The Essential Catalytic Redox Couple in Arsenate Reductase from *Staphylococcus aureus*

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ABSTRACT: Arsenate reductase (ArsC) encoded by *Staphylococcus aureus* arsenic-resistance plasmid pI258 reduces intracellular As(V) (arsenate) to the more toxic As(III) (arsenite), which is subsequently extruded from the cell. ArsC couples to thioredoxin, thioredoxin reductase, and NADPH to be enzymatically active. A novel purification method leads to high production levels of highly pure enzyme. A reverse phase method was introduced to systematically analyze and control the oxidation status of the enzyme. The essential cysteinyl residues and redox couple in arsenate reductase were identified by a combination of site-specific mutagenesis and endoprotease-digest mass spectroscopy analysis. The secondary structures, as determined with CD, of wild-type ArsC and its Cys mutants showed a relatively high helical content, independent of the redox status. Mutation of Cys 10, 82, and 89 led to redox-inactive enzymes. ArsC was oxidized in a single catalytic cycle and subsequently digested with endoproteinases ArgC, AspN, and GluC. From the peptide-mass profiles, cysteines 82 and 89 were identified as the redox couple of ArsC necessary to reduce arsenate to arsenite.

Arsenic and antimony resistance on *ars* operons are found on plasmids and chromosomes in various organisms (*1*). In *Escherichia coli*, arsenic resistance encoded by plasmid R773 is governed by a five gene operon, *arsRDABC* (*2*). In *Staphylococcus aureus*, only three of these genes are identified as *arsRBC* on plasmid pI258 (*3*, *4*).

The *arsC* gene of both bacteria encodes an arsenate reductase (ArsC) which reduces intracellular As(V) (arsenate) to the more toxic As(III) (arsenite) (*5*). The highly reactive arsenite should be pumped out at once before it reacts with thiol groups and inactivates enzymes (*5*). In *S. aureus*, the arsenite is rapidly extruded out of the cell by an ATPase-independent, proton-driven transport system (ArsB*)* (*6*−*8*). The specificity of the extrusion system is such that arsenite/antimonite(III) are transported. This may be a solution to circumvent phosphate starvation because if the *ars* system were able to transport arsenate, it might also transport the structurally very similar phosphate oxyanion (*9*).

The arsenate reductase of *E. coli* encoded by the arsenical resistance operon of plasmid R773 has two cysteinyl residues, but only one is essential for catalysis (*10*). In the *E. coli* system, arsenate reductase reaction requires gluthathione (GSH)[1] and glutaredoxin, and its action postulates the formation of a covalently linked ArsC−GSH intermediate (*11*, *12*). Arsenate reductase (ArsC) encoded by *S. aureus* arsenic-resistance plasmid pI258 is a 14.8 kDa monomeric protein with only 12% identity with ArsC from *E. coli* R773. In vitro ArsC from *S. aureus* pI258 requires coupling to thioredoxin (Trx), thioredoxin reductase (TR), and NADPH to be enzymatically active (*13*). *S. aureus* ArsC has four cysteinyl residues: Cys 10, Cys 15, Cys 82, and Cys 89. Which Cys residues are essential for arsenate reduction had not been determined. Although Cys 10 and 82 had been postulated to be involved (*14*), this point was not documented or proven.

In this paper, all four Cys residues were one-by-one altered to alanine and/or serine, and double mutant constructs were made. New plasmid constructs have led to high expression yields of soluble enzyme in *E. coli*. Wild-type ArsC and the Cys mutants were purified and enzymatically and physically characterized. The different forms (oxidized/reduced) of ArsC were separated on C4 reverse phase HPLC and characterized by mass spectroscopy. By the combination of endoproteinase digestions on substrate-oxidized ArsC together with mass spectroscopy analysis, the redox couple responsible for electron donation to arsenate was assigned.

MATERIALS AND METHODS

Overexpression and Purification of Arsenate Reductase and Its Mutants. The *E. coli* strain JM109, with the pHNarsC wild-type plasmid (*13*) (with ampicillin resistance), was grown in a Luria−Bertani broth (LB) preculture with 100 μg/mL ampicillin for 6 h at 37 °C. The culture was transferred to Terrific broth (TB) with ampicillin, and induction was carried out in the exponential phase with 1 mM IPTG for 4 h. Cells were harvested by centrifugation at 4 °C and suspended in cold 50 mM Tris/HCl, pH 8.0, 50

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[1] Abbreviations: GSH, glutathione; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride; PDA, photodiode array; RPC, reverse phase chromatography; DTT, dithiothreitol; Trx, thioredoxin; TR, thioredoxin reductase; CSH, reduced cysteinyl residue; CS−SC, oxidized cysteinyl couple; MSO, methionyl oxidation.

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mM NaCl, 2 mM 2-mercaptoethanol, 0.1 mM EDTA, 0.1 mg/mL AEBSF, and 1 μg/mL leupeptin prior to French press disruption. Then 50 μg of DNase I/mL (EC 3.1.21.1; Sigma, St. Louis, MO) and 20 mM $MgCl_2$ were added, and the solution was left for 30 min at room temperature. Cell debris was removed by centrifugation for 30 min at 12000*g* at 4 °C, and the supernatant was brought to 55% ammonium sulfate. After pH adjustment to pH 8, it was kept for 1 h at 4 °C. Precipitated proteins were removed by centrifugation (30 min at 12000*g*). The supernatant was directly loaded at 225 cm/h onto a Phenyl Sepharose Fast Flow (HS) column equilibrated in 20 mM Tris/HCl, pH 8.0, 1.5 M ammonium sulfate, 0.1 mM EDTA, 2 mM 2-mercaptoethanol to trap recombinant ArsC. The column was eluted with a 2 step gradient, 5 column volumes each of 0.5 and 0 M ammonium sulfate in 20 mM Tris/HCl, pH 8, 0.1 mM EDTA, 2-mercaptoethanol. Recombinant ArsC eluted in the 0.5 M ammonium sulfate step which was dialyzed overnight to 20 mM Hepes/NaOH, pH 8, 0.1 mM EDTA, 2 mM 2-mercaptoethanol in 3500 dalton cutoff dialysis tubing (Spectrapor, Houston, TX) to lower the conductivity to approximately 1 mS/cm. The dialyzed pool was further purified on a Source30 Q anion-exchange column equilibrated in 20 mM Hepes/NaOH, pH 8, 0.1 mM EDTA, 2 mM 2-mercaptoethanol. Sample was loaded at 300 cm/h, and after a 2-column volume wash, the column was developed with a 10-column volume linear gradient to 300 mM NaCl in the same buffer. Recombinant ArsC eluted at approximately 150 mM NaCl. The ArsC-containing fractions were pooled and concentrated on a 5 kDa cutoff Sartocon micro tangential flow cassette (Sartorius, Goettingen, Germany) up to approximately 50 mL followed by a Vivaspin 5 kDa cutoff concentration step (Vivascience, Lincoln, U.K.). The concentrated sample was further purified on a Superdex75 PG (16/90) gel filtration column in 20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.1 mM EDTA, 2 mM 2-mercaptoethanol. All columns were run at room temperature on an Äkta-Explorer except for the size exclusion column that was operated at 4 °C on an FPLC. The size exclusion column was calibrated with a gel filtration standard from Biorad (Hercules, CA), i.e., γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and Vitamin B-12 (1.35 kDa).

Separation of ArsC Forms on Anion Exchange. ArsC was concentrated on a Vivaspin 5 kDa cutoff concentrator and dialyzed to water in a Slide-A-Lyzer 3.5K (Pierce, Rockford, IL). After dialysis and pH 8.0 adjustment, ArsC was loaded onto a 1 mL Resource Q column equilibrated in 20 mM Tris/HCl, pH 8.0, operated at 745 cm/h. The column was eluted with a gradient of 200-column volumes up to 20 mM Tris/HCl, pH 8, 300 mM NaCl. N-formylated ArsC was separated from the nonformylated form under those conditions.

Site-Directed Mutagenesis. pHNArsC (*13*) was used as DNA template in PCR amplification for construction of Cys mutants. The primers used were designed to specifically introduce respectively the mutations C10A, C15A, C82A/S, and C89A and the double mutations C10SC15A/S (*15*). They incorporate a *Nco*I restriction site at the start of the *arsC* gene and a *Hin*dIII site at the end. After restriction, nuclease digestion, and purification (Qiagen, Valencia, CA), the resulting PCR fragments were cloned into the expression vector pTrc99A (Amersham Pharmacia Biotech, Uppsala, Sweden) and transformed in *E. coli* strain JM109 (*16*). Growth conditions and purification method were similar as described for wild-type ArsC.

Overexpression and Purification of Thioredoxin Reductase. Wild-type *E. coli* thioredoxin reductase was grown in a *trx*B$^-$ strain of *E. coli* containing a high copy number plasmid which carried the *trxB* gene and imparted trimethoprim resistance (k1380.pTrR1) (*15*). Cells were grown in Mueller Hilton broth at 37 °C. Cells were harvested and disrupted by the French press in 20 mM Tris/HCl, pH 7.6, 0.1 mM EDTA, 0.1 mg/mL AEBSF, and 1 μg/mL leupeptin. Then 50 μg of DNase I/mL and 20 mM $MgCl_2$ were added to the lysate, and it was left for 30 min at room temperature. After centrifugation (30 min at 12000*g*), the supernatant was dialyzed overnight at 4 °C against 2 times 2 L of 20 mM Tris/HCl, pH 7.6, 0.1 mM EDTA. This pool was directly loaded onto a Poros50 HQ (Perseptive Biosystems, Cambridge, MA) column equilibrated in the same buffer at room temperature. The enzyme was eluted with a step gradient of 0.1 M NaCl increments, with thioredoxin reductase eluting in the 0.3 M NaCl step. This main peak was pooled and dialyzed against 4 L of 10 mM potassium phosphate, pH 7.6, at 4 °C overnight. The sample was applied to a 2′,5′-ADP Sepharose 4B column equilibrated in the same buffer and washed with 0.2 M NaCl steps until thioredoxin reductase eluted at 0.6 M NaCl. Both columns were operated at room temperature.

HPLC chromatography was carried out on a 600S Controller coupled to a 996 PDA detector (Waters, Milford, MA) equipped with a Rheodyne 9125 (Cotati, CA) injector using a reverse phase C4 column (4.6 × 250 mm) (214TP54) (Vydac, Hesperia, CA) equilibrated in 15% acetonitrile, 0.1% TFA at 1 mL/min. The column was developed with a 25 min linear gradient from 15% to 50% acetonitrile at room temperature. Absorption data collection at 215 nm was performed under Millennium (Waters).

The peptides generated by endoproteinase digestion were separated on the same system using a Vydac C18 (4.6 × 250 mm) reverse phase column (218TP54) equilibrated in 10% acetonitrile, 0.1% TFA at 1 mL/min. The peptides were separated in a linear gradient of 20 min from 10% to 70% acetonitrile in 0.1% TFA at room temperature.

Peptide Mapping. Wild-type ArsC and ArsC C15A mutant both at 1.4 mg/mL were reduced in the presence of 40 mM 2-mercaptoethanol for 1 h at room temperature. The reduction was checked on reverse phase C4 under the conditions as described. Subsequently, 2-mercaptoethanol was removed, and the sample was brought under the respective buffer conditions for endoprotease digestion by running it through an analytical Superdex75 HR (10/30) gel filtration column. For GluC, 50 mM ammonium bicarbonate, pH 7.8, 100 mM KCl was used, and for AspN and ArgC, 20 mM Tris/HCl, pH 8, 150 mM NaCl was used. ArsC and ArsC C15A were oxidized in the presence of 1 mM arsenate for 30 min at 37 °C. Percentage of oxidation was checked on reverse phase C4 chromatography. Samples were boiled for 5 min and cooled on ice, and endoproteinase was added as recommended by the manufacturer. Digestion was performed overnight at 37 °C. Peptides were separated on reverse phase C18 and were analyzed with mass spectroscopy. Peptide profiles were compared with the computer-generated fragments using Peptide Mass from the SWISS PROT sequence database.

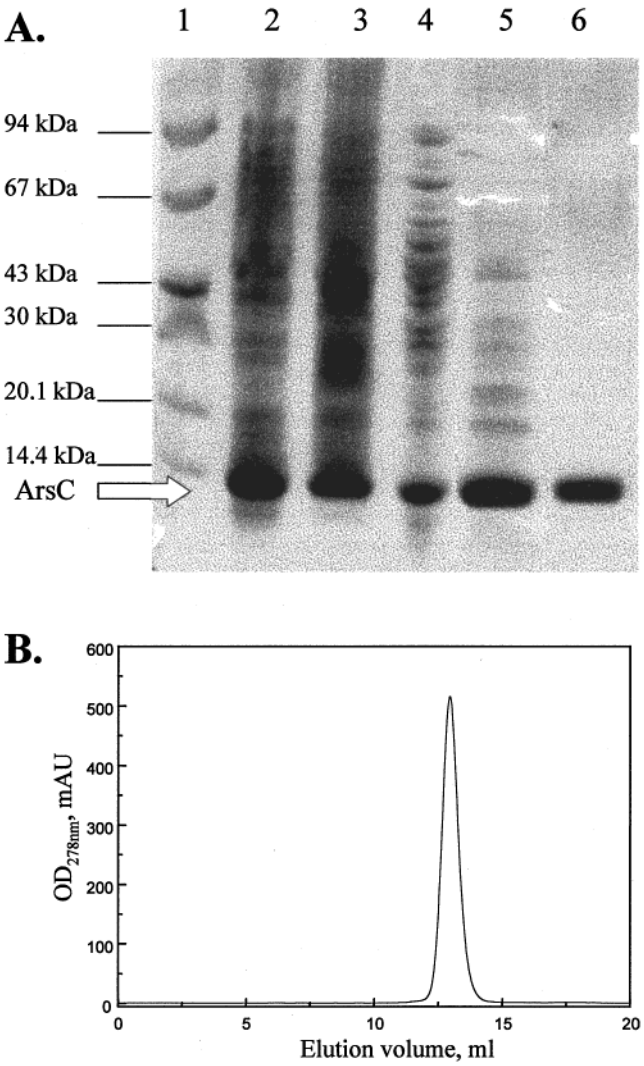

FIGURE 1: (A) ArsC purification stages shown on a reduced Tricine−SDS−PAGE gel. Lane 1: Molecular mass markers (Amersham Pharmacia Biotech): phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-LACTALBUMIN (14.4 kDa). Lane 2: Cytoplasmic cell extract. Lane 3: Sample of the supernatant from a 55% ammonium sulfate precipitation. Lane 4: Sample from pooled ArsC fractions after Phenyl-Sepharose Fast Flow. Lane 5: Sample from pooled fractions after anion-exchange Source30 Q. Lane 6: Sample from ArsC after gel filtration on Superdex75PG 16/90. (B) ArsC analyzed on an analytical gel filtration column, Superdex75 HR (10/30). The column was operated in 20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM 2-mercaptoethanol at room temperature and was calibrated with a gel filtration standard from Biorad. ArsC eluted at 12.97 mL, corresponding to 21 kDa.

Electrospray mass spectrometry was carried out in a Quattro II quadrupole mass spectrometer (Micromass, Manchester, U.K.) having a *m*/*z* range of 4000, equipped with an electrospray interface. The mass spectrometer was scanned over a *m*/*z* range appropriate for the material being examined. Calibration was performed with PEG2000 and verified with horse heart hemoglobin (Sigma, St. Louis, MO). Samples were injected directly into the electrospray source via a Rheodyne 7125 (Cotati, CA) loop injector. The mobile phase was water/acetonitrile (50/50) at a flow rate of 20 μL/min using a Harvard syringe pump (Harvard 22). The source conditions were optimized for each sample with the following average conditions: capillary potential, 3.00 kV; cone voltage, 50−90 V. The source was maintained at a temperature of 60 °C. Spectra were recorded in the continuum mode using the Masslynx NT software (Micromass) and analyzed with either the Transform or the MaxEnt algorithm.

Table 1: Observed Masses of Wild-Type ArsC (14 812 Da Is the Calculated Mass of the Reduced Form) Determined with Mass Spectroscopy

mass (Da)	reduced	oxidized, −2 Da	formylated, +28 Da	Met-oxidation,[a] +16 Da	Na^+-form, +23 Da
14810		X			
14812	X				
14826		X		1	
14842		X		2	
14858		X		3	
14865		X		2	X
14874		X		4	
14881		X		3	X
14883	X			3	X
14918		X	X	5	
14934		X	X	6	
14957		X	X	6	X

[a] Number of oxidations of the three methionines in ArsC.

CD Spectroscopy. ArsC and its Cys mutants were reduced in the presence of 40 mM 2-mercaptoethanol for 1 h at room temperature. Subsequently, buffer was changed to 50 mM boric acid, pH 8, 100 mM KF, and 2-mercaptoethanol was removed on an analytical Superdex75 HR column operated at 1 mL/min, at room temperature. Samples were diluted to approximately 0.2 mg/mL in the respective buffers, and for each sample, 30 scans were taken between 184 and 250 nm in a J-715 spectropolarimeter (Jasco, Tokyo, Japan). Secondary structure estimation was performed based on a database of 33 proteins established from crystallographic data (Dicroprot V2.3d, Gilbert Deléage, Institut de Biologie et Chemie des Protéines, Lyon, France).

Arsenate Reductase Enzyme Assay. A spectrophotometric assay measuring NADPH oxidation by a decrease in absorption at 340 nm coupled to arsenate reduction was performed in a buffer of 20 mM Tris/HCl, pH 7.5, 150 mM NaCl at 37 °C. Assays were performed with different arsenate concentrations (nanomolar to millimolar range) and arsenate reductase concentrations (nanomolar range) in the presence of 0.42 μM *E. coli* thioredoxin, 0.14 μM *E. coli* thioredoxin reductase (purified as described), and 125 μM NADPH. The K_m values were determined in a microtiter plate reader, Elx808iu (Bio-TEK Instruments, Winooski, VT), and calculated from Origin 5.0 (Microsoft). The k_{cat} values were measured in a UVIKON 923 double-beam UV/VIS spectrophotometer (KONTRON, Milano, Italy) using a 100 μL cuvette with a 10 mm path length (HELLMA, Germany).

Arsenate Reductase C10SC15A/Thioredoxin (Glutathione) Reduction Experiments. ArsC C10SC15A was oxidized by dialysis to water in a Slide-A-Lyzer 3.5K. Then 8.8 μM ArsC C10SC15A was incubated for 5 min at 37 °C in 20 mM Tris/HCl, pH 7.5, with respectively an equimolar concentration of DTT, *E. coli* Trx, reduced *E. coli* Trx, and a double molar concentration of reduced GSH. Prior to starting the 5 min incubation with ArsC C10SC15A, 17.6 μM Trx and 17.6 μM GSH were both separately incubated for 30 min at 37 °C with an equimolar amount of DTT. Oxidized ArsC C10SC15A (14 μM) was also incubated with respectively

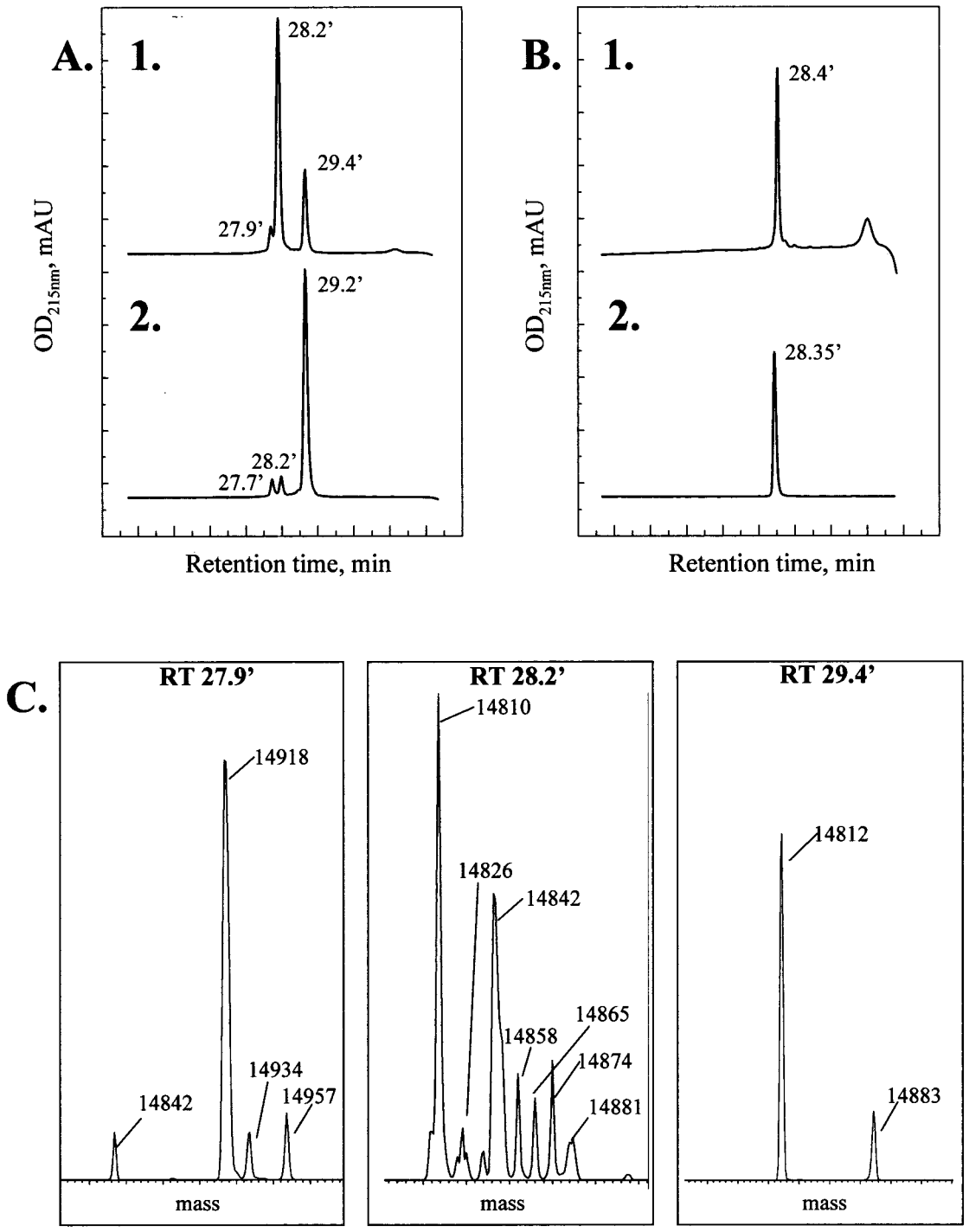

FIGURE 2: C4 reverse phase chromatographic profile monitored at 215 nm of size exclusion column purified wild-type ArsC (A1) and wild-type ArsC after reduction (40 mM 2-mercaptoethanol) or under assay conditions after incubation with thioredoxin, thioredoxin reductase, and NADPH at 37 °C (A2). Purified ArsC C82S (B1) and after its reduction with 40 mM 2-mercaptoethanol (B2). Reconstructed mass spectra of wild-type ArsC between 14 500 and 15 000 Da corresponding to peaks with retention times of 27.9, 28.2, and 29.4 min from the C4 reverse phase column shown in panel A1 (C).

20 μM DTT, 4 μM Trx, and the mixture of 20 μM DTT, 4 μM Trx for 15 min at 37 °C in 20 mM Tris/HCl, pH 7.5. After incubation, samples were analyzed on reverse phase C4 under the conditions described.

Materials. Reduced GSH, endoproteinases GluC from *Staphylococcus aureus* V8, AspN from *Pseudomonas fragi,* and ArgC from *Clostridium histolyticum* were all sequencing grade and purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). *E. coli* thioredoxin was obtained from Promega (Madison, WI). The following items were all purchased from Amersham Pharmacia Biotech (Uppsala, Sweden): Phenyl SepharoseFF (HS), Source30 Q, Superdex75 PG, Superdex75 HR (10/30), 2′,5′-ADP−Sepharose 4B, Äkta-Explorer, FPLC, pTrc99A. Acetonitrile HPLC Ultra Gradient Grade was purchased from Baker (Deventer, Holland); arsenate (Na_2HAsO_4, A-6756) was bought from Sigma. AEBSF and leupeptin are products from ICN (Aurora, OH).

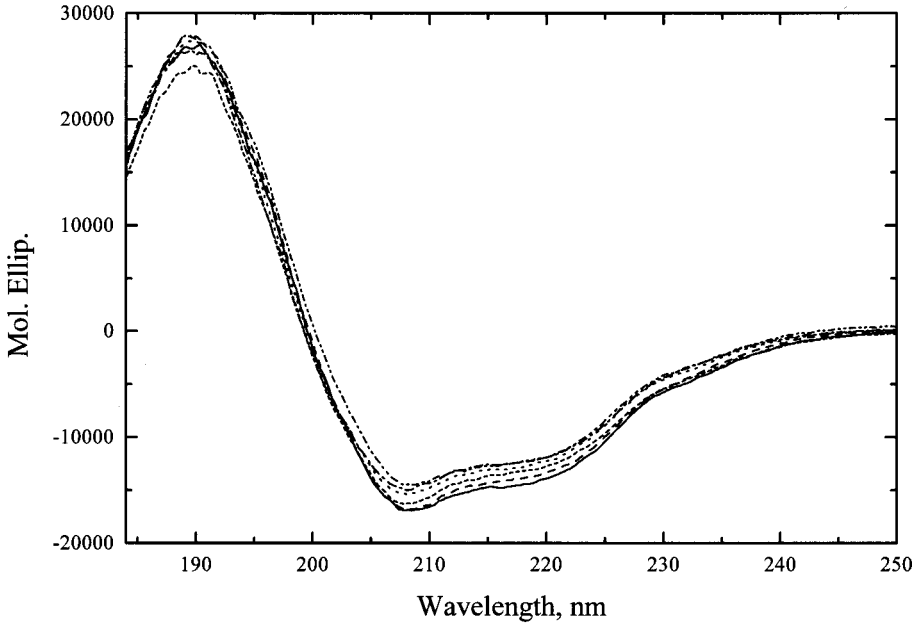

FIGURE 3: Comparison of CD spectra of ArsC between 250 and 184 nm. Reduced ArsC wild type (- - -), ArsC C10A (- · -), ArsC C15A (···), ArsC C82S (—), ArsC C89A (— —), and ArsC C10SC15A (— ·· —) after buffer change to 50 mM boric acid, pH 8.0, 100 mM KF.

RESULTS

Expression and Purification of Arsenate Reductase. Wild-type ArsC and the Cys mutants (C15A, C10A, C82A, C82S, C89A, C10SC15S, C10SC15A) were expressed in *E. coli*. Only wild-type ArsC, ArsC C15A, ArsC C10A, ArsC C82S, ArsC C89A, and ArsC C10SC15A were expressed soluble and with high yields. ArsC C82A was found in inclusion bodies, and the double mutant C10SC15S was not expressed. A purification method was designed to purify ArsC and its Cys mutants from the soluble fraction. [Previously (*13*), the refolding and purification of arsenate reductase from inclusion bodies had been described.] Briefly, ammonium sulfate precipitation followed by hydrophobic interaction chromatography and size-exclusion chromatography produced the required degree of purity. The final yields obtained after purification were 16 mg/L culture for wild-type ArsC, 25 mg/L for ArsC C15A, 36 mg/L for ArsC C10A, 23 mg/L for ArsC C82S, 37 mg/L for ArsC C89A, and for ArsC C10SC15A a yield of 58 mg/L culture. During purification, the purity was checked on a reduced Tricine−SDS−PAGE (*17*) (Figure 1A) gel. ArsC was always detected as a single, strong band at the 14.4 kDa marker position of α-lactalbumin. The protein, as eluted from the final gel filtration column, was more than 97% pure. Wild-type ArsC and the Cys mutants eluted at approximately 21 kDa from the size exclusion column (Figure 1B).

Oxidized and Reduced Arsenate Reductase. Although the ArsC protein appeared homogeneous on SDS−PAGE, reverse phase C4 revealed three major elution peaks with retention times of 27.9, 28.2, and 29.4 min (Figure 2A1). Mass spectroscopy profiles of these fractions (Figure 2C) can be interpreted as follows: the first C4 RPC peak with retention time 27.9 min as the oxidized N-formylated ArsC, the second peak with retention time 28.2 min as the nonformylated oxidized form of ArsC, and the last peak with retention time 29.4 min as the reduced ArsC. The complex pattern of each fraction can be explained as due to variations in N-formylation, methionine oxidation (sulfoxides and sulfones), oxidized and reduced ArsC, and a sodium ion bound form (Table 1). Not only wild-type ArsC but also the Cys mutants produced these spectra, and the amount of formylation or methionine oxidation was not consistent and varied with each batch as a function of time.

ArsC is highly susceptible to oxidation. An overnight incubation at 4 °C, even in the presence of a reductant (2 mM β-mercaptoethanol), resulted in a C4 RPC peak shift toward the oxidized form. Renewed addition of 2 mM DTT or 40 mM 2-mercaptoethanol, sufficient for full reduction, led to the expected reversal (Figure 2A2). C4 reverse phase chromatography was also found to be an efficient method to follow the reduction reaction under standard assay conditions, when ArsC was incubated in the presence of thioredoxin, thioredoxin reductase, and NADPH at 37 °C (Figure 2A2). This peak shift after reduction was only seen for wild-type ArsC, ArsC C15A, ArsC C10A, and ArsC C10SC15A. The ArsC Cys mutants C82S and C89A showed no peak shift after reduction with 40 mM 2-mercaptoethanol or in the presence of DTT (Figure 2B).

Secondary Structure of Arsenate Reductase. Wild-type ArsC and its Cys mutants were reduced in an excess of 40 mM 2-mercaptoethanol, and subsequently the buffer was changed on an analytical size exclusion column to an appropriate buffer for CD measurements in the far-UV. The redox status of ArsC was systematically controlled on a C4 RPC. The CD spectra of wild-type ArsC and all studied Cys mutants looked similar (Figure 3) with a high α-helical content. For wild-type ArsC and the Cys mutants, 35% α-helical content, 15% β-sheet, 21% turn, and 30% random coil were calculated. Contrary to an earlier report (*13*), we were not able to observe any significant change in the magnitude of the ellipticity after the addition of 50 μM or 2 mM arsenate or between oxidized and reduced ArsC (data not shown). In all cases, the oxidation status of ArsC was checked on C4 RPC. The secondary structure of ArsC was found to be redox-status-independent.

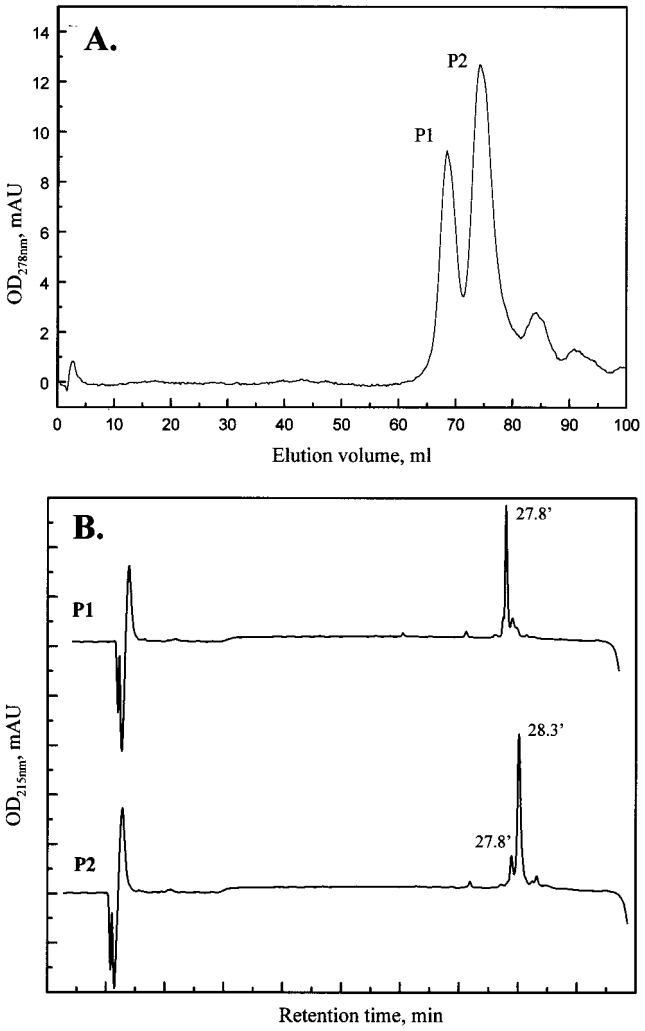

FIGURE 4: (A) Chromatographic elution profile of fully oxidized ArsC C15A on a 1 mL Resource Q column. The pH 8 adjusted sample was loaded on a 1 mL Resource Q column equilibrated in 20 mM Tris/HCl, pH 8.0, and eluted with a 200-column volume gradient to 300 mM NaCl in the same buffer. (B) P1 and P2 from part A were separately pooled and concentrated on a 5 kDa cutoff concentrator and subsequently analyzed on reverse phase C4. The chromatographic profile at 215 nm is shown.

Enzyme Activity. Wild-type ArsC and all Cys mutants were tested in a coupled thioredoxin, thioredoxin reductase, NADPH oxidation assay. Only ArsC wild type and ArsC C15A showed enzymatic activity. All other ArsC mutants (C10A, C82S, C89A, and C10SC15A) showed no enzymatic activity.

For the kinetic characterization of ArsC and in view of the heterogeneity in the samples (Figure 2C), we developed one more additional purification step. An additional Resource Q column run under nonreducing conditions, and with a shallow gradient of 200-column volumes to 300 mM NaCl, allowed the separation of N-formylated from nonformylated ArsC (Figure 4A). Both elution peaks were analyzed on C4 RPC (Figure 4B) and with mass spectroscopy. Peak 1 was the N-formylated oxidized form while the second elution peak was the oxidized ArsC.

The profile of NADPH oxidation followed Michaelis−Menten kinetics for wild-type ArsC and ArsC C15A, resulting in apparent K_m's of respectively 66 and 134 nM (Figure 5). Wild-type ArsC and ArsC C15A were both found

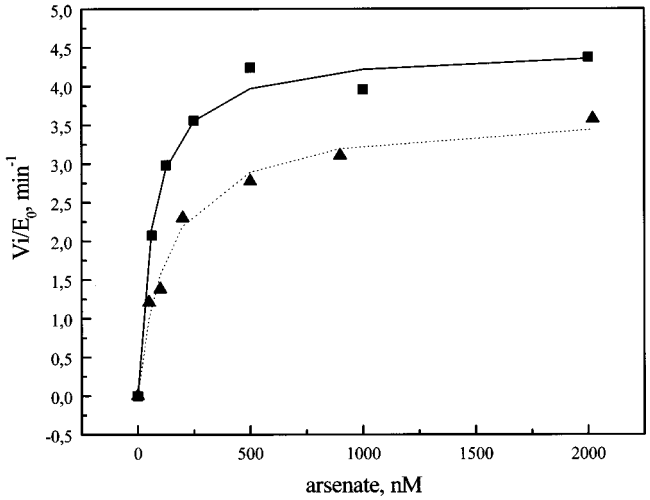

FIGURE 5: Michaelis−Menten plot, V_i/E_0 (min^{-1}) versus substrate [arsenate] (nM), of wild-type ArsC (■) (K_m = 66 nM, k_{cat} = 4.5 min^{-1}) and ArsC C15A (▲) (K_m = 134 nM, k_{cat} = 3.6 min^{-1}). Assays were performed in 20 mM Tris, pH 7.5, 150 mM NaCl at 37 °C with increasing arsenate concentrations in the presence of 30 nM ArsC, 0.42 μM *E. coli* thioredoxin, 0.14 μM *E. coli* thioredoxin reductase, and 125 μM NADPH.

to have two k_{cat} values. For wild-type ArsC, a k_{cat} of 4.5 min^{-1} at 2 μM AsO_4^{3-} and a k_{cat} of 9.9 min^{-1} at 10 mM AsO_4^{3-} were measured. This doubling of k_{cat} at a 5000-fold higher concentration of arsenate, and the similar observation in 10 mM phosphate at 10 μM arsenate concentration, confirms earlier observations (*13*). ArsC C15A showed a similar phenomenon but with slightly different k_{cat} values of respectively 3.66 min^{-1} (Figure 5) and 4.8 min^{-1}. At micromolar substrate concentrations, for wild-type ArsC a k_{cat}/K_m value of 1.17×10^6 M^{-1} s^{-1} was calculated, and for mutant C15A, a 2.6 times lower k_{cat}/K_m value of 4.41×10^5 M^{-1} s^{-1} was found. Doubling the concentration of thioredoxin and thioredoxin reductase had no effect on the NADPH oxidation rate.

Redox Couple of Arsenate Reductase. In *E. coli*, ArsC of plasmid R773 was shown to be active with only one cysteine (Cys 12), and the formation of a mixed disulfide complex between ArsC and GSH was hypothesized (*11*, *12*). When *S. aureus* wild-type ArsC was incubated for 1 h at room temperature with arsenate and thioredoxin in a 1/2/100 molar ratio, no covalently linked thioredoxin−ArsC intermediate form could be detected by C4 RPC mass spectroscopy analysis. From these results, one can conclude that the electron transfer probably involved an intramolecular disulfide pair. ArsC C15A already lacks one cysteinyl residue and is almost as active as wild type. This makes it an easier target for the determination of the redox couple essential for ArsC activity. Mutant ArsC C15A was oxidized in the presence of its substrate arsenate, allowing for a single round of reduction leading to nearly 100% of ArsC in the oxidized form. Subsequently, the oxidized ArsC was digested with respectively endoproteinases ArgC, AspN, and GluC. The peptide mixtures were separated with C18 RPC and analyzed by electrospray mass spectroscopy. Based on the peptide maps, the disulfide connectivity was worked out (Figure 6). The presence of multiple ArsC forms (oxidation, N-formylation, Na^+-salts) (Figure 2C) results in a complex of peptide masses. Nevertheless, only one disulfide-containing fragment linking two peptides was observed (2957 Da fragment after

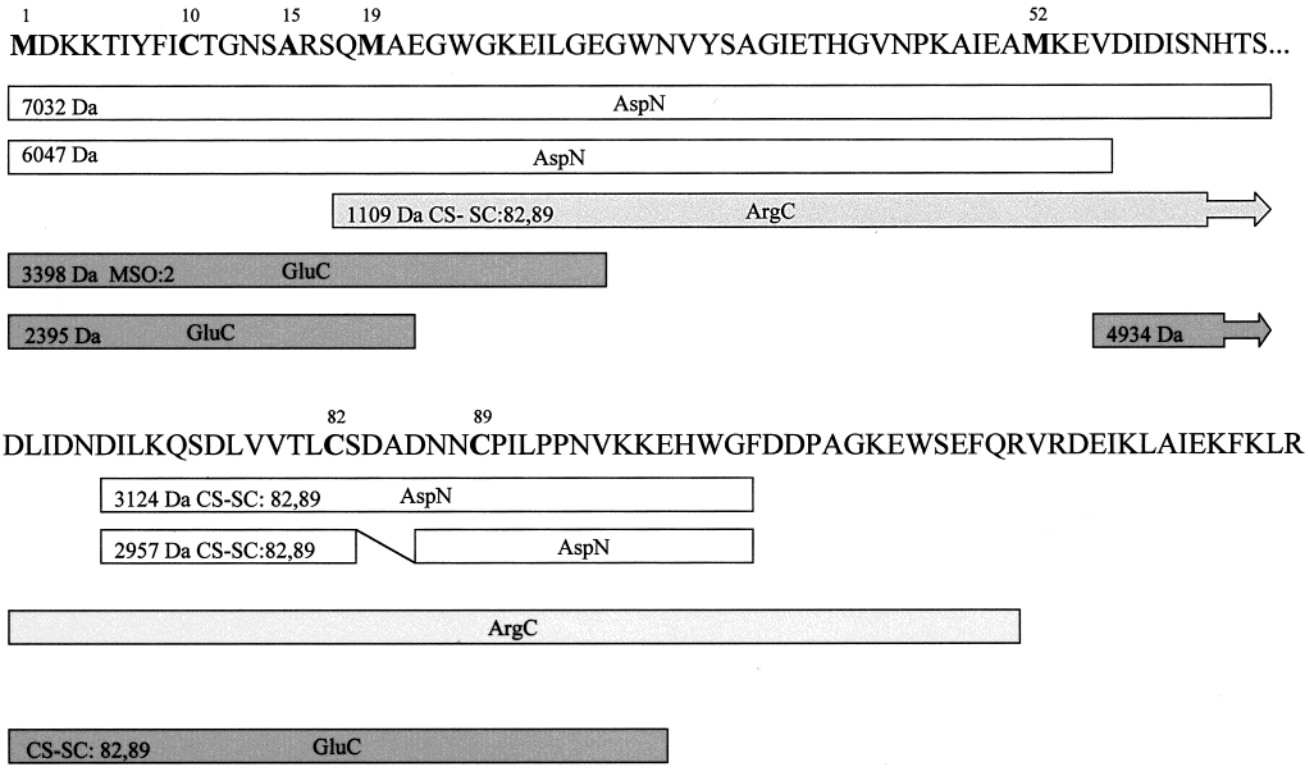

FIGURE 6: Analytical summary of endopeptidase digestion data. The top line(s) give(s) the sequence of ArsC C15A in one-letter code with the methionines and cysteines in boldface (sequence position on top). Rectangles below show relevant fragments for the determination of the redox couple identified with the name of the peptidase used. ArsC C15A was oxidized in the presence of its substrate arsenate and digested respectively with endoproteinases ArgC, AspN, and GluC. Resulting peptides were separated by C18 reverse phase chromatography and identified with electrospray mass spectroscopy. The fragments obtained by AspN, ArgC, and GluC digestion are respectively shown in different gray scales. In each fragment, the total mass in daltons is indicated. CS−SC in the box indicates the observation of a disulfide bridge between cysteinyls 82 and 89 in a fragment. MSO followed by a number indicates the number methionines oxidized in a fragment.

AspN digest) (Figure 6). Fragments containing disulfides involving Cys 10 were never observed. Cysteines 82 and 89 were identified as the redox couple of Ars C necessary for electron donation to arsenate. This experiment has been repeated for wild-type ArsC, and the results lead to the same conclusion.

The assignment of the active redox couple of ArsC was confirmed by an experiment where the oxidized ArsC double mutant C10SC15A was incubated with DTT and thioredoxin in a 3.5/5/1 molar ratio for 15 min at 37 °C. Reverse phase C4 analysis resulted in an almost complete shift toward the reduced peak of ArsC with the characteristic retention time of 29.4 min, whereas ArsC C10SC15A incubated with either DTT or thioredoxin alone showed no peak shift at all (Figure 7C,D,G). The DTT in this experiment served as reductant for thioredoxin, and under the chosen condition, it was not directly reducing the enzyme. Another experiment with equimolar amounts of ArsC C10SC15A and reduced Trx leads to the same result (Figure 7F). The specificity of Trx as electron donor for ArsC is shown, as GSH was not capable of reducing ArsC (Figure 7E,F). As such, the capacity of thioredoxin to interact with ArsC, leading to the reduction of the oxidized ArsC double mutant C10SC15A, confirmed cysteinyl 82 and 89 as the active redox couple.

DISCUSSION

In this report on arsenate reductase from *Staphylococcus aureus* plasmid pI258, the essential cysteinyl residues were defined (C10, C82, and C89) and the redox couple involved in electron donation was assigned (C82 and C89). A novel purification method was developed to obtain highly pure enzyme, and a C4 reverse phase method was introduced, allowing for the fast analysis of the oxidation status of the enzyme.

The solubly expressed ArsC migrated on SDS−PAGE in a single band indicating a molecular mass of approximately 14 kDa and gave a single sharply defined symmetrical peak of 21 kDa on an analytical gel filtration column (Figure 1). The aberrant elution position on the size exclusion column is indicative for a protein with a rather big volume and a rather extended conformation or could be due to a specific interaction with the matrix. Expression both in soluble form and in inclusion bodies occurred. In this study, however, we used only the soluble expressed, folded ArsC. The yields were high (16−58 mg/L of culture). The optimized C4 reverse phase analysis conditions resulted in a reproducible, accurate separation of three forms of ArsC: N-formylated oxidized, nonformylated oxidized, and reduced ArsC. Mass spectroscopy analysis of these different forms confirmed the molecular weight of the monomeric protein of 131 amino acids. [ArsC lacking the three N-terminal amino acids (*13*) has not been observed under our expression and purification conditions.]

ArsC is highly susceptible to oxidation, and although the purification was done in the presence of EDTA and 2-mercaptoethanol, the oxidized and the reduced form were both detected. ArsC has three methionines, and during the overproduction and purification, a fraction of these methion-

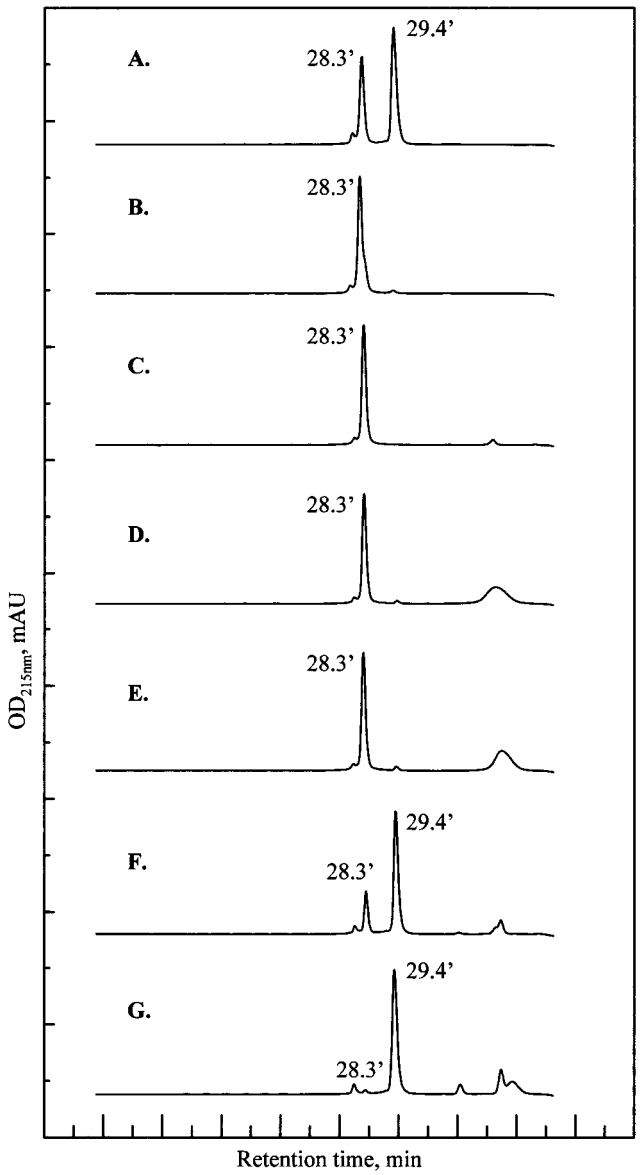

FIGURE 7: C4 reverse phase chromatographic profiles at 215 nm of ArsC C10SC15A under different incubation conditions. Size exclusion purified ArsC C10SC15A (A) was oxidized by dialysis against water (B). Oxidized ArsC C10SC15A (8.8 μM) incubated for 5 min at 37 °C with 8.8 μM DTT (C), with 8.8 μM Trx (D), with 17.6 μM reduced GSH (E), and with 8.8 μM reduced Trx (F). Oxidized ArsC C10SC15A (14 μM) incubated with a mixture of 4 μM Trx and 20 μM DTT at 37 °C for 15 min (G). Oxidized ArsC C10SC15A (14 μM) incubated with respectively 20 μM DTT and 4 μM Trx at 37 °C for 15 min resulted in similar chromatographic profiles as shown in parts C and D.

ines became oxidized. The observation of methionine sulfoxides and sulfones, the N-formylation, and the presence of both oxidized (CS−SC 82, 89) and reduced ArsC (CSH 82, CSH 89) reveal a complex mixture under the single peak on the gel filtration column (Figure 1B, Figure 2, and Table 1).

Wild-type ArsC and all ArsC mutants studied showed nearly identical CD spectra (Figure 3), indicating properly folded proteins, and this despite the observation that the majority of the mutants were enzymatically inactive. The spectra were insensitive to changes in the redox state of ArsC. Neither did the addition of arsenate produce any change to the spectrum (data not shown). In previous work (*13*), a reduction of the magnitude of ellipticity was found after the addition of arsenate. This result was not confirmed.

Wild-type ArsC has a high affinity for arsenate with a K_m of 66 nM for AsO_4^{3-} and replacing Cys 15 with Ala reduced the affinity by only a factor of 2. The soluble overexpressed ArsC had a 10 times smaller K_m as compared to the refolded ArsC from inclusion bodies in the earlier study (*13*). For both wild-type ArsC and ArsC C15A, unusually low turnover numbers of 4.5 and 3.6 per minute were found at 2 μM AsO_4^{3-} (Figure 5), confirming earlier data (*13*). At a 5000-fold higher substrate concentration (10 mM) the k_{cat} value increased 2-fold. However, this remains low for an enzyme. A similar observation (*13*) led to the hypothesis that it may be necessary to keep the ArsC activity rate below that for toxic arsenite efflux by the ArsB membrane protein.

The analysis by mass spectroscopy of the ensemble of carefully separated endoproteinase digest fragments, obtained from ArsC that underwent one round of oxidation, unequivocally identified Cys 82 and Cys 89 as catalytic cysteines (Figure 6).

Further, the systematically observed difference of 2 Da between oxidized and reduced ArsC (Figure 2C, Table 1) already indicated the involvement of one and only one intramolecular thiol pair in the oxidation process. An additional observation strongly corroborating the above findings concerned the inability of the C82S and C89A mutants to produce the characteristic shifts in retention time upon oxidation and reduction (Figure 2B).

Finally: only thioredoxin and not glutathione was able to regenerate oxidized ArsC C10SC15A (Figure 7). This confirms the thioredoxin specificity for ArsC and means that either Cys 82 or Cys 89 in oxidized ArsC C10SC15A is nucleophilically attacked by the active thiolate of Cys 32 of thioredoxin (*18*), leading to the reduced form. The oxidation interaction of the ArsC C10SC15A double mutant with thioredoxin as the redox equivalent donor not only proves the sufficiency of the Cys 82−Cys 89 pair, but also in addition proves the retention of the full interaction potential with thioredoxin. Indeed, not only the active site residues from thioredoxin (Cys 32 and Cys 35) are essential for the reduction of a wide range of oxidized proteins but also the integral structure participates in the thioredoxin−target recognition (*19*). *E. coli* thioredoxin (this study and *13*) and *Spirulina* thioredoxin (unpublished results) are possible electron donors for ArsC. The physiological electron donor for *S. aureus* ArsC has not been identified.

All our experiments indicate that arsenate reductase from *Staphylococcus aureus* uses an intramolecular thiol pair (Cys 82, Cys 89) for the reduction of arsenate. This is a completely different observation from what was found for *E. coli* arsenate reductase, where the formation of an intermediate covalently linked ArsC−GSH complex is postulated to be responsible for electron donation toward arsenate (*11*, *12*). This result may come as a surprise, but *E. coli* (Gram −) ArsC and *S. aureus* (Gram +) ArsC only have a sequence identity of 12%. This suggests they have strongly diverged or are truly different proteins with different catalytic mechanisms.

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