ArsC, an arsenate reductase encoded by the Escherichia coli R-factor R773, provides arsenate resistance by reducing As(V) (arsenate) to As(III) (arsenite) (1), which is then rapidly excreted by the cell. Reduced glutathione (GSH) and glutaredoxin 1 (Grx1) 1 (3) are required for reduction of arsenate to arsenite. The enzyme mechanism of this novel reductase.

ArsC encoded by Escherichia coli plasmid R773 catalyzes the reduction of arsenate to arsenite. The enzymatic reaction requires reduced glutathione and glutaredoxin. In this study a direct association between ArsC and glutaredoxin was demonstrated. An arsC gene with six histidine codons added to the 5' end of the gene was constructed, and the resulting ArsC enzyme was shown to be functional. Interaction of the histidine-tagged ArsC and glutaredoxin was examined by Ni²⁺ affinity chromatography. The association required the presence of reduced glutathione and the substrate arsenate or a competitive inhibitor, phosphate or sulfate. A free thiolate on glutathione was not required. A tryptophan residue was introduced into ArsC at the 11th position, immediately adjacent to the active site Cys-12. Trp-11 fluorescence was quenched upon addition of arsenate. Addition of reduced glutathione after arsenate resulted in a rapid increase in fluorescence followed by a slower decay of the signal. These spectroscopic signals were specific for arsenate and reduced glutathione; neither competitive inhibitors nor non-thiol glutathione analogs produced this effect. Cys-12 thiolate was also required. Thus the intrinsic fluorescence of Trp-11 provides a useful probe to investigate the mechanism of this novel reductase.

The role of GSH in the ArsC-catalyzed reaction was investigated. In E. coli GSH exists predominantly in the reduced form in millimolar concentrations (6, 8), functioning as a redox buffer against oxidants and xenobiotics (8). The question arises as to whether GSH participates directly in the ArsC reaction or is required only for reduction of oxidized Grx1. Native ArsC has no tryptophan residues. To examine this question a tryptophan codon was introduced into arsC, encoding an A11W ArsC. Trp-11 is adjacent to Cys-12, an essential residue postulated to transfer electrons from ArsC to arsenate (9). The intrinsic fluorescence of Trp-11 was used as a fluorescence reporter of ligand interactions. Arsenate quenched the fluorescence of Trp-11. GSH alone had no effect, but adding GSH after arsenate produced a rapid increase in fluorescence which decayed slowly. These results suggest that the first step in the reaction is the binding of arsenate, followed by the interaction of the enzyme-arsenate complex with GSH. A reaction scheme is hypothesized in which the enzyme forms a mixed disulfide between the Cys-12 thiolate of ArsC and GSH. Grx1 would then be required to resolve the mixed disulfide, regenerating reduced ArsC.

**Experimental Procedures**

**Bacterial Strains, Plasmids, and Media**—Strains and plasmids used in this study are given in Table I. For protein production, cells were grown at 37 °C with shaking in Luria-Bertani medium (10). For phenotypic measurements of arsenate resistance, E. coli strain AW10 (Δars:cam) (11) carrying the indicated plasmids were grown in a low phosphate medium containing 3 mM sodium arsenate (1). In this strain the chromosomal ars operon was disrupted and is hypersensitive to arsenate. Turbidity at 600 nm was measured after 8–12 h of growth at 37 °C with shaking. Appropriate antibiotics were added as required.

**Materials**—All restriction enzymes and nucleic acid-modifying enzymes were obtained from Life Technologies, Inc. The Altered Sites™ in vitro Mutagenesis System was purchased from Promega. Oligonucleotides were synthesized in the Macromolecular Core Facility of Wayne State University School of Medicine. T4 glutaredoxin, thioredoxin reductase, and anti-Grx1 serum were generous gifts from Dr. Fredrik Åslund, Medical Nobel Institute for Biochemistry, Sweden. All other chemicals were obtained from commercial sources.

**DNA Manipulation**—Plasmid isolation, DNA restriction endonuclease analysis, ligation, and transformation were performed as described (10, 12). Mutations in arsC were constructed by site-directed mutagenesis, as described previously (9). Mutagenic oligonucleotides were designed to change the codons for Ala-11 (GCC) to Trp (TGG), Cys-12 (TCG) to Ser (AGC), and Tyr-7 (TAT) to Trp (TGG). Either the wild type arsC or arsC<sub>A11W</sub> gene inserted into the multiple cloning site of pAL-

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1. The abbreviations used are: Grx1, glutaredoxin 1; NTA, nitrilotriacetic acid; MOPS, 4-morpholinepropanesulfonic acid.
2. J. Liu and B. P. Rosen, unpublished data.
terTM1 vector (Promega) was used as a template (9). The identity of the mutation was confirmed by DNA sequencing of the entire mutant gene. DNA was prepared using a Qiagen DNA purification system. Sequencing was performed using the internal DNA labeling (Cy5TM-dATP labeling mix) with the T7 DNA polymerase Cy5TM AutoRead™ Sequencing kit from an ALF-Express DNA Sequencing System (Pharmacia Biotech Inc.).

**Construction of the Gene for His6ArsC**—An ArsC with six histidine codons at the 5′ end was constructed by polymerase chain reaction (PCR) using primers that contained the restriction enzymes NdeI and HindIII and cloned into NdeI/HindIII-digested vector plasmid pET28a (Novagen). The construction was confirmed by restriction enzyme digestion and was transformed into E. coli strain BL21 (DE3) with selection for kanamycin resistance. An arsC encoding His6C12S was constructed similarly.

**Protein Purification**—Wild type and substituted ArsCs and Grx1 were purified as described previously (3). For purification of His6ArsC, E. coli strain BL21 (DE3) bearing the gene for the His6ArsC was grown in LB medium. At an OD<sub>660</sub> of 0.5–0.6, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.4 mM as inducer, and the culture was shaken for an additional 3 h at 37 °C. The cells were washed once with buffer A (20 mM Tris-HCl, pH 7.5, containing 5 mM imidazole) and lysed by freezing/thawing. The lysate was centrifuged at 70,000 × g for 60 min at 4 °C, and the clear supernatant solution was loaded at a flow rate of 1 ml/min onto a 5-ml Ni<sup>2+</sup>-NTA column preequilibrated with 30 mM imidazole in buffer A. Grx1-containing fractions were identified by SDS-polyacrylamide gel electrophoresis (13), pooled, and concentrated using a Millipore Ultrafree-15 BIOMAX-5K centrifugal filter (Millipore) at 2,000 × g. Purified ArsC was stored at -80 °C. The concentrations of ArsC enzymes containing Trp-7 and Trp-11 were determined from the absorbance at 210 nm using a molar extinction coefficient of 7,953 and 9,150, respectively (14). The concentrations of other proteins were determined either with a micromodification of the method of Lowry et al. (15) or using a Coomassie protein assay (Bio-Rad). The reductase assay for ArsC was performed as described previously (3). When T4 glutaredoxin was used in place of T4 glutaredoxin, the reaction contained 25 mM MOPS-NaOH, pH 6.6, 0.2 mM NADPH, 30 μM each of thioredoxin reductase and T4 glutaredoxin, 10 μg of ArsC, and 20 mM sodium ascorbate. GSH was added as indicated.

**Interaction of His6ArsC and Grx1**—Purified His6ArsC was dialyzed against buffer A adjusted to pH 6.6. Ni<sup>2+</sup>-NTA spin columns (Qiagen) of 0.1 ml of equilibrated with the same buffer were loaded with 0.1 mg of His6ArsC protein in a volume of 0.1 ml. The column was centrifuged at 700 × g for 2 min. Purified Grx1 (50 μg) in 0.1 ml was allowed to soak into the resin, and the column was centrifuged again. The column was then washed with 0.6 ml of the buffer A containing 10 mM imidazole to remove nonspecifically bound protein. Finally 0.1 ml of buffer A containing 0.5 mM imidazole, pH 8.0, was applied to the column to elute bound His6ArsC. The proteins in the eluates were separated by SDS-polyacrylamide gel electrophoresis on 16% acrylamide gels and analyzed by staining with Coomassie Blue. In the parallel assays His6ArsC was preincubated for 5 min at 37 °C with 40 mM sodium ascorbate, 40 mM sodium phosphate, 40 mM sodium sulfate or 10 mM sodium arsenite. Grx1 was preincubated for 5 min at 37 °C with 30 mM GSH or its non-thiol derivatives, S-methylglutathione or S-hexylglutathione, as indicated.

**Measurements of Fluorescence**—Fluorescence measurements were performed using an SLM 8000 spectrofluorometer using an excitation wavelength of 295 nm. The slit widths for excitation and emission were 4 mm. Reagents were added with a microliter syringe through a light-protected port to the cuvette containing 2-ml samples, and the solutions were stirred continuously during the measurement. Dilution effects were negligible. Tryptophan fluorescence intensity changes were recorded at 350 nm for A11W and A11W/C12S, 354 nm for free tryptophan, or 330 nm for Y7W. All spectra were corrected for background and Raman scattering by subtracting the buffer signal.

**RESULTS**

**Properties of His6ArsC**—Histidine-tagged ArsC was constructed to contain an additional 20 residues at the amino terminus of the protein, including six histidines and a threonin recognition site. The modified enzyme is approximately 2 kDa larger than the wild type. It exhibited a wild type rate of arsenate reductase activity, with approximately the same 〈<sub>Km</sub> for arsenate. Table I lists the properties of His6ArsC and Grx1, as well as the interactions between these two proteins.

**TABLE I**

| Strains/plasmid | Genotype and phenotype | Source or reference |
|-----------------|------------------------|---------------------|
| JM109           | recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δlac-proAB F' [traD36 proAB<sup>+</sup> lacI prophage Δ2185] | 10                  |
| AW10            |                         |                     |
| pET28a          | Cloning and expression vector for construction of His-tag labeled protein, Km<sup>+</sup> | Novagen             |
| pET-HisArsC     | PCR fragment containing whole arsC gene cloned into pET28a | This study          |
| pAlterArsC      | 1.6-kilobase HindIII-BamHI fragment containing arsC gene cloned into pAlter-1 vector (Promega) | 9                   |
| pAlt/ARS        | arsAB gene cloned and Km<sup>+</sup> cloned into pACYC184 vector | This study          |
| pAlterA11W      | 1.6-kilobase HindIII-BamHI fragment containing arsC<sub>Alt</sub> gene cloned into pAlter-1 vector | This study          |
| pAlterY7W       | 1.6-kilobase HindIII-BamHI fragment containing arsC<sub>Y7W</sub> gene cloned into pAlter-1 vector | This study          |
| pAlterA11W/C12S | 1.6 kilobase HindIII-BamHI fragment containing arsC<sub>Alt</sub><sub>C12S</sub> gene cloned into pAlter-1 vector | This study          |

**FIG. 1. Interaction of His6ArsC and Grx1.** Panel A, eluates after imidazole elution. His6ArsC (0.1 mg) was bound to Ni<sup>2+</sup>-NTA columns in buffer A followed by the addition of 50 μg of Grx1, 30 mM GSH or an excess of 40 mM sodium sulfite of oxiynsan, as indicated. The columns were washed extensively with buffer A, followed by elution with 0.1 ml of 0.5 mM imidazole in buffer A, as described under “Experimental Procedures.” Portions (10 μl) were analyzed by SDS-polyacrylamide gel electrophoresis. Lane 1, 5 μg of purified His6ArsC standard; lane 2, 1 μg of Grx1 standard; lane 3, His6ArsC + Grx1; lane 4, His6ArsC + Grx1 + GSH; lane 5, His6ArsC + Grx1 + sodium arsenite; lane 6, His6ArsC + Grx1 + sodium arsenate + GSH; lane 7, His6ArsC + Grx1 + sodium phosphate; lane 8, His6ArsC + Grx1 + sodium phosphate + GSH. Panel B, conditions and concentrations were the same as in panel A. In each lane 10 μl of the indicated fraction was analyzed by SDS-polyacrylamide gel electrophoresis. Lanes 1, 4, 7, 10, and 13, flow-through after loading of all reagents; lanes 2, 5, 8, 11, and 14, buffer wash; lanes 3, 6, 9, 12, and 15, eluates after imidazole wash. Lanes 1–3, His6ArsC + Grx1 + sodium arsenate + S-methylglutathione; lanes 4–6, His6ArsC + Grx1 + sodium arsenate + S-hexylglutathione; lanes 7–9, His6ArsC<sub>C12S</sub> + Grx1 + sodium arsenate + GSH; lanes 10–12, His6ArsC + Grx1 + sodium sulfate + GSH; lanes 13–15, His6ArsC + Grx1 + sodium arsenite + GSH.
and $V_{\text{max}}$ (data not shown). Cells expressing the mutated gene on a plasmid in trans with the arsAB genes showed normal levels of arsenate resistance (data not shown).

Interaction of His6ArsC and Grx1—Approximately 0.3 mM imidazole was required to displace His6ArsC from a Ni$^{2+}$-NTA affinity column (data not shown). When Grx1 was loaded onto the column after binding of His6ArsC, none of the Grx1 was retained (Fig. 1A, lane 3), indicating that there was no interaction between the two proteins. If Grx1 was preincubated with 30 mM GSH before loading onto a column with bound His6ArsC, no binding of Grx1 was observed (Fig. 1A, lane 4). Similarly, if His6ArsC was preincubated with 40 mM sodium arsenate before loading on the column, no binding of Grx1 was observed (Fig. 1A, lane 5). However, when His6ArsC was preincubated with sodium arsenate and Grx1 preincubated with GSH, Grx1 coeluted with His6ArsC (Fig. 1A, lane 6). Thus both arsenate and GSH were required for protein-protein interaction. Immunoblotting with anti-Grx1 serum confirmed that the lower band was Grx1 (data not shown). Similar results were obtained when the competitive inhibitor sodium phosphate (Fig. 1A, lane 8) or sodium sulfate (Fig. 1B, lane 12) or the product, sodium arsenite (Fig. 1B, lane 15), was substituted for arsenate. In contrast, there was no apparent interaction when sodium nitrate was substituted for sodium arsenate (data not shown). These results suggest that filling of an anion site on ArsC by the substrate arsenate, competitive inhibitors phosphate and sulfate, or the product arsenite in combination with GSH stabilizes ArsC-Grx1 interaction.

When non-thiol derivative S-methylglutathione or S-hexylglutathione were substituted for GSH, binding of Grx1 to His6ArsC was decreased (Fig. 1B, lanes 3 and 6). However, the fact that binding still occurred with non-thiol glutathione derivatives indicates that the glutathione thiolate is not required for ArsC to interact with Grx1. The ability of the histidine-tagged derivative of the enzymatically inactive C12S ArsC to interact with Grx1 was examined. When both sodium arsenate and GSH were present, binding of Grx1 to His6ArsC was observed (Fig. 1B, lane 9). Thus, interaction of ArsC and Grx1 does not require catalytic activity, nor is the Cys-12 thiolate essential for Grx1 binding.

Role of GSH—GSH is required to reduce oxidized Grx1 (5). From previous results it was not clear whether GSH also played a direct role in the ArsC-catalyzed reaction (3, 9). To investigate this question, T4 glutaredoxin was substituted for

![Graph](https://via.placeholder.com/150)

**Fig. 2.** Glutathione dependence of arsenate reductase activity. Panel A, thiol dependence of arsenate reductase activity. The reaction contained 0.2 mM NADPH, 30 μg of thioredoxin reductase, and 30 μg of T4 glutaredoxin in 25 mM MOPS-NaOH buffer, pH 6.6. At the time indicated by arrow a, 10 μg of purified ArsC and 20 mM sodium arsenate were added. At the time indicated by arrow b, various thiols were added to 0.5 mM. Curve 1, GSH; curve 2, dithiothreitol; curve 3, β-mercaptoethanol. Panel B, Grx1 requirement for arsenate reductase activity. The reaction contained 0.2 mM NADPH, 30 μg of glutathione reductase, 30 μg of wild type or substituted Grx1, and 1 mM GSH in 25 mM MOPS-NaOH buffer, pH 6.6. At the time indicated by arrow a, 10 μg of ArsC and 20 mM sodium arsenate were added. Curve 1, wild type Grx1; curve 2, the C14S derivative of Grx1.

![Graph](https://via.placeholder.com/150)

**Fig. 3.** Emission spectra of wild type and the single tryptophan ArsCs. Curve 1, A11W; curve 2, A11W/C12S; curve 3, Y7W; curve 4, A11W + 6 mM guanidine HCl; curve 5, A11W/C12S + 6 mM guanidine HCl; curve 6, Y7W + 6 mM guanidine HCl; curve 7, 2 μM tryptophan; curve 8, wild type ArsC. The concentration of each protein was 2 μM. The excitation wavelength was 295 nm.

![Graph](https://via.placeholder.com/150)

**Graph**
**Interaction of ArsC with Substrates and Cofactors**

*E. coli* glutaredoxin. Whereas Grx1 requires GSH for reduction, *T. fourth* Grx can be reduced directly by thioredoxin reductase using NADPH as reductant. Using *T. fourth* Grx there was no reduction of arsenate by ArsC (Fig. 2A). However, the addition of GSH stimulated arsenate reduction (Fig. 2A, curve 1), indicating that GSH participates directly in the ArsC reaction. Neither dithiothreitol (Fig. 2A, curve 2) nor β-mercaptoethanol (Fig. 2A, curve 3) could replace GSH.

If GSH is directly required for the reductase reaction, it may form a mixed disulfide with ArsC. This question was examined using a Grx1 with a C14S substitution (16). This altered glutaredoxin retains Cys-11, the critical residue in catalyzing the reduction of mixed disulfides of glutathione. However, it can no longer serve as a hydrogen donor for ribonucleotide reductase, which requires reduction of intramolecular protein disulfides. The rates of ArsC-catalyzed arsenate reduction were the same with wild type Grx1 (Fig. 2B, curve 1) and C14S Grx1 (Fig. 2B, curve 2). This result is consistent with the function of Grx1 being to reduce a mixed disulfide rather than as a direct hydrogen donor in arsenate reduction. The most likely mixed disulfide would be between the thiolate of Cys-12 in ArsC and glutathione.

**Biochemical Properties of Altered ArsC Reductases—ArsC contains no tryptophans. To develop potential probes of the mechanism of ArsC, the seventh and eleventh codons of *arsC* were individually mutated from tyrosine and alanine codons, respectively, to a tryptophan codon. These locations were chosen because of their proximity to the active site Cys-12 residue. *E. coli* strain AW10 expressing either *arsC*11W or *arsC*7W in *trans* with wild type *arsA* and *arsB* genes retained wild type arsenate resistance (data not shown). Both A11W and Y7W proteins were produced in amounts similar to that of the wild type, and both were purified. Y7W ArsC had the same *Kₚ* and *Vₚₘₐₓ* as wild type (data not shown). Purified A11W had the same *Kₚ* for arsenate, with a *Vₚₘₐₓ* approximately half that of the wild type enzyme (data not shown). The A11W substitution was combined with a C12S substitution. The double-substituted protein was, as expected, catalytically inactive.

**Fluorescent Properties of Tryptophan-containing ArsC Reductases—**The emission spectra of both A11W (Fig. 3, curve 1) and A11W/C12S (Fig. 3, curve 2) had a maximum emission wavelength at 350 nm, whereas the *λₘₐₓ* of Y7W was 330 nm (Fig. 3, curve 3). Upon denaturation with 6 M guanidine hydrochloride, the *λₘₐₓ* of each protein was 354 nm, the same as that of free tryptophan (Fig. 3, curves 4–7). These results indicate that Trp-11 is located in a relatively solvent exposed environment, whereas Trp-7 is in a relatively less polar environment.

A decrease in intrinsic fluorescence was observed upon addition of sodium arsenate to A11W (Fig. 4A, curve 1). In contrast, the fluorescence of neither Y7W nor free tryptophan was affected by addition of sodium arsenate (Fig. 4B, curves 3 and 4). The addition of a competitive inhibitor, either sulfate or phosphate, resulted in a slight increase in A11W fluorescence (Fig. 4A, curves 2 and 3). Neither arsenite, the product of the reaction, nor chloride, which is neither a substrate nor competitor, had a substantial effect on A11W fluorescence (Fig. 4B, curves 1 and 2).

The addition of GSH alone had no effect on A11W fluorescence (Fig. 5, curve 1). However, if GSH was added after arsenate, there was a rapid fluorescence enhancement followed by a decay of the signal (Fig. 4, curve 1, and Fig. 5, curve 2). This synergistic effect of arsenate and GSH was not observed with Y7W (Fig. 4B, curve 3), nor was there an effect of GSH after the addition of sulfate (Fig. 4A, curve 2) or phosphate (Fig. 4A, curve 3). If arsenate was added after GSH, fluorescence quenching was 10-fold less rapid than when arsenate was added in the absence of GSH (Fig. 5, curve 1). This reduction in arsenate effect may be the result of an additive effect of arsenate quenching and GSH enhancement of fluorescence. It appears, then, that GSH affects A11W fluorescence only after the substrate arsenate is bound to the enzyme.

On the other hand, neither the monothiol β-mercaptoethanol nor the dithiol dithiothreitol was able to replace GSH (Fig. 6A). Similarly, non-thiol analogs and derivatives of glutathione, including ophthalmic acid, S-methylglutathione, and oxidized GSSG, could not substitute for GSH (Fig. 6B). These results suggest that the rapid fluorescence enhancement represents a transient conformational change in the enzyme that is produced specifically by the combination of arsenate and the reduced form of glutathione.

The role of the Cys-12 thiolate in the arsenate and glutathione responses was examined using the noncatalytic A11W/
C12S enzyme. The intrinsic fluorescence of this double-substituted ArsC was increased slightly by arsenate (Fig. 4C, curve 1) and phosphate (Fig. 4C, curve 2). This response is similar to that of the active A11W enzyme produced by the competitive inhibitor phosphate (Fig. 4A, curve 3). This result may suggest that the initial event reported by Trp-11 is binding of the oxyanion to a site on the enzyme. Because the noncatalytic A11W/C12S does not exhibit fluorescence quenching upon arsenate addition, the decrease in Trp-11 fluorescence may reflect a conformation of the enzyme in a subsequent step of the reaction, perhaps formation of a thiol-As bond. Subsequent addition of GSH after arsenate had a negligible effect on the fluorescence of the inactive enzyme. Thus the glutathione-dependent increase in fluorescence requires prior binding of the oxyanionic substrate, a free thiol on glutathione, and a free thiol on the enzyme, suggesting that the fluorescent signals are reporting events during catalysis. However, since no covalent intermediates have been isolated, there may be alternate explanations for these data.

**DISCUSSION**

ArsC encoded by the *arsC* gene of the *ars* operon *E. coli* plasmid R773 is an enzyme that reduces arsenate (As(V)) to arsenite (As(III)). The product, arsenite, is the substrate of the ArsA-ArsB efflux pump that renders the cells resistant to arsenicals and antimonials. We have shown previously that a single cysteine residue, Cys-12, is required for catalysis (9). The reaction also requires glutathione and glutaredoxin, but their exact roles were not known (3). We postulated a model in which the dithiol pair of glutaredoxin was oxidized to a disulfide during arsenate reduction. GSH was assumed to be required for reduction of oxidized Grx1 rather than playing a direct role in ArsC catalysis. In this report we demonstrate 1) direct interaction of His6ArsC and Grx1; 2) that GSH is required for the ArsC-catalyzed reaction as well as for Grx1 reduction; and 3) that Grx1 does not have to be oxidized to the disulfide form and may function as a reductase of a mixed disulfide between the Cys-12 thiol of ArsC and GSH.

Glutaredoxin is a small thioredoxin-like protein that has a broad range of specificity. Grx1 catalyzes electron transfer to ribonucleotide reductase (4) and is involved in the reduction of sulfate (17, 18), ascorbate (19), tellurite (20), and arsenate (3). During oxidative stress protein glutathionylation and deglutathionylation, a process also referred to as S-glutathionylation, is catalyzed by Grx1 (21). The data in this paper are the first biochemical evidence of physical interaction between ArsC and...
Grx1. A polyHis-ArsC chimeric protein was bound to an immobilized Ni$^{2+}$ column. Grx1 alone did not bind to the metal column but was retained by association with the His6ArsC. The association of the two proteins depended on the presence of both GSH and the substrate, arsenate. Arsenate could be replaced with a competitive inhibitor, either phosphate or sulfate, and non-thiol derivatives of GSH could substitute for that thiol. Thus catalytic conditions are not required for the protein-protein interaction. An ArsC derivative with a C12S substitution was capable of interacting with Grx1. Although the C12S derivative still has Cys-106, the thiol of this nonessential cysteine residue is buried within the enzyme and does not exhibit reactivity with thiol reagents (9). Thus it is unlikely that ArsC and Grx1 are held together by disulfide bonds.

ArsC has low affinity for arsenate and glutathione, making direct binding measurements difficult. To examine ligand binding to ArsC intrinsic protein fluorescence was used. Native ArsC has no tryptophan. This allowed placement of tryptophan residues in locations of potential utility by site-directed mutagenesis of arsC. Tryptophan codons were introduced at positions 7 and 11, both of which are close to the essential residue Cys-12 (9). Both proteins were purified, and the effect of ligands on intrinsic tryptophan fluorescence was examined. The Y7W enzyme had normal activity, and there was no effect of substrate, product, competitive inhibitors, or thiol reagents on its fluorescence. Although this protein was of no value in defining ligand interactions, it served as a useful control for the specific ligand-induced changes observed with the A11W enzyme.

Although the data and the fluorescence results described above could be interpreted in a variety of ways, they are consistent with the following reaction scheme.

\[
\begin{align*}
ES + \text{As(V)} & \rightleftharpoons ES \cdot \text{As(V)} \quad (\text{Reaction 1}) \\
ES \cdot \text{As(V)} & \rightleftharpoons ES - \text{As(V)} \quad (\text{Reaction 2}) \\
ES - \text{As(V)} + \text{GSH} & \rightleftharpoons ES - \text{As(V)} - \text{SG} \quad (\text{Reaction 3}) \\
ES - \text{As(V)} - \text{SG} & \rightleftharpoons ES - \text{SG} + \text{As(III)} \quad (\text{Reaction 4}) \\
ES - \text{SG} + \text{Grx1SH} & \rightleftharpoons ESH + \text{GS} - \text{SGTrx1} \quad (\text{Reaction 5}) \\
\text{GS} - \text{SGTrx1} + \text{GSH} & \rightleftharpoons \text{Grx1SH} + \text{GSSG} \quad (\text{Reaction 6})
\end{align*}
\]

The fluorescence of A11W was quenched rapidly by arsenate, whereas the competitive inhibitors phosphate and sulfate produced a slight enhancement. In contrast, the fluorescence of the noncatalytic A11W/C12S enzyme exhibited the same enhancement with arsenate and phosphate. These results suggest that the initial event in the reaction is noncovalent binding of oxynion, either the substrate arsenate or a competitive inhibitor, to the enzyme (Reaction 1), with a concomitant fluorescence enhancement. The next step is proposed to be formation of a covalent enzyme-As(V) complex by arsenylation of the Cys-12 thiolate (Reaction 2). This produces a conformational change resulting in quenching of Trp-11 fluorescence. GSH, which has no effect of Thp-11 fluorescence by itself, produces a complex effect if added subsequent to arsenate. There is a rapid fluorescence enhancement followed by an apparent exponential decay of the fluorescent signal. This would suggest the formation of a transient intermediate that decays into a more stable form. We propose that the rapid event is formation of a ternary complex of enzyme, arsenate, and glutathione (Reaction 3).

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