, 736–747
19. Sambrook, J., and Russell, D. W. (2001) Molecular Cloning: A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
20. Kim, K., Kim, I. H., Lee, K. Y., Rhee, S. G., and Stadtman, E. R. (1988) J. Biol. Chem. 263, 4704–4711
21. Jiang, Z. Y., Woollard, A. C., and Wolff, S. P. (1991) Lipid 26, 853–856
22. Isikorski, R. S., and Hieter, P. (1989) Genetics 122, 19–27
23. Rose, M., and Botstein, D. (1983) Methods Enzymol. 101, 167–180
24. Bligh, E. G., and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911–917
25. Levine, R. L., Williams, J. A., Stadtman, E. R., and Schacter, E. (1994) Methods Enzymol. 233, 346–357
26. Talent, J. M., Kong, Y. L., and Gracy, R. W. (1998) Anal. Biochem. 263, 31–38
27. Choi, J., Choi, S., Choi, J., Cha, M. K., Kim, I. H., and Shin, W. (2003) J. Biol. Chem. 278, 49478–49486
28. Storz, G., Tartaglia, L. A., and Ames, B. N. (1990) Science 248, 189–194
29. de Macario, E. C., and Macario, A. J. (2000) Front. Biosci. 5, D780–D786
30. Storz, G., and Inlay, J. A. (1999) Curr. Opin. Microbiol. 2, 188–194