Mechanism of Thiol-Supported Arsenate Reduction Mediated by Phosphorolytic-Arsenolytic Enzymes

I. The Role of Arsenolysis

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Several mammalian enzymes catalyzing the phosphorolytic-arsonolytic cleavage of their substrates (thus yielding arsenylated metabolites) have been shown to facilitate reduction of arsenate (AsV) to the more toxic arsenite (AsIII) in presence of their substrate and a thiol. These include purine nucleoside phosphorylase (PNP), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and glycogen phosphorylase-a (GPa). In this work, we tested further enzymes, the bacterial phosphotransacetylases (PTAs) and PNP, for AsV reduction. The PTAs, which arsenolytically cleave acetyl-CoA producing acetyl-arsenate, were compared with GAPDH, which can also form acetyl-arsenate by arsenolysis of its nonphysiological substrate, acetyl-phosphate. As these enzymes also mediated AsV reduction, we can assert that facilitation of thiol-dependent AsV reduction may be a general property of enzymes that catalyze phosphorolytic-arsonolytic reactions. Because with all such enzymes arsenolysis is obligatory for AsV reduction, we analyzed the relationship between these two processes in presence of various thiol compounds, using PNP. Although no thiol influenced the rate of PNP-catalyzed arsenolysis, all enhanced the PNP-mediated AsV reduction, albeit differentially. Furthermore, the relative capacity of thiols to support AsV reduction mediated by PNP, GPa, PTA, and GAPDH apparently depended on the type of arsenylated metabolites (i.e., arsenate ester or anhydride) produced by these enzymes. Importantly, AsV reduction by both acetyl-arsenate–producing enzymes (i.e., PTA and GAPDH) exhibited striking similarities in responsiveness to various thiols, thus highlighting the role of arsenylated metabolite formation. This observation, together with the finding that PNP-mediated AsV reduction lags behind the PNP-catalyzed arsenolysis lead to the hypothesis that arsenolytic enzymes promote reduction of AsV by forming arsenylated metabolites which are more reducible to AsIII by thiols than inorganic AsV. This hypothesis is evaluated in the adjoining paper.

Arsenic compounds are ubiquitous in nature. Chronic exposure to drinking water and food contaminated with inorganic arsenicals poses major health concern because it induces tissue injuries and cancer (Hughes et al., 2007; Styblo et al., 2002; Tseng, 2007). The environmentally prevalent form of arsenic, the pentavalent arsenate (AsV), enters the body through inorganic phosphate (Pi) transporters and can replace Pi in biochemical processes due to close structural similarity of AsV to Pi (Hughes, 2006). Thereafter, AsV can readily be reduced in a glutathione (GSH)–dependent manner to the trivalent arsenite (AsIII; Csanaky and Gregus, 2005), which is much more toxic because of its covalent reactivity with essential thiol groups (Kitchin and Wallace, 2008; Knowles and Benson, 1983). Further metabolism of AsIII in the body yields pentavalent and trivalent methylated metabolites, with the former being relatively nontoxic, whereas the latter even more toxic than AsIII (Styblo et al., 2002; Thomas, 2007). Therefore, reduction to AsIII is not only the first step in the biotransformation of AsV but also an important toxification reaction. Several microbial enzymes carry out thiol-dependent reduction of AsV to AsIII, thereby making it recognizable for proteins responsible for its sequestration or export from the cell (DeMel et al., 2004; Messens and Silver, 2006; Mukhopadhyay and Rosen, 2002). However, the mechanism of AsV reduction in mammals is still unknown.

Four mammalian enzymes have been demonstrated to facilitate the thiol-dependent reduction of AsV to AsIII in vitro, namely purine nucleoside phosphorylase (PNP; Gregus and Németi, 2002; Radbaugh et al., 2002), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Gregus and Németi, 2005), glycogen phosphorylase-a (GPa; Gregus and Németi, 2007; Németi and Gregus, 2007), and ornithine carbamoyltransferase (OCT; Németi and Gregus, 2009). In a process termed phosphorolysis, these enzymes cleave their substrates with Pi into two products, one of which is a stable phosphorylated metabolite. Importantly, AsV can substitute Pi in these cleavage processes, which are then termed arsenolytic reactions and which yield two products, one of which is a purportedly

Key Words: arsenate; reduction; thiols; glutathione; arsenolysis.
unstable arsenylated metabolite, an AsV ester or an AsV anhydride. Each of the four aforementioned phosphorolytic enzymes facilitated reduction of AsV, provided its substrate that becomes cleaved in the arsenolytic reaction and a thiol compound, such as GSH, were present. This phenomenon has prompted us to hypothesize that the thiol-dependent reduction of AsV to AsIII takes place in the course, or as a result, of arsenolysis. Nevertheless, the mechanism whereby the two processes, that is, reduction of AsV to AsIII and the arsenolytic cleavage reaction, are coupled has been elusive, although it has been speculated that a specific protein thiol group at the Pᵢ binding site of PNP and at the catalytic center of GAPDH may be involved in AsV reduction mediated by these enzymes (Gregus and Németh, 2002, 2005).

Two objectives have dictated the present investigations. The first is to further substantiate the growing confidence fuelled by our successive studies with PNP, GAPDH, GPa, and OCT that the capacity to promote thiol-dependent AsV reduction is a general property of the enzymes that catalyze phosphorolytic and arsenolytic reactions. For this purpose, we tested further enzymes for AsV-reducing activity, including a bacterial PNP and two phosphotransacetylases (PTAs), which originate from Clostridium kluyveri and Methanosarcina thermophila. Like its mammalian counterpart (Gregus and Németh, 2002), the bacterial PNP also catalyzes the arsenolysis of inosine into ribose-1-arsenate and hypoxanthine; however, it lacks the cysteine at its phosphate binding site. PTA enzymes cleave acetyl-CoA into coenzyme A and acetyl-phosphate in presence of Pᵢ; however, they produce the unstable acetyl-AsV in presence of AsV (Stadtman, 1952, 1955; Fig. 1). Incidentally, acetyl-AsV can also be produced when GAPDH, the well-known glycolytic enzyme, is supplied with acetyl-phosphate and AsV (Krimsky and Racker, 1955; Slocum and Varner, 1960; Fig. 1). Therefore, it was of high theoretical interest whether or not this arsenolytic reaction was also coupled to AsV reduction in presence of thiols.

Our second objective was to analyze the arsenolytic enzyme-mediated thiol-supported AsV reduction in order to decipher its mechanism. To this end, we investigated the effects of various thiol compounds, including the monothiol GSH and 2-mercaptoethanol (2-ME) and the dithiol dithiothreitol (DTT), dimercaptosulphanesulfonic acid (DMSA), and dimercaptosuccinic acid (DMSA), on the AsV reduction mediated by six phosphorolytic-arsenolytic enzymes. In addition, using PNP as a model enzyme, we analyzed the quantitative relationship between the two coupled reactions, that is, the arsenolysis of inosine and the reduction of AsV, with respect to their responsiveness to the abovementioned thiol compounds and their time courses. The findings of these studies collectively indicate that for AsV reduction mediated by phosphorolytic-arsenolytic enzymes supplied with thiols neither the arsenolytic enzyme nor the arsenolytic process per se is important, but rather the formation of an arsenylated metabolite.

MATERIALS AND METHODS

Chemicals. Inosine, hypoxanthine, 2-ME, DTT, DMPS, glycogen (type III from rabbit liver), glyceraldehyde-3-phosphate diethylacetal monobarium salt, glycerol-2-phosphate, DMSA, acetic anhydride, acetyl-phosphate, and coenzyme A (CoA-SH) were from Sigma. GSH, nicotinamide adenine dinucleotide (NAD), and disodium hydrogen arsenate (AsV) were from Reanal, Ltd. (Budapest, Hungary). Sodium arsenite (AsIII) was purchased from Carlo Erba (Milan, Italy). Koningic acid was generously provided by Professor Keiji Hasumi (Tokyo Noko University, Tokyo, Japan). Acetyl-coenzyme A (acyl-CoA) was synthesized from acetyl-CoA and AsV (Krimsky and Racker, 1955; Slocum and Varner, 1960; Fig. 1). Therefore, it was of high theoretical interest whether or not this arsenolytic reaction was also coupled to AsV reduction in presence of thiols.

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![Phosphotransacetylase-catalyzed arsenolysis of acetyl-coenzyme A](image1)

Phosphotransacetylase-catalyzed arsenolysis of acetyl-coenzyme A

\[
\begin{align*}
\text{Acetyl-CoA} & \quad \text{As} = \text{O} \\
\text{Acetyl-AsV} & \quad \text{Coenzyme A}
\end{align*}
\]

![GAPDH-catalyzed arsenolysis of acetyl-phosphate](image2)

GAPDH-catalyzed arsenolysis of acetyl-phosphate

\[
\begin{align*}
\text{Acetyl-phosphate} & \quad \text{As} = \text{O} \\
\text{Acetyl-AsV} & \quad \text{Phosphate}
\end{align*}
\]

**FIG. 1.** Arsenolytic reactions catalyzed by PTA and GAPDH. Note that the GAPDH-catalyzed arsenolysis of acetyl-phosphate shown above is an artificial reaction reported by Krimsky and Racker (1955). Physiologically, GAPDH catalyzes the phosphorolysis of GAPDH-bound 3-phosphoglyceroyl adduct; however, in presence of AsV instead of P₃, the adduct is removed by arsenolysis. This latter reaction, whose scheme is depicted in the companion paper (Gregus et al., 2009), is the subject of the experiments depicted in Figure 10.
according to methods described previously (Simon and Shemin, 1953; Srere et al., 1963). The sources of chemicals used in arsenic speciation have been given elsewhere (Csanaky et al., 2003; Németi and Gregus, 2002). All other chemicals were of the highest purity commercially available.

**Enzymes and assays.** PTAs from *Clostridium kluyveri* and *Methanosarcina thermophila*, rabbit muscle GPα, GAPDH, and calf spleen PNP were from Sigma. PNP of bacterial origin produced by Toyobo Co., Ltd. (Osaka, Japan) was purchased from Sorachim (Paris, France). Before use, these enzymes were assayed for their known biochemical activity, based on which they were used in subsequent assays measuring their AsV-reducing activity. The activities of PTAs were assayed by the spectrophotometric method of Klotzsch (1969) that measures the thioester bond formation in presence of CoA-SH and acetyl-phosphate. GPα activity was assayed in the direction of glycogenolysis, as described by Kaiser et al. (2001). The assay measures the formation of NADPH during the GP-limited conversion of glycogen to glucose-1-phosphate, which is converted first to glucose-6-phosphate then to 6-phosphogluconate by phosphoglucomutase and glucose-6-phosphate dehydrogenase, respectively. The activity of GAPDH was assayed spectrophotometrically as described earlier (Gregus and Németi, 2005). This procedure measures the rate of the reverse reaction (rather than the forward reaction in the direction of glycolysis) by quantifying the decrease of NADH concentration during the GAPDH-limited

![FIG. 2. PTA-mediated reduction of AsV—Dependence on time and enzyme concentration. PTA from *C. kluyveri* or *M. thermophila* (0.5 U/ml or as indicated in the right panel) was preincubated in sucrose-Hepes buffer containing ammonium chloride (25mM) with GSH (10mM) at 37°C for 5 min. Then acetyl-CoA (0.5mM) and AsV (50μM) were added to start the incubation lasting for the times indicated in the left panel or for 30 min. Symbols represent AsIII formation rates (mean ± SEM) in three incubations.](image)

![FIG. 3. PTA-mediated reduction of AsV—Dependence on glutathione and ammonium chloride concentrations. PTA from *C. kluyveri* or *M. thermophila* (0.5 U/ml) was preincubated in sucrose-Hepes buffer containing ammonium chloride (at concentrations indicated in the left panel or 25mM) with GSH (10mM or as indicated in the right panel) at 37°C for 5 min. Then acetyl-CoA (0.5mM) and AsV (50μM) were added to start the 30-min incubation. Symbols represent AsIII formation rates (mean ± SEM) in three incubations.](image)
conversion of 3-phosphoglycerate to glyceraldehyde-3-phosphate in the presence of excess phosphoglycerate kinase and ATP. The activities of PNPs were measured according to the method of Kalckar (1947) that records the formation of uric acid from hypoxanthine by xanthine oxidase in the PNP-limited conversion of inosine to hypoxanthine.

Assaying arsenolytic enzymes for thiol-dependent AsV-reducing activity. AsV-reducing activities of purified PTA and GAPDH were assayed in sucrose-Hepes buffer (containing 250mM sucrose, 25mM Hepes, 5mM MgCl2, 2mM EDTA, pH 7.4), whereas those of GPa and PNP were measured in glycerol-2-phosphate buffer (containing 25mM glycerol-2-phosphate, 1mM EDTA, pH 7.4). The enzymes were preincubated with a thiol compound at 37°C for 5 min then AsV was added to start the incubation in a final volume of 0.3 ml. The thiol used and other details of the incubation conditions are specified in the figure legends. The incubations were stopped by sequential addition of 100 μl of 50mM CdSO4 solution followed by 100 μl of 1.5M perchloric acid solution containing

FIG. 4. PTA-mediated reduction of AsV—Dependence on AsV and acetyl-CoA concentrations. PTA from C. kluyveri or M. thermophila (0.5 U/ml) was preincubated with GSH (10mM) in sucrose-Hepes buffer containing ammonium chloride (25mM) at 37 °C for 5 min. Then acetyl-CoA (0.5mM or at concentrations indicated in the right panel) and AsV (at concentrations indicated in the left panel or 50μM) were added to start the 30-min incubation. Symbols represent AsIII formation rates (mean ± SEM) in three incubations.

FIG. 5. PTA-mediated reduction of AsV—Effects of coenzyme A (CoA-SH), acetyl-phosphate, and Pi. PTA from C. kluyveri or M. thermophila (0.5 U/ml) was preincubated with GSH (10mM) in sucrose-Hepes buffer containing ammonium chloride (25mM) at 37°C for 5 min. Then CoA-SH, or acetyl-phosphate, or Pi (at the concentrations indicated) and acetyl-CoA (0.5mM) plus AsV (50μM) were added to start the 30-min incubation. Symbols represent AsIII formation rates (mean ± SEM) in three incubations. Asterisks indicate AsIII formation rates significantly different (p < 0.05) from that observed in the absence of added compounds (i.e., at concentration zero).
50 mM HgCl₂. The rationale for this procedure has been given elsewhere (Némethi and Gregus, 2004). Briefly, Hg²⁺ ions effectively displace the thiol-bound AsIII; however, they oxidize it at neutral pH but not in acid. Therefore, we added Cd²⁺ first, which binds to thiol groups at neutral but not at acidic pH, and which displaces thiol-bound AsIII, but does not oxidize the released AsIII. The incubates thus treated were stored at −80°C until arsenic analysis.

FIG. 6. AsV reduction mediated by GAPDH catalyzing arsenolysis of acetyl-phosphate (acetyl-P)—Effects of omission of reagents, addition of the GAPDH inhibitor koningic acid (KA), and substitution of GSH with other thiols. To perform the complete assay (A), GAPDH from rabbit muscle (2 U/ml) was preincubated in sucrose-Hepes buffer with GSH (10 mM) and NAD (1 mM) at 37°C for 5 min. Then acetyl-P (5 mM) and AsV (100 μM) were added to start the 30-min incubation. To examine the effect of KA (30 μM), this inhibitor was added into preincubation mix. To test the effect of DMSA, DTT, DMPS, and 2-ME (B), GSH in the complete assay was replaced with one of these thiols (all at 10 mM SH-group concentration). Bars represent AsIII formation rates (mean ± SEM) in three to six incubations. All AsIII formation rates are significantly lower (p < 0.05) than those observed in the complete incubation (A) or in presence of GSH (B).

FIG. 7. Effect of thiols on the PNP-mediated AsV reduction. PNP of calf spleen or bacterial origin was preincubated in glycerol-2-phosphate buffer with inosine (1 mM) and the thiol compound indicated (1–15 mM) at 37°C for 5 min. Then AsV (50 μM) was added to start the 10-min incubation. The effects of GSH and DMSA, which are also depicted in the insert, were tested at 0.5 U/ml PNP concentration, whereas the effects of the other thiols were examined at 0.02 U/ml PNP concentration. Symbols represent AsIII formation rates (mean ± SEM) in three incubations.
Because in presence of a thiol AsV is reduced nonenzymatically to a small extent, the nonenzymatic AsIII formation rates were regularly determined from incubations lacking enzyme but containing the appropriate thiol compound, and subtracted from the rates measured when the incubation contained both enzyme and thiol. The enzymatic AsV-reducing activity thus calculated was expressed as the amount of AsIII formed per minute and unit enzyme.

**Assaying PNP for arsenolytic and AsV-reducing activities.** In order to compare the AsV-reducing activity (i.e., AsIII formation from AsV) and arsenolytic activity (i.e., hypoxanthine formation from inosine) of PNP, bacterial PNP (25 mU/ml) was incubated with AsV (100µM) in presence of inosine (1mM) and a thiol compound (i.e., GSH, 2-ME, DMSA, DMPS, or DTT) in sucrose-Hepes buffer at 37°C for 10 min, as described above. Immediately before the incubations were stopped, the incubates were divided into two samples. AsV reduction in the sample to be used for arsenic analysis was stopped as described above, whereas arsenolysis in the sample for hypoxanthine analysis was stopped by adding 1/6 volume of 2.1M perchorlic acid solution. Other details of these assays are given in the legends of Figures 12–15.

**Arsenic analysis.** After having been subjected to protein precipitation as described above, the incubates from the AsV reductase assays were centrifuged at 10,000 × g, 4°C for 10 min. AsIII and AsV in the resultant supernatants were separated and quantified by HPLC-hydride generation-atomic fluorescence spectrometry, using a strong anion exchange guard column and an analytical column (both Hamilton PRP X-100) and eluted isocratically with 60mM sodium phosphate buffer (pH 5.75). The details of this analysis have been given elsewhere (Gregus et al., 2000; Németh et al., 2003).

**Hypoxanthine analysis.** Hypoxanthine in the supernatant of the deproteinized incubates was quantified using an HPLC-UV method. Briefly, samples were injected through a Rheodyne 7125 injector equipped with a 20-µl sample loop onto a guard column (3.9 × 20 mm) followed by an analytical column (3.9 × 50 mm; both Novapack C18, particle size 4 µm), and eluted isocratically with 60mM sodium phosphate buffer (pH 5.75) at a flow rate of 1 ml/min. The absorbance of the effluent was monitored at 254 nm by a flow-through spectrophotometer (Waters 486) and recorded by Millenium 3.2 (Waters, Milford, MA). Hypoxanthine and inosine eluted at 1.2 min and 3.3 min, respectively. Quantification was based on peak areas of samples and authentic standards.

**Statistics.** Data were analyzed using one-way ANOVA followed by Duncan’s test or Student’s t-test with p < 0.05, as the level of significance.

**RESULTS**

**Bacterial Phosphotransferases, which Arsenolytically Cleave Acetyl-Coenzyme A and Form Acetyl-Arsenate, also Mediate Thiol-Supported AsV Reduction Coupled to Arsenolysis**

PTA enzymes originating from *Clostridium kluyveri* and *Methanosarcina thermophila* catalyze the phosphorolysis or arsenolysis of acetyl-CoA (Fig. 1). As shown in Figures 2–5, the two enzymes mediated AsV reduction with very similar activities when supplied with their substrate acetyl-CoA, the activator NH$_4^+$ ion, and GSH. Formation of AsIII from AsV increased asymptotically as a function of incubation time (Fig. 2, left) and of the concentrations of the enzymes (Fig. 2, right), NH$_4$Cl (Fig. 3, left), AsV (Fig. 4, left), and acetyl-CoA (Fig. 4, right). Figure 3 (right) demonstrates that AsIII was not formed in absence of GSH, and GSH supported the AsV-reducing activities of both enzymes in a concentration-dependent fashion.

Free CoA-SH, the product of PTA-catalyzed phosphorolysis/arsenolysis (Fig. 1), inhibited AsV reduction mediated by either PTA enzyme (Fig. 5). In contrast, acetyl-phosphate, the other product of PTA-catalyzed phosphorolysis, significantly stimulated AsV reduction by both PTA enzymes at low concentrations (0.25 and 0.5mM), and inhibited it only at 1.5mM and above. Interestingly, the responsiveness to Pi of the PTA-mediated AsV reduction differed with respect to the enzymes. Formation of AsIII from AsV by PTA from *C. kluyveri* was not influenced significantly by Pi (Fig. 5, left), whereas Pi diminished AsV reduction by PTA from *M. thermophila* significantly above 1mM (Fig. 5, right).

**GADPH, which Arsenolytically Cleave Acetyl-Phosphate and Forms Acetyl-Arsenate, also Mediates Thiol-Supported AsV Reduction Coupled to Arsenolysis**

It is known that GAPDH can be acylated on its active site cysteine not only by 1,3-bisphosphoglycerate (the regular substrate of GAPDH) but also by acetyl-phosphate. The thus-formed thioester bond between the enzyme and the acetyl group can be cleaved arsenolytically resulting in acetyl-arsenate formation (Krimsky and Racker, 1955; Slocum and Varner, 1960). Therefore, we tested whether GAPDH could facilitate reduction of AsV to AsIII when catalyzing the arsenolysis of...
acetyl-phosphate, the reaction depicted in Figure 1. As shown in Figure 6, GAPDH mediated the reduction of AsV if supplied with its non-physiological substrate acetyl-phosphate, the activator NAD, and the thiol GSH. Omission of any of these reactants or addition of the GAPDH inhibitor koningic acid strongly diminished AsIII formation (Fig. 6, left). Because AsV reduction depended on the presence of GSH, we tested if other thiols could also support formation of AsIII during the GAPDH-catalyzed arsenolysis of acetyl-phosphate. As demonstrated in Figure 6 (right), DTT, 2-ME, DMPS, and DMSA also supported AsV reduction, albeit less effectively than GSH.

**Thiols Differentially Support Phosphorolytic/Arsenolytic Enzyme-Mediated AsV Reduction**

The observation that phosphorolytic/arsenolytic enzymes can uniformly facilitate the reduction of AsV to AsIII in a strictly thiol-dependent manner prompted us to compare the concentration-dependent effects of different thiols on the formation of AsIII by six purified arsenolytic enzymes, including two PNP enzymes (i.e., PNP from calf spleen and from a bacterial species), GPa and GAPDH from rabbit muscle, and the two bacterial PTA enzymes. Each enzyme was incubated with AsV in presence of its arsenolytic substrate, its activator (if applicable), and a thiol (GSH, 2-ME, DTT, DMSA, or DMPS) at 0–15mM SH-group concentrations.

The AsV-reducing activities of the PNP enzymes and of GPa, which form AsV esters of sugars (i.e., ribose-1-arsenate and glucose-1-arsenate, respectively) during their arsenolytic reaction, were best supported by DTT, and in presence of this dithiol the calf spleen and bacterial PNP exhibited 50 and 30 times higher AsV-reducing activities, respectively, than GPa (Figs. 7 and 8). In contrast, AsV reduction by PTAs and GAPDH, which produce AsV anhydrides (i.e., acetyl-AsV and 3-phosphoglyceroyl-AsV) during their arsenolytic reaction, was best supported by GSH (Figs. 9 and 10, respectively). Irrespective of the enzymes, however, the concentration-dependent effects of thiols on AsV reduction exhibited similar patterns: with respect to the thiol concentration, DTT and DMPS asymptotically, 2-ME sigmoidally, DMSA linearly, whereas GSH quadratically supported the reduction of AsV to AsIII.

**Divergence in Arsenolysis of Inosine and Reduction of AsV as Revealed by Studies with PNP in Presence of Thiols**

In order to better understand the mechanism of arsenolytic enzyme-mediated thiol-supported AsV reduction, we characterized the relationship between the two coupled reactions, that is, the arsenolytic reaction and the reduction of AsV to AsIII, in presence of different thiols. These experiments were carried out with PNP, the most active AsV-reducing arsenolytic enzyme hitherto identified. The PNP-catalyzed arsenolysis was quantified as the amount of hypoxanthine produced by the arsenolytic cleavage of inosine (Fig. 11), whereas the coupled AsV reduction was measured as the amount of AsIII formed.

Figure 12 (top) demonstrates that the formation of hypoxanthine (i.e., the rate of PNP-catalyzed arsenolysis of inosine) was similar in the absence and the presence of any of the five thiol compounds. In contrast, the formation of AsIII...
was abolished in absence of thiols (Fig. 12, bottom), and different thiol compounds, though added at equimolar SH-group concentration (10mM -SH), differentially increased AsV reduction, with DTT providing the strongest and GSH the weakest support.

To analyze the relationship between the PNP-catalyzed arsenolysis and AsV reduction further, the concentration-dependent effects of DTT and GSH were examined. In the SH-group concentration range tested (0–15mM), neither GSH nor DTT altered hypoxanthine production (Fig. 13, top). In contrast, the DTT-supported AsV reduction increased asymptotically, reaching maximum at 1.25mM DTT concentration (i.e., at 2.5mM SH-group concentration). In presence of DTT, the maximal AsIII formation rate was approximately 80% of the rate of hypoxanthine production (Fig. 13, bottom). In presence of GSH, AsV reduction increased quadratically with the rise of GSH concentration up to 15mM, and the highest rate of AsIII formation reached only 7% of the rate of hypoxanthine production.

The time course of the PNP-catalyzed arsenolysis and the coupled AsV reduction was also examined in presence of GSH (10mM) or DTT (0.25, 0.5, or 5mM). In presence of GSH, hypoxanthine production linearly declined with time (Fig. 14, top), whereas AsV reduction linearly increased (Fig. 14, bottom). In presence of DTT, hypoxanthine formation rates were comparable and similarly decreased with time, irrespective of the thiol concentration (Fig. 15, top). In contrast, the AsV reduction increased initially when DTT was present at low concentrations (0.25 or 0.5mM) then it declined with time. At 5mM DTT, however, the initially much higher AsIII formation rate decreased steadily from the start (Fig. 15, bottom). Nevertheless, the AsIII formation rates declined less rapidly than the rates of hypoxanthine production at any DTT concentration tested.

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DISCUSSION

The observations presented in this work permit four main conclusions to be drawn. First, we can now assert with great confidence that facilitation of thiol-dependent AsV reduction is a general property of the enzymes which catalyze phosphorolytic and arsenolytic reactions. To the list of such enzymes with known AsV-reducing activity (i.e., the mammalian PNP, GAPDH, GP, and OCT), three more can be added as a result of the present research, namely the bacterial PNP and PTAs, as well as the mammalian GAPDH, the latter in this case acting as a catalyst of the arsenolysis of a non-physiological substrate shown in Figure 1. Importantly, these enzymes, like the previously identified ones (Gregus and Németi, 2002, 2005; Németi and Gregus, 2007, 2009), also mediate reduction of AsV to AsIII exclusively in presence of a thiol compound and strictly in conjunction with the arsenolytic reaction they catalyze. For example, AsV reduction mediated by PTAs and GAPDH (1) required not only GSH, but also their respective substrate that feeds the arsenolytic reaction (i.e., acetyl-CoA and acetyl-phosphate; Figs. 4 and 6), and (2) was enhanced by the respective activator of these enzymes (i.e., NH4+ -ions and NAD; Figs. 3 and 6), and (3) was diminished by the product of

FIG. 11. Arsenolysis of inosine mediated by PNP.
the arsenolytic reaction (i.e., CoA-SH; Fig. 5) or by the inhibitor of the enzyme (i.e., konicic acid; Fig. 6) that counteract the arsenolysis. Even the biphasic effect of acetyl-phosphate on AsV reduction by PTA enzymes, that is, enhancement at low and diminution at high acetyl-phosphate concentrations (Fig. 5), can be attributed to similar changes in the PTA-catalyzed arsenolytic reaction illustrated in Figure 1, because acetyl-phosphate at low concentration may predominantly act by facilitating conversion of CoA-SH (that had been produced by the arsenolysis of acetyl-CoA) back to acetyl-CoA in the reverse reaction, thereby forming acetyl-CoA that fuels the arsenolysis and eliminating CoA-SH that hinders the arsenolysis; however, at high concentration acetyl-phosphate would act predominantly as a product inhibitor of the PTA-catalyzed arsenolytic reaction.

A second conclusion drawn from the present findings is that thiol compounds are obligate supporters of AsV reduction mediated by phosphorolytic-arsenolytic enzymes; however, their supporting potency depends on both the type of thiol (i.e., GSH, 2-ME, DTT, DMSA, and DMPS) and, ostensibly, the type of the arsenylated metabolite formed. Despite the structural differences among the enzymes investigated, AsV reduction by enzymes forming ester derivatives of AsV (i.e., the two PNPs and GP) was uniformly best supported by DTT and least supported by GSH and DMSA (Figs. 7 and 8), whereas AsV reduction by enzymes producing anhydrides of AsV (i.e., GAPDH and the two PTAs) was best supported by GSH (Figs. 9 and 10), although the causal explanation for this divergence is not immediately apparent.

The third set of conclusions can be drawn from the results of the experiments with bacterial PNP as a model enzyme, in which we analyzed the relationship between the PNP-catalyzed arsenolytic reaction (as measured by hypoxanthine production; see scheme in Fig. 11) and the PNP-mediated AsV reduction (as measured by AsIII formation) in presence of various thiols (Figs. 12–15). Seemingly at variance with earlier findings (discussed above) indicating that the arsenolytic process is a prerequisite for the arsenate reduction, and thus AsV reduction is coupled with and dependent on the arsenolysis, these studies demonstrate that the rate of arsenolysis alone does...
not determine the rate of AsV reduction, making these two processes appear independent and uncoupled. The following specific observations support this latter conclusion. (1) The rate of arsenolysis was clearly independent of either the presence of a thiol or the type of thiol being present (i.e., GSH, 2-ME, DTT, DMSA, or DMPS; Fig. 12, top), whereas AsIII formation occurred exclusively in presence of a thiol and strongly depended on the type of thiol compound (Fig. 12, bottom). (2) Although the rate of arsenolysis was not influenced by DTT or GSH at a wide range of concentrations (0–15mM with respect to SH-groups; Fig. 13, top), the rate of AsV reduction was markedly increased by the rise in thiol concentration, albeit in a different fashion with DTT than with GSH (Fig. 13, bottom). Figures 12 and 13 also reveal an illuminating observation with respect to the quantitative relationship between the two processes: the rate of AsV reduction under all conditions remains below the rate of arsenolysis. For example, the maximal rate of AsIII formation in presence of DTT and GSH represented only 80 and 7%, respectively, of the rate of hypoxanthine formation. (3) In presence of either DTT or GSH, the rate of arsenolysis invariably decreased steadily and significantly with time in the first 10 minutes (top panels in Figs. 14 and 15), whereas the rate of AsV reduction increased steadily with GSH present at 10mM (Fig. 14, bottom), or increased transiently before declining slowly with DTT present at 0.25–0.5mM (Fig. 15, bottom). These latter observations reveal yet another informative difference between the two reactions, that is, a striking dissimilarity in their dynamics: arsenolysis is an early rapid and soon declining process, whereas the arsenolysis-dependent AsV reduction is a comparatively delayed and protracted process.

Fourthly, we can conclude that it is the formation of the arsenylated metabolite, rather than the arsenolytic enzyme or the arsenolytic process per se, that is crucial in the phosphorolytic-arsenolytic enzyme-mediated thiol-supported AsV reduction. Several observations corroborate this assumption. For example, the two different bacterial PTA enzymes mediated AsV reduction at very similar rates (Figs. 2–4) and with very similar responsiveness to various metabolites (Fig. 5).
as well as to various thiols (Fig. 9). In addition, both the calf spleen PNP and the bacterial PNP exhibited similar characteristics with respect to AsV reduction coupled to arsenolysis of inosine, including comparable rates and responsiveness to thiols (Fig. 7) and to the PNP-inhibitor BCX-1777 (Gregus and Németh, 2002; Gregus et al., 2009), despite the fact that there are critical differences in their structures. The mammalian PNP, for example, contains a CX_3R sequence at its P_{i} (and AsV)–binding site, whose cysteine has been assumed to play a role in PNP-mediated AsV reduction (Gregus and Németh, 2002). This speculation, however, can now be discounted as the bacterial PNP lacks a CX_3R sequence, as stated by the manufacturer (personal communication) and as deduced by Bzowska’s group, who tentatively identified this enzyme as PNP from Cellulomonas sp. (Bzowska et al., 2000; Tebbe et al., 1999). The most compelling evidence for the decisive role of arsenylated metabolite formation, rather than the arsenolytic enzyme or the arsenolytic process themselves, in arsenolysis-coupled AsV reduction comes from the comparison of PTAs with GAPDH. These enzymes are completely different structurally, and also catalyze different arsenolytic reactions, that is, arsenolysis of acetyl-CoA and acetyl-phosphate, respectively (Fig. 1), yet both enzymes catalyze arsenolytic reactions that yield, incidentally, the same product, acetyl-arsenate (Fig. 1; Krímsky and Racker, 1955; Stadtman, 1955). As demonstrated in Figures 6 and 9, AsV reduction mediated by the two types of acetyl-phosphate forming enzymes (i.e., the PTAs and GAPDH) exhibits strikingly similar responsiveness to various thiols at 10 mM, as the potency of thiols to support AsV reduction is ranked as GSH > DTT > 2-ME > DMPS > DMSA for the PTAs and GAPDH alike.

In summary, apparently all phosphorolytic-arsenolytic enzymes can mediate thiol-supported reduction of AsV to AsIII. Experimental evidence indicates that the arsenolytic reaction is prerequisite, but is not the sole rate determinant in AsV reduction; reduction of AsV to AsIII also depends on the quality and the quantity of the thiol compound present. This observation together with several additional findings of this study (e.g., difference in the dynamics of arsenolysis and AsV reduction, the rate of AsV reduction lagging behind the rate of arsenolysis, and the importance of arsenylated metabolite formation rather than the arsenolytic process or the enzyme per se) collectively point to the following hypothetical mechanism for arsenolysis-coupled thiol-supported AsV reduction: arsenolytic enzymes mediate reduction of AsV in presence of thiols because they form arsenylated metabolites in which the arsenic is more reducible by thiols to AsIII than in the inorganic arsenate. This hypothesis is tested in the accompanying paper (Gregus et al., 2009).

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