Reactivity of Glutaredoxins 1, 2, and 3 from Escherichia coli Shows That Glutaredoxin 2 Is the Primary Hydrogen Donor to ArsC-catalyzed Arsenate Reduction

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In Escherichia coli ArsC catalyzes the reduction of ars enate to arsenite using GSH with glutaredoxin as electron donors. E. coli has three glutaredoxins: 1, 2, and 3, each with a classical Cys-Pro-Tyr-Cys active site. Glutaredoxin 2 is the major glutathione disulfide oxidoreductase in E. coli, but its function remains unknown. In this report glutaredoxin 2 is shown to be the most effective hydrogen donor for the reduction of arsenate by ArsC. Analysis of single or double cysteine-to-serine substitutions in the active site of the three glutaredoxins indicated that only the N-terminal cysteine residue is essential for activity. This suggests that, during the catalytic cycle, ArsC forms a mixed disulfide with GSH before being reduced by glutaredoxin to regenerate the active ArsC reductase.

Glutaredoxins (Grx) are glutathione-dependent dithiol hydrogen donors for Escherichia coli enzymes such as ribonucleotide reductase, 3'-phosphodeoxyribonuclease, and ars enate reductase. The product of the grxA gene, glutaredoxin 1 (Grx1), was originally identified as a hydrogen donor to ribonucleotide reductase (1). In searching for alternate reductants of ribonucleotide reductase, two new glutaredoxins, Grx2 and Grx3, were identified, both of which catalyze GSH-disulfide reduction of 2-hydroxyethylidisulfide (HED) in a coupled system with NADPH and glutathione reductase (2). Grx2 was shown to be the predominant glutaredoxin in E. coli cells, with an intracellular concentration of 5 µM compared with 0.2 µM for Grx1 and 2.4 µM for Grx3. Grx2 also has a higher turnover number than the other two glutaredoxins for reduction of a mixed disulfide of GSH and HED. As a consequence of its high concentration and turnover, Grx2 provides over 80% of the GSH-oxidoreductase activity in E. coli for reduction of GSH mixed disulfides. However, Grx2 is not a hydrogen donor to ribonucleotide reductase, and no function has yet been assigned for this protein (3).

Resis tance to arsenicals and antimonials in E. coli is conferred by arsenic resistance (ars) operons (4). The arsC gene product of the ars operon of plasmid R773 is an ars enate reductase (5). ArsC reduces arsenate to arsenite, which is subsequently extruded from the cell, conferring resistance. In vitro reductase activity requires both GSH and Grx1 (6). Thioredoxin is not able to couple to the R773 ars enate reductase. In contrast, the unrelated ars enate reductase of Staphylococcus aureus plasmid p2585 requires thioredoxin and cannot use GSH and glutaredoxin (7).

In this study, we examined the relative efficiency of Grx1, Grx2, and Grx3 to serve as hydrogen donor for the reduction of arsenate by ArsC. Each glutaredoxin supported ars enate formation, with the relative efficiencies being Grx2 > Grx3 > Grx1. This is the first demonstration of a role of Grx2 in a physiological reaction. Glutaredoxins have two active site cysteine residues in the sequence Cys-Pro-Tyr-Cys. The N-terminal cysteine has been shown to be required for both protein disulfide reduction and reduction of mixed protein-glutathione disulfides (8). The other cysteine residue is required for the former but not for the latter. Here the codon for either of the two cysteine residues in grxA, grxB, and grxC was mutated to a serine codon, and the effect on ars enate reductase activity examined with the altered Grx1, Grx2, and Grx3. Mutation of the codon for the C-terminal active site cysteine of any of the three glutaredoxins had no effect on ArsC activity. In contrast, Grx1, Grx2, or Grx3 with a cysteine to serine substitution in the N-terminal residue were unable to serve as hydrogen donors to ArsC-catalyzed ars enate reduction. The results are consistent with a reaction cycle in which ArsC forms a mixed disulfide with glutathione, where the role of glutaredoxin would be to reduce the mixed disulfide, regenerating reduced ars enate reductase.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Media—Strains and plasmids used in this study are described in Table I. Cells were grown in Luria-Bertani medium (9) at 37 °C supplemented when necessary with 20 µg/ml chloramphenicol, 10 µg/ml tetracycline, or 50 µg/ml kanamycin.

DNA Manipulations—All restriction enzymes and nucleic acid modifying enzymes were obtained from Life Technologies, Inc. Plasmid isolation, DNA restriction endonuclease analysis, ligation, and transformation were performed as described previously (9, 10) The Wizard™ Plus miniprep DNA purification system and Wizard™ DNA clean-up system from Promega were used to prepare plasmid DNA for restriction enzyme digestion and recovering DNA fragment from low melting agarose gels, respectively.

Cloning of grxC—The grxC gene from E. coli strain JM109 was amplified by PCR to introduce a Ndel site at the 5' end and EcoRI site at the 3' end. The forward primer was 5' -CATATGGCATGGAAATCTATACC-3', which introduced a Ndel site at the 5'-end of the fragment. The reverse primer was 5'-GAAATCTATATTTGCAGGG-GATCCATCC-3'. A 30-cycle PCR reaction (94 °C for 1 min, 55 °C for 0.5 min, and 72 °C for 1 min) was run with DNA from E. coli strain JM109. The amplified product was cloned into pGEM-T vector and sequenced (11) for GSSG and HED. The other cysteine residue is required for the former but not for the latter. Here the codon for either of the two cysteine residues in grxA, grxB, and grxC was mutated to a serine codon, and the effect on ars enate reductase activity examined with the altered Grx1, Grx2, and Grx3. Mutation of the codon for the C-terminal active site cysteine of any of the three glutaredoxins had no effect on ArsC activity. In contrast, Grx1, Grx2, or Grx3 with a cysteine to serine substitution in the N-terminal residue were unable to serve as hydrogen donors to ArsC-catalyzed ars enate reduction. The results are consistent with a reaction cycle in which ArsC forms a mixed disulfide with glutathione, where the role of glutaredoxin would be to reduce the mixed disulfide, regenerating reduced ars enate reductase.

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sequenced, then subcloned into pALTEr-Ex2 and pET28a for mutagenesis or expression of Grx3, respectively.

**Oligonucleotide-directed Mutagenesis—Mutations in grxA, grxB, and grxC were introduced by site-directed mutagenesis using the Altered Sites™ system (Promega). Plasmid pALTER-Grx1, pALTER-Grx2, and pALTER-Grx3 containing the grxA, grxB, and grxC genes were used as the template to obtain mutants (Table I). The mutagenic oligonucleotides used were as follows: grxA C11S, GGGTTAGCCCTTAC; grxB C12S, GCCCTTACAGTGTGCGT; and grxC C15S, ACCAAAGAAACCAGCCCGT.**

**Construction of Vectors for the Overexpression of Grx2—**The grxB gene was amplified using primers containing NdeI and HinI sites of pET28a and cloned into the pET28a vector. The amplified fragment was used as a template for a second PCR reaction using primers G2-F and G2-RC to obtain mutants (Table I). The amplified fragment was subcloned into pALTEr-Ex2 and pET28a for mutagenesis or expression of Grx3, respectively.

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Kinetic constants for glutaredoxin as hydrogen donor for reduction of arsenate by ArsC

| Glutaredoxin | $K_m$ | $V_{max}$ | $k_{cat}$ | $k_{cat}/K_m$ |
|--------------|------|---------|--------|-------------|
| Grx1         | 4.2  | 1118    | 0.3    | 0.071 x 10^6 |
| Grx2         | 0.003 | 514     | 0.14   | 46.7 x 10^6  |
| Grx3         | 0.3  | 339     | 0.09   | 3.0 x 10^6   |

$K_m$ for glutaredoxin at 6.3 µM ArsC and 10 mM sodium arsenate.

$V_{max}$ was calculated per milligram of ArsC.

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Glutaredoxin 2 and Arsenate Reduction

RESULTS AND DISCUSSION

Purification of Glutaredoxins—To allow for rapid purification of recombinant glutaredoxins, the sequence for 20 codons was added to the 5'-end of each glutaredoxin gene, including the sequence for a thrombin recognition site and six histidine codons, adding approximately 2 kDa to the molecular mass of each glutaredoxin species. Each protein was purified by chelate affinity chromatography. In saturating amounts, the three His-tagged glutaredoxins reduced arsenate in rates identical to those of their wild type counterparts lacking the six histidine tag (data not shown). Because of the ease of purification, all subsequent assays were performed with the His-tagged glutaredoxins.

Grx2 Is the Major Hydrogen Donor for Arsenate Reduction—Each of the three glutaredoxins was able to serve as a hydrogen donor to ArsC-catalyzed arsenate reduction (Fig. 1). At saturating concentrations, the turnover numbers ($k_{cat}$) of the three proteins were within a factor of two to three of each other (Table II). In contrast, the $K_m$ of ArsC for the three glutaredoxins differed by 3 orders of magnitude. ArsC exhibited a 100-fold lower $K_m$ for Grx2 than Grx3 and more than 1,000-fold lower when compared with Grx1. Thus, the catalytic efficiency ($k_{cat}/K_m$) for Grx2 is approximately 2 orders of magnitude greater than that for Grx3 and approximately 3 orders of magnitude greater than that for Grx1. These results suggest that Grx2 is most likely the major hydrogen donor for the reduction of arsenate to arsenite, the first credible physiological role found for Grx2. Since it is the major GSH-disulfide oxidoreductase in E. coli, the identification of an electron acceptor is important for understanding the function of Grx2.

It should be pointed out, however, that disruption of the gene for any of the three glutaredoxins does not eliminate arsenate resistance conferred by the $ars$ operon (data not shown). This is not surprising in light of the redundancy of glutaredoxins and glutaredoxin-like proteins (16) and suggests the presence of an additional yet unidentified glutaredoxin activity. Similarly, in vivo reduction of ribonucleotides by ribonucleotide reductase is not affected by single or multiple disruptions of the trxA or grxA genes. However, cells lacking both Trx1 and Grx1 have highly induced ribonucleotide reductase (17). Later work resulted in the isolation of a second thioredoxin (Trx2) encoded by the trxC gene (18, 19). On the other hand, 3'-phosphoadenylsulfate reduction is more specifically coupled to Trx1, Trx2 or Grx1 (20), and a double disruption of the trxA and grxA genes resulted in cysteine auxotrophy (21).

Role of Glutaredoxin in Arsenate Reduction—Glutaredoxins can reduce either intramolecular disulfides (e.g. ribonucleotide reductase) or mixed disulfides between a thiol compound and GSH (e.g. the complex between HED and GSH). Grx1, Grx2, and Grx3 each have the conserved active site sequence Cys-Pro-Tyr-Cys. Both cysteine residues are required for protein disulfide reduction (8). For reducing glutathione-containing mixed disulfides, however, the N-terminal cysteine is sufficient (8, 22). In the monothiol mechanism, the N-terminal cysteine of glutaredoxin reacts with the glutathionyl moiety of the mixed disulfide, forming the mixed disulfide intermediate GrxS-SG. Simultaneously, the non-glutathione component is released in its reduced form. The GrxS-SG can be further reduced by GSH to give reduced glutaredoxin and GSSG.

To determine which glutaredoxin mode was involved in ArsC-catalyzed arsenate reduction, mutant glutaredoxin genes encoding single and double cysteine-to-serine substitutions of
the three E. coli glutaredoxins were constructed. Glutaredoxins with their N-terminal cysteine changed to serine (Grx1 C11S, Grx2 C9S, or Grx3 C12S) were unable to serve as hydrogen donors for the reduction of arsenate (Fig. 2). The three double substitutions (Grx1 C11/14S, Grx2 C9/12S, or Grx3 C12/15S) exhibited the same phenotype as the single substitutions. In contrast, glutaredoxins with intact N-terminal cysteines but serine substitutions in the C-terminal cysteines (Grx1 C14S, Grx2 C9S, or Grx3 C12S) retained nearly complete wild type activity. Thus, for ArsC catalysis, Cys11 for Grx1, Cys9 for Grx2, C12S, or Grx3 C15S) retained nearly complete wild type activity. These results are consistent with formation of a mixed protein-SG disulfide during the reaction cycle, as indicated in the following proposed reaction scheme (where ES\(^-\) represents the Cys\(^{12}\) thiolate of ArsC).

\[
\begin{align*}
ES^+ + As(V) & \rightleftharpoons ES^- \cdot As(V) \quad \text{(Reaction 1)} \\
ES^- \cdot As(V) + GS^- & \rightleftharpoons ES-SG + As(III) \quad \text{(Reaction 2)} \\
ES-SG + Grx2-S & \rightleftharpoons ES^- + GS-SGrx2 \quad \text{(Reaction 3)} \\
GS-SGrx2 + GS & \rightleftharpoons Grx2S^+ + GSSG \quad \text{(Reaction 4)} \\
GSSG + NADPH + H^+ & \rightleftharpoons 2 \text{ GSH} + NADP^+ \quad \text{(Reaction 5)}
\end{align*}
\]

In this proposed minimal reaction scheme, Reaction 1 shows noncovalent binding of arsenate to ArsC. Since ArsC is competitively inhibited by other tetrahedral oxyanions such as phosphate and sulfate (6), the first step represents binding at an oxyanion binding site. In Reaction 2, reduction of As(V) to As(III) is achieved by one electron donated by the thiolate of Cys\(^{12}\) of ArsC and another electron derived from the thiolate of Grx2. Thus, Reaction 2 is likely to be the sum of several steps, one possibly involving a protein thyl radical. In Reaction 3 Grx2 is indicated as the hydrogen donor for reduction of the Grx2-SG mixed disulfide. This is followed by regeneration of reduced Grx2 by GSH, forming GSSG in reaction (4). The GSSG will finally be reduced to 2 mol of GSH by NADPH and glutathione reductase in Reaction 5.

Since Grx2 has a catalytic efficiency that is 1 or 2 orders of magnitude greater than Grx3 or Grx1, what governs the glutaredoxin specificity of arsenate reduction? Since the three E. coli glutaredoxins have similar activities in an assay using reduction of the mixed disulfide between 2-hydroxyethylidisulfide and GSH (23), it is reasonable to speculate that specific protein-protein interactions between ArsC and each of the three glutaredoxins are involved, and the efficiency of those interactions determines the rate of reaction (3). Thus, the role of Grx2 in the ArsC reduction system is the first example of a monothiol mechanism in a substrate reduction involving electron transport by a glutaredoxin.

REFERENCES

1. Holmgren, A. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 2275–2279
2. Åslund, F., Ehn, B., Miranda-Vizuete, A., Pueyo, C., and Holmgren, A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9815–9817
3. Vlaminck-Gardikas, A., Åslund, F., Spyrou, G., Bergman, T., and Holmgren, A. (1997) J. Biol. Chem. 272, 11236–11243
4. Oden, B. P. (1999) Trends Microbiol. 7, 207–212
5. Åslund, F., Ehn, L., and Rosen, B. P. (1994) Mol. Microbiol. 12, 301–306
6. Gladysheva, T. B., Oden, K. L., and Rosen, B. P. (1994) Biochemistry 33, 7288–7293
7. Bushweller, J. H., Åslund, F., Wuthrich, K., and Holmgren, A. (1992) Biochemistry 31, 9288–9293
8. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
9. Chung, C. T., Niemela, S. L., and Miller, R. H. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2172–2175
10. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
11. Liu, J., and Rosen, B. P. (1997) J. Biol. Chem. 272, 21084–21089
12. Laemmli, U. K. (1970) Nature 227, 680–685
13. Gill, S. C., and von Hippel, P. H. (1989) Anal. Biochem. 182, 319–326
14. Jordan, A., Åslund, F., Pontis, E., Reichard, P., and Holmgren, A. (1991) Protein Expression Purif. 2, 287–295
15. Bjornberg, O., and Holmgren, A. (1991) J. Biol. Chem. 266, 18044–18050
16. Miranda-Vizuete, A., Martinez-Galisteo, E., Åslund, F., Lopez-Barea, J., Pueyo, C., and Holmgren, A. (1994) J. Biol. Chem. 269, 16631–16637
17. Miranda-Vizuete, A., Damdimopoulos, A. E., Gustafsson, J., and Spyrou, G. (1997) J. Biol. Chem. 272, 20841–20847
18. Stewart, E. J., Åslund, F., and Beckwith, J. (1998) EMBO J. 17, 5543–5550
19. Green, C. H., Prior, A., Schwenn, J. D., Åslund, F., Ritz, D., Vlamis-Gardikas, A., and Holmgren, A. (1999) J. Biol. Chem. 274, 7685–7688
20. Russel, M., Model, P., and Holmgren, A. (1990) J. Bacteriol. 172, 1923–1929
21. Grassino, C., and Mieyal, J. J. (1993) J. Biol. Chem. 268, 16631–16637
22. Holmgren, A., and Åslund, F. (1995) Methods Enzymol. 252, 283–292