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Purification of an oxidation-sensitive enzyme, pI258 arsenate reductase from *Staphylococcus aureus*

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Abstract

Arsenate reductase (ArsC) from *Staphylococcus aureus* pI258 is extremely sensitive to oxidative inactivation. The presence of oxidized ArsC forms was not that critical for NMR, but kinetics and crystallization required an extra reversed-phase purification to increase sample homogeneity. The salt ions observed in the X-ray electron density of ArsC were investigated. Carbonate was found to have the lowest dissociation constant for activation (K_a=1.1 m*M*) and potassium was stabilizing ArsC (ΔT_m=+6.2 °C). Also due to the use of these salt ions, the final yield of the purification had improved with a factor of four, i.e. 73 mg/l culture.

Keywords: *Staphylococcus aureus*; Arsenate reductase; Enzymes

1. Introduction

Quite often, the technical aspects of protein purification are succinctly mentioned, if at all, in a short and cryptic paragraph of the material and methods section of a scientific paper. All small bits and pieces that together make up the purification protocol are almost always ignored, incomplete or hidden. We have found that seemingly unimportant details can make the difference between failure and success in the case of oxygen sensitive enzymes. Here, arsenate reductase (ArsC) encoded by *Staphylococcus aureus* arsenic-resistance plasmid pI258 is used to demonstrate how feeding back the results from several biophysical techniques may result in considerable downstream maturation of the purification protocols.

ArsC reduces arsenate(V) to arsenite(III) as part of an arsenic detoxification process in *S. aureus*. ArsC requires thioredoxin (Trx), thioredoxin reductase (TR) and NADPH to be enzymatically active [1,2]. As a soluble, monomeric, 131 residue protein (M_r 14 812.7), ArsC features four cysteinyl residues (Cys 10, 15, 82, 89) which have to be in their reduced state for enzymatic activity. A flexible P-loop [3], three cysteines (Cys 10, 82 and 89) [1], a substrate polarizing arginine (Arg^{16}) and a transition state stabilizing aspartate (D105) [4] are essential for arsenate reductase activity. For catalysis, ArsC makes use of a phosphatase-like nucleophilic attack followed by a unique intramolecular disulfide cas-

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cade mechanism [4] resulting in the formation of a Cys^{82}–Cys^{89} disulfide bond [1]. This oxidation is also accompanied with sizeable conformational changes in the Cys 82–89 region: the α-helix bearing both cysteines is looped out, allowing a 10 Å translation of Cys^{89} towards Cys^{82}, necessary for mutual disulfide bridge formation [4,5]. At the same time the disulfide bridge is exposed at the surface of ArsC, ready for interaction with thioredoxin [5]. Tetrahedral oxyanions (50 m*M* sulfate, phosphate or perchlorate) stabilize ArsC by binding to the $C–X_5–R$ catalytic P-loop motif characteristic for phosphotyrosine [5]. The largest stabilizing effect is observed for arsenate, itself a tetrahedral oxyanion, which binds to the P-loop as the first step in its reduction process. The structure of ArsC shows that it belongs to the family of proteins with a PTPase I fold. Astonishingly, ArsC also catalyses dephosphorylation in addition to reduction, albeit with reduced efficiency [5]. This additional function requires only one cysteine, Cys^{10} from the P-loop [5]. The presence of functionally relevant thiol groups turns pI258 ArsC into an extremely oxidation-sensitive enzyme that will easily be converted into different inactivated forms during its purification [1,6]. In this paper, the modifications as a result of the feedback from NMR, kinetics, and crystal growth and from electron density analysis, which led to the maturation of a purification protocol of this oxidation-sensitive enzyme are rationalized and reported in detail.

2. Experimental

2.1. Culture condition

The *Escherichia coli* strain BL21 (DE3) with the pET-11a *arsC* wild type plasmid [3] was grown in a Luria–Bertani broth (LB) preculture with 1% glucose (a precautionary adaptation that avoids possible pre-induction expression) and 100 μg/ml ampicillin at 37 °C. The culture was transferred to Terrific broth (TB) and was grown for 4 h at 37 °C with 0.1% glucose and 100 μg/ml ampicillin. Induction at a cell density of approximately 1.5 was carried out overnight with 1 m*M* isopropylthiogalactoside (IPTG) at 28 °C.

2.2. Purification of ArsC

The purification method described in this section is the method obtained following the optimization described and discussed in this paper. Cells were harvested by centrifugation at 4 °C and suspended in cold 100 m*M* Tris–HCl, pH 8.0, 50 m*M* NaCl, 20 m*M* 2-mercaptoethanol, 0.1 m*M* EDTA, 0.1 mg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) and 1 μg/ml leupeptine prior to French Press disruption. A 50 μg DNase I/ml (EC 3.1.21.1, Sigma, St. Louis, MO, USA) and 20 m*M* $MgCl_2$ were added and the solution was left to stand for 30 min at room temperature. Cell debris were removed by centrifugation for 30 min at 12 000 *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 12 000 *g*). The supernatant was directly loaded at 20 ml/min onto a Phenyl Sepharose Fast Flow (HS) column (140×26 mm) from Amersham Biosciences (Uppsala, Sweden), equilibrated in 20 m*M* Tris–HCl, pH 8.0, 1.7 *M* ammonium sulfate, 0.1 m*M* EDTA, 1 m*M* dithiothreitol (DTT) to trap ArsC. The column was developed with a two-step gradient, 5 column volumes each of 0.5 and 0 *M* ammonium sulfate in 20 m*M* Tris–HCl, pH 8, 0.1 m*M* EDTA, 1 m*M* DTT. Recombinant ArsC eluted in the 0.5 *M* ammonium sulfate step which was dialyzed overnight to 20 m*M* ammonium hydrogencarbonate, pH 7.8, 0.1 m*M* EDTA, 1 m*M* DTT in dialysis tubing with a M_r cut off of 3500 (Spectrapor, Houston, TX, USA) to lower the conductivity to approximately 1 mS/cm. The dialyzed pool was further purified on a Source30 Q anion-exchange column equilibrated in the same buffer. The sample was loaded at 600 cm/h and after a 2-column volume wash the column was developed with a 10-column volume linear gradient to 300 m*M* NaCl. The ArsC containing fractions were pooled and concentrated on a Vivaspin concentrator with a M_r 5000 cut off (Vivascience, Lincoln, UK). The concentrated sample was further purified on a Superdex75 PG 16/90 gel filtration column in

20 m*M* Tris–HCl, pH 8.0, 150 m*M* KCl, 50 m*M* K_2SO_4, 0.1 m*M* EDTA, 1 m*M* DTT. After the gel filtration run, ArsC was concentrated, flash frozen in separate aliquots in liquid nitrogen and stored at −20 °C.

When needed, an aliquot was defrosted, reduced with 20 m*M* DTT during 30 min at room temperature and injected on a Jupiter C_{18} (10 μm, 300 Å, 250×10 mm) reversed-phase column (Phenomenex, St. Torrance, CA, USA). The column was equilibrated with 20 m*M* Tris–HCl, pH 8.0, 10 m*M* K_2SO_4, 1 m*M* DTT, 15% acetonitrile and eluted with a 20-column volume gradient to 45% acetonitrile in the same buffer at 8 ml/min. The different peaks were collected separately and dialyzed against 20 m*M* Tris pH 8.0, 50 m*M* K_2SO_4, 0.1 m*M* EDTA, 1 m*M* DTT to remove acetonitrile. All columns were run at room temperature on an Äkta-Explorer except for the size-exclusion column that was operated at 4 °C on a fast protein liquid chromatography (FPLC) system. All buffer solutions were argon flushed (15 min) prior to use.

2.3. Mass spectrometry

The different ArsC samples for mass spectrometry were taken after Jupiter C_{18} reversed-phase liquid chromatography. Samples were concentrated by Speedvac prior to analysis. Electrospray mass spectrometry was carried out with a Quattro II quadrupole mass spectrometer (Micromass, Manchester, UK) as described previously [6].

2.4. Circular dichroism (CD) spectroscopy

ArsC wild type was dialyzed against 20 m*M* Tris–HCl, 1 m*M* DTT, pH 8.0, 200 m*M* NaCl and 20 m*M* Tris–HCl, 1 m*M* DTT, pH 8.0, 200 m*M* KCl. Thermal denaturation curves with ArsC wild type (0.2 mg/ml) were recorded in a J-715 spectropolarimeter (Jasco, Tokyo, Japan) in a 1-mm cuvette using the CD signal at 220 nm. The temperature of the sample was controlled during the measurements by a sensor built into the cuvette holder and connected to a Haake N3 circulating bath (Gebr. Haake, Karlsruhe, Germany), which allows temperature stability of the sample within 0.1 °C.

2.5. Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis

ArsC was analyzed on pre-casted 10% Bis–Tris NuPage SDS–PAGE with a SDS–2-(*N*-morpholino)ethane sulfonic acid (MES)–running buffer solution in a Xcell II mini-Cell using the manufacturer's recommendations (Novex, San Diego, CA, USA).

2.6. Kinetic assays and Selwyn's test of enzyme inactivation

The procedures for kinetic assays and the Selwyn test of enzyme inactivation [7] were performed in a SPECTRAmax340PC (Molecular Devices, Sunnyvale, CA, USA) and described in full in Messens et al. [3]. Activation and inhibition were measured in 50 m*M* Tris pH 8.0, 150 m*M* KCl buffer solution with 200 μ*M* arsenate as substrate. ArsC wild type (100 n*M*) was pre-incubated (5 min at 37 °C) with increasing concentrations of *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), K_2HPO_4, K_2SO_4, NH_4HCO_3 and $NaClO_4$ before measuring the activity in a coupled Trx, TR, NADPH enzymatic assay under standard condition [3]. For activation, the velocity data points were plotted against increasing activator concentrations and fitted with the hyperbolic equation:

$$v_i = \frac{V_{max}}{a/K_a + 1} + \frac{V'_{max} a}{K_a + a}$$

where a is the activator concentration, K_a is the dissociation constant of activation, V_{max} is the maximum velocity without activator and V'_{max} is the maximum velocity due to activation. Inhibition was characterized by fitting the rate of enzyme deactivation to the usual equation for irreversible inhibition [8]. The Selwyn tests of enzyme inactivation were performed in the presence of either 50 m*M* TES, 50 m*M* MES, 50 m*M* *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) HEPES or 50 m*M* $(NH_4)HCO_3$ with 100 μ*M* arsenate as substrate. Progress curves were obtained with various concentrations of ArsC but otherwise identical assay conditions plotted against an abscissa of time multi-

plied by enzyme concentration. If those curves were not superimposable, the rate depending on the enzyme concentration changes throughout the reaction and so the active ArsC concentration must be variable.

3. Results and discussion

Previously, ArsC has been purified with an ammonium sulfate cut (55% ammonium sulfate) followed by a capture step on a hydrophobic interaction matrix (Phenyl Sepharose FF 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), an intermediate anion-exchange purification step (Source 30 Q equilibrated in 20 m*M* HEPES/NaOH, pH 8.0, 0.1 m*M* EDTA, 2 m*M* 2-mercaptoethanol) and a polishing step on a size-exclusion column (20 m*M* Tris–HCl, pH 8.0, 150 m*M* NaCl, 0.1 m*M* EDTA, 2 m*M* 2-mercaptoethanol). Under these purification conditions, final protein yields of 16 mg/l culture for wild type ArsC were obtained [1,6]. Using ArsC with the degree of purity obtained from this protocol, we previously established that Cys^{82} and Cys^{89} act as the redox couple in arsenate reductase. The purity was also sufficient to follow and evaluate the redox-state of ArsC [1] and to initiate structural analysis by NMR using ^{13}C, ^{15}N-labelled ArsC [9].

The process of purification has its own dynamics, because it precedes biophysical investigation. The initial purification protocol should continuously be submitted to revision as soon as more data on the protein of interest are becoming available. For ArsC, the continuous feedback has included data from both structural and functional investigations resulting in a highly modulated downstream process.

3.1. ArsC for NMR experiments

Successful NMR studies of medium sized (M_r 10 000–30 000) proteins like ArsC require ^{13}C, ^{15}N enriched protein, soluble at concentrations above 0.2 m*M*, preferably at pH 4 to 5 [10]. For ArsC a complication results from the fact that it precipitates below pH 6.5, at least in the concentrated solutions required for NMR. Using a phosphate buffer at pH 6.7, a protein solution up to 2.5 m*M* was stable. The use of 1 m*M* DTT is required in order to maintain the cysteinyl residues reduced. EDTA was added to remove trace amounts of divalent metals, which might induce oxidation. When concentrators stored in glycerol are to be used, it is necessary to wash the membranes extensively, as glycerol generates disturbing resonances in the ^1H-NMR spectrum. Small contributions from different post-translationally modified ArsC forms with formylation or with oxidized methionines/cysteines did not prove to be that critical as long as the polypeptide is a single molecular entity. Extra purification steps to remove post-translationally modified ArsC forms were therefore not necessary.

NMR study of ArsC only required a buffer change towards a 50 m*M* potassium phosphate buffer, pH 6.7, 150 m*M* KCl, 0.1 m*M* EDTA, 1 m*M* DTT. Before sample preparation, argon gas was bubbled through the solution to minimize the oxygen content. Using these measuring conditions approximately 90% of the expected amide resonances were present in the spectra and could be assigned to their residues (Fig. 1). The missing 10% of the residues include isolated residues, but also a stretch of seven residues involving Ser^{11} to Ser^{17} [9]. In view of the pH limit of 6.7, the loss of 10% of the residues is most likely attributed to hydrogen-exchange broadening. All the absent residues are indeed located in surface exposed loops, as deduced from the X-ray structure [5]. The addition of extra tetrahedral oxyanions to the phosphate buffer solution introduced nearly all missing amide resonances as a result of binding interactions with the P-loop (Fig. 2) [3,9]. Therefore, KCl (150 m*M*) in the buffer solution was replaced by a salt with a tetrahedral oxyanion: K_2SO_4 (50 m*M*).

3.2. Purification conditions for kinetics

Accurate kinetic studies require a strictly defined ArsC form to ensure reproducible k_{cat} values for each produced batch. To prevent ArsC from any possible irreversible oxidation, the concentration of 2-mercaptoethanol was increased to 20 m*M* in the extraction buffer solution. Here, the use of DTT was not possible as it precipitated ArsC in the crude

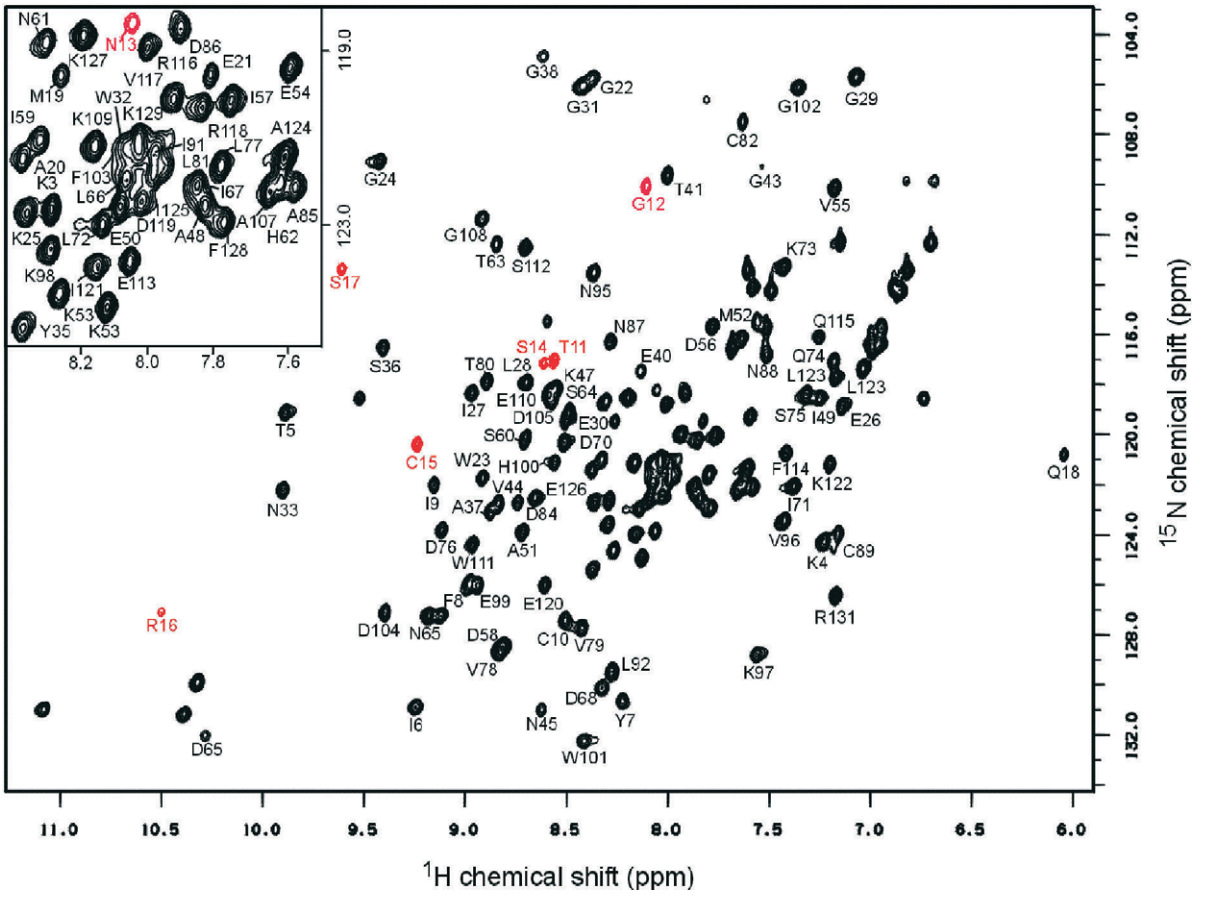

Fig. 1. ^{1}H–^{15}N HSQC spectrum of wild type ArsC in its reduced state. This spectrum of 1.8 m*M* ArsC was recorded at 25 °C and 500 MHz in 50 m*M* potassium phosphate buffer, 50 m*M* K_2SO_4, 0.1 m*M* EDTA, 0.02% NaN_3, 1 m*M* DTT, pH 6.7 in 5% $^{2}H_2O$, 95% H_2O [9]. Unlabelled cross-peaks correspond to the side-chain amide (Gln, Asn) or indole NH (Trp) resonances. Correlation peaks, which only become visible when K_2SO_4 is present in solution, are highlighted in red.

lysate [6]. The switch from 2 m*M* 2-mercaptoethanol to 1 m*M* DTT in all other buffer solutions and the flushing with argon (15 min) were introduced to protect ArsC from possible oxidation in all other steps.

The introduction of an extra reversed-phase separation step at pH 8.0 on an inert silica RPLC column stable up to pH 10 (Jupiter C_{18}) resulted in the separation of three ArsC fractions (P1, P2, P3) (Fig. 2). Fraction P3 had the highest V_{max} for reductase activity [3]. The P2 fraction contained still some activity, but also contained part of the most active fraction P3. No reductase activity was detected in fraction P1.

Each fraction was analyzed with electrospray mass spectrometry (Table 1). The different forms observed might be due to formylation ($M_r + 28$) and multiple oxidation [$M_r + (n \cdot 16)$ (oxygen), with $n = 2$, 5, 6 and 7]. Especially, the irreversible formation of sulfinic acid (Cys–SO_2H) and sulfonic acid (Cys–SO_3H) on one of the crucial cysteines would result in enzymatically dead ArsC. The faster elution results from decreased hydrophobicity as a result of the multiple oxidation (Fig. 2, Table 1). The most hydrophobic

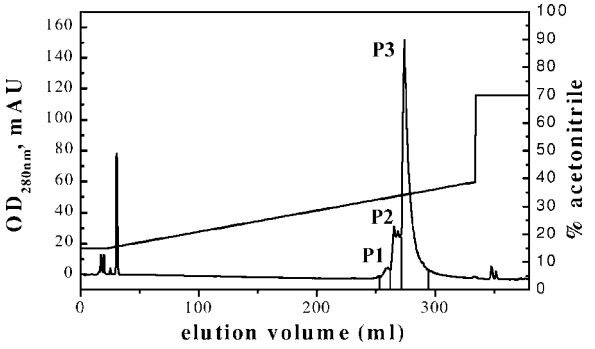

Fig. 2. Reversed-phase liquid chromatography to increase ArsC homogeneity. Chromatographic elution profile at 280 nm of ArsC injected on a Jupiter C_{18} column at pH 8.0. The column was equilibrated with 20 m*M* Tris–HCl, pH 8.0, 10 m*M* K_2SO_4, 1 m*M* DTT, 15% acetonitrile (argon flushed) and eluted with a 20-column volume gradient to 45% acetonitrile in the same buffer at 8 ml/min.

fraction, P3, contained mainly (97%) ArsC with a single M_r of 14 812, consistent with the calculated mass of reduced ArsC.

To conclude, measures to prevent ArsC from oxidizing (DTT and argon flushing) combined with removal of residual oxidized ArsC forms by RPLC were essential to guarantee kinetic parameter reproducibility for each produced batch.

Table 1
The molecular masses of the ArsC (14 812 is the calculated M_r of the reduced form) species observed in fractions P1, P2 and P3 after RPLC on Jupiter C_{18}

Fractions	M_r	Redox-state[a]	Formylation (+28)	Number of oxygens[b] (+16)
P1	14 952	R	X	7
	14 925	R		7
	14 932	DO	X	6
	14 920	R	X	5
P2	14 920	R	X	5
	14 844	R		2
	14 842	SO		2
	14 812	R		0
	14 760	?	?	?
P3	14 840	R	X	0
	14 812	R		0

[a] R, reduced; SO, single oxidized with one disulfide bridge formed; DO, double oxidized with two disulfide bridges formed; ?, unidentified component.

[b] Oxidation products of methionines and/or cysteines.

3.3. The crystallization conditions of an oxidation sensitive redox-enzyme

The successful growth of suitable X-ray diffraction quality crystals probably sets the strictest requirement for a highly homogeneous sample. Initially, crystallization of ArsC failed, and only a C10SC15A double mutant [1] purified through an extra RPLC step [5] could be crystallized. The reduction by half in the number of oxidation sensitive cysteines allowed a considerable downsize in the number of oxidated ArsC forms, which could then be removed by the extra RPLC step.

However, when ArsC C10SC15A came into contact with TES buffer solution during either purification, dialysis or in the crystallization buffer solution, crystallization failed, indicating this should be considered as a prohibited additive. Oxidized and reduced ArsC C10SC15A were crystallized under different crystallization conditions, resulting in crystals of the same space group but with different unit cell parameters [5]. The crystals of reduced and oxidized ArsC diffracted to an atomic resolution of 1.1 and 2.0 Å, respectively, making the electron density of sufficient quality to visualize the ligands and many water molecules (Fig. 3).

Further optimization of the crystallization conditions resulted in the crystallization of ArsC wild type with its four cysteines in the reduced state [4]. The key to success consisted in refraining from the immediate addition of precipitant solution [5]. Instead, the ArsC containing drop was left to equilibrate in the presence of the bottom solution for 20 h [100 m*M* Tris–HCl pH 8.0, 50 m*M* ammonium hydrogen carbonate, 42.5% (w/v) PEG 4000 and 10 m*M* DTT] [4]. After this period a volume of bottom solution precipitant equal to the initial volume of the drop was added to achieve super saturation conditions and within the next 16 h crystals were formed. A short incubation period with a DTT containing precipitant solution minimized the chance that ArsC oxidizes and forms various oxidized species (Table 1). As such, high protein sample

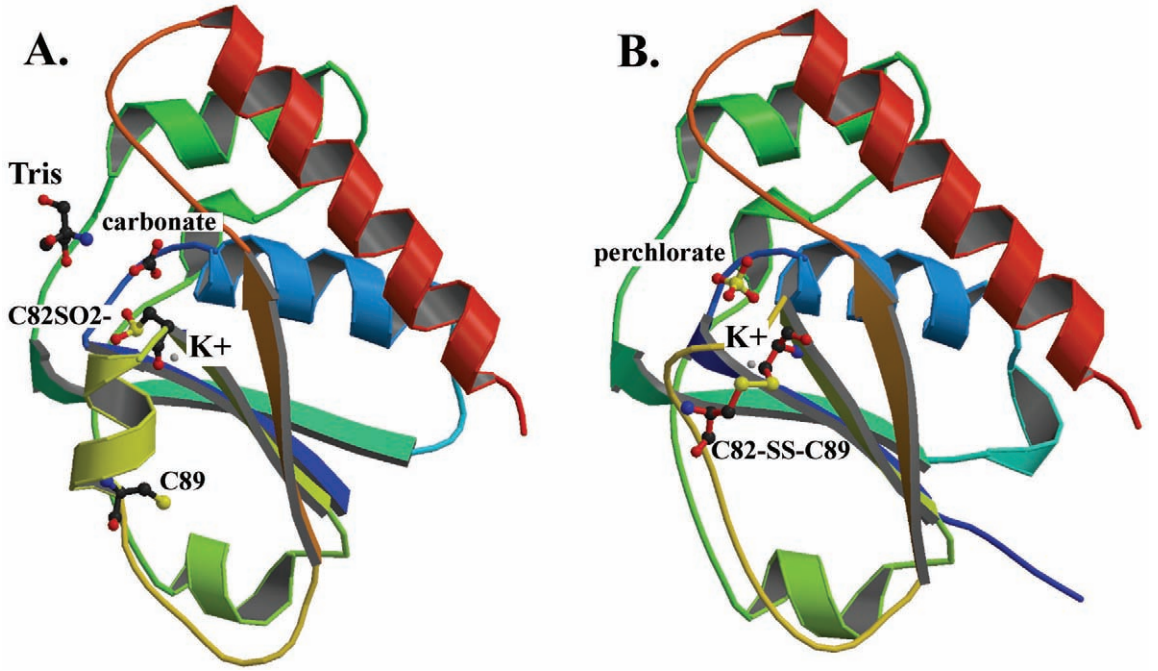

Fig. 3. The crystal structure of ArsC with ligands. (A) The structure of ''reduced ArsC C10SC15A''. A sulfinic acid was formed on Cys^{82} ($C82SO_2^-$), a Tris molecule is sitting at the entrance of the P-loop and carbonate is bound more deeply into this loop. (B) The structure of ''oxidized ArsC C10SC15A''. In this structure perchlorate is bound in the active site P-loop and also here a K^+ ion was well defined in the electron density.

homogeneity of this oxidation-sensitive enzyme during crystallization was maintained.

3.4. The purification history of each ArsC form

For each protein batch of ArsC slightly different crystallization conditions were required [4,5]. A ClO_4^- ligand interacting with the P-loop residues was observed in the electron density of oxidized ArsC C10SC15A, while CO_3^{2-} and Tris were found in reduced ArsC C10SC15A (Fig. 3). In addition, the electron densities in both structures and in those wild type, C15A and C89L ArsC revealed a coordinated potassium ion [4]. The importance of these ligands was further assessed with various techniques.

The kinetic stability of ArsC in the presence of 50 m*M* $(NH_4)HCO_3$ was tested with Selwyn's test of enzyme inactivation [7]. Ammonium hydrogen carbonate was slightly stabilizing ArsC, as enzyme concentrations >100 n*M* were necessary for all data points to fall on a single curve (Fig. 4). This is weaker than already established before for the tetrahedral oxyanions sulfate, phosphate, perchlorate and arsenate [3].

The dissociation constant for activation (K_a) and the k_{cat} of $(NH_4)HCO_3$ for ArsC wild type were compared with the parameters obtained with the tetrahedral oxyanions, sulfate and phosphate. Carbonate was only weakly activating (a k_{cat} increase with a factor of 1.3) and has a $K_a = 1.1$ m*M*. Sulfate and phosphate were observed to be better activators, increasing the k_{cat} of the arsenate reductase activity of ArsC wild type with a factor of approximately four, but with higher K_a values of 16.1 and 10.8 m*M*, respectively. For the enzymatic stabilization and activation a tetrahedral conformation is therefore found to be an advantage, apparently at the expense of binding affinity.

Sulfate and phosphate are isosteric with arsenate and several examples of their presence in crystal

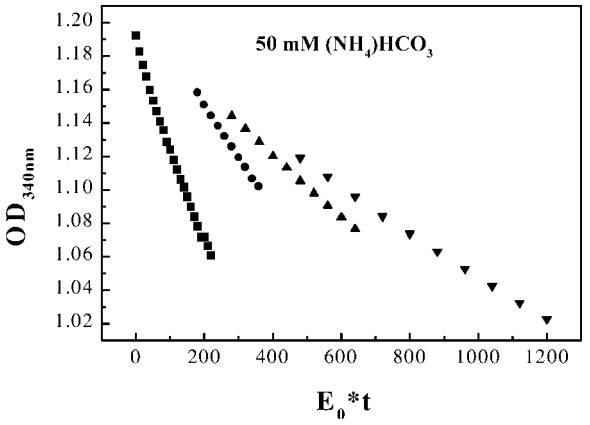

Fig. 4. Stabilizing effect of carbonate during the enzymatic assay. Selwyn's test of enzyme inactivation: progress curves plotted against initial enzyme concentration (E_0) multiplied by time (t) at different enzyme concentrations (■ = 25 n*M*, ● = 50 n*M*, ▲ = 100 n*M*, ▼ = 200 n*M*).

structures of ArsC from *Bacillus subtilus* [11], R773 ArsC from *E. coli* [12] and in low-M_r PTPase [13] have been published. Protection towards oxidation of the crucial cysteine in the P-loop of low-M_r PTPases by the competitive inhibitor phosphate has also been reported [14,15].

Not surprisingly, the highly oxidative perchlorate ($E_0' = +1.19$ V) found in the structure of oxidized ArsC C10SC15A was slightly inhibiting the arsenate reductase reaction of ArsC wild type ($K_i = 81$ m*M*), but was stabilizing at 50 m*M* in the Selwyn test [3].

Quite remarkably, Cys^{82} is observed to form a sulfinic acid in the structure of the reduced form of ArsC C10SC15A (Fig. 3A) with Cys^{82} and Cys^{89} not engaged in disulfide bonding (1JF8). These crystals of ArsC C10SC15A were obtained before the need for argon flushing had been realized, allowing such oxidation to occur. An explanation for this non-disulfide bridged, yet oxidized ArsC form might be that this in vitro derivatization of Cys^{82} in the absence of physiological redox mechanisms protects it from the formation of a Cys^{82}–Cys^{89} disulfide bridge (Fig. 3B). The occurrence of this uncontrolled irreversible oxidation was fatal for ArsC's activity, but necessary to obtain crystals of non-disulfide-bonded ArsC C10SC15A [5].

Oxidation of sulfenic acids to sulfinic acids occurs spontaneously. The formation of sulfenic acid (Cys–SOH) derivatives are known to play an important role in the redox regulation of transcription factors [16] and in tyrosine phosphorylation-dependent signal transduction events [17]. So far, no evidence is suggesting such a role in ArsC.

Also potassium was definitely present in the electron density of all the crystallized ArsC's (Fig. 5A) (1JFV and 1JF8) [5] and (1LJL, 1LJU and 1LK0) [4]. Potassium was used in the buffer solution during the purification procedure. In combined

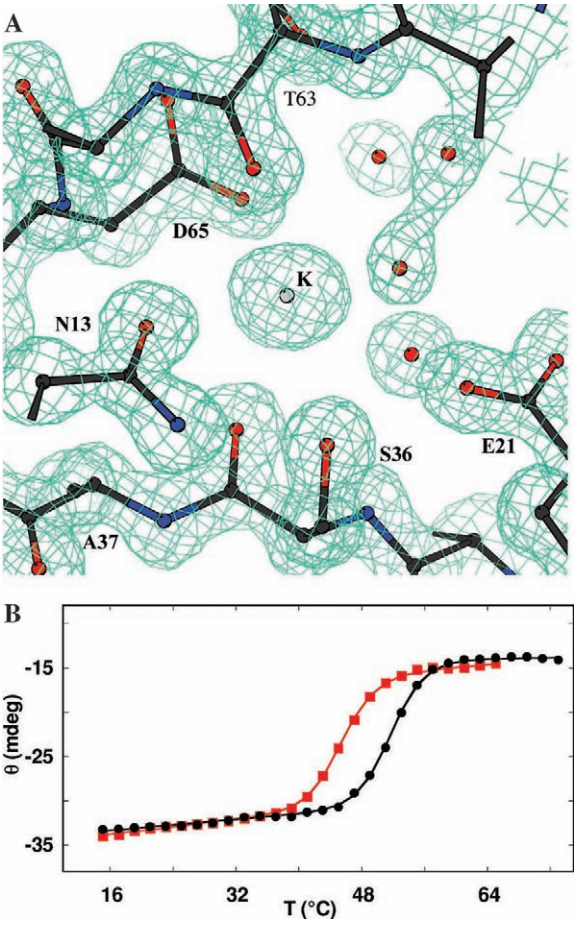

Fig. 5. Stabilizing effect of K^+ on ArsC. (A) $2F_o$–F_c electron density map in the region of the K^+ binding-site of the reduced form of ArsC at 1.1 Å resolution. The map shows potassium (grey) making contact with residues D65, N13, S36 and two water molecules (red) [5]. (B) Circular dichroism (CD) thermal denaturation measurement at 220 nm of ArsC wild type in 20 m*M* Tris–HCl, 1 m*M* DTT, pH 8.0 in the presence of 200 m*M* NaCl (red) and 200 m*M* KCl (black).

dialysis–mass spectrometry experiments, K^+ was easily replaced by Na^+ and *vice versa*, making thermal denaturation CD experiments with wild type ArsC in the presence of either Na^+ or K^+ possible (Fig. 5B). An increase in the ArsC melting temperature (T_m) of 6.2 °C in the presence of K^+ as compared to Na^+ containing buffer solution was observed. This is an extreme ΔT_m that warrants a further in depth study. Potassium seems to have a very strong global stabilizing effect on pI258 ArsC and therefore it is not surprising that also this ion was found in its structure. Potassium will be the preferable cation to be used during the purification in order to stabilize ArsC.

3.5. Improving the purification

The impact of the stabilizing ions of ArsC from several applications on the different purification steps (capture, intermediate purification and polishing) was investigated.

Sulfate has already previously been used as a lyotropic salt during capture on Phenyl Sepharose, but by increasing the concentration of ammonium sulfate of the binding buffer solution from 1.5 to 1.7 *M* complete capture was ensured.

The addition of tetrahedral oxyanions, like K_2SO_4, to the anion-exchange buffer solution to stabilize ArsC during this intermediate purification step was not possible, because the conductivity for binding became to high. The presence of HEPES in the crystal structure of low-M_r PTPase was shown [18], and therefore, the possible stabilizing effect of sulfonic acid buffers (MES, HEPES and TES) in a Selwyn test [7] was investigated. Neither MES, HEPES and TES buffer salts were stabilizing ArsC, making them irrelevant for further use during purification. Also, the effect of different buffer salts with a pK_a around pH 8 on the chromatographic behavior of ArsC during the anion-exchange-run was tested. Phosphate (stabilizing tetrahedral oxyanion [3]), carbonate (found in the structure of ArsC C10SC15A (Fig. 3A), activator and stabilizing tetrahedral oxyanion (Fig. 4)) and Tris [found in the ArsC structure (Fig. 3A)] were compared (Fig. 6). The chromatographic profile of the anion-exchange run in the presence of Tris resulted in ArsC eluting in a wide, broad area. In the presence of phosphate and ammonium hydrogen carbonate as buffer component, ArsC eluted in a much sharper peak. The ionic strength of the 20 m*M* phosphate buffer (4 mS/cm) is two times higher compared with 20 m*M* carbonate buffer (2 mS/cm), making that in the presence of 20 m*M* phosphate buffer ArsC eluted in the beginning of the salt gradient. Therefore, in order to guarantee full binding and stabilization during the anion-exchange intermediate purification step, carbonate buffer solution was preferred. A possible explanation for this behavior might be that during the desorbing process, the chance that another patch of the protein could interact with the matrix requiring higher salt concentrations to compete with, will be higher for a more flexible than for a more rigid, stabilized protein. A flexible protein will have the tendency to elute in a broad area, while a more rigid protein will elute in a sharp peak.

For the polishing step by size-exclusion chromatography (SEC) it was obvious to replace 150 m*M* NaCl by the stabilizing potassium and sulfate salts. For the extra polishing step on RPLC that was needed for kinetics and crystallization, K_2SO_4 was included in the buffer solution.

A combination of these changes in the downstream process of ArsC resulted in an increase of the final purification yield up to 73 mg/l culture, i.e. an improvement by a factor of four.

4. Conclusion

We have shown that the purification demands are modulated by the nature of each required application of ArsC. As long as the homogeneity and the redox-state of the ArsC sample are good enough to perform the necessary analyses no extra purification is required. For NMR, pH close to the solubility limit, tetrahedral oxyanions and high protein concentrations are necessary. For crystallization, each purified ArsC sample, with its complete history, has to be considered as a new molecular entity. Kinetics and crystallization require an extra RPLC purification step. The salt ions observed in the structure of ArsC have been used to improve the purification protocol. In general, the importance of each salt ion in the buffer solutions and their impact on the final purified protein are often ignored and need to be emphasized.

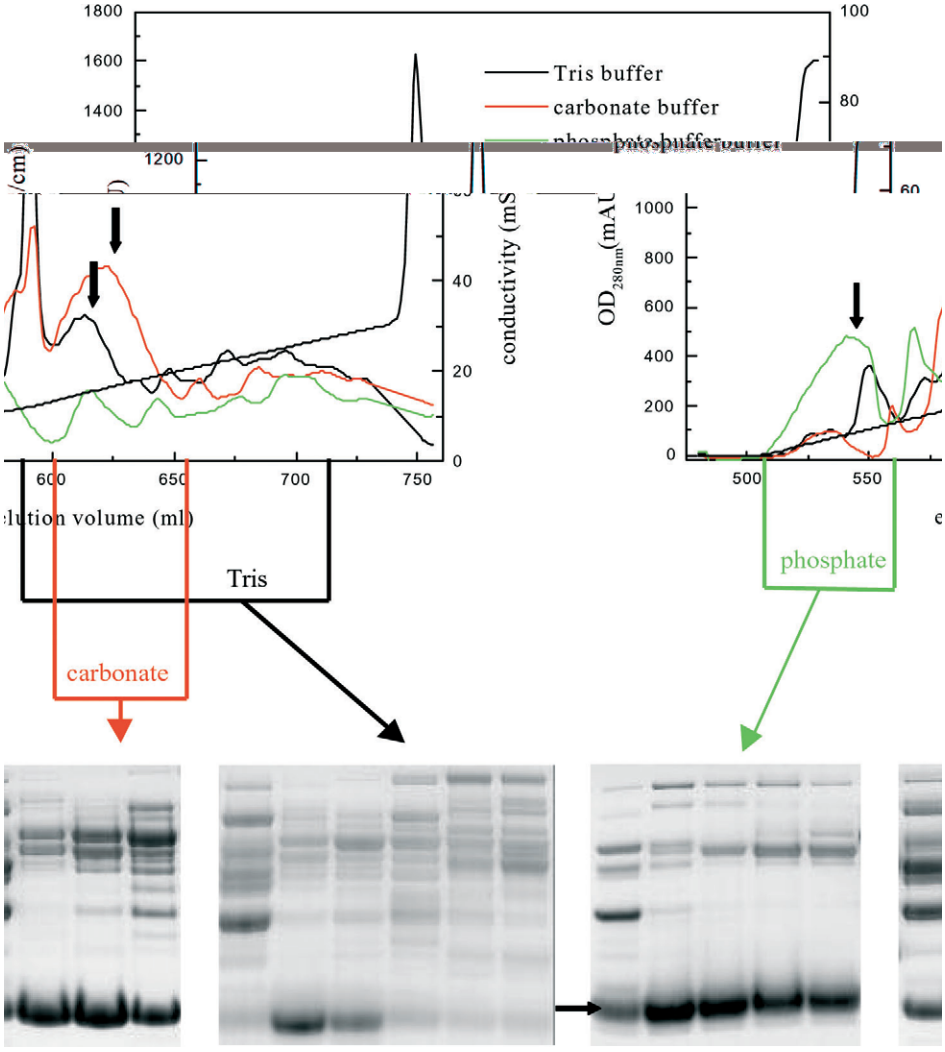

Fig. 6. Comparison of the buffer conditions on Source 30 Q. ArsC wild type, after the capture step on Phenyl Sepharose FF, evaluated under different buffer conditions [20 m*M* Tris pH 8.0, 0.1 m*M* EDTA, 1 m*M* DTT, 20 m*M* K phosphate pH 7.8, 0.1 m*M* EDTA, 1 m*M* DTT; 20 m*M* $(NH_4)HCO_3$, pH 7.8, 0.1 m*M* EDTA, 1 m*M* DTT] on Source 30 Q (16×120 mm). The column was developed with a 10-column volume gradient to 300 m*M* NaCl at 20 ml/min in the respective buffers at room temperature. The gradient parts of the chromatographic profiles at 280 nm are shown. The black arrows indicate the elution position of ArsC. Fractions (15 μl of each) were evaluated by SDS–PAGE.

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