Arsenate Reductase, Mycothiol, and Mycoredoxin Concert Thiol/Disulfide Exchange *[S]

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We identified the first enzymes that use mycothiol and mycoredoxin in a thiol/disulfide redox cascade. The enzymes are two arsenate reductases from Corynebacterium glutamicum (Cg_ArsC1 and Cg_ArsC2), which play a key role in the defense against arsenate. In vivo knockout studies showed that the genes for Cg_ArsC1 and Cg_ArsC2 and those of the enzymes of the mycothiol biosynthesis pathway confer arsenate resistance. With steady-state kinetics, arsenate analysis, and theoretical reactivity analysis, we unraveled the catalytic mechanism for the reduction of arsenate to arsenite in C. glutamicum. The active site thiolate in Cg_ArsCs facilitates adduct formation between arsenate and mycothiol. Mycoredoxin, a redox enzyme for which the function was never shown before, reduces the thiol-arseno bond and forms arsenite and a mycothiol-mycoredoxin mixed disulfide. A second molecule of mycothiol recycles mycoredoxin and forms mycothione that, in its turn, is reduced by the NADPH-dependent mycothione reductase. Cg_ArsCs show a low specificity constant of ~5 M⁻¹ s⁻¹, typically for a thiol/disulfide cascade with nucleophiles on three different molecules. With the in vitro reconstitution of this novel electron transfer pathway, we have paved the way for the study of redox mechanisms in actinobacteria.

The frequent abundance of arsenic in the environment has guided the evolution of enzymes for the reduction of arsenate (As(V))¹ (1). Arsenate reductases (ArsCs) are unusual among well studied enzyme classes, because there is not a single family of evolutionarily related sequences. The structural folds and mechanisms that they are using are fundamentally different and arose independently during evolution (2). Arsenate reductases are small cytoplasmic redox enzymes that reduce arsenate to arsenite (As(III)) by the sequential involvement of three different thiolate nucleophiles that function as a redox cascade. As such, arsenate reductases from different organisms often work together with the thiol/disulfide mechanism in the cell.

The major and most ubiquitous system for protection against oxidative stress and to maintain the intracellular thiol homeostasis is the thioredoxin system that is composed of Trx (thioredoxin) and TrxR (thioredoxin reductase) (3). In addition to the thioredoxin system, most living organisms contain low molecular weight thiol compounds that serve as a buffer to avert disulfide stress. In eukaryotes and Gram-negative bacteria, the redox level is maintained by redox cycling of glutathione (GSH) with Grx (glutaredoxin) and glutathione reductase (4). Gram-positive bacteria, like Staphylococcus aureus, produce no glutathione, but millimolar quantities of reduced coenzyme A is the predominant thiol, which is kept reduced with a NADPH-dependent flavoenzyme, coenzyme A disulfide reductase (5). Also actinobacteria, like Corynebacterium glutamicum, produce no GSH, but instead they contain millimolar concentrations of MSH (mycothiol; chemically 1D-myo-inositol-2-[N-acetyl-L-cysteinyl] amido-2-deoxy-β-D-glucopyranoside), a pseudodisaccharide containing a cysteine moiety as a reactive thiol (6). Its oxidized form is mycothione (MSSM). In actinobacteria, MTR (mycothione reductase) is the NADPH-dependent flavoenzyme that reduces MSSM in order to maintain the intracellular redox homeostasis to allow the proper functioning of a variety of biological functions (7).

Arsenate reductases are part of a defense mechanism of the cell against toxic arsenate. Their genes are most of the time found in an operon together with arsenite sensing and efflux genes (8). Based on the mechanism used to reduce arsenate to arsenite, two distinct ArsC classes can be defined. The first one is the thioredoxin-coupled ArsC class represented by S. aureus pl258 ArsC and Bacillus subtilis ArsC (9–11). Both enzymes use the structural fold of low molecular weight tyrosine phosphatase and need Trx to start a second catalytic cycle (12–14). The second class is the GSH/glutaredoxin-coupled class represented by Escherichia coli plasmid R773 ArsC (15, 16), the eukaryotic Acr2p reductase from Saccharomyces cerevisiae (17), and ArsC from Leishmania major (18). In this second class, two different structural folds are found; E. coli R773 ArsC partially resembles glutaredoxin (19), whereas the eukaryotic ArsCs have a rhodanese fold like the Cdc25a cell cycle control phosphatase (20). Notably, all arsenate reductases have a thiolate nucleophile at the N-terminal end of an α-helix. The active

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** The abbreviations used are: As(V), arsenate; As(III), arsenite; MSSM, mycothione.
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site of the ArsCs with a phosphatase-like scaffold is conserved (root mean square deviation of 0.54 Å) with a catalytically important Arg on position Cys

In *C. glutamicum*, there are four *ars* genes located on different places in the chromosome (21): one orphan *ars* gene (*arsC4*) and three *ars* genes (*arsC1-arsC1*’ and *arsC2*) present in two *ars* operons. We show here that two of the encoded proteins, Cg_ArsC1 and Cg_ArsC2 (with 66% sequence identity) are members of a new third class, the mycothiol- and mycoredoxin-dependent arsenate reductases. Both the genes of *arsC1* and *arsC2*, together with the genes for the enzymes of the mycothiol biosynthesis pathway are involved in arsenate resistance in *C. glutamicum*. We have reconstituted in vitro a novel electron transfer network containing, next to Cg_ArsC1 or Cg_ArsC2, mycothiol, mycoredoxin, and mycothione reductase. As such, the mechanism for the reduction of arsenate by *C. glutamicum* could be unraveled.

**EXPERIMENTAL PROCEDURES**

**Knockouts Involved in Mycothiol Biosynthesis—MshB, MshC, and MshD mutant strains from *C. glutamicum* were described previously (22).** Mutant strains *C. glutamicum* MshA and Mtr were kindly supplied by Dr. Kalinowski (Bielefeld, Germany). In all of these mutants, the *msh/mtr* structural genes were removed by the site-specific gene deletion system based on the plasmid pK18mobSacB (23); recombinant plasmids containing the up and down regions of the *msh/mtr* genes were mobilized to the recipient strain *C. glutamicum* RES167 and integrated into a specific site of the chromosome, allowing for marker-free deletion of the target genes when antibiotic pressure and sucrose was adequate.

**As(V) and As(III) Resistance Assays—Single colonies of the *C. glutamicum* strains were inoculated into fresh MMC or TSB and grown for 16 h at 30 °C in aerobic conditions.** Exponential phase cells were diluted 100-fold into fresh, prewarmed low concentrations of As(V) or As(III) in the form of sodium arsenate, in the assay buffer solution to obtain the final concentrations, except Cg_ArsC1 (or Cg_ArsC2) and its substrate arsenite, in the assay buffer solution to obtain the final concentration of 10 μM Mrt1_wt, 3 μM MTR, 0.47 mM MSH, and 250 μM NADPH (component mixture) taking into account the subsequent addition of arsenate and the respective Cg_ArsC enzyme.

**Arsenate Reductase Activity Assay—Cg_ArsCs were injected in 20 mM Tris/HCl, pH 6.5, to obtain 250 μM NADPH, 3 μM MTR, 10 μM Mrt1, 0.47 mM MSH, 200 mM Cg_ArsCs, and 100 mM As(V) (varying concentrations) incubated for different times at 37 °C.** A pH of 6.5 guarantees most arsenate in its diionic form and arsenite as As(OH)2+. The reaction was stopped by removing the proteins on a solid phase extraction cartridge (Waters Oasis HLB). The excess of arsenate was removed on Dowex 21K/XLT anion exchange resin (Supelco) pretreated with NaCl in Tris/HCl, pH 6.5, and thoroughly washed with water. The flow-through fraction was 0.2-μm filtered, argon-flushed, and injected on a Hamilton PRP-X100 anion exchange column (250 × 4.1 mm) operated in 20 mM KH2PO4/K2HPO4, pH 6.0, at 1 ml/min. The high pressure liquid chromatography effluent was mixed with 1.5 M HCl and 2.5% NaBH4, 2% NaOH at 1 ml/min to form gaseous arsine (AsH3). The arsines were analyzed and quantified using an atomic fluorescence spectrometer (Excalibur, PS Analytical, Orpington, UK) calibrated with a standard of arsenite and arsenate.

**Softness Difference Calculation**—The ArsC-arseno adduct is modeled as CH3S-AsO32− and CH3S-HAsO42−. The geometries of MSH, GSH, HAsO42−, CH3S-AsO32−, and CH3S-HAsO42− were optimized at the B3LYP/6-31+G** level. Subsequent single point calculations were performed in a polarized continuum (24) aqueous solvent model at the B3LYP/6-31+G** level. All calculations were performed with the Gaussian package (25).

The preferred reactivity between the attacking nucleophilic sulfur atom of MSH and GSH and the accepting electrophilic
When the single cysteine in the gene were able to complement the arsenate reductase activity.

arsC2 levels were obtained as for wild type (Fig. 1). Functional plasmids containing the constitutively expressed strains (28). With single ArsC mutant (ArsC1-C2), however, was very sensitive to arsenate (28). With single ArsC mutant (ArsC1-C2), however, was very sensitive to arsenate (28). With single ArsC mutant (ArsC1-C2), however, was very sensitive to arsenate (28). With single ArsC mutant ( ArsC1-C2), however, was very sensitive to arsenate (28). With single ArsC mutant ( ArsC1-C2), however, was very sensitive to arsenate (28). With single ArsC mutant ( ArsC1-C2), however, was very sensitive to arsenate (28). With single ArsC mutant ( ArsC1-C2), however, was very sensitive to arsenate (28). With single ArsC mutant ( ArsC1-C2), however, was very sensitive to arsenate (28).

FIGURE 1. The arsC1 and arsC2 genes and the genes of the MSH pathway confer As(V) resistance. In all panels, the cell density (A600 nm) of the culture as a function of increasing concentration of arsenate or arsenite is shown. A, resistance to arsenate of C. glutamicum strains: RES167 (wild type), mutant ArsC1 (arsC1'), mutant ArsC2 (arsC2'), and mutant ArsC1-C2 (arsC1' arsC2'). B, resistance to arsenate of strain ArsC1-C2 after homologous complementation analyses using the genes arsC1 (+pECarsC1), arsC2 (+pECarsC2), arsC1' (+pECarsC1'), and arsC4 (+pECarsC4). Also, mutant copies of the arsC1 and arsC2 genes with the Cys codon replaced by Ser were used for the complementation analyses (+pECarsC1* and +pECarsC2*). C and D, resistance to arsenate (C) or arsenite (D) of C. glutamicum wild type (RES167) and mutant strains involved in mycothiol biosynthesis or its regeneration (msh/mtr). See Table S1 for details.

Arsenate or sulfur atom of HAsO₄²⁻, CH₃S-AsO₃²⁻, and CH₃S-HASO₅³⁻ can be extracted from the difference in the local softness between the reacting partners (hard and soft acids and bases principle) (26). The smaller this difference, the higher the reactivity.

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\Delta s = s^-(As/S)_{electrophile} - s^-(S)_{nucleophile}
\]  
(Eq. 1)

The local softness was calculated as described (27).

RESULTS

Cg_ArsC1 and Cg_ArsC2 Are Involved in the Defense against Arsenate—Resistance analyses of C. glutamicum strains showed a high tolerance of As(V) (28). With single ArsC mutant strains (C. glutamicum ArsC1 or ArsC2), the same resistance levels were obtained as for wild type (Fig. 1A). The double mutant (ArsC1-C2), however, was very sensitive to arsenate and showed resistance levels, which were 20–30 times lower. This double disruption mutant strain was further used as a host for homologous complementation analyses (Fig. 1B). Bifunctional plasmids containing the constitutively expressed arsC1, arsC2, arsC1', and arsC4 genes from C. glutamicum (pECars derivatives; Table S1) were transferred to this double knock-out strain. Only strains containing either the arsC1 or the arsC2 gene were able to complement the arsenate reductase activity. When the single cysteine in the arsC1 and the arsC2 genes was mutated to a serine, the complementation was lost (Fig. 1B). As such, the single cysteine in Cg_ArsCs is involved in the reaction mechanism.

We also checked the effect of the absence of Cg_ArsC1 and Cg_ArsC2 on As(III) resistance. Removing arsC1, arsC2, or both genes had no effect on the resistance against As(III), and resistance levels equivalent to those for wild type were obtained (data not shown). As such, the arsC1 and arsC2 genes confer only As(V) resistance.

To test whether these four arsC genes could rescue arsenate reductase activity across different species, we did a heterologous complementation analysis. Cloning of the four respective arsC genes from C. glutamicum (arsC1, -C2, -C1', and -C4) in a WC3110 E. coli mutant lacking arsenate reductase activity (21, 29) was successful. However, none of the arsC genes increased the survival of the WC3110 strain in arsenate (data not shown). This might indicate that the genes for arsenate reductases in C. glutamicum have evolved to become host-specific enzymes, which depend on the proper cellular environment. It suggests that next to Cg_ArsC1 or Cg_ArsC2, there are C. glutamicum-specific molecules involved in the enzymatic reduction mechanism of arsenate.

Mycothiol Is Involved in Arsenate Defense—Since C. glutamicum produces no glutathione, unlike E. coli, but instead millimolar concentrations of mycothiol, we tested the influence of removing the genes of the biosynthesis pathway of MSH on the arsenate and arsenite resistance of C. glutamicum. In Mycobacterium species, the genes mshA, mshB, mshC, and mshD were found to encode for the enzymes that sequentially catalyze the formation of MSH starting from UDP-N-acetyl-glucosamine and inositol-P (30). Orthologs of these msh genes have been located in the genome data of C. glutamicum (31, 32).

With mutants from C. glutamicum (mshA, mshB, mshC, mshD, and mtr), the arsenate and arsenite resistance was tested. In all mutants, clear differences in resistance to arsenate were observed (Fig. 1C). Some of the msh/mtr mutants have a similar susceptibility to As(V) as observed for the ArsC1-C2 double mutant strain, suggesting a possible relation between MSH and the arsenate reductase activities. For arsenite, this was not the case, and only a slight decrease of the resistance level as compared with wild type was observed (Fig. 1D).

Next to the mycothiol redox system, C. glutamicum has a transcriptional thioredoxin unit consisting of trxB-trxA (thioredoxin reductase and thioredoxin; NCgl2984-NCgl2985)
In the case of *S. aureus* plasmid pI258 ArsC, TrxR and Trx are the redox enzymes responsible for electron transport from NADPH (12, 33). By the construction of *trx* gene disruption mutants, we checked the possible involvement of *trxB* and *trxA* in As(V) resistance in *C. glutamicum*. Unfortunately, the inactivation of the *trx* genes seems to be lethal, because no transconjugants were obtained after many attempts, suggesting their importance and essential role for *C. glutamicum*. Their involvement in the defense mechanism against arsenate could not be ruled out in vivo.

**Electron Transfer Pathways in Vitro Reconstructed**—In *C. glutamicum*, we located two hypothetical mycoredoxins: Mrx1 (NCgl0808) and Mrx2 (NCgl2445). The corresponding *mrx* genes were identified by homology with the *E. coli* glutaredoxin genes (grx) (16). Mrx2 shows 76% sequence identity (NCBI BLAST program) (34) with NrdH-redoxin from *Corynebacterium ammoniagenes* for which the structure shows a domain-swapped dimer (35). Mycoredoxin1 (Mrx1) is characterized with the same active site sequence motif as Grx (i.e. CPYC). Grx is a redox enzyme with a high specificity for the tripeptide GSH (36). Based on this knowledge, the *in vitro* electron transfer functionality of two pathways was tested, the Trx/TrxR pathway and the MSH/Mrx1 pathway (Fig. 2, A and B). Whether Mrx2 could accept electrons from MTR or TrxR was also tested. For this kinetic study, we used only enzymes of *C. glutamicum*, because it is well known that although the active site cysteines of redox enzymes are essential for protein reduction, the participation of the integral structure in the target recognition process modulates its efficiency in doing so (37). All enzymes of both pathways were recombinantly overexpressed in *E. coli* and purified to homogeneity (see supplemental material).

The redox enzymes Trx, Mrx1, and Mrx2 were oxidized with a 10-fold molar excess of diamide, which was subsequently removed on a size exclusion column. By monitoring the consumption of NADPH as a function of time, the electron transfer chains were tested for all three enzymes were tested in both pathways (Fig. 2). Mrx1 was found to be specific for the MSH/Mrx1 pathway (Fig. 2B), whereas the function of Mrx2 and Trx is restricted to accept electrons from TrxR (Fig. 2A). We have reconstituted two thiol/disulfide redox pathways from *C. glutamicum* and shown for the first time the functionality of two hypothetical mycoredoxins: Mrx1 and Mrx2.

*Cg_ArsC1* and *Cg_ArsC2* Receive Electrons from the Mycothiol/Mycoredoxin 1 Pathway—The following objective was to investigate whether *Cg_ArsCs* reduce arsenate to arsenite with electrons coming from the Trx/TrxR-pathway or from the MSH/Mrx1 pathway. Both *Cg_ArsC1* and *Cg_ArsC2* were recombinantly overexpressed in *E. coli* (see supplemental material). With progress curves using varying concentrations of enzymes, arsenate, and NADPH, we found that both *Cg_ArsC1* and *Cg_ArsC2* are only connected with the MSH/Mrx1 pathway (Fig. 2C), whereas the function of Mrx2 and Trx is restricted to accept electrons from TrxR (Fig. 2A). We have reconstituted two thiol/disulfide redox pathways from *C. glutamicum* and shown for the first time the functionality of two hypothetical mycoredoxins: Mrx1 and Mrx2.
The Cg_ArsCs Kinetics Are Slow—In order to correctly interpret the kinetic parameters generated in an enzymatic assay with several components, it is of uppermost importance that the enzymes stay active during the course of the enzymatic assay (14). Especially for enzymes that contain oxidation sensitive cysteines and in assays where products are formed that could react with cysteines (like arsenite), extra attention is needed (14). We showed that millimolar concentrations of As(III) are inhibiting, whereas As(V) is activating the MSH/Mrx1 pathway (Fig. 3A). To ensure that product inhibition by As(III) is negligible, initial velocities were measured.

In the assay, the concentration of MSH, Mrx1, and MTR have to be high enough so that their action is not rate-limiting. Otherwise, the progress curves will show a lag phase, and the use of initial rates for calculating kinetic plots will lead to false cooperativity. We varied the concentrations of Cg_ArsC, MSH, Mrx1, and MTR with a constant concentration of 250 μM NADPH. After concentration optimization, the various components in the coupled enzyme assay necessary to yield Michaelis-Menten kinetics (Hill factor of 1) were found to be 200 nm Cg_ArsC1, 0.47 mM MSH, 3 μM MTR, and 250 μM NADPH. Since arsenate is already inducing nonenzymatic background electron transfer in the MSH/Mrx1 pathway (Fig. 3B), the reaction was started with the addition of Cg_ArsC. Removing the components one by one and testing different combinations resulted in lower initial velocities. As such, all components contribute to the electron transfer pathway.

Thiol/disulfide exchange reactions are pH-dependent. We tested the pH dependence of the reaction by comparing the initial rates in the presence of 10 mM arsenite at various pH values (6.5, 7.0, 7.5, and 8.0). Increasing pH resulted in increasing initial velocities, with the highest value at pH 8.0.
Finally, we measured the kinetic parameters of Cg_ArsC1 (Fig. 3C) and Cg_ArsC2 (Fig. 3D) under the optimized conditions in the presence and absence of the oxidant (Table 1). Phosphate and sulfate have been shown to stabilize pI258 ArsC from S. aureus and increase its $k_m$ and $k_{cat}$ values (14). For Cg_ArsCs, the stabilizing effect is only marginal (for the Selwyn test, see Fig. S2). The $k_m$ drops with a factor of 2, and the effect on the $k_{cat}$ is insignificant. All together, Cg_ArsCs have slow kinetics ($k_{cat}$ of 32 and 17 min$^{-1}$; see Table 1) with a specificity constant of $\approx 5$ M$^{-1}$ s$^{-1}$.

Cg_ArsC and Mycoredoxin 1 Show Specificity to Mycothiol, and Mycoredoxin 1 Functions as a Monothiol Oxidoreductase—Mycoredoxin 1 (Mrx1) seems to function in C. glutamicum as a glutaredoxin. Therefore, we tested whether electron transfer is also possible when mycothiol is replaced by glutathione. We analyzed NADPH consumption at 340 nm in the presence of MTR, Mrx1, GSH, Cg_ArsC, and arsenate (Fig. 4A). Progress curves showed no electron transfer in the presence of GSH. The coupled redox cascade reaction is strictly linked to MSH.

Glutaredoxins are functioning either with one or two cysteines in the active site (38, 39). In the case of a monothiol, first a mixed disulfide between glutaredoxin and glutathione is formed. By a subsequent thiol-disulfide exchange with reduced glutathione, the enzyme is regenerated. In the case of a dithiol, a mixed disulfide between glutaredoxin and glutathione is formed. By a subsequent thiol-disulfide exchange with reduced glutathione, the enzyme is regenerated. In the case of a dithiol, a mixed disulfide between glutaredoxin and glutathione is formed. By a subsequent thiol-disulfide exchange with reduced glutathione, the enzyme is regenerated.

To check whether Mrx1 is using one or two active site cysteines in the reaction mechanism related to Cg_ArsCs, we mutated the first, the second, and both cysteines of the CXXC motif to alanines. Their functionality was tested in progress curves and compared with wild type Mrx1 (Fig. 4B). Mutant Mrx1 CXXA is almost as functional as wild type. Its initial velocity dropped less than 10% (Figs. 3B and 4B). On the other hand, electron transfer was drastically reduced to background levels when Mrx1 AXXC or Mrx1 AXXA was present. As such, Mrx1 is functioning as a monothiol mixed disulfide reductase with an essential N-terminal nucleophilic cysteine.

Cg_ArsC Catalyzes the MSH/Mrx1-dependent Reduction of Arsenate to Arsenite—In a comparative study, we analyzed the catalyzed versus the noncatalyzed formation of arsenite as a function of time using the optimized assay conditions (see above) (Fig. 5A). Cg_ArsC1 is clearly decreasing the activation energy toward product formation during the reaction. Nevertheless, noncatalyzed As(III) formation is also observed, and this phenomenon is even more striking during an overnight experiment at 37 °C (Fig. 5C). Cg_ArsCs incubated with arsenate do not produce As(III). At least MSH is needed, but the reaction is more efficient in the presence of MSH and Mrx1. Under the latter conditions, the importance of the N-terminal nucleophilic cysteine of Mrx1 is confirmed.

Both Cg_ArsC1 and Cg_ArsC2 produce about the same amount of As(III) in a 2-h incubation experiment at 37 °C (Fig. 5B). Increasing the concentration of wild type Mrx1 from 10 to 465 μM (equivalent to the concentration of MSH in the reaction) in the absence of MTR and NADPH increases the level of As(III) (Fig. 5B). To produce arsenite, MTR and NADPH are not explicitly needed, and with a reaction mixture of Cg_ArsC, arsenate, MSH, and Mrx1, similar high levels of As(III) are obtained. As such, the functional role of MTR and NADPH is most probably only to recycle the formed mycothione or the mixed disulfide between mycothiol and mycoredoxin.

Cg_ArsC Forms an Arseno Adduct to Facilitate As(V)-SM Formation—To scrutinize the reaction, MSH was tested as neutral and thiolate entity (pK$_{a2}$ = 8.3 (40)) for its nucleophilic attack toward arsenate and toward a Cg_ArsC arseno-thiol adduct. We address the question whether MSH is performing a nucleophilic attack toward the sulfur or toward the arsenic atom in the arseno-thiol adduct.

With a pK$_{a2}$ of 6.9, the majority of arsenate is present as HAsO$_2^-$ at pH 8. The protonation state of the Cg_ArsC arseno-thiol adduct is not known but will be mono- or dianionic; as such, both CH$_3$S-AsO$_2^-$ and CH$_3$S-HAsO$_4^-$ are considered as simplified models for the enzyme arseno-thiol adduct (Table 2). The reactivity of MSH toward HAsO$_4^-$ is compared with the reactivity toward CH$_3$S-AsO$_2^-$ and CH$_3$S-HAsO$_5^-$. We found that according
to the principle of minimal softness (hard and soft acids and bases principle), the reactivity to the arsenic atom is larger (lower difference in softness) than to the sulfur atom in both CH$_3$S-AsO$_3^2^-$ and CH$_3$S-HAsO$_4^-$ (Table 2). We also observed that the reactivity of MSH toward the thiol-arseno adduct is higher than toward arsenate. These data suggest that Cg_ArsCs form an arseno adduct that facilitates As(V)-SM formation. Similar results were obtained with GSH (Table 2).

DISCUSSION

Most of the cellular arsenate resistance is associated with the presence of cellular arsenate reductases (ArsC/Acr2p). In C. glutamicum, we have found a completely new enzymatic mechanism for the reduction of arsenate in which the electron transfer is coupled to mycothiol (MSH) and mycoredoxin1 (Mrx1) (Fig. 6). No link with the Trx/TrxR-pathway was observed.

With MSH biosynthesis pathway mutants (22), we showed a clear link between the production of mycothiol and the level of arsenate resistance. Newton and Fahey (41) showed that MshA and MshC activities are essential for MSH production, whereas MshB and MshD mutants are still producing trace amounts of MSH in Mycobacterium. The arsenate resistance levels observed for C. glutamicum mutants (Msha, Mshb, Mshc, and Mshd) are consistent with their findings. Msha and Mshc mutants of C. glutamicum displayed equivalent resistance levels as observed for the double disruption ArsC1-C2 mutant, whereas in the MshB and MshD mutants, the resistance to As(V) was not so dramatically decreased (Fig. 1 D). In a C. glutamicum Mtr mutant strain with the MSSM reduction blocked, a constitutive production of MSH maintains the arsenate resistance at a basal level of 3 mM.

Although As(III) is a stronger oxidant and more toxic than As(V), the resistance levels to As(III) for the mutants of the MSH biosynthetic pathway did not decrease to the same extent as observed for As(V) (Fig. 1 D). In these mutants, the slight decrease in resistance at higher As(III) concentration might be associated with the lack of the redox buffer capacity in the absence of MSH. In E. coli, a similar gradual decrease of the resistance to As(III) was observed in the absence of GSH (42).

We showed that for the arsenate reduction mechanism, Cg_ArsC1 and Cg_ArsC2 depend not only on the presence of MSH but also on the presence of the N-terminal cysteine in both Cg_ArsCs and Mrx1. As such, the reaction proceeds via a thiol/disulfide exchange mechanism. This is also confirmed by the necessity of the presence of MTR and NADPH to reduce MSSM or the MSH-Mrx1 mixed disulfide. In the absence of MTR and NADPH, no electrons are consumed, and the reaction cannot be followed spectrophotometrically at 340 nm. Adding As(V), MTR, and NADPH to MSH and Mrx1, in the absence of Cg_ArsCs, resulted already in a background level reaction (Fig. 3 B). Arsenate reacts with MSH and forms arsenite (Fig. 5). For C. glutamicum, the reaction of arsenate with millimolar concentrations of MSH in the cell might even be the first line of defense against arsenate entering the cell via the phosphate uptake system (2). Arsenite induces the ars operon, since the Cg_ArsR repressor can only be

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**FIGURE 5.** Cg_ArsC1 catalyzes the MSH/Mrx1-dependent reduction of arsenate to arsenite. A, in 20 mM Tris/HCl, pH 6.5, as buffer solution, 100 mM As(V), 0.47 mM MSH, 10 μM MTR, and 250 μM NADPH were incubated without (gray) and with 200 nM Cg_ArsC1 (black) at 37 °C for 30, 60, and 120 min. Relative percentages of the produced As(III) are shown. B, relative percentages of the produced As(III) after 2 h of incubation with varying sample compositions are shown. The same concentrations as in A were used, except when indicated. ArsC2 was used at 200 nM. C, same as in B but after 16 h of incubation.

**TABLE 2**

Softness difference calculation

| Electrophiles | Sulfur atom of GSH Δs | Sulfur atom of MSH Δs | Sulfur atom of MS$^{-}$ Δs |
|---------------|------------------------|------------------------|---------------------------|
| Arsenic in HAo$_4^{2-}$ | 1.47 | 1.37 | 1.15 |
| Arsenic in CH$_3$S-HAsO$_4^{2-}$ | 0.002 | 0.09 | 2.62 |
| Sulfur in CH$_3$S-AsO$_3^{2-}$ | 1.19 | 1.28 | 3.81 |
| Arsenic in CH$_3$S-AsO$_3^{2-}$ | 0.87 | 0.78 | 1.74 |
| Sulfur in CH$_3$S-AsO$_3^{2-}$ | 1.35 | 1.44 | 3.96 |

*a.u., atomic units.*
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released from the ars operator/promoter region with As(III) and not with As(V) (43). A slightly different model has been suggested for Desulfovibrio desulfuricans (44); here, the constitutively expressed orphan arsC gene is producing arsenite to induce the ars operon.

In the absence of Cg_ArsCs, MSH is capable of reducing As(V) to As(III). This reaction strongly suggests the formation of a MS-As(V) adduct (Fig. 6A) that will be reduced by MSH to generate As(III) (Fig. 5) and MSSM. MSH in its thiolate form seems to be the preferred nucleophile for this reaction (Table 2). MSSM will be further reduced by MTR with the consumption of NADPH. The reduction of MS-As(V) is more efficient in the presence of Mrx1 and causes the background level in the kinetic and the arsenite assays. Mrx1 uses its N-terminal nucleophilic cysteine to attack the MS-As(V) adduct with the formation of an Mrx1-S-SM complex. Subsequently, Mrx1-S-SM enters the MSH/MTR-pathway (Fig. 6B). Unfortunately, we do not have experimental evidence for As(V)-SM adduct and the Mrx1-S-SM mixed disulfide complex formation, because these transiently occurring forms are instantly reduced by MSH and/or Mrx1.

It looks as if Cg_ArsCs are not necessary to reduce As(V) to As(III), although its catalytic role was clearly shown in a short time range, where the electron transfer and the production of arsenite is increased in the presence of Cg_ArsCs (Fig. 5A).

The thiol/disulfide-linked reaction mechanism of Cg_ArsC. A, the gas phase-optimized (hf/6–31G level) mycothiol-arseno structure is shown. MSH is shown in a stick representation, and the sulfur (S), arsenic (As) and the As(V)-oxygen are shown in ball representations. The optimized S–As interatomic distance is 2.5 Å. B, Cg_ArsC-catalyzed mycothiol-arseno adduct formation. Mrx1 reduces the thiol-arseno bond and forms As(III) and a mixed mycothiol-mycoredoxin disulfide (Mrx1-S-SM). A second molecule of MSH recycles Mrx1 and forms MSSM; (iv) MSSM in its turn is reduced by the NADPH-dependent MTR. At a first glance, the mechanism with the formation arseno-glutathione intermediate looks similar to what has been proposed for E. coli R773 ArsC, which is coupled to GSH and Grx (19, 45). However, in the mechanism suggested for E. coli, a monohydroxy positively charged arsenite intermediate is formed on R773 ArsC, and arsenite is only released from the enzyme after hydroxylation. In C. glutamicum, the mechanism is different; the active site thiolate in Cg_ArsC lowers the energy barrier to facilitate adduct formation between arsenate and mycothiol. Here, arsenite is released after a nucleophilic attack of Mrx1.

Cg_ArsC1 and Cg_ArsC2 are very slow arsenate reductases (Table 1). We compared the kinetic constants of both Cg_ArsCs with other small cytoplasmic arsenate reductases (Table 3). Low specificity constants are also observed for the GSH/Grx-coupled arsenate reductases. Common is that arsenate reductases are using the sequential involvement of three different thiolate nucleophiles that function as a redox cascade. In pl258 ArsC from S. aureus (11), with all three thiolate nucleophiles in a single molecule, a higher catalytic efficiency is obtained; the specificity constant is several orders of magnitude higher. All arsenate reductases produce arsenite with relatively small $k_{\text{cat}}$ values. Low $k_{\text{cat}}$ values might be explained by the fact that arsenite is more toxic compared with the substrate arsenate (46). To guarantee an immediate efflux of the reactive arsenite, the Acr3p efflux pump (28, 47) has to work in concert with the Cg_ArsCs.

GSH and MSH are low molecular weight redox buffer components for which evolution might have selected a similar active site in glutaredoxin and mycoredoxin. We showed that Cg_ArsC and

![Image](61x241 to 397x389)

**FIGURE 6. The thiol/disulfide-linked reaction mechanism of Cg_ArsC. A, the gas phase-optimized (hf/6–31G level) mycothiol-arseno structure is shown. MSH is shown in a stick representation, and the sulfur (S), arsenic (As) and the As(V)-oxygen are shown in ball representations. The optimized S–As interatomic distance is 2.5 Å. B, Cg_ArsC-catalyzed mycothiol-arseno adduct formation. Mrx1 reduces the thiol-arseno bond and forms As(III) and a mixed mycothiol-mycoredoxin disulfide (Mrx1-S-SM). A second molecule of MSH recycles Mrx1 and forms MSSM that is reduced by the NADPH-dependent MTR.**

**TABLE 3**

| ArsCs** | Nucleophiles | $K_m$ | $k_{\text{cat}}$ | $k_{\text{cat}}/K_m$ | $H$ | References |
|---------|--------------|------|-------------------|---------------------|-----|------------|
| Cg_ArsC1 | MSH/Mrx | 142 ± 19 | 32 ± 2.3 | 3.8 | 1 | This work |
| Cg_ArsC2 | MSH/Mrx | 82 ± 13 | 17 ± 1.2 | 3.4 | 1 | This work |
| Sc_Acr2p | GSH/Grx | 35 | 6 | 2.8 | 2.7 | Mukhopadhyay et al. (29) |
| Ec_ArsC R773 | GSH/Grx | 15 | 32 | 35 | 1 | Gladysheva et al. (15) |
| Os_Acr | GSH/Grx | 12 | 20 | 27 | 1 | Duan et al. (55) |
| Lm_Acr2 | GSH/Grx | 10 | 5.5 × 10⁻³ | 10⁻² | 1 | Zhou et al. (18) |
| Sa_ArsC pl258 | Trx | 68 × 10⁻³ | 215 | 5.2 × 10⁴ | 1 | Messens et al. (14) |

**a Cg, C. glutamicum; Sc, S. cerevisiae; Ec, E. coli; Os, Oryza sativa; Lm, L. major; Sa, S. aureus.**

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Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., Biscachi, D., Zhou, Y., Armirotti, A., and Bordo, D. (2009) J. Mol. Biol. 386, 1229–1239.

Mukhopadhyay, R., Phung, L. T., and Silver, S. (2002) J. Biol. Chem. 277, 26102–26109.

Mukhopadhyay, R., Rosen, B. P., Phung, L. T., and Silver, S. (2002) FEMS Microbiol. Rev. 26, 311–325.

Mukhopadhyay, R., Rosen, B. P., Phung, L. T., and Silver, S. (2002) J. Biol. Chem. 277, 26102–26109.

Mukhopadhyay, R., and Wyna, L. (2002) J. Biol. Inorg. Chem. 7, 1465–1473.

Mukhopadhyay, R., and Rosen, B. P. (1998) FEBS Lett. 420, 415–419.

Mukhopadhyay, R., and Rosen, B. P. (2008) FEBS Lett. 582, 2012–2016.

Mukhopadhyay, R., Phung, L. T., and Silver, S. (2002) J. Biol. Chem. 277, 26102–26109.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) Biochemistry 37, 17145–17156.

Mukhopadhyay, R., and Rosen, B. P. (2000) J. Biol. Chem. 275, 30311–30319.

Mukhopadhyay, R., and Rosen, B. P. (2002) J. Biol. Chem. 277, 26102–26109.

Mukhopadhyay, R., Phung, L. T., and Silver, S. (2002) J. Biol. Chem. 277, 26102–26109.

Mukhopadhyay, R., and Rosen, B. P. (2000) J. Biol. Chem. 275, 30311–30319.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.

Mukhopadhyay, R., and Rosen, B. P. (1998) J. Biol. Chem. 273, 9474–9478.
A Mycothiol-Mycoredoxin-dependent Arsenate Reductase

48. Newton, G. L., Fahey, R. C., Cohen, G., and Aharonowitz, Y. (1993) *J. Bacteriol.* 175, 2734–2742
49. Bzymek, K. P., Newton, G. L., Ta, P., and Fahey, R. C. (2007) *J. Bacteriol.* 189, 6796–6805
50. Newton, G. L., Av-Gay, Y., and Fahey, R. C. (2000) *Biochemistry* 39, 10739–10746
51. Misset-Smits, M., van Ophem, P. W., Sakuda, S., and Duine, J. A. (1997) *FEBS Lett.* 409, 221–222
52. Vogt, R. N., Steenkamp, D. J., Zheng, R., and Blanchard, J. S. (2003) *Biochem. J.* 374, 657–666
53. Wang, R., Yin, Y. J., Wang, F., Li, M., Feng, J., Zhang, H. M., Zhang, J. P., Liu, S. J., and Chang, W. R. (2007) *J. Biol. Chem.* 282, 16288–16294
54. den Hengst, C. D., and Buttner, M. J. (2008) *Biochim. Biophys. Acta* 1780, 1201–1216
55. Duan, G. L., Zhou, Y., Tong, Y. P., Mukhopadhyay, R., Rosen, B. P., and Zhu, Y. G. (2007) *New Phytol.* 174, 311–321