His-8 Lowers the pKₐ of the Essential Cys-12 Residue of the ArsC Arsenate Reductase of Plasmid R773*

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The 141-residue ArsC arsenate reductase of plasmid R773 has an essential cysteine residue, Cys-12. The pKₐ of Cys-12 was determined to be 6.4, compared with a pKₐ of 8.3 for free cysteine. The possibility of the formation of an ion pair between Cys-12 and a basic residue was investigated. Enzymatic activity was rapidly inactivated by the histidine-modifying reagent diethylpyrocarbonate. The codons for the two histidine residues in ArsC, His-8 and His-88, were changed by site-directed mutagenesis. Cells expressing arsC_His8, arsC_His88, or arsC_His888 genes retained arsenate resistance, and the purified proteins had wild type level of reductase activity. Cells expressing arsC_His8, arsC_His88 arsC_His888 or arsC_His888 genes were each sensitive to arsenite, and the purified H3P, H8G, and H8R proteins each lacked enzymatic activity. Using the single histidine proteins it was shown that both histidines react with diethylpyrocarbonate but that only reaction with His-8 resulted in inactivation. The pKₐ value of Cys-12 was determined to be 6.3 in the H8R enzyme and 8.3 in the H8G enzyme. These results indicate that His-8 is essential for catalytic activity and that a positively charged residue is required at position 8 to lower the pKₐ of the cysteine thiolate at position 12.

Arsenical resistance (ars) operons of both Gram-positive and Gram-negative bacteria confer resistance to the toxic oxyanions arsenite (As(III)), antimonite (Sb(III)), and arsenate (As(V))(1). In Escherichia coli resistance to trivalent arsenicals and antimonials conferred by the ars operon of plasmid R773 results from their active extrusion from the cells by an ATP-coupled arsenite pump (1–3). Resistance to arsenite in both Gram-positive and Gram-negative organisms requires the product of the arsC gene, an arsenate reductase that generates arsenite, the substrate of the resistance pump (4–6). The arsC gene is essential for in vitro arsenite reductase activity (7). The pKₐ value of free cysteine in solution is 8.3, considerably higher than pH optimum of the ArsC-catalyzed reaction, which is in the range of 6.3 to 6.6 (6). The pKₐ value of cysteine residues in the active site of a number of enzymes have been shown to be lowered by the formation of an ion pair with a proximate basic residue (8–10). ArsC reductase activity was inhibited by treatment with diethylpyrocarbonate (DEPC),1 suggesting the involvement of histidine residues in ArsC function, perhaps in ion pairing with Cys-12. For that reason the role of histidine residues was investigated.

ArsC has only two histidine residues, His-8 and His-88. The codons for these two residues were individually changed by site-directed mutagenesis. The phenotype of cells expressing arsC_His88V mutants (where X can be one of several substitutions) was indistinguishable from wild type, and the corresponding purified gene products each had wild type ArsC properties. Cells expressing arsC_His88 mutants lost arsenate resistance, and the purified gene products were each catalytically inactive. When the ArsC enzymes were reacted with DEPC, there was a correlation between the rate of DEPC inactivation and formation of N-carbethoxylated His-8. The pKₐ of Cys-12 thiolate was nearly 2 units lower in the wild type enzyme than in a H8G enzyme. These results indicate that His-8 is an essential residue of the reductase, with one critical function being to lower the pKₐ of Cys-12.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Media—The strains and plasmids used in this study are given in Table I. For protein production cells were grown at 37 °C with shaking in 2 × YT medium (11). For photolytic measurements of arsenate resistance E. coli strain AW10 (∆ars:cam) (12) carrying the indicated plasmids were grown in a low phosphate medium (5). Sodium arsenate was added at the indicated concentrations. In this strain the chromosomal ars operon was disrupted to produce hypersensitivity 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.

DNA Manipulation—Plasmid isolation, DNA electrophoresis, restriction endonuclease analysis, ligation, and transformation were performed as described (11, 13).

Construction of Mutant arsC Genes—Mutations in the sequence of the arsC gene were introduced by site-directed mutagenesis using the Altered Sites™ in vitro mutagenesis system (Promega). The arsC gene inserted into the multiple cloning site of pALTER-1 vector (Promega) was used as the template (7). Degenerate oligonucleotides were used to introduce mutations in the codons for His-8 and His-88. The identity of the mutations was confirmed by DNA sequencing of each mutant gene (14). Single-stranded plasmid DNA was prepared using a QIAGEN DNA purification system (Qiagen Inc., Chatsworth, CA). Sequencing was performed by using the internal DNA labeling (CyS™-dATP labeling mix) with the T7 DNA polymerase CyS™ AutoRead™ Sequencing kit and an ALF-express DNA Sequencer (Pharmacia Biotech Inc).

To construct the double mutants arsC_His8_Cys8 and arsC_His88_Cys888 plasmids containing the arsC_His8, arsC_Cys8, arsC_His88, or arsC_Cys888 genes were individually digested with restriction enzymes AluI and Eco47III. The fragments containing the two desired mutations were ligated together and transformed into E. coli strain JM109, with selection for ampicillin resistance. The mutations were confirmed by DNA sequencing.

1 The abbreviations used are: DEPC, diethylpyrocarbonate; DTNB, 5,5'-dithiobis(2-nitrobenzoic) acid; MOPS, 4-morpholinepropanesulfonic acid; MES, 4-morpholinethanesulfonic acid.

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Strains and plasmids

| Strains        | Genotype and phenotype | Source or Ref. |
|----------------|------------------------|----------------|
| JM109          | recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi (Δlac-proAB) F' [traD36 proAB' lacI' lacZAM15] | 11 |
| AW10           | Δars::cam dam dcm supE44 hsdR17 thi leu rpsL lacY galK galT ara tnaA thr ttx Δlact::proAB F' [traD36 proAB' lacI' lacZAM15] | 12 |

Plasmids

| Plasmid       | Description                          | Source or Ref. |
|---------------|--------------------------------------|----------------|
| pArsC         | arsC gene in plasmid pUC18           | 7              |
| pArsB200      | arsRΔ2AB genes in pACYC184, KmR     | 7              |
| pArsC_H8X     | pArsC with codon for His-8 changed by site-directed mutagenesis, where X indicates one of several substitutions | This study |
| pArsC_H8R     | pArsC with codon for His-88 changed by site-directed mutagenesis, where X indicates one of several substitutions | This study |
| pArsC_C12S    | pArsC with codon for Cys-12 changed by site-directed mutagenesis to serine codon | 7              |
| pArsC_C106S   | pArsC with codon for Cys-106 changed by site-directed mutagenesis to serine codon | 7              |
| pArsC_H8R_C12S| 1.5-kb AluNI-Eco47III fragment from pArsC_C12S, religated with a 2.8-kb AluNI-Eco47III fragment from pArsC_H8R | This study |
| pArsC_C106S   | 1.5-kb AluNI-Eco47III fragment from pArsC_C106S, religated with a 2.8-kb AluNI-Eco47III fragment from pArsC_H8R | This study |
| pArsC_H8R_C106S| 1.5-kb AluNI-Eco47III fragment from pArsC_H8R_C12S, religated with a 2.8-kb AluNI-Eco47III fragment from pArsC_C106S | This study |

Mutagenesis of Histidine Codons in ArsC—Site-directed mutagenesis. Cells of strain AW10 bearing the arsC gene on a compatible plasmid were examined for arsC genes on one plasmid and wild type arsA and arsB genes on a compatible plasmid were examined for arsenate resistance (Fig. 2). AW10 (Δars::cam) is sensitive to 0.1 mM sodium arsenate (12). Cells expressing the wild type arsC

Inhibition of ArsC arsenate reductase activity by DEPC. Purified ArsC (50–250 μM) was incubated with 1.6 mM DEPC at room temperature. Additions: ■, none; ●, 20 mM sodium arsenate; ○, 50 mM sodium phosphate; ▲, control with no DEPC.

RESULTS

Inactivation of the ArsC Arsenate Reductase by Diethylpyrocarbonate—Reductase activity was rapidly inactivated by DEPC. Purified ArsC (50–250 μM) was incubated with 1.6 mM DEPC at room temperature. Additions: ■, none; ●, 20 mM sodium arsenate; ○, 50 mM sodium phosphate; ▲, control with no DEPC.

To reverse DEPC inactivation DEPC-inactivated ArsC (50 μM) was rapidly diluted 2-fold with 0.1 mM sodium phosphate buffer, pH 7.0, containing 1 mM hydroxylamine. After incubation at 25 °C for the indicated times, excess reagents were removed with a spin column, and enzymatic activity was assayed. As a control unmodified ArsC was given the same treatment.

Mutation of Histidine Codons in arsC—DEPC is known to modify histidine residues in proteins. His-8 and His-88 was individually changed to several different residues by site-directed mutagenesis. Cells of E. coli strain AW10 bearing the mutated arsC genes on one plasmid and wild type arsA and arsB genes on a compatible plasmid were examined for arsenate resistance (Fig. 2). AW10 (Δars::cam) is sensitive to 0.1 mM sodium arsenate (12). Cells expressing the wild type arsC

The number of "N-carbethoxyhistidine residues formed during the reaction with DEPC was determined by difference spectroscopy at 240 nm. Tyrosine modification was estimated from the decrease in absorbance at 278–280 nm in the presence and absence of 6 mM guanidine hydrochloride (19). The number of cysteine residues in ArsC was estimated from the reaction with 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB) in 6 mM guanidine hydrochloride using molar extinction coefficient of 13,600 M⁻¹ cm⁻¹ (20).
gene in trans with the arsA and arsB genes are resistant to at least 10 mM sodium arsenate (shown only to 1.5 mM in Fig. 2), but cells bearing only arsC gene or only arsA and arsB genes are sensitive to 0.1 mM sodium arsenate (7). Cells bearing the arsC gene or only arsB genes with mutations in the codon for His-88 were as resistant to arsenate as the wild type (Fig. 2B). Two double mutants were constructed by combining individual mutations by molecular cloning: arsC<sub>H8P</sub>, arsC<sub>H8G</sub>, or arsC<sub>H8R</sub> genes with arsA and arsB genes in trans were sensitive to arsenate (Fig. 2A). In contrast, cells expressing arsC genes with mutations in the codon for His-88 were as resistant to arsenate as the wild type (Fig. 2B).

Analysis of the Altered ArsC Proteins—The steady state level of expression of the altered ArsC proteins was approximately the same as a wild type ArsC protein, as was determined by Western blot analysis using ArsC-specific antibody (data not shown). No differences were observed in the mobility on SDS-polyacrylamide gel electrophoresis between wild type and any of the altered ArsC proteins. The purification procedure produced about the same amount of each mutant protein at the same level of purity as wild type ArsC (data not shown). The elution from a molecular sieve column and circular dichroism spectra were essentially identical for all of the altered arsenates (data not shown). Although local changes in structure cannot be excluded, there do not appear to be gross alterations in tertiary structure of the ArsC enzymes used in this study.

Arsenate Reductase Activity of the Altered ArsC Proteins—Purified H88W, H88S, H88V, and H88R enzymes each had nearly the same specific activity and pH dependence of arsenate reductase activity as wild type enzyme (data not shown). In contrast, no measurable activity was found for the H8P, H8G, H8R, H8R/C106S, and C12S/H88R enzymes, even at a protein concentration 100-fold greater than wild type. The assay is not sufficiently sensitive to detect activity less than 10⁻³ that of the wild type, so the possibility that these enzymes have very low turnover rates cannot be excluded. These results are consistent with the phenotypic properties of the mutants (Fig. 2) and suggest that His-88 in ArsC protein is not involved in the ionization of the imidazolium group of His-8 as described under “Experimental Procedures.” Pseudo-first order rate constants for the inactivation (k<sub>obs</sub>) were calculated from the slopes of semilogarithmic plots of activity against the time of preincubation at varying pH values. The line is a theoretical curve calculated assuming a single ionizing group of pKₐ = 7.0.

Inactivation of H88R by DEPC—Since H88R exhibited wild type ArsC properties, this protein was used for further characterization of the reaction with DEPC. The slopes of the lines from the concentration dependence of DEPC inactivation as a function of time (Fig. 3A) were used to determine values for the pseudo-first order rate constants of the inactivation, k<sub>obs</sub>, as described (23). A linear relationship of k<sub>obs</sub> with DEPC concentration was observed (Fig. 3B).

pH Dependence of DEPC Inactivation of H88R—The pH-dependent increase of k<sub>obs</sub> at 1 mM of DEPC over the pH range from 5.5 to 8.2 was determined (Fig. 3C). The experimental values fit a theoretical curve calculated for pKₐ of 7.0. The data indicate that the unprotonated form of an ionizable group reacts with DEPC to inactivate the enzyme. The values of k<sub>obs</sub> were independent of the buffer concentration over a range of 5 mM to 0.1 M sodium or potassium phosphate at pH 6.5, demonstrating that the observed pKₐ was not due to titration of buffer components. While a pKₐ of 7.0 is about 1 pH unit higher than that of free histidine in solution, it most likely reflects the ionization of the imidazolium group of His-8, as the data below indicate.

Kinetics of DEPC Modification—Binding of diethylpyrocarbonate with ArsC was monitored spectrophotometrically. The differences in absorbance between unmodified and DEPC-
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Hydroxylamine reactivation of DEPC-inactivated H88R

| Incubation time with 0.5 M NH2OH | Residual activity* |
|----------------------------------|--------------------|
| k                               | %                  |
| 0                               | 21                 |
| 0.5                             | 30                 |
| 1                               | 60                 |
| 2                               | 98                 |
| 3                               | 23                 |
| 5                               | 26                 |
|                                 | 31                 |

* 0.03 mM H88R was inactivated with either 0.3 or 3 mM DEPC for 25 min prior to treatment with hydroxylamine. At the indicated times portions of 50 μl of the reaction mixture were passed through a Sephadex G-25 spin column, and reductase activity was assayed as described under "Experimental Procedures."

Determination of the pK_a of the Cys-12—The rate of inactivation of the C106S enzyme, which has only Cys-12, by DTNB was measured (Fig. 5A). The dependence of the pseudo-first order rate constants for inactivation on pH was measured (Fig. 5B). Pseudo-first order rate constants for the inactivation (k_obs) were calculated from the slopes of semilogarithmic plots of activity against the time of preincubation at varying pH values. The line is a theoretical curve calculated assuming pK_a = 6.5.

The rate of inactivation of C106S by DTNB, A, inhibition of C106S (5–50 μM) with DTNB and measurement of residual activity was performed as described under "Experimental Procedures." Purified ArsC was incubated with the following concentrations of DTNB: A, 1.5 μM; C, 3.0 μM; D, 3.5 μM; E, 5.0 μM; F, 7.0 μM; G, 8.0 μM; H, 15.0 μM; I, 20.0 μM. B, dependence of the pseudo-first order rate constants for inactivation on the DTNB concentration. Values for k_obs were determined from the slopes of semilogarithmic plots of activity against the time of preincubation at varying pH values. The line is a theoretical curve calculated assuming pK_a = 6.5.

The peak of absorbance at about 240 nm was used to calculate the concentration of N-carbethoxylated histidine residues (Fig. 4A). The peak of absorbance at about 240 nm was used to calculate the concentration of N-carbethoxylated histidine residues (Fig. 4A). For the wild type enzyme two phases were observed (Fig. 4A). In the fast phase of formation of N-carbethoxylated histidine reductase activity was not greatly affected. In the slow phase activity was lost (Fig. 4B). In completely inactivated ArsC 1.9 histidine residues were modified. Modification of tyrosine residues was detected spectrophotometrically from the difference spectra in the range of 278 to 283 nm, and no loss of tyrosine was observed (data not shown). Similarly, the two cysteine residues were unmodified, as determined by titration with DTNB (data not shown). Thus, under these experimental conditions no cysteine or tyrosine residues were modified.

These results indicate that both histidine residues, His-8 and His-88, are modified by DEPC. To determine which histidine residue reacted in the fast phase and which in the slow phase, the time courses of modification were measured in single histidine proteins. The His-R-substituted ArsC, which is inactive and has only His-88, reacted rapidly with DEPC (Fig. 4A). The active H88R enzyme, with only His-8, lost activity at the same rate as the wild type enzyme (Fig. 4B), and His-8 was N-carbethoxylated slowly (Fig. 4A). Preincubation with the substrate arsenate protected H88R from inactivation by DEPC (data not shown). In contrast, the product, arsenite, had little effect. These results suggest that modification of His-8 leads to loss of reductase activity.

The reductase activity of H88R reacted with a 10-fold excess of DEPC could be fully restored by treatment with neutral hydroxylamine for 2 h (Table II). During the same period the disappearance of a single N-carbethoxylated histidine was observed (data not shown). When the enzyme was incubated with a 100-fold excess of DEPC, it was completely inactivated, and only partial restoration of activity by hydroxylamine with time was observed (Table II). Restoration of activity eliminates possible modification of the lysines and the N terminus of the enzyme in DEPC inactivation, since modification of amino groups are not readily reversed with hydroxylamine. These results are consistent with modification of His-8 being solely responsible for inactivation of reductase activity by diethylpyrocarbonate.

**Determination of the pK_a of the Cys-12**—The rate of inactivation of the C106S enzyme, which has only Cys-12, by DTNB was determined (Fig. 5A). The dependence of k_obs on the concentration of DTNB was linear (Fig. 5B). The pH-dependent increase of k_obs at 25 μM DTNB indicates a group with a pK_a of 6.5 is involved in the reaction (Fig. 5C). DTNB is specific for cysteine thiolates. Since the only thiol present in C106S is Cys-12, that thiolate has a pK_a of 6.5.
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Thus His-88 is unrelated to catalysis. Cells bearing four different *arsC* genes with mutations in the codon for His-88 were each arsenate-sensitive. Three of the gene products were purified and shown to be catalytically inactive.

The kinetics of DEPC modification indicated that both histidine residues were modified. Through the use of altered proteins having only His-8 or His-88, it was shown that His-88 reacted rapidly with DEPC. His-8 reacted more slowly but with complete loss of activity. The activity of the DEPC-modified enzyme could be restored by treatment with neutral hydroxylamine, and spectroscopic determination of N-carboxyhistidine residues demonstrated regeneration of His-8 by treatment with hydroxylamine.

The substrate arsenate protected His-8 from DEPC modification, suggesting that it is in or near the active site. Since the source of reducing equivalents for arsenate reduction are glutathione and the thiol carrier glutaredoxin (6), it is reasonable that a cysteine residue would be involved in catalysis, and Cys-12 has previously been identified as an active site residue (7). What is the relationship between His-8 and Cys-12? The reductase has a pH optimum of about 6.5, considerably lower than the *K*_µ* for 8.3 for free cysteine. Other proteins with active site cysteine thiolates have acidic pH optimum, and in such proteins there is a basic residue near the cysteine that forms a stable ion pair with it (8–10). Therefore the possibility of His-8 and Cys-12 forming a thiolate-imidazolium charge pair was investigated. The *K*_µ* of Cys-12 was found to be approximately 6.5, the same as the pH optimum of the reductase reaction. When another basic residue, arginine, was substituted for His-8, the *K*_µ* remained the same. Although catalytic activity is not maintained in the HSR enzyme, charge pairing is. When a neutral residue, glycine, was substituted for His-8, the *K*_µ* of Cys-12 increased to 8.3. These results are consistent with His-8 and Cys-12 forming a charge pair that lowers the *K*_µ* of the cysteine thiolate. This increases the nucleophilicity of Cys-12 at the pH optimum of the enzyme.

### DISCUSSION

The 141-residue ArsC enzyme reduces arsenate (As(V)) to arsenite (As(III)), the substrate of the ATP-coupled Ars pump that extrudes arsenite from cells of *E. coli* (6). Coupling of the reductase to the pump expands the range of resistance to include both the oxidized and reduced oxynitrides of arsenic. ArsC has a single essential cysteine residue, Cys-12 (7). In this report we demonstrate that reductase activity was inactivated by diethylpyrocarbonate, a histidine modifying reagent. ArsC has just two histidine residues, His-8 and His-88. By site-directed mutagenesis using degenerate oligonucleotides, mutations were introduced into the codons for His-8 and His-88. Cells expressing four different mutations in the codon for His-88 were each arsenate-resistant, and the purified substituted His-88 ArsC enzymes had wild type catalytic activity.

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