A Novel Peroxiredoxin Activity Is Located within the C-Terminal End of *Rhodospirillum rubrum* Adenylyltransferase[∇]

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Adenylyltransferase (GlnE) catalyzes the reversible adenylylation of glutamine synthetase. In this report we present, for the first time, evidence for a peroxiredoxin activity of *Rhodospirillum rubrum* GlnE, through the carboxyl-terminal AhpC/thiol-specific antioxidant (TSA) domain. The combination of GlnE and AhpC/TSA domains within the same polypeptide constitutes a unique domain architecture that has not previously been identified among proteobacteria.

The photosynthetic purple free-living bacterium *Rhodospirillum rubrum* can use a number of nitrogen sources, including molecular dinitrogen, which is reduced to ammonium ions in the reaction catalyzed by the metalloenzyme complex nitrogenase (13). Ammonium ions are further assimilated into glutamate/glutamine, mainly through the glutamine synthetase (GS)-glutamate synthase pathway (10).

GS, one of the key enzymes in nitrogen assimilation, is strictly regulated in bacteria by feedback inhibition, transcriptional regulation, and/or covalent modification with AMP groups (1, 8, 14). Recently we have investigated the regulation of GS activity by the bifunctional enzyme adenylyltransferase (ATase, or GlnE), the product of the *glnE* gene in *R. rubrum*. We have shown that the adenylylation activity of GlnE is dependent on the presence of one of the signal transduction P_{II} proteins and also that this reaction is inhibited by 2-oxoglutarate (6). Interestingly, we have found that the domain architecture of *R. rubrum* GlnE is different from those of the previously reported GlnE proteins, as a BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST/>) indicates. A complementary search in Pfam (<http://pfam.sanger.ac.uk/>) reveals that *R. rubrum* GlnE contains, in addition to the adenylylation and deadenylylation domains, a novel C-terminal alkyl hydroperoxide reductase (AhpC)/thiol-specific antioxidant (TSA)-like domain that belongs to the peroxiredoxin family, a family of ubiquitous antioxidant enzymes (4, 16). The closest relative to this domain is found in the *Magnetospirillum magnetotacticum* bacterioferritin comigratory protein (BCP), with 68% sequence identity. This AhpC from *M. magnetotacticum*, however, is probably encoded by a separate gene, located downstream of *glnE*, although the biochemical evidence for this has not been reported (<http://genome.ornl.gov/microbial/mmag/>). Another homolog of the *R. rubrum* AhpC/TSA domain (designated Ahp below) is the *Escherichia coli* BCP, which has been shown to act as a general hydroperoxide peroxidase, with the cysteine residue at position 45 being required for catalytic

activity (5). One of the assays used to assess the activity of *E. coli* BCP (and other peroxiredoxins) is the ability to protect *E. coli* GS against oxidative inactivation in vitro (5). This inactivation is due to reactive oxygen species generated in vitro in the presence of a transition metal such as Fe³⁺, oxygen, and a reducing agent such as dithiothreitol (DTT) or ascorbate (12). It has been shown previously for *E. coli* that the loss in GS activity is due to in situ oxidation of His269 and Arg344, destroying the integrity of the n2 Mn-binding site (9).

To further investigate the role of the Ahp domain in *R. rubrum* adenylyltransferase, we have separately analyzed GlnE, a truncated GlnE lacking Ahp (GlnEΔAhp), and Ahp alone with respect to the ability to protect purified *R. rubrum* GS against oxidative inactivation. The protocols used for purification of GlnE, GlnEΔAhp, and GS have been described previously (6). The gene sequence encoding the Ahp domain corresponding to a truncated version of *glnE* lacking positions 1 to 2994 was obtained by PCR using *Pfx* polymerase with pETglnE (6) as a template. A BamHI site was introduced before the methionine codon at position 2994 of the *glnE* gene, which is in frame with the start codon of the native *glnE*. The PCR product was subcloned into the pCR-Blunt II-TOPO vector (Invitrogen) before ligation into the pGEX-6P-3 vector (GE Healthcare), giving pGEX-Ahp. The plasmid was transformed into *E. coli* BL21(DE3)pLysS, and the cells were grown in Luria-Bertani (LB) medium at 37°C to an optical density at 600 nm of 0.6. Protein expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside for 1 h. Cell breakage was performed as previously described (6), and purification was carried out according to the manufacturer's instructions (GE Healthcare). The glutathione S-transferase tag was removed by on-column cleavage with Precision protease (GE Healthcare). GS protection assays were carried out at 30°C in a reaction mixture (60 μl) containing 7.5 μM (or the indicated concentration) protector protein (GlnE, GlnEΔAhp, or Ahp), 33 nM GS (dodecamer), 10 mM Tris-HCl (pH 7.6), 3 μM FeCl₃, and 10 mM DTT or 10 mM ascorbate (as indicated). At the time points indicated, samples were withdrawn, and GS activity was measured by the transferase assay as previously described (3). Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were routinely grown aerobically in LB medium with the addition of antibiotics at the following final

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or relevant characteristics ^a	Reference or source
Strains		
<i>E. coli</i>		
TOP10	Host for pCR-Blunt II-TOPO derivatives	Invitrogen
BL21(DE3)pLysS	Host for overexpression of pGEX derivatives; Cm ^r	Invitrogen
<i>R. rubrum</i>		
S1	Wild type	17
UR 1660	$\Delta glnE::aacC1$ (opposite orientation); Gm ^r	
Plasmids		
pCR-Blunt II-TOPO	Subcloning vector; Km ^r	Invitrogen
pGEX-6P-3	GST-tagged (N-terminal) overexpression vector, Ap ^r	GE Healthcare
pGEX-GlnEΔAhp	pGEX-6P-3 derivative giving 3' deletion of <i>glnE</i>	6
pGEX-Ahp	pGEX-6P-3 derivative giving 5' deletion of <i>glnE</i> cloned into the BamHI site; Ap ^r	This report
pGEX-Ahp48	pGEX-Ahp derivative encoding recombinant Ahp(C48S); Ap ^r	This report
pGEX-Ahp102	pGEX-Ahp derivative encoding recombinant Ahp(C102S); Ap ^r	This report
pGEX-Ahpdouble	pGEX-Ahp derivative encoding recombinant Ahp(C48S C102S); Ap ^r	This report
pETGlnE	pET101 derivative containing <i>glnE</i> ; Ap ^r	6

^a GST, glutathione *S*-transferase; Cm^r, Gm^r, Km^r, and Ap^r, resistant to chloramphenicol, gentamicin, kanamycin, and ampicillin, respectively.

concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 34 μg/ml.

The results in Fig. 1 clearly show that *R. rubrum* GS is inactivated by the Fe³⁺/DTT system, and that this inactivation is prevented by GlnE, in a concentration-dependent fashion. While Ahp alone is able to retain the protective activity, the truncated GlnEΔAhp is not. As shown in Fig. 2, the protection activity of the *R. rubrum* Ahp domain is retained in the presence of ascorbate instead of DTT, indicating that the protec-

tion is not thiol specific. Taken together, the results in Fig. 1 and 2 clearly show that the recombinant *R. rubrum* GlnE exhibits a peroxiredoxin activity that is dependent on the presence of the C-terminal domain. This protein is, to our knowledge, the first reported GlnE with peroxiredoxin activity, and its domain architecture is unique among bacteria. To further characterize the peroxiredoxin activity of GlnE, we replaced the two cysteine residues in the Ahp domain with serine and analyzed the abilities of the single and double variants to pro-

FIG. 1. Effects on GS activity of exposure to Fe³⁺/DTT in the presence and absence of different GlnE variants. GS (33 nM) was incubated with FeCl₃ (3 μM) and DTT (10 mM), with additions as indicated. After 30 min, samples were taken for determination of GS activity. Results from three independent experiments are shown.

FIG. 2. Effects on GS activity of exposure to Fe^{3+} /ascorbate in the presence and absence of the Ahp domain and variants. GS (33 nM) was incubated with FeCl_3 (3 μM) and ascorbate (10 mM), with additions as indicated. After 30 min, samples were taken for determination of GS activity. Results from three independent experiments are shown.

protect GS. Appropriate primers with a 1-bp mismatch converting the codon for cysteine to that for serine were designed to generate C48S, C102S, and C48S C102S substitutions in Ahp by standard PCR-mediated site-directed mutagenesis using *Pfu* polymerase (Stratagene). For convenience in the comparison with other peroxiredoxins, the residues were numbered from Met998 in GlnE and correspond to C1046 and C1100 in the full-length GlnE protein. The template used was pGEX-Ahp, and the plasmids produced were named pGEX-Ahp48, pGEX-Ahp102, and pGEX-Ahpdouble. All constructs were verified by sequencing. The Ahp variants were purified as described above for Ahp, and their abilities to protect GS were analyzed.

The results depicted in Fig. 1 and 2 show that the change of

cysteine to serine at position 48 completely abolishes the protection activity, while the variant with a substitution at position 102 still retained some, although low, activity. The variant containing both substitutions showed no protection activity. The same pattern was observed whether DTT (Fig. 1) or ascorbate (Fig. 2) was used. Our results differ from those of the experiments reported for *E. coli* BCP (5), in which the protective effect was exclusively thiol dependent.

We have also analyzed the ability of the Ahp domain (and variants) to degrade H_2O_2 in the presence of DTT. The assays were carried out at room temperature in a reaction mixture (60 μl) containing 10 mM Tris-HCl (pH 7.6), 7.5 μM protector protein, 10 mM DTT, and 60 μM H_2O_2 . The remaining H_2O_2

FIG. 3. Peroxidase activities of the GlnE Ahp domain and variants. Ahp (7.5 μM) was incubated with 60 μM H_2O_2 and 10 mM DTT. After 5 min, the percentage of H_2O_2 consumption was calculated. Results from three independent experiments are shown.

was measured by the oxidation of xylenol orange, essentially according to reference 11. Figure 3 shows that the Ahp domain has the ability to degrade H_2O_2 and also that the peroxidase activity is abolished when Cys48 is replaced by serine but that the C102S variant is still active.

Taken together, the results from Fig. 1 and 2 would suggest that Cys48 is the catalytic site. This residue is the so-called peroxidatic cysteine, and the absence of a strong effect of the Cys102 substitution suggests that *R. rubrum* GlnE belongs to the 1-Cys peroxiredoxin class. In this class only the peroxidatic cysteine residue is conserved (16). In this context it is noteworthy that a yeast mitochondrial 1-Cys peroxiredoxin (yPrx1, belonging to the PrxIV group) was recently shown to protect GS in the presence of ascorbate, contradicting the idea of thiol-specific antioxidant protection (11). However, sequence analysis reveals that the characteristic residues H40 and R199 are not conserved between yPrx1 and the *R. rubrum* GlnE Ahp domain (data not shown). Due to the ascorbate-dependent activity, it is tempting to compare these two proteins, although they might not share the same structural features.

We have also tried to investigate the reason for the dramatic loss of GS activity observed in the presence of Fe^{3+} and DTT, and we detected two putative degradation products under these conditions. However, due to the low abundances of these products, the overall effect on activity seems to be negligible (data not shown). It has been shown for *E. coli* that the loss of activity is due partly to degradation of GS (7) but also to a subtle structural instability of the oxidized protein (2, 15), and it is likely that that is also the case for *R. rubrum* GS.

Previous studies performed with *E. coli* GlnE showed that this enzyme can, like GS, be degraded in vitro after oxidation with Fe^{3+} /DTT (7). We therefore investigated whether *R. rubrum* GlnE activity also was affected by the presence of Fe^{3+} /DTT. For this purpose, we incubated GlnE or GlnEΔAhp (0.7 μ M) with 3 μ M $FeCl_3$ and 10 mM DTT for 30 min. EDTA (final concentration, 200 μ M) was added to stop the reaction, and then the GS adenylation activities of the enzymes were analyzed as previously described (6). Under the conditions tested, the adenylation activities of both enzymes were unaffected (data not shown). This indicates that the Ahp domain is not required for autoprotection of GlnE.

We also attempted to study the role of the Ahp domain in vivo by measuring the effect of oxygen exposure on GS activity in both wild-type *R. rubrum* and a Δ glnE strain (17). However, no difference could be detected (data not shown), indicating that the Ahp activity alone is not essential for protection against radicals, most likely due to the existence of other protection mechanisms. Since the Ahp domain is part of GlnE, it could be suggested that it offers a specific protection mechanism for GS. However, the lack of a specific oxygen-sensitive phenotype for the Δ glnE mutant suggests that the Ahp domain in GlnE is probably only one among several radical protection mechanisms, e.g., glutathione peroxidase, other peroxiredoxins, catalase, and superoxide dismutase. Genes for all these enzymes have been identified in the *R. rubrum* genome (<http://genome.ornl.gov/microbial/rub/>).

In conclusion, we provide, for the first time, evidence for a third activity of an adenylyltransferase, i.e., protection against oxidative radicals, in addition to the adenylation/deadenylation activities. The discovery of this unique combination of GlnE and Ahp domains in a single protein can open new lines of research on the interplay between nitrogen metabolism and cellular redox control in proteobacteria.

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REFERENCES

- Eisenberg, D., H. S. Gill, G. M. Pfluegl, and S. H. Rotstein. 2000. Structure-function relationships of glutamine synthetases. *Biochim. Biophys. Acta* **1477**:122–145.
- Fisher, M. T., and E. R. Stadtman. 1992. Oxidative modification of *Escherichia coli* glutamine synthetase. Decreases in the thermodynamic stability of protein structure and specific changes in the active site conformation. *J. Biol. Chem.* **267**:1872–1880.
- Hammarström, A., A. Soliman, and S. Nordlund. 1991. Low- and high-activity forms of glutamine synthetase from *Rhodospirillum rubrum*: sensitivity to feed-back effectors and activation of the low-activity form. *Biochim. Biophys. Acta* **1080**:259–263.
- Hofmann, B., H. J. Hecht, and L. Flohe. 2002. Peroxiredoxins. *Biol. Chem.* **383**:347–364.
- Jeong, W., M. K. Cha, and I. H. Kim. 2000. Thioredoxin-dependent hydroperoxide peroxidase activity of bacterioferritin comigratory protein (BCP) as a new member of the thiol-specific antioxidant protein (TSA)/alkyl hydroperoxide peroxidase C (AhpC) family. *J. Biol. Chem.* **275**:2924–2930.
- Jonsson, A., P. F. Teixeira, and S. Nordlund. 2007. The activity of adenylyltransferase in *Rhodospirillum rubrum* is only affected by α -ketoglutarate and unmodified PII proteins, but not by glutamine, in vitro. *FEBS J.* **274**:2449–2460.
- Kim, K., S. G. Rhee, and E. R. Stadtman. 1985. Nonenzymatic cleavage of proteins by reactive oxygen species generated by dithiothreitol and iron. *J. Biol. Chem.* **260**:15394–15397.
- Kingdon, H. S., and E. R. Stadtman. 1967. Two *E. coli* glutamine synthetases with different sensitivities to feedback effectors. *Biochem. Biophys. Res. Commun.* **27**:470–473.
- Liaw, S. H., J. J. Villafranca, and D. Eisenberg. 1993. A model for oxidative modification of glutamine synthetase, based on crystal structures of mutant H269N and the oxidized enzyme. *Biochemistry* **32**:7999–8003.
- Merrick, M. J., and R. A. Edwards. 1995. Nitrogen control in bacteria. *Microbiol. Rev.* **59**:604–622.
- Monteiro, G., B. B. Horta, D. C. Pimenta, O. Augusto, and L. E. Netto. 2007. Reduction of 1-Cys peroxiredoxins by ascorbate changes the thiol-specific antioxidant paradigm, revealing another function of vitamin C. *Proc. Natl. Acad. Sci. USA* **104**:4886–4891.
- Netto, L. E., and E. R. Stadtman. 1996. The iron-catalyzed oxidation of dithiothreitol is a biphasic process: hydrogen peroxide is involved in the initiation of a free radical chain of reactions. *Arch. Biochem. Biophys.* **333**:233–242.
- Nordlund, S., and P. W. Ludden. 2004. Post-translational regulation of nitrogenase in photosynthetic bacteria, p. 175–196. In W. Klipp, B. Masephol, J. R. Gallon, and W. E. Newton (ed.), *Genetics and regulation of nitrogen fixation in free-living bacteria*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Rhee, S. G., P. B. Chock, and E. R. Stadtman. 1989. Regulation of *Escherichia coli* glutamine synthetase. *Adv. Enzymol. Relat. Areas Mol. Biol.* **62**:37–92.
- Rivett, A. J., and R. L. Levine. 1990. Metal-catalyzed oxidation of *Escherichia coli* glutamine synthetase: correlation of structural and functional changes. *Arch. Biochem. Biophys.* **278**:26–34.
- Wood, Z. A., E. Schroder, J. Robin Harris, and L. B. Poole. 2003. Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem. Sci.* **28**:32–40.
- Zhang, Y., E. L. Pohlmann, M. C. Conrad, and G. P. Roberts. 2006. The poor growth of *Rhodospirillum rubrum* mutants lacking PII proteins is due to an excess of glutamine synthetase activity. *Mol. Microbiol.* **61**:497–510.