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Development of a downstream process for the isolation of *Staphylococcus aureus* arsenate reductase overproduced in *Escherichia coli*

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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). In order to study the structure of ArsC and to unravel biochemical and physical properties of this redox enzyme, wild type enzyme and a number of cysteine mutants were overproduced soluble in *Escherichia coli*. In this paper we describe a novel purification method to obtain high production levels of highly pure enzyme. A reversed-phase method was developed to separate and analyze the many different forms of ArsC. The oxidation state and the methionine oxidized forms were determined by mass spectroscopy.

Keywords: Purification; *Staphylococcus aureus*; *Escherichia coli*; Enzymes; Arsenate reductase

1. Introduction

Bacterial resistance to metals provides good model systems for transport-mediated resistance and has been thoroughly documented [1–6]. The determinants for metal resistance have been found on transmissible plasmids [7,8], transposons [9] and chromosomes [10]. This also applies to arsenic and antimony resistance, where *ars* operons are found on plasmids and chromosomes in various organisms [11]. In *Escherichia coli* arsenic resistance encoded by plasmid R773 is governed by a five-gene operon *ArsRDABC*. In *Staphylococcus aureus* only three of these genes are identified as *arsRBC* on plasmid pI258 [12,8]. The *arsC* gene of both bacteria encodes an arsenate reductase (ArsC) which reduces intracellular As(V) (arsenate) to the more toxic As(III) (arsenite) [13]. The accumulation of As(III) within the cytosol is undesirable and the highly reactive arsenite should be pumped out at once before it reacts with thiol groups and inactivates enzymes [13]. In *Staphylococcus aureus*, the arsenite is rapidly extruded out of the cell by an ATPase-independent, proton-driven transport system (*arsB*), while in *Escherichia coli* an ATP-dependent transport system (*arsAB*) is responsible for the transport [14–16]. The metal specificity of the extrusion system is such that the arsenite (III) and antimonite (III) are transported. This may be a wise solution to circumvent defense against phosphate starvation because if the *ars* system were able to transport

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arsenate, it might also transport the structurally very similar phosphate oxyanion [1].

Arsenate reductase (ArsC) encoded by *Staphylococcus aureus* arsenic-resistance plasmid pI258 is a soluble, heat-stable monomeric protein (M_r 14 800) [17,18]. A previous report on arsenate reductase described the refolding and purification of arsenate reductase from inclusion bodies [17]. In vitro ArsC requires coupling to thioredoxin, thioredoxin reductase and NADPH to be enzymatically active. The mechanism of catalysis and structure of ArsC are not known.

In order to study the structure of ArsC from *Staphylococcus aureus* with ^{15}N-, ^{13}C-nuclear magnetic resonance (NMR) and X-ray crystallography and to unravel its catalytic mechanism, ArsC and some of its cysteine mutants were overproduced in *Escherichia coli*. All four cysteine residues were one-by-one altered to alanine. The chosen plasmid constructs have led to high expression yields of soluble enzyme and a novel purification method was developed to purify ArsC to a high purity. During the purification the purity of ArsC was evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The different forms (oxidized/reduced) of ArsC were separated by C_4 reversed-phase high-performance liquid chromatography (HPLC) and characterized by mass spectroscopy (MS). After the gel-permeation-polishing step an extra anion-exchange column, developed with an extremely shallow salt-gradient, was introduced to increase the homogeneity of the ArsC sample for X-ray crystallographic applications.

2. Experimental

2.1. Chemicals

Acetonitrile HPLC ultra gradient grade was purchased from Baker (Deventer, The Netherlands). Tris ultra pure, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) and leupeptine were products from ICN (Aurora, OH, USA). Isopropylthiogalactoside (IPTG), dioxane-free, high purity was purchased from Calbiochem (La Jolla, CA, USA). *N*-(Hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid (HEPES), 2-mercaptoethanol and DNase I (EC. 3.1.21.1) were obtained from Sigma (St. Louis, MO, USA) and $MgCl_2$, HCl and NaOH were from Merck (Darmstadt, Germany).

2.2. Site-directed mutagenesis

pHNArsC [17] was used as the DNA template in polymerase chain reaction (PCR) amplification for construction of Cys mutants. The primers used were designed to specifically introduce, respectively, the mutations C10A, C15A, C82A, C89A [19]. They incorporate a NcoI restriction site at the start of the *arsC* gene and a HindIII site at the end. After restriction and purification (Qiagen, Valencia, CA, USA), the resulting PCR fragments were cloned into the expression vector pTrc99A (Amersham Pharmacia Biotech, Uppsala, Sweden) and transformed in *E. coli* strain JM109 [20].

2.3. Culture conditions

The *E. coli* strain JM109 [20], with the pHNarsC wild type plasmid or the Cys mutant constructs (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 m*M* IPTG for 4 h.

2.4. Equipment

Cells were broken with a French Pressure Cell press (Spectronic Instruments, Rochester, NY, USA) in a 20 K Cell and compared with sonication in a Soniprep 150 (Sanyo, Leicester, UK). Cells were harvested and precipitated proteins were removed by centrifugation in a J2-MI centrifuge in, respectively, a JA10 and JA20 rotor (Beckman Instruments, Palo Alto, CA, USA).

For filtration of protein samples a 0.22-μm Falcon Bottle Top Filter (Beckton Dickinson, NJ, USA) was used. Conductivity and pH were measured with, respectively, a Consort P107 and a Consort K620 (Turnhout, Belgium).

For protein dialysis a relative molecular mass (M_r) 3500 cut-off dialysis tubing (Spectrapor, Houston, TX, USA) or a Slide-A-Lyzer 3.5K (Pierce, Rock-

ford, IL, USA) was used. Protein concentration was performed with a M_r 5000 cut-off Sartocon micro tangential flow cassette (Sartorius, Goettingen, Germany) and a Vivaspin M_r 5000 cut-off concentrator (Vivascience, Lincoln, UK).

All liquid chromatography (LC) runs were performed at room temperature on an Äkta-Explorer (Amersham Pharmacia Biotech) except for the size-exclusion column Superdex 75PG (16/90) that was operated at 4°C on an fast protein liquid chromatography (FPLC) system (Amersham Pharmacia Biotech). HPLC was carried out on a 600S Controller coupled to a 996 photo diode array (PDA) detector (Waters, Milford, MA, USA) equipped with a Rheodyne 9125 (Cotati, CA, USA) injector. Data collection at 215 nm was performed under Millennium (Waters). Final protein concentrations were measured at 278 nm in an UV–Vis spectrophotometer (Kontron, Milan, Italy).

2.5. Chromatographic columns

The following resins were all purchased from Amersham Pharmacia Biotech: Phenyl Sepharose Fast Flow (high substitution), Source30 Q, 1 ml Resource Q, Superdex75 PG, Superdex75 HR (10× 300 mm). Poros20 HQ was bought from Perseptive Biosystems (Framingham, MA, USA). For the HPLC runs a Vydac reversed-phase C_4 column (4.6×250 mm) (214TP54) (Hesperia, CA, USA) was used. The Superdex75 HR (10/30) column was calibrated with a gel-permeation standard from Bio-Rad (Hercules, CA, USA).

2.6. Mass spectrometry

Electrospray MS was carried out in a Quattro II quadrupole mass spectrometer (Micromass, Manchester, UK) 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 poly ethylene glycol (PEG2000) and verified with horse heart hemoglobin (Sigma). Samples were injected directly into the electrospray source via a Rheodyne 7125 loop injector. The mobile phase was water–acetonitrile (50:50, v/v) at a flow-rate of 20 μl/min using a Harvard syringe pump (Harvard 22). The source conditions were optimized for each sample with 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 trashed out with either the Transform or MaxEnt algorithm.

3. Results and discussion

In order to study the structure of a protein it is favorable to start from soluble protein, which reduces the risk of using improperly folded protein. In a previous report [17] the refolding and purification of arsenate reductase out of inclusion bodies was described for wild type ArsC. New expression and growth conditions were used and a novel purification method was designed to purify ArsC and its Cys mutants from the soluble fraction. Cys mutants were chosen because they will be used in future experiments to unravel the catalytic mechanism of this redox enzyme. Wild type ArsC and its Cys mutants (C15A, C10A, C82A, C89A) were expressed in *E. coli*, but only ArsC wild type, ArsC C15A, ArsC C10A and ArsC C89A were expressed in soluble form. The Cys mutant C82A was found in inclusion bodies. Expression and cellular localization were evaluated by SDS–PAGE [21]. The following purification method was designed with overexpressed soluble wild type ArsC and was applicable to all soluble Cys mutants of ArsC.

3.1. Cell harvesting conditions

A good purification method starts with a proper cell harvesting step in the right buffer conditions based on the intrinsic properties of the protein. Wild type ArsC contains 131 amino acids, has a molecular mass of 14 812 and a calculated isoelectric point (p*I*) of 4.9. Wild type ArsC has four cysteinyl residues and they are thought to play an essential role in the catalysic mechanism of this redox enzyme. ArsC precipitated out of solution below pH 6.5 and was kept in solution in crude lysate above pH 7. 50 m*M* Tris, pH 8 was the minimum buffer capacity necessary to avoid a pH drop during cell breakage followed by ArsC precipitation. AEBSF, leupeptine

and EDTA were added as protease inhibitors. AEBSF was preferred to phenylmethylsulfonyl fluoride (PMSF) because AEBSF is water-soluble and was found to be more stable at mildly alkaline pH as compared to PMSF. During the purification the redox enzyme was preferably kept in its reduced form to finally end up with catalytically active enzyme after purification. Although dithiothreitol (DTT) is known to be a better reductant than 2-mercaptoethanol, ArsC precipitates in the crude lysate in the presence of DTT and therefore, 2-mercaptoethanol was chosen as reductant. These observations resulted in the following harvesting step. *E. coli* cells overexpressing wild type ArsC were harvested by centrifugation at 10 000 *g* for 15 min at 4°C and were suspended in cold 50 m*M* Tris–HCl, pH 8.0, 50 m*M* NaCl, 2 m*M* 2-mercaptoethanol, 0.1 m*M* EDTA, 0.1 mg/ml AEBSF and 1 μg/ml leupeptine. For cell disruption a three-stroke French Press disruption at 103 MPa in a 20K cell resulted in more efficient cell breakage than sonication.

3.2. Sample clarification

After an efficient cell breakage, DNA was contaminating the crude lysate. Before removing the cell debris a DNase I treatment reduced the viscosity of the lysate and resulted in a compact cell debris pellet after a single centrifugation run. Therefore, 50 μg DNase I/ml and 20 m*M* $MgCl_2$ were added and the crude lysate was left for 30 min at room temperature. The amount of $MgCl_2$ necessary for DNase I activity was doubled to compensate for the presence of EDTA. Cell debris was removed by centrifugation for 30 min at 12 000 *g* at 4°C.

ArsC was found to be soluble at pH 8 in the presence of 55% ammonium sulfate. Higher concentrations of ammonium sulfate resulted in ArsC precipitation and lower yields. Addition of ammonium sulfate up to 55% resulted in a pH drop below pH 6.5 and subsequently ArsC starts to precipitate out of solution. It is highly important to adjust the pH of the lysate to pH 8 after the addition of ammonium sulfate to guarantee final high yields. After removal of the cell debris the lysate was brought to 55% ammonium sulfate, the pH was adjusted to pH 8 and the solution was kept for at least 1 h at 4°C. Precipitated contaminating proteins were removed by centrifugation for 30 min at 12 000 *g*.

3.3. ArsC capture

The design of a fast capture step to separate ArsC from contaminants such as proteases is a necessity in a good purification protocol. The major fraction of the contaminating proteins was removed with the ammonium sulfate step. As the supernatant, after removal of the contaminating proteins, contains 55% ammonium sulfate, hydrophobic interaction chromatography (HIC) to trap ArsC became the logical choice. Phenyl Sepharose 6 Fast Flow (high substitution) with a high protein binding capacity (>30 mg/ml human serum albumin) was found to be a good chromatographic medium to quickly trap ArsC (Fig. 1). Nevertheless, the column dimensions were chosen to be large enough (26×150 mm) to avoid possible ArsC displacement by highly hydrophobic components in the medium. The supernatant, after removal of the precipitated contaminants, was 0.2-μm filtrated. Subsequently the supernatant was loaded onto a Phenyl Sepharose Fast Flow (high substitution) column in down-flow mode at a flow-rate of 225 cm/h (flow through detection: the first peak in Fig. 1). The column was equilibrated in 20 m*M* Tris–HCl, pH 8.0, 1.5 *M* ammonium sulfate, 0.1 m*M* EDTA, 2 m*M* 2-mercaptoethanol to trap recombinant ArsC and was washed until baseline optical density (O.D.) conditions occurred. Subsequently, the column was developed with a two-step gradient, five column volumes each of 0.5 *M* and 0 *M* ammonium sulfate in 20 m*M* Tris–HCl, pH 8, 0.1 m*M* EDTA, 2-mercaptoethanol in up-flow mode to reduce the elution volume of the ArsC containing fractions. Recombinant ArsC eluted in the 0.5 *M* ammonium sulfate step (Fig. 1).

3.4. Intermediate purification step

For the intermediate purification step where resolution and capacity are the important purification parameters ion-exchange chromatography was chosen. Because ArsC was insoluble below pH 6.5 and has a calculated p*I* of 4.9, an anion-exchange resin was the only choice. Two anion-exchange

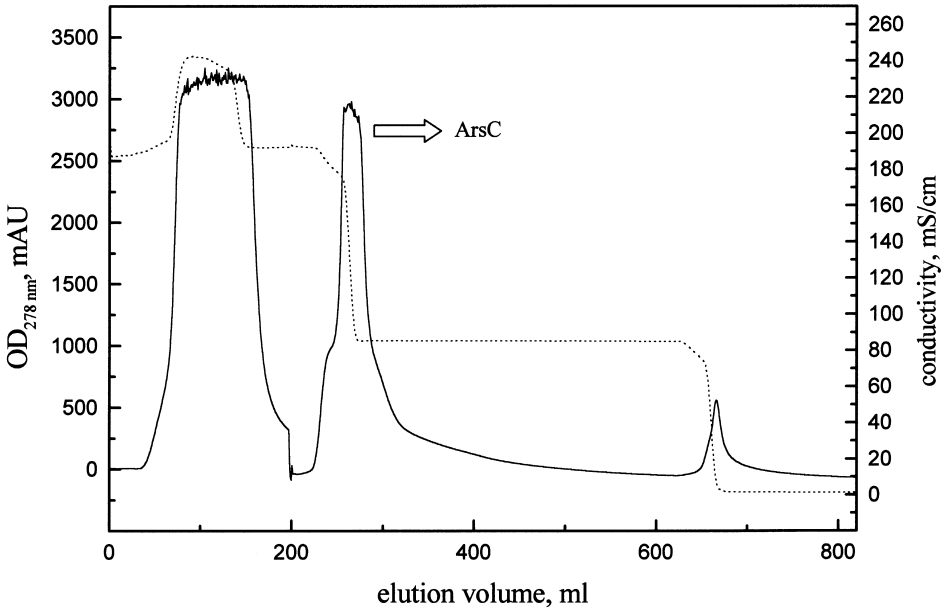

Fig. 1. Elution profile of ArsC from phenyl Sepharose Fast Flow (high substitution) (26×150 mm) operated at room temperature at a flow-rate of 20 ml/min. The conductivity was monitored during the run (dot line). The column was equilibrated in 20 m*M* Tris–HCl, pH 8.0, 1.5 *M* ammonium sulfate, 0.1 m*M* EDTA, 2 m*M* 2-mercaptoethanol to trap ArsC. The column was developed with a two-step gradient, five column volumes each of 0.5 *M* and 0 *M* ammonium sulfate in 20 m*M* Tris–HCl, pH 8, 0.1 m*M* EDTA, 2-mercaptoetnanol. Recombinant ArsC eluted in the 0.5 *M* ammonium sulfate step.

stationary phases were tested, a Poros20 HQ resin and a Source30 Q medium, each in two different buffer systems, Tris–HCl, pH 8 and HEPES–NaOH, pH 8. During the runs on both matrices the pH was monitored online. It was found that the use of Tris–HCl resulted in a drop in pH during the protein desorption with NaCl and that Source30 Q showed the best pH stability. For Poros20 HQ the pH was dropping below pH 6.5, resulting in ArsC precipitation on the column. With the HEPES–NaOH, pH 8 buffer system we found an increase in pH during the protein desorption process with NaCl and here as well Source30 Q was the most pH stable resin. As ArsC was known to be unstable below pH 6.5, the best anion-exchange chromatographic step for ArsC was on Source30 Q in a HEPES–NaOH, pH 8 buffer system.

Not only a proper resin and buffer system needs to be chosen for ArsC, also the binding conditions on Source30 Q were not obvious. It turned out that in order to insure complete binding of ArsC on the anion-exchange column the conductivity needs to be approximately 1 mS/cm. The ArsC containing fractions from the HIC column were pooled based on SDS–PAGE analysis and subsequently dialyzed overnight to 20 m*M* HEPES–NaOH, pH 8.0, 0.1 m*M* EDTA, 2 m*M* 2-mercaptoethanol in a M_r 3500 cut-off dialysis tubing 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 m*M* HEPES–NaOH, pH 8, 0.1 m*M* EDTA, 2 m*M* 2-mercaptoethanol. Sample was loaded and ran with a flow-rate of 300 cm/h and after a two-column volume wash the column was eluted with a 10-column volume linear gradient to 300 m*M* NaCl in the same buffer. Recombinant ArsC eluted in a broad peak at approximately 150 m*M* NaCl (Fig. 2).

3.5. Polishing

In the polishing phase of a purification protocol the focus is on removal of trace contaminants to achieve final purity. Next to removal of the last

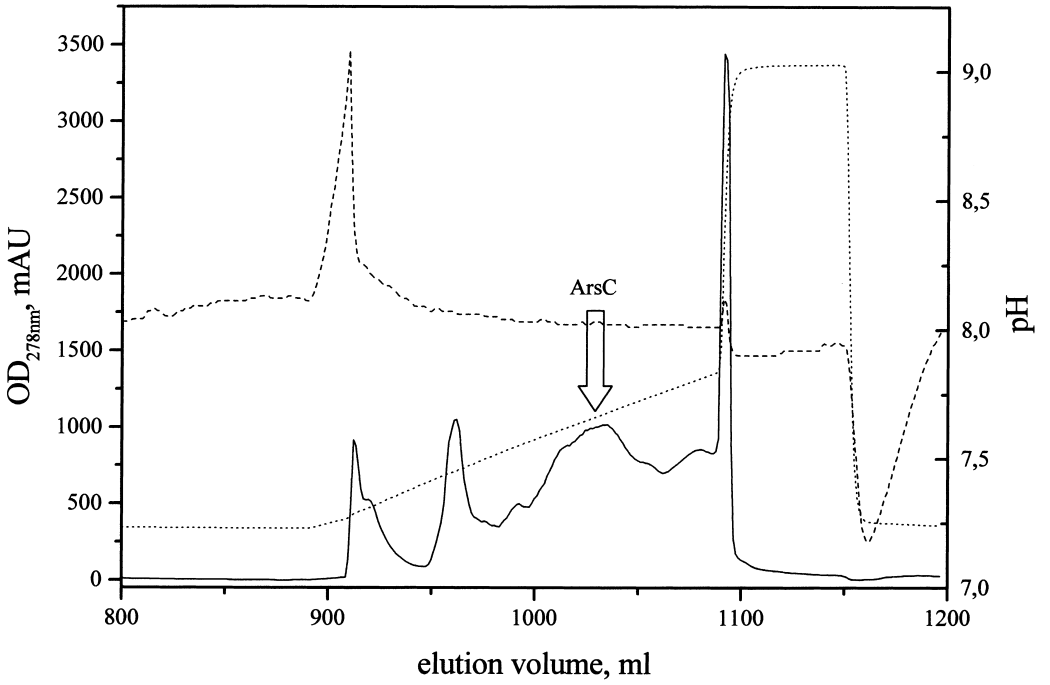

Fig. 2. Elution profile at 278 nm of ArsC from Source30 Q (16×100 mm) operated at room temperature with a flow-rate of 10 ml/min. The conductivity (dot line) and pH (dash line) were monitored during the run. The dialyzed pool of ArsC post HIC capture was further purified on a Source30 Q anion-exchange column equilibrated in 20 m*M* HEPES–NaOH, pH 8, 0.1 m*M* EDTA, 2 m*M* 2-mercaptoethanol. Sample was loaded and after a two-column volume wash the column was developed with a 10-column volume linear gradient to 300 m*M* NaCl in the same buffer. Recombinant ArsC eluted in a broad peak at approximately 150 m*M* NaCl in the run.

contaminants a buffer adjustment to bring the protein in the right storage or analysis conditions is important during this stage. Therefore, a high-resolution resin with high recovery is a necessity. Size-exclusion chromatography on a Superdex75PG was fulfilling this requirement for ArsC. One drawback of this method is the maximum loading volume of a size-exclusion column, being only 2% of the total column volume to assure high resolution purification. The volume of the pool of the ArsC containing fractions after ion-exchange (IEX) was rather large when a relatively slow elution gradient was applied. Therefore, an extra concentration step prior to size-exclusion purification was necessary. The ArsC containing pool was concentrated on an M_r 5000 cut-off Sartocon micro tangential flow cassette up to approximately 50 ml followed by a Vivaspin M_r 5000 cut-off concentration step. Concentration of ArsC was never found to be a problem, as concentrations up to 2.6 m*M* (38 mg/ml) were possible without protein precipitation. The concentrate (12 ml) was run in four sequential runs on a Superdex75 PG (16×900 mm) gel-permeation column in 20 m*M* Tris–HCl, pH 8, 150 m*M* NaCl, 0.1 m*M* EDTA, 2 m*M* 2-mercaptoethanol at 4°C (Fig. 3). This buffer was found to be a good buffer for storage of ArsC at 4°C and did not interfere with activity measurements.

3.6. Analysis

The final protein concentrations were determined with $O.D._{278\ nm}$ measurements based on the calculated extinction coefficient [22] of ArsC, i.e., $\epsilon^{0.1\%}_{278\ nm}=1.7$. The final protein yields after purification were: 16 mg/l culture for wild type ArsC, 25 mg/l for ArsC C15A, 36 mg/l for ArsC C10A and 37 mg/l for ArsC C89A. The yields are higher as compared to what was found in previous work [17] where a final yield of 5 mg/l culture was found after the purification from inclusion bodies.

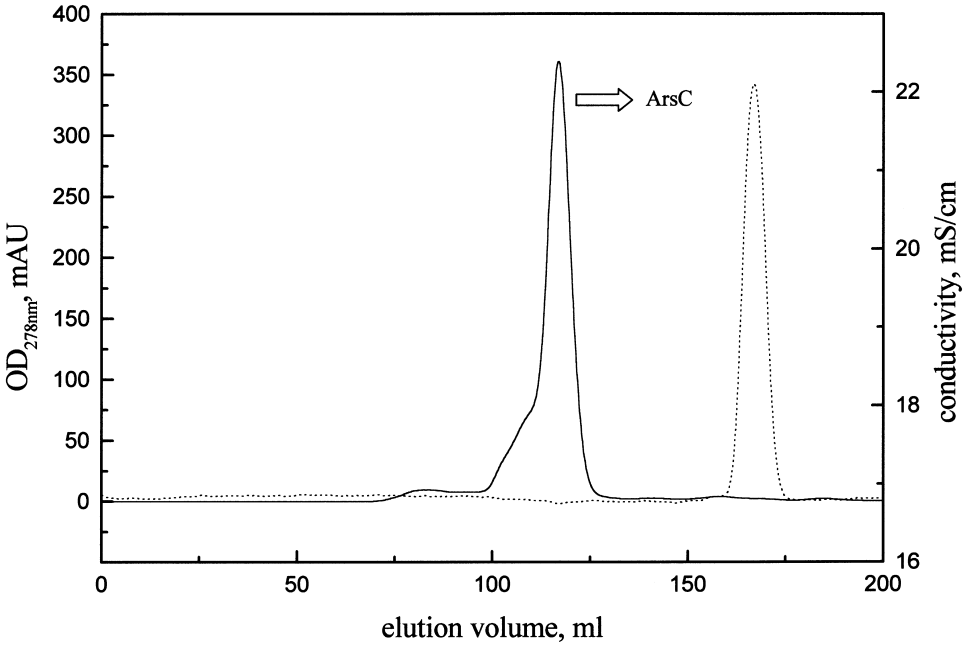

Fig. 3. Elution profile at 278 nm of ArsC from Superdex75 PG (16×900 mm) operated at 4°C at 45 cm/h in 20 m*M* Tris–HCl, pH 8, 150 m*M* NaCl, 0.1 m*M* EDTA, 2 m*M* 2-mercaptoethanol. The conductivity was monitored during the run (dot line).

During the purification the sample purity was checked on a reduced Tricine-SDS–PAGE gel [23] (Fig. 4). Due to the complexity of the coupled enzyme assay [17] it was impossible to follow ArsC activity in a reliable way during the downstream process. SDS–PAGE analysis to screen for the enzyme during the purification became a necessity. ArsC was always detected, as a single band, at the position of the α-lactalbumin marker protein (14 400). After the final gel-permeation column, the wild type ArsC pool and the pools of the Cys mutants were evaluated on an analytical Superdex75HR column (Fig. 5). ArsC eluted with a molecular mass of approximately 21 000 from the size-exclusion column. The size-exclusion column was calibrated with a gel-permeation standard, i.e., γ-globulin (158 000), ovalbumin (44 000), myoglobin (17 000) and vitamin B-12 (1350). The aberrant elution position on size-exclusion column is indicative for a protein with a rather big volume, a rather extended conformation or could be due to aspecific interaction with the matrix (although 150 m*M* NaCl was included in the elution buffer).

The purified ArsC was further analyzed on re-

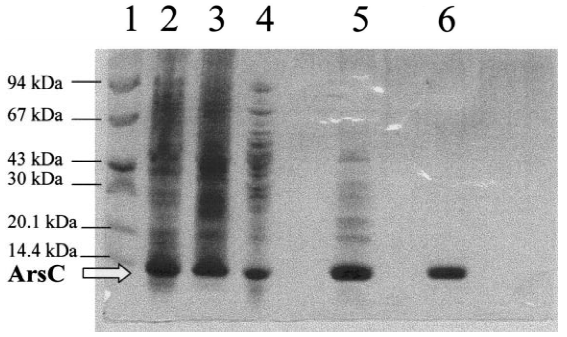

Fig. 4. ArsC purification stages shown on a reduced Tricine-SDS–PAGE gel. Lane 1: low-molecular-mass marker (Amersham Pharmacia Biotech), phosphorylase b (94 000), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100) and α-lactalbumin (14 400); lane 2: cytoplasmic cell extract; lane 3: sample of the supernatant from a 55% ammonium sulfate precipitation step; lane 4: sample from pooled ArsC fractions after HIC on Phenyl Sepharose FF; lane 5: sample from pooled fractions after anion-exchange Source30 Q, lane 6: Sample from ArsC after gel permeation chromatography on Superdex75PG 16×900 mm.

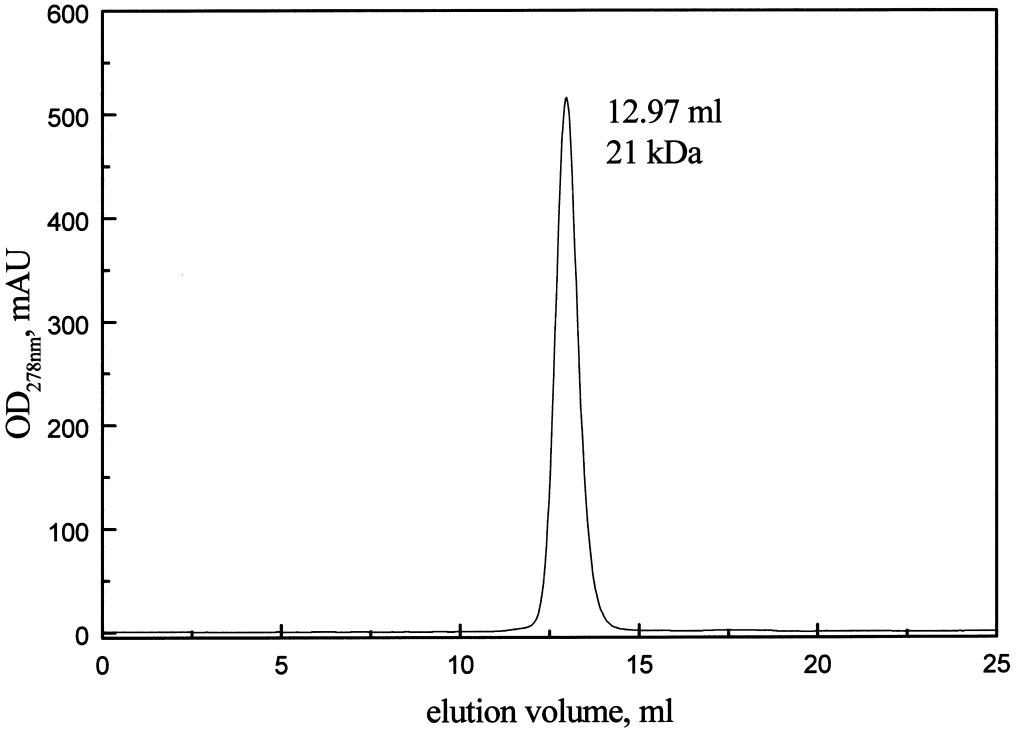

Fig. 5. ArsC analyzed on an analytical gel-permeation column Superdex75 HR (10×300 mm). The column was operated in 20 m*M* Tris, pH 8.0, 150 m*M* NaCl, 2 m*M* 2-mercaptoethanol at room temperature and was calibrated with a gel-permeation standard from Bio-Rad.

versed-phase C_4. Concentrated ArsC was diluted with 0.1% trifluoroacetic acid (TFA) and immediately injected onto a C_4 reversed-phase column equilibrated in 15% acetonitrile, 0.1% TFA at 1 ml/min. The fast pH drop below the p*I* of ArsC to approximately pH 2 gave no ArsC precipitation. The column elution conditions were optimized to obtain an accurate, reproducible, high-resolution separation. This resulted in the following final conditions: after sample injection the column was ran isocratically for 5 min followed by a 25 min linear gradient from 15% to 50% acetonitrile at room temperature. Although the protein appeared to be fairly pure based on SDS–PAGE analysis (Fig. 4) and eluted as a single, symmetrical peak from the size-exclusion column (Fig. 5), the analysis on reversed-phase C_4 revealed three major elution peaks with retention times of 27.9, 28.2 and 29.4 min (Fig. 6A). After the addition of 2 m*M* DTT or 40 m*M* 2-mercaptoethanol it was possible to shift the peak with a retention time of 28.2 min for more than 90% to the one with a retention time of 29.4 min (Fig. 6B). This indicates that the last two peaks observed on C_4 are, respectively, the oxidized and reduced forms of ArsC.

3.7. Mass spectroscopy

MS analysis of the three elution peaks detected on reversed-phase C_4 revealed that all three peaks are ArsC forms with a slightly different molecular mass (Fig. 7). MS analysis of the first C_4 reversed-phase chromatography (RPC) peak with a retention time of 27.9 min (Fig. 7A) was interpreted as oxidized, *N*-formylated ArsC (Table 1). In the second C_4-RPC peak with a retention time of 28.2 min (Fig. 7B) different non-formylated oxidized forms of ArsC were found (Table 1). For the last C_4-RPC peak with a retention time of 29.4 min (Fig. 7C) MS analysis was interpreted as reduced ArsC (Table 1). In oxidized ArsC only one disulfide bridge was formed resulting in a relative molecular mass difference of 2 (Table 1). Those mass spectra results were not only

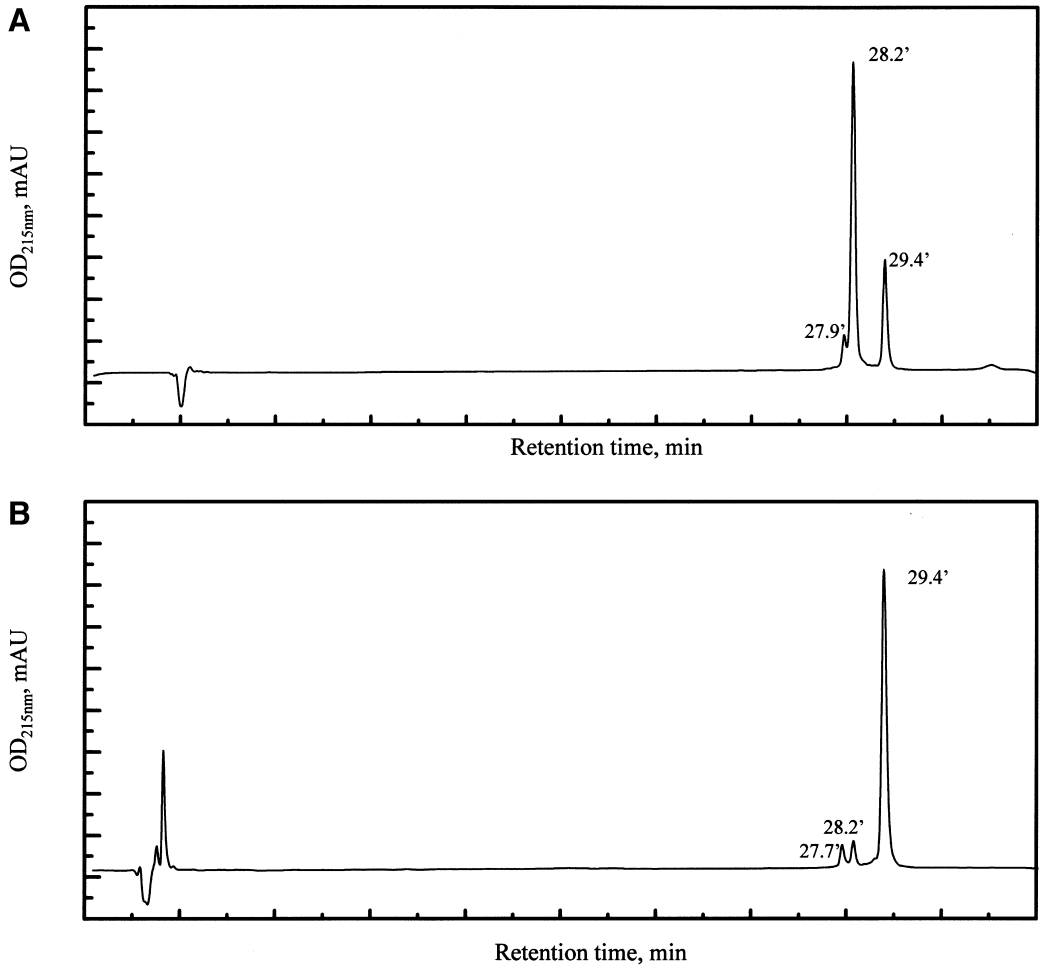

Fig. 6. (A) C_4 reversed-phase chromatographic profile at 215 nm of size-exclusion column purified wild type ArsC. (B) C_4 reversed-phase chromatographic profile at 215 nm of wild type ArsC after reduction 40 m*M* 2-mercaptoethanol or 2 m*M* DTT.

found for wild type ArsC but also for the Cys mutants and the amount of formylation or methionine oxidation was not batch consistent and varies as a function of time.

An overnight incubation at 4°C even in the presence of a reductant (2 m*M* mercaptoethanol), resulted in a peak shift towards the oxidized form. This enzyme 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 at position 1, 19 and 52 and during the overproduction and purification a fraction of the methionines became oxidized and the N-terminal methionine was partially formylated. The formation of methionine sulfoxides and sulfones, the formylation, the presence of both oxidized and reduced ArsC results in a highly inhomogeneous mixture which is hidden under the single peak on the gel-permeation column (Fig. 7, Table 1).

3.8. An extra polishing step

An accurate and reproducible analytical method was developed on reversed-phase C_4 to separate the reduced and oxidized form of ArsC. The oxidation state of ArsC was evaluated with this analytical method but to generate a highly homogeneous

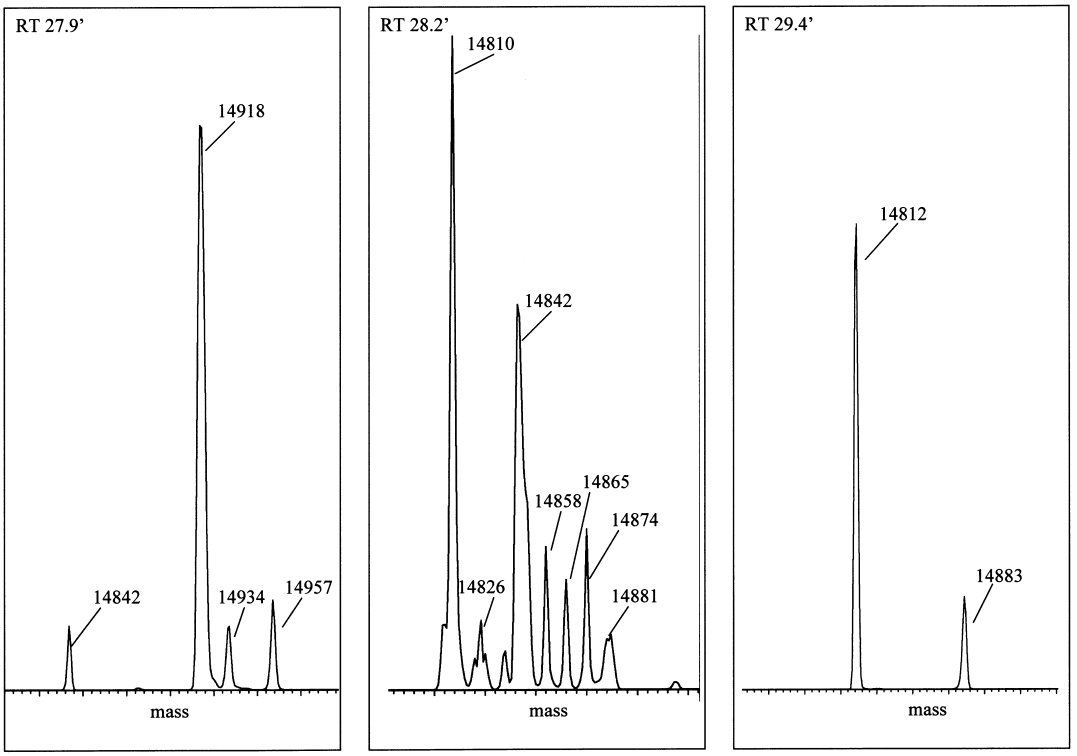

Fig. 7. Reconstructed mass spectra window of wild type ArsC between 14 500 and 15 000 corresponding to retention times 27.9 min (A), 28.2 min (B) and 29.4 min (C) from the C_4 reversed-phase column.

sample for structure determination an extra polishing step needs to be introduced.

Therefore, purified ArsC was concentrated on a Vivaspin M_r 5000 cut-off concentrator and extensively dialyzed to water to reduce the conductivity and to completely oxidize the enzyme. After dialysis

Table 1
Observed relative molecular masses of wild type ArsC (14 812 is the calculated M_r of the reduced form) determined with MS

M_r	Reduced	Oxidized −2	Formylated +28	Met-oxidation[a] +16	Na^+ form +23
14 810		X			
14 812	X				
14 826		X		1	
14 842		X		2	
14 858		X		3	
14 865		X		2	X
14 874		X		4	
14 881		X		3	X
14 883	X			3	X
14 918		X	X	5	
14 934		X	X	6	
14 957		X	X	6	X

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

and adjustment to pH 8 the oxidized ArsC was loaded onto a 1-ml Resource Q column equilibrated in 20 m*M* Tris–HCl, pH 8.0 and operated at a flow-rate of 745 cm/h. The column was eluted with an extremely shallow gradient of 200 column volumes up to 20 m*M* Tris–HCl, pH 8, 300 m*M* NaCl (Fig. 8A). Both obtained elution peaks, although not completely separated, were evaluated on reversed-phase C_4 under the same conditions as described before. The first peak was identified as oxidized *N*-formylated ArsC and the second peak was oxidized ArsC (Fig. 8B). It was only possible to separate both peaks under oxidizing conditions. In the presence of reductant no separation was possible.

This extremely flat elution gradient (200 column volumes up to 300 m*M* NaCl) on a Resource Q was

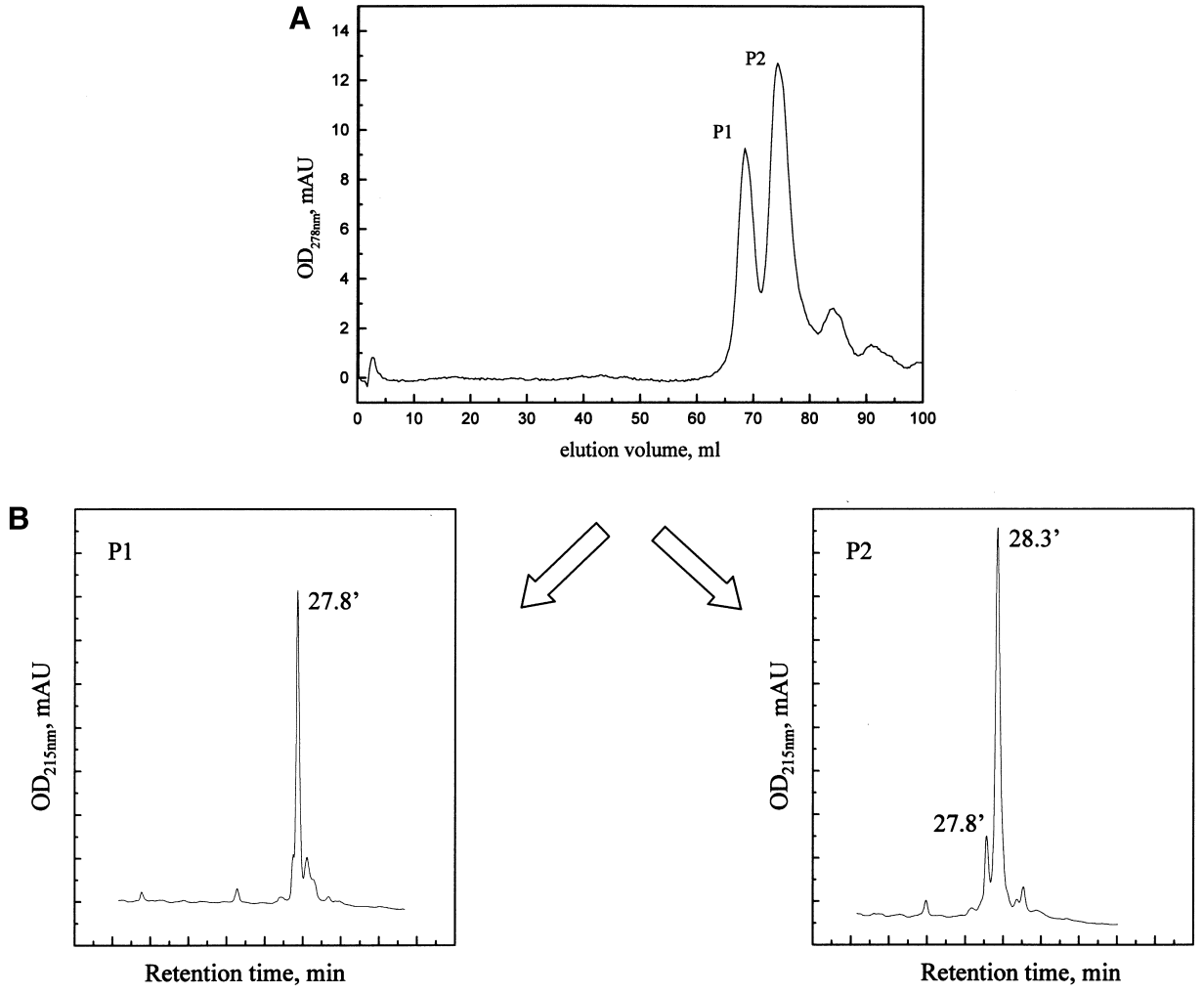

Fig. 8. (A) Part of the chromatographic elution profile at 278 nm of ArsC C15A on a 1-ml Resource Q column. ArsC C15A was first oxidized by an intensive dialysis to water. The pH 8 adjusted sample was loaded on a 1-ml Resource Q column equilibrated in 20 m*M* Tris–HCl, pH 8.0 and eluted with a 200-column volume gradient to 300 m*M* NaCl in the same buffer. The column was run at a flow-rate of 4 ml/min at room temperature. (B) P1 and P2 were separately pooled and concentrated on a Vivaspin (M_r 5000 cut-off) concentrator and subsequently analyzed on reversed-phase C_4. For each peak a part of the chromatographic profile at 215 nm is shown.

not enough to baseline separate both peaks. Lowering the steepness of the slope did not improve separation. To increase resolution another anion-exchange column with a particle size lower than 15 μm and with a high protein binding capacity might do the job.

4. Conclusion

ArsC is a protein highly susceptible to oxidation and under the chosen growth conditions the N-terminal methionine was partially formylated. This makes the development of a downstream process to obtain highly pure homogeneous material for structure determination not obvious. The optimized C_4 reversed-phase analysis conditions resulted in a reproducible, accurate separation of essentially three forms of ArsC identified by MS as: *N*-formylated oxidized, non-formylated oxidized and reduced ArsC.

The high expression levels obtained and the solubility of ArsC at m*M* level have benefits for the preparation of mmol quantities needed for ^{15}N-, ^{13}C-NMR structure analysis. The partial *N*-formylation and the presence of methionine sulfoxide and sulfones in ArsC will only minimally interfere in the NMR work. For X-ray crystallographic studies the introduction of an extra polishing step on anion-exchange to increase sample homogeneity by excluding the *N*-formylated forms of ArsC is a necessity.

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