Identification of an Essential Cysteinyl Residue in the ArsC Arsenate Reductase of Plasmid R773†

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ABSTRACT: The ArsC protein encoded by the arsenical resistance operon of plasmid R773 catalyzes the reduction of arsenate to arsenite in *Escherichia coli*. The reductase has been shown to require glutathione and glutaredoxin, suggesting that thiol chemistry might be involved in the reaction mechanism. The ArsC arsenate reductase has two cysteinyl residues, Cys12 and Cys106. By a combination of random and site-specific mutagenesis, Cys12 was altered to four other amino acid residues. Cells expressing any of those *arsC* genes were sensitive to arsenate. The $ArsC_{C12S}$ protein was purified and found to be catalytically inactive. Cys106 was altered separately to seryl, glycyl, and valyl residues. Cells expressing $arsC_{C106S}$, $arsC_{C106G}$, and $arsC_{C106V}$ genes retained arsenate resistance, and the purified C106S and C106G proteins had reductase activity. Both wild-type ArsC and C106S proteins were inactivated by iodoacetate. In the native enzyme only Cys12 was alkylate by iodoacetate; Cys106 was alkylated only if the enzyme was first denatured. In the presence of the substrate, arsenate, or competitive inhibitors, phosphate or sulfate, the rate of alkylation was reduced. Reductase activity was inhibited by *N*-ethylmaleimide and could be protected by arsenate. These results suggest Cys12 is an active-site residue essential for catalysis by the arsenate reductase.

The *ars* operon of plasmid R773 confers resistance to the toxic oxyanions arsenite [As(III)], antimonite [Sb(III)], and arsenate [As(V)] in *Escherichia coli* (Hedges & Baumberg, 1973; Kaur & Rosen, 1992). The operon encodes three proteins responsible for the resistance (Chen et al., 1986). The *arsA* and *arsB* gene products form an arsenite- (antimonite) translocating ATPase that produces resistance to arsenite and antimonite by extruding those oxyanions from the cells. In addition to the ArsA and ArsB proteins, the *arsC* gene product is required for arsenate resistance (Chen et al., 1985). Other *arsC* genes have been identified on plasmids of Gram-positive organisms (Ji & Silver, 1992; Rosenstein, 1992) and in the chromosome of *E. coli* (Sofia et al., 1994; Carlin et al., 1995). In *E. coli in vivo* arsenate resistance was shown to require reduced glutathione (GSH)[1] (Oden et al., 1994), and the purified ArsC protein was shown to be a reductase requiring reduced glutathione and glutaredoxin for the conversion of arsenate to arsenite (Gladysheva et al., 1994). The involvement of thiols in reduction suggested the participation of cysteinyl residues in the ArsC protein. The ArsC protein contains two cysteinyl residues, Cys12 and Cys106. In this paper we report the mutagenesis of the *arsC* gene to alter Cys12 and Cys106 to other residues. Cells expressing four different $arsC_{C12}$ mutants were all sensitive to arsenate. Cells expressing three different $arsC_{C106}$ mutants were resistant to arsenate. Purified C12S protein was catalytically inactive, while the C106S and C106G proteins retained activity. The thiol reagents iodoacetate (IAA) and *N*-ethylmaleimide (NEM) inactivated the ArsC protein, and arsenate protected the enzyme from inactivation. Under nondenaturing conditions only Cys12 reacted with IAA. These results provide strong evidence that Cys12 is located at the active site and is required for catalysis.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media. The strains and plasmids used in this study are given in Table 1. For protein production, cells were grown at 37 °C with shaking in LB medium (Sambrook *et al.*, 1989). For phenotypic measurements of cultures of the arsenate resistance, *E. coli* strain AW10 (Δ*ars::cam*) (Carlin *et al.*, 1995) carrying the indicated plasmids was grown in a low-phosphate medium consisting of 80 mM NaCl, 20 mM KCl, 20 mM NH_4Cl, 3 mM $(NH_4)_2SO_4$, 1 mM $MgCl_2$, 2 μM $ZnSO_4$, 0.12 M Tris base, supplemented with 0.5% (w/w) either glycerol or glucose, 2 μg/mL thiamine, 1% peptone, and 0.1 mM $CaCl_2$, adjusted to pH 7.0−7.4 with HCl (Oden *et al.*, 1994). In this strain the chromosomal *ars* operon was disrupted to produce hypersensitivity to arsenate. Resistance was tested in liquid cultures using the low-phosphate medium. Turbidity was monitored at OD_{600nm} after 8−12 h of growth at 37 °C with shaking in medium containing varying concentrations of sodium arsenate. Appropriate antibiotics were added as required.

Materials. All restriction enzymes and nucleic acid modifying enzymes were obtained from Gibco-BRL. The Altered Sites *in vitro* mutagenesis system was purchased from Promega. Oligonucleotides were synthesized in the

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[1] Abbreviations: GSH, reduced glutathione; IAA, iodoacetate; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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Table 1: Strains and plasmids

strain/plasmid	genotype and phenotype	source of reference
	E. coli Strains	
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ*(lac−proAB)F′[traD36 proAB⁺ lacI^q lacZΔM15]*	Sambrook *et al.*, 1989
AW10	Δ*ars::cam dam dcm supE44 hsdR17 thi leu rpsL lacY galK galT ara tonA thr tsx* Δ*(lac−proAB) F′[traD36 proAB⁺ lacI^q lacZΔM15]*[a]	Carlin *et al.*, 1995
HMS174(DE3)	*recA1 hsdR rif^r* (λcI*ts*857 *imd*1 Sam7 *nin5 lac* UV5-T7 gene 1)	Studier & Moffat, 1986
	Plasmids	
pUC18	cloning vector, Ap^r	Yanisch-Perron *et al.*, 1985
pWSU1	entire *ars* operon cloned into pBR322	San Francisco *et al.*, 1990
pArsC	1.6-kb *Hind*III−*Bam*HI fragment from pWSU1 containing the *arsC* gene cloned into plasmid pUC18	this laboratory
pT7-5	expression vector with the T7 promoter, Ap^r	Tabor & Richardson, 1977
pT7-5-ArsC	1.6-kb *Hind*III−*Bam*HI fragment from pWSU1 containing the *arsC* gene cloned into plasmid pT7-5	this study
pUM3	*arsABC* genes of R773 cloned into the *Hind*III site of pBR322 (*arsABC*)	Mobley *et al.*, 1983
pUM11	4.3-kb *Hind*III fragment of plasmid pUM3 cloned into plasmid pACYC184	Karkaria *et al.*, 1990
pArsAB	deletion of a 840-bp *Kpn*I fragment containing 78% of the *arsC* gene from plasmid pUM11	this study
pJBS633	*blaM* gene fusion vector, Km^rTc^r	Broome-Smith & Spratt, 1986
pArsAB200	*Nde*I−*Eco*RI fragment containing the *km^r* gene from pJBS633 inserted into *Nco*I−*Eco*RI-digested pArsAB[b]	this study

[a] P1 transduction of Δ*ars::cam* from AW3110 to JM110. [b] The *Nde*I and *Nco*I sites were filled in with the Klenow fragment of DNA polymerase prior to blunt-end ligation.

Macromolecular Core Facility of Wayne State University School of Medicine. All other chemicals were obtained from commercial sources.

DNA Manipulation. Plasmid isolation, DNA restriction endonuclease analysis, ligation, and transformation were performed as described (Sambrook *et al.*, 1989; Chung *et al.*, 1989).

Mutagenesis of the arsC Gene. Mutations in the sequence of the *arsC* gene were introduced by site-directed mutagenesis using the Altered Sites *in vitro* mutagenesis system (Promega). The 1.6-kb *Bam*HI−*Hind*III fragment containing the *arsC* gene from pArsC was inserted into the multiple cloning site of pALTER −1 vector (Promega) and used as the template. Degenerate oligonucleotides were used to introduce mutations in the codon for the Cys12 and Cys106. The identity of the mutations was confirmed by DNA sequencing of each mutant gene. Double-stranded plasmid DNA was prepared using the Wizard Minipreps DNA purification system (Promega) and denatured with 0.2 M NaOH and 0.2 mM EDTA for 30 min at 37 °C. Sequencing was done using the Sequenase version 2.0 DNA sequencing kit (U.S. Biochemical Corp.) by the method of Sanger *et al.* (1977).

Hydroxylamine was used to introduce random mutations into the *arsC* gene, as described by Humphreys *et al.* (1976). The reaction mixture contained 3 μg of DNA from plasmid pArsC and 0.19 mL of a solution consisting of 0.4 M NH_2-OH, 50 mM Na_3PO_4, and 1 mM EDTA, pH 7.0, in a final volume of 0.2 mL. The reaction was carried out at 70 °C for 30 min. The DNA was precipitated with 100% ethanol, washed once with 70% ethanol, and dried. The DNA was dissolved in 10 μL of a buffer consisting of 10 mM Tris-HCl and 1 mM EDTA, pH. 8.0, and transformed into cells of strain AW10 bearing plasmid pArsAB200 (*arsA arsB*), with selection for Ap^r and Km^r. Transformants were screened for arsenate sensitivity on solid low-phosphate medium containing 3 mM sodium arsenate.

Purification and Assay of the ArsC Protein. The wild-type and altered ArsC proteins were purified from 3-L cultures of *E. coli* strain JM109 bearing plasmid pArsC or strain HMS174DE3 bearing plasmid pT7-5-ArsC. Cultures were grown in LB medium at 37 °C to an OD_{600} of 0.6, and then expression of the *arsC* gene was induced by addition of 50−500 μM isopropyl β-D-thiogalactopyranoside. The ArsC proteins were purified as described previously (Gladysheva *et al.*, 1994). The protein concentration was determined by the method of Lowry *et al.* (1951) or from the absorbance at 280 nm using the calculated molar extinction coefficient as 3960 for native ArsC. Arsenate reductase activity was assayed as described (Gladysheva *et al.*, 1994). Activities were corrected for nonenzymatic reduction of arsenate by GSH.

Reaction of the ArsC Protein with Thiol Reagents. Dithiothreitol (DTT) was removed from the ArsC protein by gel filtration in a spin column (Penefsky, 1977). A syringe (1 mL) was nearly filled with Sephadex G-25 and equilibrated with buffer. The sample was added to the top of the syringe, which was then centrifuged for 2 min at 1000 rpm using a swinging bucket rotor. DTT was retained in the spin column. The eluant, in which approximately 70% of the added ArsC protein was recovered, was mixed under argon at room temperature with freshly prepared iodoacetic acid (IAA) to a final concentration of 0.8 mM in a buffer consisting of 50 mM MES and 50 mM MOPS adjusted to pH 6.5 with NaOH. Aliquots were withdrawn periodically for the reductase assay. The transferred IAA had no inhibitory effect on the coupling system. In the case of substrate protection experiments, ArsC protein was incubated with 50 mM arsenate, phosphate, sulfate, or nitrate salts at room temperature for 10 min before addition of IAA. Similar experiments was done for reactions between ArsC protein and varying concentrations of *N*-ethylmaleimide (NEM), as indicated. Thiol content was measured using 5 mM 5,5′-dithiobis(2-nitrobenzoic acid)(DTNB) according to the method of Ellman (1959).

NMR Spectra. The NMR experiments were conducted on a Varian UNITY 500-MHz NMR spectrometer at the University of Western Ontario using a triple-resonance probe.

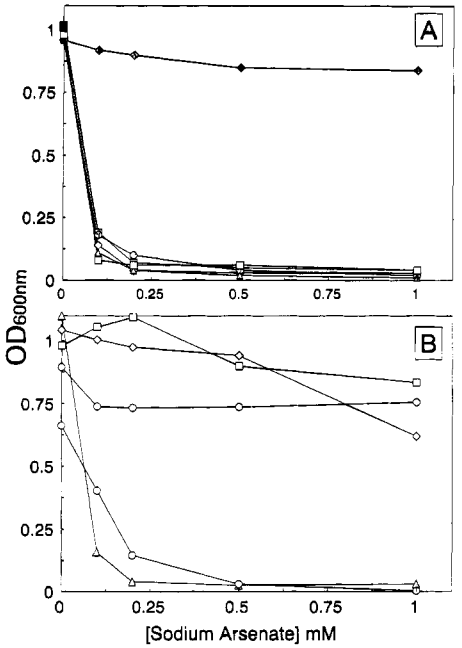

FIGURE 1: Arsenite resistance in *E. coli* strain AW10 strain expressing wild-type and mutant *ars*C genes in trans with *ars*AB200. Overnight cultures of *E. coli* strain AW10 bearing the indicated *ars* plasmids were diluted 100-fold into fresh low-phosphate medium containing varying concentrations of sodium arsenate. Expression of the *ars* genes was induced by 0.1 mM isopropyl β-D-thiogalactopyranoside. Growth was measured at OD_{600nm} after 8 h of growth at 37 °C in cells bearing plasmids with the following *ars* genes: panel A, (♦) *arsA arsB* $arsC_{WT}$, (■) $arsC_{WT}$, (◇) *arsA arsB* $arsC_{C12S}$, (□) *arsA arsB* $arsC_{C12Y}$, (▲) *arsA arsB* $arsC_{C12G}$, and (●) *arsA arsB* $arsC_{C12A}$; panel B, (▲) *arsA arsB*, (●) *arsA arsB* $arsC_{C106S}$, (♦) *arsA arsB* $arsC_{C106G}$, (□) *arsA arsB* $arsC_{C106V}$, and (○) *arsA arB* $arsC_{C106R}$.

The samples contained 1 mM ArsC protein, 0.2 M NaCl, 1 mM DTT, and 10 μM EDTA, pH 6.5, in 10% D_2O. The temperature was controlled at 25 °C. A total of 512 scans/spectrum were acquired. VNMRSQI software was used on a Silicon Graphics Indigo to process the data. The chemical shifts were measured relative to the principal resonance of sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

RESULTS

Arsenate Resistance Phenotype of Cells with Mutations in the arsC Gene. By random mutagenesis with hydroxylamine, one mutation was found in the codon for Cys12 that produced a change to tyrosine. Using oligonucleotide-directed mutagenesis, Cys12 was changed to serine, alanine, and glycine, and Cys106 was changed to serine, glycine, arginine, and valine.

Strains expressing the mutant or native *arsC* genes in trans with wild-type *arsA* and *arsB* genes were examined for their ability to confer resistance to arsenite or arsenate. Each strain retained arsenite resistance (data not shown), consisting with the observation that only the *arsA* and *arsB* genes are required for resistance to arsenite (Chen *et al.*, 1985). Cells bearing the wild-type *arsC* gene in trans with the *arsA* and *arsB* genes could grow in low-phosphate medium containing 1 mM sodium arsenate, while cells harboring either *arsA* and *arsB* genes or only the *arsC* gene were sensitive to sodium arsenate (Figure 1). Cells expressing *arsC* genes with mutations in the codon for Cys12 exhibited a fully sensitive phenotype (Figure 1A). In contrast, cells expressing

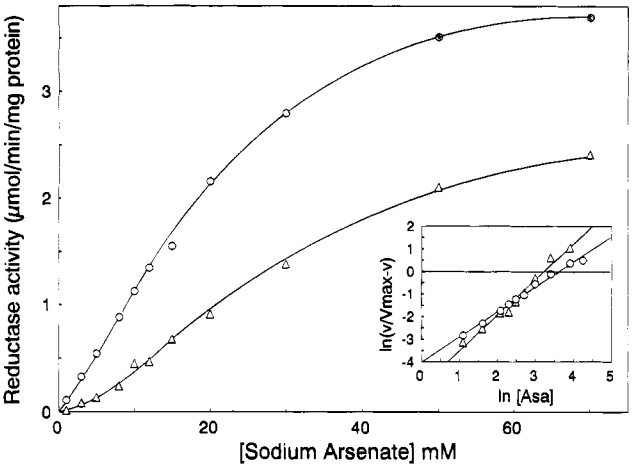

FIGURE 2: Kinetics of arsenate reduction in wild-type and $ArsC_{C106S}$ proteins. Reductase assays were performed as described under Experimental Procedures. Initial velocities are expressed as micromoles of NADPH oxidized per minute per milligram protein. (●) Wild-type ArsC protein; (▲) C106S. The data were fitted using nonlinear regression (Enzfitter, Elsevier-BIOSOFT, Cambridge, U.K.). Inset, Hill plot of the data.

arsC genes with mutations in the codon for Cys106 position ($arsC_{C106S}$, $arsC_{C106G}$, or $arsC_{C106V}$) exhibited resistance to arsenate (Figure 1B). At higher concentrations of arsenate, strains expressing these mutant *arsC* genes were somewhat more sensitive than the wild type but still resistant compared to strains without an *arsC* gene (data not shown). Only an $arsC_{C106R}$ mutation resulted in loss of arsenate resistance (Figure 1B).

Arsenate Reductase Activity in Wild-Type and Altered ArsC Proteins. The C12S and C106S proteins were expressed in *E. coli* in approximately the same amounts as the wild-type protein and did not appear to be abnormally unstable, as shown by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (data not shown). The C12S, C106S, and C106G proteins were purified, and their reductase activity was assayed. Purified C12S protein exhibited no arsenate reductase activity over background, even when assayed at a protein concentration 10-fold higher than wild type. Both the wild-type and C106S proteins exhibited a slightly sigmoidal relationship between initial velocity and arsenate concentration, with a Hill coefficient of 1.1 for the wild type and 1.6 for the C106S enzyme (Figure 2). Purified C106G enzyme was also found to exhibit sigmoidal kinetics, with a Hill coefficient of 1.5 (data not shown). The V_{max} values of the two altered enzymes were approximately half that of the wild type.

Structural Analysis of the Altered ArsC Proteins. To determine whether the cysteine to serine substitutions introduced major structural changes in the altered proteins, ^1H NMR spectra were obtained for the wild-type, C12S, and C106S proteins (data not shown). The spectrum of the C12S protein was approximately the same as that of the wild-type ArsC protein, while the spectrum of the C106S protein showed some differences, suggesting structural alterations. The similarities in the chemical shifts and line widths of the ^1H NMR spectra of the wild-type enzyme and the C12S protein suggest that their overall structures are similar. However, differences in the resonances observed at 7.05 and 8.20 ppm for the C106S protein suggest that the structure of this enzyme has some differences from the wild-type ArsC protein.

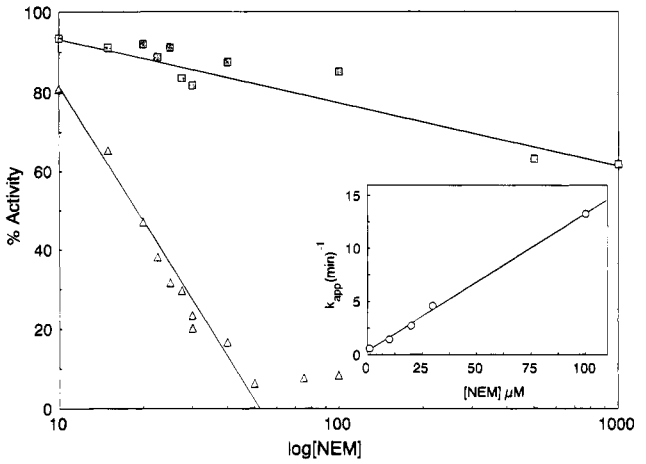

FIGURE 3: Inactivation of the ArsC arsenate reductase by NEM. After removal of DTT by passage through a spin column, the purified ArsC protein was incubated with increasing concentrations of NEM for 6 min at room temperature, following which reductase activity was assayed. (▲) No additions; (■) with 0.2 M sodium arsenate. Inset: Plot of apparent first-order rate constants for inactivation obtained at various concentrations of NEM against inhibitor concentration, yielding a second-order rate constant of 0.8 μM^{-1} min^{-1}.

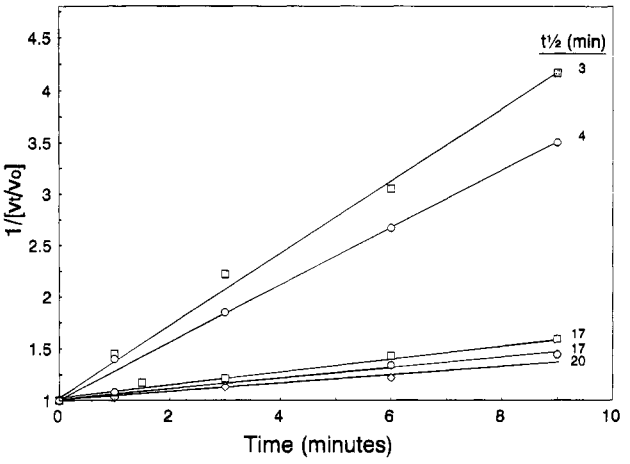

FIGURE 4: Effect of arsenate and competitive inhibitors inactivation of the ArsC reductase by iodoacetate. Treatment with IAA and assays of reductase activity were performed as described under Experimental Procedures. Following removal of DTT with a spin column, the purified ArsC protein was incubated with 50 mM of the sodium salt of each oxyanion for 10 min at 23 °C. IAA was added and the incubation continued. At the indicated times the enzyme was diluted into the assay mixture, and reductase activity was measured. Each reaction was adjusted to a constant ionic strength with NaCl and to a pH of 6.5. Additions: (■) none; (○) nitrate; (□) arsenate; (●) phosphate; (◇) sulfate.

In addition, the results of limited trypsin digestion analysis were consistent with NMR results: the wild-type and $ArsC_{C12S}$ proteins were resistant to trypsin digestion, as determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis, while the $ArsC_{C106S}$ protein was degraded by trypsin to smaller peptides within 5 min (data not shown). These results imply that the amino acid substitutions at position 12 did not produce significant structural changes, indicating that the loss of function of the C12S protein was due to the replacement of an essential residue. On the other hand, the altered enzymatic properties of the C106S enzyme may result from structural changes.

Effect of Thiol Reagents on the ArsC Protein. The effects of treatment with the cysteine-reactive reagents *N*-ethylmaleimide (NEM) and iodoacetate (IAA) on reductase activity were examined. The activity of the ArsC enzyme was rapidly inhibited by NEM, exhibiting a second-order rate constant of inactivation of 0.8 μM^{-1} min^{-1} (Figure 3). Nearly complete inhibition was observed in 5 min with 50 μM NEM, but the enzyme was almost completely protected by the addition of the substrate arsenate. Preincubation with arsenite had no effect on NEM inactivation of the enzyme (data not shown).

Treatment of the ArsC protein with iodoacetate similarly resulted in rapid inactivation. The reaction between equal concentrations of protein and iodoacetate was plotted as $1/[v_t/v_0]$ against time, giving a straight line that is consistent with a second-order reaction with a single rate constant (Figure 4), where v_t is the rate at different times of inhibition by iodoacetate and v_0 is the uninhibited rate. Addition of 50 mM sodium arsenate or 50 mM sodium phosphate or sodium sulfate, both of which have been shown to competitively inhibit reductase activity (Gladysheva *et al.*, 1994), resulting in substantial protection from IAA inactivation (Figure 4). At an ionic strength corresponding to that of 50 mM sodium arsenate (adjusted with NaCl), the $t_{1/2}$ of inactivation by IAA was 3 min; with arsenate, phosphate, or sulfate, the $t_{1/2}$ increased to 17−20 min). On the other hand, 50 mM sodium nitrate, which is neither a substrate nor an inhibitor, had little effect on IAA inactivation. Arsenite, the product of the reductase reaction, also did not protect when added at 0.3 mM, a concentration 3-fold higher than the K_i for arsenite (Gladysheva *et al.*, 1994). The activity of the C106S enzyme was also inhibited by IAA, and arsenate similarly protected (data not shown), suggesting that alkylation of Cys12, the remaining cysteinyl residue in the C106S protein, was responsible for the inhibition.

To examine in more detail which cysteinyl residue was responsible for loss of activity, the wild-type ArsC, C12S, and C106S proteins were treated with an equal molar concentration of IAA. The wild-type enzyme was also treated with a 2-fold excess of IAA, corresponding to the number of free thiols in the protein. The proteins were then denatured with guanidine hydrochloride, and the amount of remaining free thiol was quantified with DTNB. In the wild-type enzyme 56% of the thiols remained, indicating that only one of the two cysteinyl residues reacted with IAA. In the C106S protein 15% of the thiols remained, indicating that the single remaining cysteinyl residue in the C106S protein also reacted with IAA. In contrast, 93% of the thiols of the C12S protein remained, indicating that the single cysteinyl residue in the C12S protein did not react with IAA. These results indicate first that only Cys12 reacted with iodoacetate, and second that alkylation of Cys12 resulted in inactivation of the enzyme, suggesting that Cys12 is an active-site residue.

DISCUSSION

The ArsC protein is a small protein of 16 kDa that catalyzes reduction of As(V) to As(III) (Gladysheva *et al.*, 1994). No cofactors or prosthetic groups have been identified for the ArsC protein. *In vitro* both GSH and glutaredoxin are required, although it is not clear whether the requirement for GSH is simply to rereduce oxidized glutaredoxin or whether it also participates in ArsC-catalyzed arsenate reduction. Since the reducing equivalents derive from those cysteine thiolates, it was reasonable to consider

the possibility that cysteinyl residues in the protein are involved in catalysis. The ArsC protein has only two cysteines, Cys12 and Cys106. Using a combination of random and site-specific mutagenesis, four mutants in the codon for Cys12 were isolated: C12S, C12A, C12G, and C12Y. Cells expressing the mutant $arsC_{C12}$ genes were all arsenate-sensitive, and the purified C12S was catalytically inactive. These results strongly indicate a role for Cys12 in the activity of the ArsC reductase.

Chemical modification of the ArsC protein with sulfhydryl reagents provided another line of evidence supporting the idea that Cys12 is within the active site. The maleimide NEM and the alkylating reagent iodoacetate both rapidly inactivated the enzymatic activity of the ArsC protein. The substrate arsenate protected the enzyme from inactivation by both reagents, consistent with the possibility of a cysteine in or conformationally coupled to the substrate binding site. Examination of the stoichiometry of alkylation by IAA demonstrated that only one cysteinyl residue reacted in the wild-type protein, one in the C106S and none in the C12S proteins. This result strongly indicates that Cys12 but not Cys106 is alkylated and that modification of Cys12 leads to loss of catalytic activity. We would postulate that the thiolate group of Cys12 participates in catalysis, although a structural role cannot be ruled out from the data presented here.

Substitution of either serine or glycine for Cys106 resulted in active ArsC enzymes that had maximal velocities about half that of the wild-type enzyme and an increase in the apparent positive cooperativity. However, structural changes were noted in the two that might account for catalytic differences. The fact that substitution of Cys106 does not result in loss of resistance or enzymatic activity is an important demonstration that this residue is not essential for catalysis.

REFERENCES

Broome-Smith, J. K., & Spratt, B. G. (1986) *Gene 49*, 341−349.
Carlin, A., Shi, W., Dey, S., & Rosen, B. P. (1995) *J. Bacteriol. 177*, 981−986.
Chen, C. M., Mobley, H. L. T., & Rosen, B. P. (1985) *J. Bacteriol. 161*, 758−763.
Chen, C. M., Misra, T., Silver, S., & Rosen, B. P. (1986) *J. Biol. Chem. 261*, 15030−15038.
Chung, C. T., Niemela, S. L., & Miller, R. H. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 2172−2175.
Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70−77.
Gladysheva, T. B., Oden, K. L., & Rosen, B. P. (1994) *Biochemistry 33*, 7287−7293.
Hedges, R. W., & Baumberg, S. (1973) *J. Bacteriol. 115*, 459−460.
Humphreys, G. O., Willshaw, G. A., Smith, H. R., & Anderson, E. S. (1976) *Mol. Gen. Genet. 145*, 101−108.
Ji, G., & Silver, S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 7974−7978.
Karkaria, C. E., Chen, C. M., & Rosen, B. P. (1990) *J. Biol. Chem. 265*, 7832−7836.
Kaur, P., & Rosen, B. P. (1992) *Plasmid 27*, 29−40.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
Mobley, H. L. T., Chen, C. M., Silver, S., & Rosen, B. P. (1983) *Mol. Gen. Genet. 191*, 421−426.
Oden, K. L., Gladysheva, T. B., & Rosen, B. P. (1994) *Mol. Microbiol. 12*, 301−306.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891−2899.
Rosenstein, R., Peschel, A., Wieland, B., & Götz, F. (1992) *J. Bacteriol. 174*, 3676−3683.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
San Francisco, M. J. D., Hope, C. L., Owolabi, J. B., Tisa, L. S., & Rosen, B. P. (1990) *Nucleic Acids Res. 18*, 619−624.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
Sofia, H. J., Burland, V., Daniels, D. L., Plunkett, G., III, & Blattner, F. R. (1994) *Nucleic Acids Res. 22*, 2576−2586.
Studier, F. W., & Moffatt, B. A. (1986) *J. Mol. Biol. 189*, 113−130.
Tabor, S., & Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1074−1078.
Yanisch-Perron, C., Vieira, J., & Messing, J. (1985) *Gene 33*, 103−119.

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