The Significance of Type II and PrxQ Peroxiredoxins for Antioxidative Stress Response in the Purple Bacterium Rhodobacter sphaeroides

Two peroxiredoxins, classified as Type II and PrxQ, were characterized in the purple non-sulfur photosynthetic bacterium Rhodobacter sphaeroides. Both recombinant proteins showed remarkable thioredoxin-dependent peroxidase activity with broad substrate specificity in vitro. Nevertheless, PrxQ of R. sphaeroides, unlike typical PrxQs studied to date, does not contain one of the two conserved catalytic Cys residues. We found that R. sphaeroides PrxQ and other PrxQ-like proteins from several organisms conserve a different second Cys residue, indicating that these proteins should be categorized into a novel PrxQ subfamily. Disruption of either the Type II or PrxQ gene in R. sphaeroides had a dramatic effect on cell viability when the cells were grown under aerobic light or oxidative stress conditions created by exogenous addition of reactive oxygen species to the medium. Growth rates of the mutants were significantly decreased compared with that of wild type under aerobic but not anaerobic conditions. These results indicate that the peroxiredoxins are crucial for antioxidative stress response in this bacterium. The gene disruptants also demonstrated reduced levels of photopigment synthesis, suggesting that the peroxiredoxins are directly or indirectly involved in regulated synthesis of the photosynthetic apparatus.

All organisms exposed to a variety of environments must acquire adaptation systems to sustain homeostasis. Under aerobic conditions, an organism’s metabolism unavoidably produces reactive oxygen species (ROS), such as the superoxide radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and reactive nitrogen species (RNS) such as nitric oxide (NO) (1, 2). O$_2^-$ is the first product in ROS metabolism and is generated by the incomplete reduction of oxygen to water during respiration. Although O$_2^-$ has low reactivity for lipid oxidation and cannot permeate the cytoplasmic membrane, it causes iron reduction (from Fe$^{3+}$ to Fe$^{2+}$) as well as the H$_2$O$_2$-prompted Fenton reaction that results in production of the highly reactive hydroxyl radical (OH$^-$). H$_2$O$_2$, which is enzymatically produced from O$_2^-$, is stable and can pass through the cytoplasmic membrane. The molecular properties of NO are similar to those of H$_2$O$_2$, but it is highly reactive and can cause oxidative damage. Furthermore, NO reacts with O$_2^-$ to generate peroxynitrite (OONO$^-$). It has been shown that these ROS and RNS cause lipid peroxidation and inhibition of DNA synthesis as well as inactivation of enzymes through oxidation of heme, Fe$^{2+}$, and/or sulfhydryls (1–3). To avoid such oxidative damage, all aerobic organisms have antioxidant systems that include catalase, superoxide dismutase (SOD), and various peroxidases (1–4). ROS and RNS at low levels also function as intracellular messengers in response to various stresses, and the antioxidant systems play a role in sustaining the proper ROS and RNS levels for the signaling cascade (5, 6).

Recently, peroxiredoxin (Prx) has been extensively studied as a core member of the antioxidant system conserved in many organisms (7–9). Prx was initially discovered as a 25-kDa enzyme in yeast and protects cellular components against oxidative damage (10). Later studies established that Prxs are a family of thiol-specific peroxidases and peroxynitrite reductases that can reduce various peroxides, such as H$_2$O$_2$, alkyl hydroperoxides, and peroxynitrite, with broad substrate specificity. Based on primary structure, Prxs are classified into four groups (1-Cys, 2-Cys, Type II, and PrxQ) (9). All types of Prx contain a conserved N-terminal peroxidatic Cys residue that acts as the primary catalytic center. The peroxide substrate interacts with the peroxidatic sulfhydryl group of the catalytic center to oxidize it to sulfenic acid (SOH). This sulfenic acid returns to a sulfhydryl group in a manner dependent on the Prx class. In the case of 2-Cys Prxs, a second catalytic cysteinyl group, a so-called resolving thiol, is located in the C terminus and forms an intramolecular disulfide bond within the Prx homodimer. In contrast, PrxQ and Type II Prxs function as monomeric proteins and form an intramolecular disulfide bridge between the peroxidatic Cys and the resolving thiol. The disulfide bond formed in the Prx molecules is reduced by vari-
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ous electron donors, such as thioredoxin (Trx), glutathione (GSH), and cyclophilins. 1-Cys Prx does not have the resolving thiol, and the sulfenic acid derivative of the peroxidatic Cys residue is re-reduced by an unknown interacting partner before the next catalytic cycle (7). These Prxs are conserved in both prokaryotes and eukaryotes and function not only as antioxidants but also as modulators of oxidative signaling pathways and as a redox sensor (7–9). In eukaryotic organisms, many Prxs are localized in plastid and/or mitochondria, suggesting that they were introduced into host cells during symbiosis of photosynthetic and/or aerobic bacteria. The prokaryotic Prxs have been characterized in only a limited number of bacterial species, and the physiological significance of these proteins is not fully elucidated.

In light of the evolutionary considerations mentioned above, we studied the Prx proteins in the purple bacterium *Rhodobacter sphaeroides* because this bacterium exhibits remarkably versatile bioenergetics such that it can grow by photosynthesis as well as by aerobic and anaerobic respiration under various environmental conditions (11). Genetic manipulations for this organism are also well established (12). Under high oxygen conditions, the bacterium achieves aerobic respiration with a respiratory chain that is similar to that of mitochondria, whereas under low oxygen conditions it produces photosynthetic complexes to grow by photosynthesis (11, 13, 14). The synthesized photosynthetic apparatus can produce another ROS, singlet oxygen ($^1$O$_2$) in the presence of both oxygen and light (15). Therefore, the bacterium is exposed to various ROS produced by both respiratory and photosynthetic metabolism. These properties suggest that the bacterium has acquired more sophisticated antioxidant systems than those of other bacteria studied to date. So far, several factors within the antioxidant systems of this bacterium had been analyzed (16–20). We now report that *R. sphaeroides* has two Prxs that can be classified into Type II and PrxQ Prxs and that are critical for growth under aerobic conditions. The PrxQ family protein was further categorized into a subfamily we defined based on the position of a critical cysteine.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—*R. sphaeroides* strain 2.4.1 was used as a wild-type strain. The wild-type and Prx mutant strains were grown at 30 °C in PYS medium (21). For anaerobic conditions, cells were grown in completely filled vessels. Illumination was provided by 100-watt tungsten lamps. For semiaerobic conditions cells were grown in 100-ml vessels. Illumination was provided by 100-watt tungsten lamps. For aerobic conditions cells were grown in 150-ml conical flasks by rotational incubation. For semiaerobic conditions cells were grown in 100-ml vessels. Illumination was provided by 100-watt tungsten lamps.

**Prx mutant strains** were grown at 30 °C in PYS medium (21). The wild-type and *R. sphaeroides* strain 2.4.1 was used as a wild-type strain. The wild-type and *R. sphaeroides* strain 2.4.1 was used as a wild-type strain. The wild-type and *R. sphaeroides* strain 2.4.1 was used as a wild-type strain. The wild-type and *R. sphaeroides* strain 2.4.1 was used as a wild-type strain. The wild-type and *R. sphaeroides* strain 2.4.1 was used as a wild-type strain. The wild-type and *R. sphaeroides* strain 2.4.1 was used as a wild-type strain. The wild-type and *R. sphaeroides* strain 2.4.1 was used as a wild-type strain.

**Expression and Purification of Type II and PrxQ Prxs and Thioredoxin A (TrxA) of *R. sphaeroides***—The coding regions for genes encoding RsPrxT, RsPrxQ, and TrxA (accession numbers YP_353982, ABA79134, and YP_351571, respectively) were amplified by PCR using genomic DNA of *R. sphaeroides* as a template with the following combination of primers: for RsPrxT, 5'-GGGGGGCATATGTTGATTTCGGTACGGCA-3' (Ndel) and 5'-GGGGGTTCGACTCAGATCGCGGCCAGCAG-3' (Sall); for RsPrxQ, 5'-GGGGGCGCATATGAGCGGGCCCGAGCAGAAGAAGC-3' (Ndel) and 5'-GGGGGTTCGACTCTAGTGGGCGGCCGACGGCG-3' (Sall); for TrxA, 5'-GGCATATGTCACCGTTTCCGTTCA-3' (Ndel) and 5'-GGGAATTCTCAGACGGCCGACATGTA-3' (EcoRI). The restriction sites shown in parentheses are underlined in the sequences. Each amplified DNA fragment was digested with Ndel and Sall (or EcoRI) and cloned into Ndel-Sall- (or EcoRI)-digested pTYB12 vector (New England Biolabs). Sequences of the insert DNA were confirmed by sequencing, and the obtained plasmids were named pTYTypeII, pTYPPrxQ, and pTYPTrx for RsPrxT, RsPrxQ, and TrxA expression plasmids, respectively. Expression plasmids for two mutant RsPrxQs, C44S and C80S, in which Cys-44 and Cys-80 were replaced individually by Ser, were generated by standard PCR-mediated site-directed mutagenesis with use of pTYPPrxQ as template DNA and complementary primers containing a 1-bp mismatch that converts the codon for Cys to one for Ser. The plasmids were transferred into E. coli BL21(DE3), and each chitin-tagged protein was overexpressed by induction with 1 mM isopropyl $\beta$-d-maltoside (IPTG) at 30 °C. The expressed recombinant proteins were purified with chitin beads (New England Biolabs) according to the manufacturer’s instructions. Purified proteins were dialyzed in storage buffer (20 mM Tris/HCl, pH 8.0, 10 mM NaCl, 0.1 mM EDTA, 20% glycerol). Protein was quantified using the Bradford assay kit (Bio-Rad).

**Peroxidase Activity Assay**—Trx-dependent peroxidase activity was measured by the coupled oxidation of NADPH using NADPH-Trx-reductase from *Arabidopsis thaliana* (AtNTR) and *R. sphaeroides* TrxA at 25 °C. For measurement of the activity of Prxs, a reaction containing 50 mM Hepes/NaOH, pH 7.3, 20 μM TrxA, 5 μM AtNTR (recombinant protein expressed in *E. coli*) (22), and 0.2 mM NADPH was used. To measure GSH-dependent peroxidase activity, a reaction containing 50 mM Tris/HCl, pH 8.0, 2 mM Na$_2$S, 0.1 mM EDTA, 0.23 units/ml NADPH-glutathione reductase from *E. coli* (Sigma), 1 mM NADPH, and 70 μM GSH (Sigma) was used. After the addition of various concentrations of Prx, the reaction was initiated by addition of various concentrations of H$_2$O$_2$ or cumene hydroperoxide (CHP), and the decrease of absorbance at 340 nm of the reaction was monitored.

**In Vitro Reduction of Prx Proteins**—Oxidation of Prxs was induced by incubation with 50 μM CuCl$_2$. The oxidized Prxs were incubated with a combination of DTT and TrxA as indicated in Fig. 3 (top of each photograph). The redox states of Prx proteins were determined as follows. Briefly, the protein was precipitated with 10% (w/v) trichloroacetic acid (final concentration) and collected by centrifugation. The obtained protein precipitates were then washed with acetone, and the proteins were dissolved in freshly prepared solution containing 1% SDS, 50 mM Tris/HCl, pH 7.5, and 10 mM 4-acetamido-4'-maleimidostibene-2',2'-disulfonic acid (Molecular Probes). Proteins were then separated by non-reducing SDS-PAGE.
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Construction of R. sphaeroides Prx-disrupted Strains—The R. sphaeroides RsPrxT-disrupted strain, TYPEII, was constructed as follows. The DNA fragment that includes the RsPrxT coding region and the 500-bp up- and downstream regions was amplified by PCR using genomic DNA as a template. The primers used were 5'-GGGGTGTTCTAGACGCCCCCTGTGAAGGCCG-3' and 5'-GGGGAGCTCTTGCCGAAATGCCGCCAAA-3'. The PCR product was digested with XbaI and SacI and then cloned into pUC118 vector (TAKARA), and the plasmid was named pUCTypeII. The plasmid pUCTypeII was digested with HincII, and a 1.5-kilobase StuI-digested kanamycin-resistant cassette derived from plasmid pBSL86 (23) was inserted into the HincII site. The resulting plasmid was named pUCKmTypeII. Then the insert DNA of plasmid pUCKmTypeII was removed by digestion with XbaI and Sall and introduced into XbaI-Sall-digested pZJL929A (24), a gentamicin-resistant suicide vector. The obtained plasmid was transferred into R. sphaeroides cells by conjugation with the mobilizing strain S17-1 lysogenized with pir (25, 26). Kanamycin-resistant cells on plates containing 5% sucrose were selected as double-crossover candidates, and the chromosomal insertion was confirmed by Southern hybridization.

The R. sphaeroides RsPrxQ-disrupted strain, PRXQ, was constructed as follows. The DNA fragment including the RsPrxQ coding region and the 500-bp up- and downstream regions was amplified by PCR using genomic DNA as a template. The primers used were 5'-GGGGGTCTAGACCGCCCTCACTCGACCCGGATCGGGAAGTCGACGGACAGGGCGGAGCCGC-3'. The PCR product was digested with XbaI and Sall and then cloned into pUC118 vector (TAKARA), and the plasmid was named pUCKmTypeII. Then the insert DNA of plasmid pUCKmTypeII was removed by digestion with XbaI and Sall and introduced into XbaI-Sall-digested pZJL929A (24), a gentamicin-resistant suicide vector. The obtained plasmid was transferred into R. sphaeroides cells by conjugation with the mobilizing strain S17-1 lysogenized with apir (25, 26). Kanamycin-resistant cells on plates containing 5% sucrose were selected as double-crossover candidates, and the chromosomal insertion was confirmed by Southern hybridization.

The R. sphaeroides RsPrxT/RsPrxQ double-disrupted strain, PRXQII, was constructed as follows. The plasmid pZJDSmpPrxQ was transferred into the TYPEII cells by conjugation with the mobilizing strain S17-1 lysogenized with apir (25, 26). Streptomycin/kanamycin-resistant cells were selected as double-crossover candidates, and the chromosomal insertion was confirmed by Southern hybridization.

Determination of Survival Rates—Cell densities of cultures grown under semiaerobic conditions until mid-logarithmic growth phase were adjusted to $A_{660}$ of 0.50. A dilution series was made, and then cell suspension was spotted onto PSY plates containing varying concentrations of $H_2O_2$, t-BHP, CHP, 1-chloro-2,4-dinitrobenzene (CDNB), paraquat, rose bengal, or sodium nitroprusside (SNP), as indicated in the legend to Fig. 4. The spots were allowed to dry, and the plates were incubated at 30 °C for 3–4 days (for Fig. 4, A and C) or 7 days (for Fig. 4B). The harvested cells grown until mid-logarithmic phase ($A_{660}$ of 0.5–0.6) were suspended in ICM buffer (10 mM KH$_2$PO$_4$/K$_2$HPO$_4$ and 1 mM EDTA, pH 7.2) and disrupted by sonication. The disrupted cultures were centrifuged to remove cell debris. Absorption spectra of the cell-free extracts were measured with a Hitachi U-080D spectrophotometer. Levels of light-harvesting complexes after spectroscopic analysis of the samples were determined as described (29).

RESULTS

Identification of Genes Encoding Prx-like Proteins from the Genomic Sequence of R. sphaeroides—Genes encoding Prx-like proteins in the R. sphaeroides genome were found by a computer-aided similarity search of the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov). There were two candidate open reading frames with accession numbers YP_353982 (RSP2973), annotated as an AhpC/ TSA family protein gene and a peroxiredoxin gene, respectively. From phylogenetic tree analysis based on the deduced amino acid sequences of the two proteins with those of other Prx proteins, the gene products of YP_353982 and YP_353982 were categorized as Type II and PrxQ Prxs, respectively (supplemental Fig. 1). Therefore we designated them as RsPrxT and RsPrxQ.

RsPrxT conserves the two catalytic Cys residues including peroxidatic Cys and resolving Cys residues at the position of 49 and 74, respectively (Fig. 1A). In contrast, RsPrxQ conserves only the peroxidatic Cys residue at position 44 (Fig. 1B). Although catalytic Cys residues of typical PrxQ identified so far are spaced by four amino acids (30–32), RsPrxQ lacks the latter resolving Cys residue at that position (Fig. 1B). In addition, when the amino acid sequences of various PrxQ were aligned, the position of another Cys residues was found at various positions or not found on the sequences. Within them, Cys-80 of RsPrxQ is well conserved in PrxQ proteins from some prokaryotes (Synechocystis sp. PCC6803, Anabaena variabilis ATCC29413, and Thermosynechococcus elongatus BP-1). Therefore, we designated a subgroup of PrxQs that have two conserved Cys residues with a 35-residue gap as PrxQβ. To date, nothing is known of the biochemical properties of PrxQβs. Given that PrxQα family proteins form a catalytic site by forming an intramolecular disulfide bridge (30–32) and that RsPrxQ has only two Cys
residues (peroxidatic Cys-44 and Cys-80), Cys-80 may function as a resolving Cys residue. To investigate whether the Cys-80 could be a resolving Cys, we attempted to estimate the position of the Cys from the crystal structure of a 2-Cys Prx of *Crithidia fasciculata*, which has the highest similarity based on predicted secondary structure with that of *Rs* PrxQ as determined by Genomes TO Protein structures and functions from the Laboratory for Gene-Product Informatics at the National Institute of Genetics, Japan (data not shown). As shown in Fig. 1C, Ile-53 (corresponding to the resolving Cys of PrxQ) in *C. fasciculata* 2-Cys Prx is vicinal to the peroxidatic Cys-48 with an intervening distance of ~8 Å within a same helix. In contrast, Thr-84 (corresponding to Cys-80 of *Rs* PrxQ) in *C. fasciculata* 2-Cys Prx locates in a different helix, although it is still vicinal to the peroxidatic Cys-48 (Fig. 1C). Thus, Cys-44 and Cys-80 of *Rs* PrxQ could, potentially, create an active site by forming an intramolecular disulfide bond.

To test this hypothesis we tested the *in vitro* redox properties of *Rs*PrxT wild-type and mutant proteins that contain Cys-44 changed to Ser (C44S) or Cys-80 changed to Ser (C80S) point mutations. The recombinant *Rs*PrxQ wild-type, C44S, and C80S proteins were purified to homogeneity (see “Experimental Procedures”). As shown in Fig. 2A, the oxidized form of the 2-Cys Prx of *C. fasciculata* (Protein Data Bank 1E2Y).
reported to have broad substrate-specific peroxidase activity using Trx or GSH as an electron donor (7–9). In this measurement, we examined the two different pathways for electron donation to Prxs. One is provided by the combination of NADPH, Trx from R. sphaeroides (TrxA), and Trx reductase from A. thaliana (AtNTR). The second is provided by the combination of NADPH, GSH, and glutathione reductase from E. coli. The reaction was initiated by adding substrates such as H₂O₂ or CHP. In the Trx-dependent peroxidase activity assay, NADPH oxidation was not induced without the addition of RsPrxT or RsPrxQ. The addition of RsPrxT or RsPrxQ resulted in significant oxidation of NADPH with decomposition of oxidant (Table 1). The $k_{\text{cat}}$ values for the reduction of H₂O₂ and CHP by RsPrxT were very similar to those observed for RsPrxQ. However, the two peroxides showed an approximate 2-fold higher affinity for RsPrxT than for RsPrxQ. Thus, the catalytic efficiencies ($k_{\text{cat}}/K_m$) of RsPrxT toward H₂O₂ and CHP were ~2- and 1.5-fold higher, respectively, than those of RsPrxQ (Table 1). We also measured the GSH-dependent peroxidase activity of RsPrxT and RsPrxQ; however, these were not detectable (Fig. 2B for RsPrxQ; data not shown for RsPrxT). Hence, both proteins exhibited peroxidase activities to decompose H₂O₂ and CHP with electrons donated from TrxA, although the activity of RsPrxT was slightly higher than that of RsPrxQ.

We next tested the peroxidase activity of the RsPrxQ C44S and C80S mutants. Site-specific mutation in the peroxidatic Cys residue (C44S) resulted in complete loss of peroxidase activity (Fig. 2B). On the other hand, the mutant RsPrxQ containing a Ser at the resolving Cys position (C80S) still had significant peroxidase activity with electrons donated from TrxA (but not from GSH), although the activity was approximately half that of the wild-type protein. This indicates that the resolving Cys is not essential for the peroxidase activity in RsPrxQ.

To directly confirm the reduction of RsPrxT and RsPrxQ by TrxA, we next studied the redox states of the thiol groups in the RsPrxT and RsPrxQ molecules. Previous studies indicated that ROS-induced oxidation of both PrxQu and Type II Prxs results in the formation of an intramolecular disulfide bond between the peroxidatic and resolving Cys residues (7, 9, 22, 30, 33, 34). Before incubation with TrxA, both proteins were oxidized in the presence of 50 μM CuCl₂. The oxidized RsPrxT and RsPrxQ formed an intramolecular disulfide bond (Fig. 3). Some RsPrxT also ran electrophoretically as a dimer, suggesting formation of an intramolecular disulfide bond (Fig. 3B). Given that RsPrxT proteins have three Cys residues, the intramolecular disulfide bond seems to be formed by the free thiol that is outside of the active site. The intramolecular disulfide bond formed in the oxidized RsPrxQ molecule was very stable and was not reduced even in the presence of 1 mM DTT (Fig. 3A, upper panel). The oxidized RsPrxQ was easily reduced in the presence of TrxA, and only 10 μM DTT was sufficient to completely reduce the protein. DTT-dependent reduction of RsPrxT was also greatly stimulated by TrxA (Fig. 3B). These results indicated that TrxA could function as an efficient electron donor to both RsPrxT and RsPrxQ.

Characterization of Knock-out Strains of RsPrxT and RsPrxQ—To assess the roles of RsPrxT and RsPrxQ in vivo, we constructed Prx-disrupted strains TYPEII, PRXQ, and PRXQII that lack genes encoding RsPrxT, RsPrxQ, or both RsPrxT and RsPrxQ, respectively, using conjugation techniques (see “Experimental Procedures”). Double-crossover events in the disrupted strain candidates were confirmed by Southern blotting (data not shown).

To study the effects of gene disruption of these Prxs on cell damage caused by ROS and RNS, spot assays were performed with various oxidative stresses as induced by addition of H₂O₂, t-BHP, CHP, CDNB, paraquat, SNP, or rose bengal. t-BHP and CHP induce lipid peroxidation. CDNB inactivates GSH. Paraquat and SNP produce O₂⁻ and NO⁻, respectively. Rose bengal produces ¹O₂ in the presence of light. We first tested the concentration dependence of wild-type cells for these ROS/RNS, and the concentrations of each ROS/RNS used in the assays are the maximum levels that do not affect wild-type viability. These conditions allow detection of slight effects on the viability upon Prx mutations. In aerobic-dark and anaerobic-light conditions without ROS/RNS production, cell viability was not affected by disruption of the Prx genes (Fig. 4A, control and anaerobic, respectively). However, under aerobic-light conditions, the TYPEII and PRXQII strains showed reduced viability compared with wild type even in the absence of ROS/RNS (+ light). The TYPEII strain was the most sensitive to oxidative stress.

### TABLE 1

| Substrate Type of Prx | $K_m$ (μM) | $k_{\text{cat}}$ (min⁻¹) | $k_{\text{cat}}/K_m$ |
|----------------------|----------|----------------|-------------------|
| H₂O₂ RsPrxT          | 4.75 ± 1.81 | 17.08 ± 0.86 | 3.59              |
| H₂O₂ RsPrxQ          | 8.56 ± 1.06 | 17.07 ± 0.36 | 1.99              |
| CHP RsPrxT           | 6.65 ± 0.72 | 16.45 ± 0.34 | 2.47              |
| CHP RsPrxQ           | 8.48 ± 0.31 | 16.85 ± 0.13 | 1.98              |

### FIGURE 3. Redox state of thiols in RsPrxQ and RsPrxT.

The amount of reduced RsPrxQ (A) and RsPrxT (B) was determined by 4-acetamido-4'-maleimidylstibene-2',2'-disulfonic acid labeling. The purified Prx proteins were first oxidized by incubation with CuCl₂. The proteins were then incubated with the indicated concentrations of DTT in the absence or presence of 3 μM TrxA.
induced by H$_2$O$_2$, t-BHP, SNP, or CHP of all the strains tested. Cell viability was moderately reduced in PRXQII, and PRXQ had the highest tolerance for the oxidative stress of the disrupted strains (Fig. 4A). Both TYPEII and PRXQII, but not PRXQ, were also sensitive to 1$\text{O}_2$ produced by rose bengal (Fig. 4C). In contrast, GSH depletion by CDNB and O$_2^*$ production by paraquat did not significantly affect cell viability (Fig. 4A). However, one could assume that higher concentrations of CDNB and paraquat influence the viabilities. To test this hypothesis, we did the spot assays with 10-times higher concentrations of CDNB and paraquat (Fig. 4B). At the higher concentrations of paraquat or CDNB, not only viability but also the growth rate of wild-type cells were significantly reduced. At the higher concentrations of paraquat, viabilities of all mutant strains were not significantly different from that observed in wild-type (Fig. 4B, paraquat $\times 10$). Viabilities of the mutants were also similar to that of wild type at the higher concentrations of CDNB (Fig. 4B, CDNB $\times 10$), although colonies of TYPEII were smaller than those of wild type.

We next determined the growth profiles of the Prx-disrupted strains under respiratory or photosynthetic growth conditions. Under anaerobic-light conditions, there was no significant difference in growth rates between wild type and the disruptants (Fig. 5A). In contrast, under aerobic growth conditions, all mutant strains grew slower than wild type (Fig. 5B). In the growth conditions an exponential growth phase could be seen only when cell densities were low ($A_{600} < \sim 0.5$), because available oxygen concentrations for each single cell were reduced with increasing cell densities. The calculated doubling times of all mutant strains in the exponential growth phase were increased by $\sim 2$-fold compared with that of wild type. These results indicated that either RsPrxT or RsPrxQ is important for aerobic growth of this bacterium.

The spot assays (Fig. 4) indicated that loss of resistance to oxidative stresses in the disrupted cells depended on ROS/RNS species. This could imply that other antioxidative factors can somehow compensate for loss of Prx functions. To test this hypothesis, we used northern hybridization to test for up-regulation of antioxidants that could produce such an adaptive response. The candidates were chosen from the major putative antioxidant enzymes and based on the H$_2$O$_2$-dependent expression profiles from microarray data of R. sphaeroides (17) and included catalase, Cu/ZnSOD, FeSOD, glutathione peroxidase, and cytochrome c peroxidase (CCP). The enzymatic reactions of these proteins are as follows; catalase and CCP decompose H$_2$O$_2$, SODs convert O$_2^*$ to H$_2$O$_2$, and glutathione peroxidase mediates a glutathione-dependent H$_2$O$_2$ decomposition reaction. The total RNAs were prepared from cells grown under two different growth conditions. One is an aerobic condition in which all Prx-mutant strains show reduced growth rates (Fig. 5B). The other condition was achieved by aerobic-to-anaerobic growth transitions, which may be affected by Prx functions. Under aerobic conditions, expression of CCP in TYPEII, PRXQ, and PRXQII strains were increased by $\sim 4.0$-, 4.5- and 3.0-fold, respectively, compared with wild type based on the band intensities (Fig. 6). Expression of FeSOD was also up-regulated in TYPEII, PRXQ, and PRXQII strains by $\sim 3.0$-fold compared with wild type when growth was shifted from

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FIGURE 4. Spot assays of the effects of ROS and RNS on cell viability for wild-type and Prx-disrupted strains. A, serially diluted wild-type (WT), RsPrxQ disruptant PRXQ, RsPrxT disruptant TYPEII, and RsPrxQ-RsPrxT double mutant PRXQII cells were spotted on PYS plates containing no ROS/RNS (control, + light, and anaerobic), 0.4 mM H$_2$O$_2$, 1.0 mM t-BHP, 0.2 mM SNP, 0.6 mM CHP, 0.1 mM paraquat, or 0.2 mM CDNB. The cells were grown for 3–4 days under aerobic dark conditions except for two plates indicated as + light and anaerobic, which were grown under aerobic-light and anaerobic-light conditions, respectively. B, serially diluted cultures were spotted onto plates containing 1.0 mM paraquat or 2.0 mM CDNB. Cells were grown for 7 days under aerobic-dark conditions. C, serial diluted cultures of wild type, TYPEII, PRXQ, and PRXQII were spotted onto plates containing 0.2 mM rose bengal. The plates were illuminated with white light ($\sim 50$ μmol m$^{-2}$ s$^{-1}$) for 30 min and then incubated under aerobic dark conditions. The percentage of survival rate is normalized by setting wild type rate as 100%. The results are based on two independent assays.
aerobic conditions to photosynthetic conditions (Fig. 6). However, expression of catalases, glutathione peroxidase, and Cu/ZnSOD was not significantly changed in the Prx-disrupted mutants under any growth conditions tested (data not shown). These results suggest that antioxidant factors, including at least Prxs, FeSOD, and CCP, cooperatively diminish ROS produced in the cells.

Interestingly, the Prx-disrupted strains appeared to repress the synthesis of photopigments under aerobic dark conditions (Fig. 7A). TYPEII cells showed less pigmented colonies com-

FIGURE 5. Growth profiles of Prx-disrupted strains. Cell cultures grown until late-logarithmic phase of wild type (circles), RsPrxQ disruptant PRXQ (squares), RsPrxT disruptant TYPEII (triangles), and RsPrxQ-RsPrxT double disruptant PRXQII (diamonds) were transferred into fresh medium and the growth was measured under anaerobic light (A) or aerobic dark (B) conditions.

FIGURE 6. Northern hybridization analysis of transcripts of the antioxidant enzymes CCP and FeSOD in Prx-disrupted strains. To detect CCP mRNA, total RNA was isolated from cells grown under aerobic dark conditions. To detect FeSOD mRNA, dark-aerobically grown cells were shifted to semiaerobic light conditions for 3 h, and then total RNA was isolated. rRNA is shown as a loading control. WT, wild type.

FIGURE 7. Pigmentation phenotypes of wild-type and Prx-disrupted strains. A, growth of wild-type (WT) and Prx-disrupted strains on PYS plates under aerobic dark conditions. B, absorption spectra of crude cell extracts of wild-type (black), RsPrxT disruptant TYPEII (red), RsPrxQ disruptant PRXQ (blue), and RsPrxQ-RsPrxT double disruptant PRXQII (green). C, absorption spectra of crude cell extracts of wild-type cells grown in the absence of ROS/RNS (black) and in the presence of 0.2 mM SNP (red) or 0.1 mM paraquat (blue). Protein concentrations of the measured samples were adjusted to 880 μg/ml.
pared with wild type. PRXQ gave pale pink colonies, and PRXQII gave white-pink colonies. Fig. 7B shows the absorption spectra of wild-type and the Prx-disrupted strains grown under semiaerobic dark conditions. PRXQ- (blue) and PRXQII- (green) mutants showed significant reduced synthesis of photopigments compared with that of wild-type (black). In the TYPEII mutant (red), the synthesis of photopigments was also reduced to a lesser extent than compared in PRXQ and PRXQII. Interestingly, levels of two light-harvesting complexes (B800–850 and B875) were differentially affected by the loss of Prx functions. Specifically, amounts of B800–850 light-harvesting complexes in semiaerobically grown cells of PRXQ, TYPEII, and PRXQII were ∼8-, 3-, and 14-times lower, respectively, than that of wild type (Table 2). On the other hand, the levels of B875 light-harvesting complexes in PRXQ, TYPEII, and PRXQII were reduced by 4-, 3-, and 9-fold, respectively, compared with that of wild type (Table 2). Therefore, Prx function may be needed to sustain the proper amounts of light-harvesting complexes in this bacterium.

The wild-type strain exhibited a range of phenotypes in spot assays for pigment formation in the presence of ROS or RNS (Fig. 4, A and B). This variation was also seen in the absorption spectra (Fig. 7C). The amount of light-harvesting complexes in wild-type cells (black) was significantly decreased by SNP treatment (red). Cells treated with paraquat also substantially decreased the amount of the light-harvesting complexes (blue). These results indicate that photopigment synthesis in this bacterium is strongly affected by cellular ROS/RNS accumulation, and oxidative stress in the cells easily changes the cellular levels of the photosynthetic apparatus. The level of B800–850 light-harvesting complex is severely reduced, more than that of B875, in the presence of SNP or paraquat (Fig. 7C, red and blue, respectively). This indicates that SNP and paraquat differentially affect the levels of the two light-harvesting complexes.

**DISCUSSION**

In this study we provide the first biochemical evidence showing that the two Prx proteins of R. sphaeroides, RsPrxT and RsPrxQ, have peroxidase activity with broad substrate specificity (Table 1). Mutant strains lacking either or both Prxs were hypersensitive to ROS and RNS (Fig. 4) and had lower growth rates under aerobic growth conditions (Fig. 5). These findings clearly indicate that these Prxs are important members of antioxidative systems in this bacterium.

RsPrxT contains two conserved catalytic Cys residues, a peroxidatic and a resolving Cys (Fig. 1A), that form an intramolecular disulfide bond in the oxidized state as observed in other type II Prxs (Fig. 3B). However, our in vitro analysis indicated that RsPrxT can form an intermolecular disulfide bond as well (Fig. 3B). This may be caused by an in vitro redundancy effect of CuCl₂ oxidation. In contrast, RsPrxQ conserves only the peroxidatic Cys residue (Fig. 1B). Another Cys (Cys-80) is a candidate for the resolving Cys residue of RsPrxQ and may be close to the peroxidatic Cys based on the crystal structure of a Prx with the highest similarity in its predicted secondary structure, C. fasciculate 2-Cys Prx (Fig. 1C). Furthermore, RsPrxQ has only two Cys residues, both of which are necessary to form an intramolecular disulfide bond in vitro (Fig. 2A). In addition, there are other PrxQ-like proteins that have similar conserved Cys residues (Fig. 1B). Therefore, we propose here that the RsPrxQ-like proteins form a subfamily, PrxQB, and typical PrxQ proteins that have (CX₄C) in the active site are a subfamily we named PrxQα. Given that the $K_m$ value of RsPrxQ for various peroxides is ∼8 μM (Table 1) and is about 50-fold lower than the $K_m$ values of PrxQα proteins studied so far (30), RsPrxQ may have evolved to obtain higher affinities for peroxides that form an active site with a pair of Cys residues that differ from those of PrxQα proteins. Note that other PrxQ-like proteins contain a second Cys at various positions or contain only one Cys. Study of Prx activity and substrate specificity of these PrxQ family proteins is important to understand the molecular evolution of Prx proteins.

The C80S mutant RsPrxQ containing a mutation at the resolving Cys residue still showed peroxidase activity when electrons were donated from TrxA (Fig. 2B). Given this C80S mutant formed an intermolecular disulfide bond between two peroxidatic Cys-44 in vitro (Fig. 2A), the mutant protein may function as a homodimer that forms an atypical active site with two peroxidatic Cys residues from each of the subunits. A similar observation was reported for a mutant PrxQα from poplar (30). The poplar mutant PrxQ, containing a Cys to Ser mutation at the resolving Cys residue (C51S), showed strong peroxidase activity with donating electrons from Trx. This observation suggests that the resolving Cys is not essential for peroxidase activity in PrxQ family proteins but may be important for maximum enzymatic activity and to avoid overoxidation of the peroxidatic Cys residue during a restricted supply of reducing substrate such as Trx.

Spot assays indicated that under aerobic-light conditions RsPrxT mutant (TYPEII) and RsPrxT—RsPrxQ double mutant (PRXQII) had decreased cell viability (Fig. 4, + light). This phenomenon was not observed when cells were grown under aerobic-dark (Fig. 4, control) or anaerobic-light (Fig. 4, anaerobic) conditions. Given that the co-existence of light, oxygen, and bacteriochlorophyll potentially produces $\text{O}_2$ (15), the observed reduced cell viability in the mutants may be a result of increased cellular levels of $\text{O}_2$. A similar reduced cell viability in the mutant strains was observed when cells were treated with rose bengal, which produces $\text{O}_2$ (Fig. 4C), thus, supporting this idea. These results indicate that at least RsPrxT has an important role in protecting cells from damage not only from ROS produced from respiratory components but also from photooxidative stress in R. sphaeroides.

Prx mutant strains showed compensational overexpression of FeSOD and CCP (Fig. 6), suggesting that Prxs cooperate with

| Strain       | B800–850 | B875 |
|--------------|----------|------|
| Wild type    | 8.44     | 1.79 |
| PRXQ         | 1.01     | 0.42 |
| TYPEII       | 2.89     | 0.51 |
| PRXQII       | 0.60     | 0.19 |
such antioxidant factors. Specifically, on plates containing paraquat (a producer of O$_2^-$) or CDNB (an inducer of GSH depletion), all mutant strains exhibited cell viability comparable to that of wild type (Fig. 4). Under such conditions, FeSOD and CCP could compensate for the loss of Prx functions. On the other hand, on the plates containing r-BHP, CHP (inducers of lipid peroxidation), or SNP (a producer of NO$^-$), RsPrxT-disrupted strain (TYPEII) and RsPrxT-RsPrxQ double disruptant (PRXQII) showed significant and distinct reduction, respectively, of cell viability (Fig. 4). This indicates that the increased levels of FeSOD and CCP are not enough to completely compensate for the loss of function of Prxs under such conditions. It is of note that although the expression of CCP and FeSOD in PRXQII are lower and similar, respectively, than those of TYPEII (Fig. 6), the viability of PRXQII for various ROS/RNS is higher than that of TYPEII (Fig. 4A). This suggests that other antioxidant factors are up-regulated in PRXQII, although these are still unknown.

FeSOD and CCP of purple bacteria function in the cytoplasm and periplasm, respectively (20, 35, 36). Similar compensatory expression was reported in the yeast Saccharomyces cerevisiae such that yeast mutants lacking multiple Prxs exhibited over-expression of genes for other antioxidant systems, including SOD and glutathione peroxidase (37). The S. cerevisiae CCP and SOD homologs localize to the intermembrane and matrix space of mitochondria, respectively (36, 38, 39). R. sphaeroides is classified in the α3 subgroup of proteobacteria and is thought to be phylogenetically close to the ancestor of mitochondria (40). In fact, the components of the respiratory chains are very similar between this bacterium and mitochondria (11, 13). In general, generation of ROS is tightly coupled to the activity of each respiratory component, although additional sources may exist (2, 41). These facts suggest that mitochondrial Prxs may work in concert with other antioxidant enzymes, including CCP and SOD, as in R. sphaeroides, although the mechanisms are unknown. Further studies of R. sphaeroides Prxs may be useful to understand the antioxidant systems in not only prokaryotes but also eukaryotes.

The reasons why Prx-disrupted mutants show a slow growth phenotype as well as hypersensitivities to various ROS/RNS is open to speculation. Prx-disrupted strains may increase ROS concentration in cells, as Prxs are one of the main ROS quenchers. An increase of ROS induces DNA damage and lipid peroxidation as well as inactivation of enzymes through oxidation of heme, Fe$^{2+}$, and/or sulfhydryls (2, 41). In fact, Prx-lacking strains of other organisms are reported to trigger phenomena such as free iron accumulation, DNA damage, storage of lipid peroxide, and genome instability (37, 42). Furthermore, up-regulation of genes for protein folding, proteolysis, DNA repair, and Fe-S cluster repair were found in the microarray profiles of R. sphaeroides exposed to H$_2$O$_2$ (17). These facts suggest that loss of Prxs may cause protein inactivation, DNA damage, Fe-S cluster destruction, and lipid peroxidation, which may result in a slow-growth phenotype and reduced oxidative stress resistance in the Prx-disrupted strains.

The mutational loss of Prxs resulted in decreased amounts of photopigments (Fig. 7, A and B, and Table 2). Furthermore, even in wild type, the addition of ROS or RNS affected photopigmentation (Fig. 7C). These observations indicate that depression of photopigment synthesis probably results from increased levels of intracellular ROS/RNS concentration in R. sphaeroides. Proper control of ROS/RNS levels in the bacterium (perhaps, partly through the activity of Prxs) should be important for regulated expression of photosynthesis genes. It is of note that PRXQ showed decreased photopigments more than TYPEII (Fig. 7, A and B, and Table 2), although PRXQ viability against various ROS/RNS was higher than that of TYPEII (Fig. 4). This suggests that RsPrxT is severely required, more than RsPrxT, for proper control of photopigment synthesis. Indeed, several redox-sensing proteins have been identified that control photosynthetic gene expression in this bacterium, including the PrrA/B (RegA/B) two-component system, AppA-PpsR antirepressor-repressor system, and FnL transcription factor (14, 43). Perhaps some of these factors sense intracellular concentrations of specific or nonspecific ROS/RNS levels to repress photopigment synthesis in R. sphaeroides. To determine whether the Prx-dependent modulation mechanisms actually control photosynthetic gene expression, further experiments should be required. Such studies are in progress in authors’ laboratories.

In conclusion, Prxs in R. sphaeroides have high peroxidase activity and broad substrate specificity, and they play an important role in the antioxidant stress response with coordination by other antioxidant factors. Prxs are also suggested to be directly or indirectly involved in the modulation of photopigment synthesis in response to oxidative conditions.

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