Glutathione-Dependent Reduction of Arsenate by Glycogen Phosphorylase—A Reaction Coupled to Glycogenolysis

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ArSENate (As(V)) is reduced in the body to the more toxic arsenite (As(III)). We have shown that two enzymes catalyzing phosphorolytic cleavage of their substrates, namely purine nucleoside phosphorylase and glyceraldehyde-3-phosphate dehydrogenase, can reduce As(V) in presence of an appropriate thiol and their substrates. Another phosphorolytic enzyme that may also reduce As(V) is glycogen phosphorylase (GP). With inorganic phosphate (P<sub>i</sub>), GP catalyzes the breakdown of glycogen to glucose-1-phosphate; however, it also accepts As(V). Testing the hypothesis that GP can reduce As(V), we incubated As(V) with the phosphorylated GPa and the dephosphorylated GPb purified from rabbit muscle and quantified the As(III) formed from As(V) by high-performance liquid chromatography–hydride generation–atomic fluorescence spectrometry. In the presence of adenosine monophosphate (AMP), glycogen, and glutathione (GSH), both GP forms reduced As(V) at rates increasing with enzyme and As(V) concentrations. The As(V) reductase activity of GPa was 10-fold higher than that of GPb. However, incubating GPb with GP kinase and ATP (that converts GPa to GPb) increased As(V) reduction by phosphorylase up to the rate produced by GPa incubated under the same conditions. High concentration of inorganic sulfate, which activates GPb like phosphorylation, also promoted reduction of As(V) by GPb. As(V) reduction by GPb (like As(V) reduction in rats) required GSH. It also required glycogen (substrate for GP) and was stimulated by AMP (allosteric activator of GP) even at low micromolar concentrations. P<sub>i</sub>, substrate for GP competing with As(V), inhibited As(III) formation moderately at physiological concentrations. Glucose-1-phosphate, the product of GP-catalyzed glycogenolysis, also decreased As(V) reduction. Summarizing, GP is the third phosphorolytic enzyme identified capable of reducing As(V) in vitro. For reducing As(V) by GP, GSH and glycogen are indispensable, suggesting that the reduction is linked to glycogenolysis. While its in vivo significance remains to be tested, further characterization of the GP-catalyzed As(V) reduction is presented in the adjoining paper.

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substitute for $P_i$ in these phosphorolytic reactions, and then arsenolytic cleavage occurs. Reduction of As(V) by these enzymes in the presence of thiols is apparently coupled to the arsenolytic reaction.

Theoretically, other enzymes catalyzing phosphorolytic/arsenolytic cleavage of their substrates may also reduce As(V). Such an enzyme is glycogen phosphorylase (GP). In cells, GP exists in two interconvertible forms, the phosphorylated glycogen phosphorylase-a (GPa, active) and dephosphorylated glycogen phosphorylase-b (GPb, inactive), and is under the control of phosphorylase kinase and phosphatase (responsible for the formation of GPa and GPb, respectively), as well as of allosteric effectors (Johnson, 1992). GP catalyzes the phosphorolytic breakdown of the stored fuel glycogen into glucose-1-phosphate units (Fig. 1), which may eventually be channeled into glycolysis, or after dephosphorylation in hepatocytes, the resultant glucose may be released into the bloodstream. For catalyzing glycogen breakdown, GP can accept As(V) instead of phosphate, while producing the purportedly unstable glucose-1-arsenate (Helmreich and Cori, 1964; Klein et al., 1982). It was, therefore, hypothesized that GP can reduce As(V), similar to the above-mentioned two enzymes. To test this hypothesis, GPa or GPb purified from rabbit muscle was incubated with As(V) in the presence of glycogen, AMP, and GSH. As preliminary experiments supported the hypothesis, we decided (1) to compare the As(V)-reducing activity of GPa and GPb; (2) to characterize the GPa-catalyzed As(V) reduction with respect to its dependence of GSH, AMP (allosteric activator), and glycogen (substrate) concentrations as well as its responsiveness to $P_i$ and glucose-1-phosphate (i.e., the substrate competitive with As(V) and the product of the GP-catalyzed glycogenolysis); and (3) to examine whether activation of GPb by phosphorylase kinase or inorganic sulfate influences the As(V)-reducing activity of this enzyme. While the findings of these studies are presented in this paper, the adjoining article (Gregus and Németi, 2007) characterizes the chemical responsiveness of As(V) reduction mediated by the muscle and hepatic forms of GPa. Our studies employed high-performance liquid chromatography–hydride generation–atomic fluorescence spectrometry (HPLC-HG-AFS) to separate and quantify As(III) formed from As(V).

MATERIALS AND METHODS

**Chemicals.** Glycogen (type III from rabbit liver), purified glycogen phosphorylase-a (GPa) and -b (GPb, both from rabbit muscle), phosphorylase kinase (from rabbit muscle), adenosine monophosphate sodium salt, glucose-1-phosphate, and glucose-1,6-bisphosphate were from Sigma. Reduced glutathione and disodium hydrogen arsenate (As(V)) were from Reanal Ltd. (Budapest, Hungary). The sources of chemicals used in arsenic speciation have been given elsewhere (Csánaky et al., 2003; Németi and Gregus, 2002). All other chemicals were of the highest purity commercially available.

**Assays.** GPa and GPb were dissolved at 400 U/ml nominal concentrations in a buffer containing 25mM glycercol-2-phosphate (G2P), 1mM ethylenediamine tetraacetic acid (EDTA), 1mM DTT, and 50% glycerol (vol/vol) (pH 7.4) and stored at $-20^\circ$C. In these stock solutions, the enzymes retained their original activity for up to 2 months. Nevertheless, because we often observed that the activity of GPa and GPb, as determined by us, differed from the nominal value, we routinely assayed these enzymes and set their activity in the original activity for up to 2 months. Nevertheless, because we often observed that the activity of GPa and GPb, as determined by us, differed from the nominal value, we routinely assayed these enzymes and set their activity in the arsenolysis reaction.

**FIG. 1.** Glycogen phosphorylase-catalyzed phosphorolysis of glycogen into glucose-1-phosphate. In the presence of arsenate instead of phosphate, the enzyme catalyzes arsenolysis of glycogen into the unstable glucose-1-arsenate.
continued for 60 min. Variation from these general conditions are given in the figure legends. The incubation was terminated by sequential addition of 100 μl 50mM CdSO₄ solution followed by 100 μl 1.5M perchloric acid solution containing 50mM HgCl₂. The rationale for this procedure has been given elsewhere (Németh and Gregus, 2004). The incubates thus treated were stored at –80°C until arsenic analysis. Because As(V) is reduced nonenzymatically in the presence of GSH to a small extent, the nonenzymatic As(III) formation rates were regularly determined from incubations lacking GP but containing GSH and subtracted from the rate measured when the incubation contained both GP and GSH. The net enzymatic As(V) reductase activity thus calculated was expressed as the amount of As(III) formed per minute and U phosphorylase.

To determine whether phosphorylase kinase could affect the As(V)-reducing activity of GP a or GP b, these enzymes (2 U/ml) were separately preincubated at 37°C with phosphorylase kinase (4, 8, or 16 U/ml) for 10 min, in 75 μl G2P buffer containing GSH (40mM), glycogen (2%, wt/vol), and Mg-ATP (20mM). Thereafter, the samples underwent a fourfold dilution by adding G2P buffer, AMP (200μM), and As(V) (50μM), and were incubated for additional 60 min to assay the As(V) reductase activity of the phosphorylase kinase–treated GP. The incubations were stopped as described above.

Arsenic analysis. Arsenic in the incubates was speciated and quantified by HPLC-HG-AFS. After having been subjected to protein precipitation, the incubates from the As(V) reductase assays were centrifuged at 10,000 × g, 4°C for 10 min. As(III) and As(V) in the resultant supernatants were separated on a strong anion exchange guard column and 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).

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

As(V) Reduction by GP—Comparison of GPa and GPb

Rabbit muscle GP a or GP b was incubated with As(V) in the presence of glycogen (substrate), AMP (activator), and GSH. Under these conditions, both GP a and GP b exhibited As(V) reductase activity (Fig. 2). In 60-min incubations, formation of As(III) from As(V) increased with the enzyme concentration (0.1–1.5 U/ml) almost linearly (Fig. 2, left). The rate of As(V) reduction catalyzed by either GP a or GP b was directly related to the concentration of As(V) within the 25–200μM range (Fig. 2, right). Nevertheless, the rate of As(V) reduction by GP a was 8–10 times as high as that by GP b; therefore, further experiments were carried out with GP a.

Characterization of As(V) Reduction by GPa

In order to characterize the GPa-catalyzed As(V) reduction, we determined its dependence on time and the concentrations of GSH, glycogen, and AMP. In these experiments, the complete incubations contained the latter three substances in addition to GP a and As(V). Interestingly, the time curve of As(V) reduction mediated by GPa exhibited a slightly sigmoid shape (Fig. 3), as the As(III) formation rates were lower both initially and after 90 min than between 15 and 90 min (Fig. 3, insert). Therefore, incubations were generally performed for 60 min.

Figure 4 demonstrates that in the absence of GSH, As(III) formation could not be observed. GSH enhanced As(V) reduction by GPa exponentially in a concentration-dependent fashion (Fig. 4). The concentration-dependent effect of AMP, a strong allosteric activator of GP, on the As(V)-reducing activity of GPa was demonstrated in Fig. 5.

FIG. 2. Reduction of As(V) by purified GPa and GPb as a function of enzyme and As(V) concentration. Rabbit muscle GP a or GP b at the indicated concentrations (left) or at 0.5 U/ml (right) was preincubated with glycogen (1%) plus GSH (10mM) at 37°C for 5 min. Then AMP (200μM) and As(V) at 50μM (left) or at the indicated concentrations (right) were added to start the 60-min incubation. Symbols represent As(III) formation rates (mean ± SEM) in three incubations.
was remarkable. GPa reduced As(V) even in the absence of AMP at a rate of 40 pmol/min and U phosphorylase (Fig. 5, right). However, the presence of AMP even at a concentration as low as 25 μM tripled the rate of As(V) reduction catalyzed by GPa, which remained at this level irrespective of the increase in AMP concentration.

Pi, the other physiological substrate of GP, caused an inhibition of As(III) formation from As(V) (Fig. 6, left) that exhibited a linear correlation with its concentration (0.1–2 mM). As(V) reduction was decreased by Pi significantly at 0.5 mM concentration and by 50% at 2 mM Pi. Glucose-1-phosphate, the product of GP-catalyzed phosphorolytic glycogen breakdown, also inhibited As(V) reduction by rabbit muscle GPa in a concentration-dependent fashion in the range of 0.1–1 mM. Although it caused a significant diminution of As(III) formation at a concentration as low as 0.1 mM (Fig. 6, right), glucose-1-phosphate decreased As(III) formation only by 30% even at 1 mM.

Effects of Phosphorylase Kinase and Sulfate on the As(V)-Reducing Activity of GPb

In the presence of Mg-ATP, GP kinase phosphorylates GPb into GPa. Therefore, we tested if preincubation of GPb with GP kinase and/or Mg-ATP affected its As(V) reductase activity. For comparison, these experiments were also performed with GPa, the phosphorylated form of the enzyme. We found that neither ATP nor GP kinase alone could enhance As(V) reductase activity of GPb (Fig. 7, right). However, in the combined presence of both ATP and GP kinase, As(V) reduction rates were significantly increased. Nevertheless, even when the kinase was in eightfold excess to GPb, the observed As(III) formation rates were only half of those observed with incubations containing GPa without ATP. Figure 7 (left) also demonstrates that ATP halved the As(III) formation by GPa, whereas GP kinase did not influence it significantly either in the absence or in the presence of ATP. Thus, As(III) formation in the presence of GPb, GP kinase, and Mg-ATP leveled with that produced by GPa incubated under the same conditions.

Sulfate at high concentrations mimics the effect of phosphorylation of Ser14 on GPb (Barford and Johnson, 1989). Therefore, we tested the effects of sulfate on the As(V)-reducing activity of GPb. Again, similar incubations were carried out with GPa for comparative reasons. The GPb-catalyzed As(V) reduction was not influenced by sulfate below 250 mM (Fig. 8). However, at 500 mM concentration, sulfate increased As(III) formation rates to approximately 35% of those found in GPa incubations without sulfate. In contrast, reduction of As(V) mediated by GPa was markedly diminished by sulfate, and at 500 mM concentration, As(III) formation rate decreased to approximately 35% (Fig. 8). Thus, As(III) formation in the presence of GPb and 500 mM sulfate leveled with that mediated by GPa incubated under the same conditions.

DISCUSSION

This study supports the hypothesis that glycogen phosphorylase can function as an As(V) reductase, as it demonstrates that in the presence of glycogen, AMP, and GSH, both GPa and GPb reduce As(V) to As(III) at rates increasing as a function of enzyme and As(V) concentrations. Interestingly, GPa, the dephosphorylated form of GP, has been tested for As(V)-reducing activity and reported, without specifying the assay conditions, to be inactive (Radabaugh et al., 2002). In contrast, GPb exhibited As(V) reductase activity in our experiments, although its As(V)-reducing activity was negligible.
when compared to that of GPa, the phosphorylated form of GP. This finding clearly demonstrates that the As(V)-reducing activity of GP is not a mere function of the enzyme concentration but is also influenced by the functional state or conformation of the enzyme determined partly by covalent phosphorylation of its Ser14.

Physiologically, phosphorylase b kinase activates GPb by phosphorylating it into GPa using Mg-ATP (Johnson, 1992). As expected, treatment of GPb with phosphorylase kinase and Mg-ATP enhanced As(V) reductase activity of GPb most likely by converting GPb to GPa. Indeed, at the highest kinase level and in the presence of ATP, the GPa- and GPb-mediated As(III) formation rates were equal, whereas the kinase at a lesser excess produced more moderate activation of As(V) reduction by GPb. It has been demonstrated both in vitro and in vivo that when the kinase activity is not sufficient to fully phosphorylate GPb into GPa, GPb becomes partially phosphorylated into a mixed ab form (Bot et al., 1974; Gergely et al., 1974). This ab form mimics GPb in that it is more responsive to stimulation by AMP and to inhibition by ATP

FIG. 5. Reduction of As(V) by GPa as a function of glycogen and AMP concentration. Rabbit muscle GPa was preincubated with glycogen at the indicated concentrations (left) or 1% (right) and GSH (10mM) at 37°C for 5 min. Then AMP at 200μM (left) or at the indicated concentrations (right) and As(V) (50μM) were added to start the 60-min incubation. Symbols represent As(III) formation rates (mean ± SEM) in three incubations.

FIG. 6. Effects of Pi and glucose-1-phosphate on the As(V)-reducing activity of GPa. Rabbit muscle GPa (0.5 U/ml) was preincubated with glycogen (1%), GSH (10mM), and Pi, or glucose-1-phosphate (at the indicated concentrations) at 37°C for 5 min. Then, AMP (200μM) and As(V) (50μM) were added to start the 60-min incubation. Symbols represent As(III) formation rates (mean ± SEM) in three incubations. Asterisks indicate significant difference (p < 0.05) in As(III) formation rate observed in the absence of Pi and glucose-1-phosphate.
than GPa but exhibits higher glycogenolytic activity than GPb. Formation of this ATP-sensitive ab form of GP may be responsible for the observation that upon treatment of GPb with Mg-ATP and two- or fourfold excess of GP kinase As(V) reduction increased only moderately, not reaching the rate provided by either GPa or by GPb treated with phosphorylase kinase in eightfold excess.

When GPb becomes phosphorylated, Ser14 phosphate makes ionic contacts with specific arginines from either subunit forming the Ser14 phosphate acceptor site, thereby inducing a conformational change and allosteric activation of the enzyme. However, not only Ser14 phosphate but also divalent anions high in the Hofmeister series, such as sulfate, can bind to the Ser14 phosphate acceptor site in GPb, and at high concentrations, they mimic the effect of Ser14 phosphate (Barford and Johnson, 1989; Lorek et al., 1984), thereby activating the enzyme. Most likely, this activation by sulfate (0.5M) accounts for the increase in the rate of As(V) reduction by GPb up to the GPa-catalyzed rate obtained under the same conditions. However, sulfate can also bind to the active site (Barford and Johnson, 1989), thus competing with Pi or As(V). This explains the concentration-dependent inhibitory effect of sulfate on As(V) reduction by GPb.

Besides phosphorylation, GP activity can also be regulated by noncovalent allosteric effectors, among which AMP is the most important physiologically. In the absence of AMP, GPa can still catalyze glycogen breakdown (Johnson, 1992) and also As(V) reduction; however, AMP binding to GPa enhances both its glycogenolytic activity (Barford and Johnson, 1989) and As(V) reductase activity. Although AMP augments the glycogenolytic activity of GPa at saturating Pi concentrations only by 25% (Barford and Johnson, 1989), we observed an AMP-induced threefold increase in GPa-supported As(III) formation. This apparent discrepancy can be readily explained by considering that the $K_M$ of As(V) for GPa is in the millimolar range (Helmreich and Cori, 1964), whereas the concentrations of As(V) we used in the As(V) reductase assays (typically 50μM) were far below the $K_M$, that is, still in the

**FIG. 7.** Effects of phosphorylase kinase on the As(V)-reducing activity of GPa and GPb. Rabbit muscle GPa or GPb (2 U/ml) were preincubated with phosphorylase kinase (at the indicated concentrations), glycogen (2%), GSH (40mM), and ATP (0 or 4mM) at 37°C for 10 min in a final volume of 75 μl buffer containing 25mM G2P, 1mM EDTA (pH 7.4). Then buffer, AMP (200μM), and As(V) (50μM) were added successively to start the 60-min incubation in a final volume of 300 μl (thus the enzyme and reagent concentrations in the final incubation volume were one-fourth of those during preincubation). Bars represent As(III) formation rates (mean ± SEM) in three incubations.

**FIG. 8.** Effects of sulfate on the As(V)-reducing activity of GPa and GPb. Rabbit muscle GPa or GPb (0.5 U/ml) were preincubated with glycogen (1%), GSH (10mM), and sodium sulfate (at the indicated concentrations) at 37°C for 5 min. Then AMP (200μM) and As(V) (50μM) were added to start the 60-min incubation. Symbols represent As(III) formation rates (mean ± SEM) in three incubations. Asterisks indicate significantly lower rates of GPa-catalyzed As(III) formation ($p < 0.05$) than that observed in the absence of sulfate. The cross indicates significantly higher rate of GPb-catalyzed As(III) formation ($p < 0.05$) than that observed in the absence of sulfate.
range where the formation rate of the product (here As(III)) increases near-linearly with the concentration of the substrate (here As(V)). The observation that As(V) reduction by GPa and GPb increases linearly with the concentration of As(V) indicates that GPa and GPb must have a $K_M$ well above 200$\mu$M not only for the glycogenolytic activity but also for the As(V)-reducing activity. Because AMP markedly lowers the $K_M$ for As(V) in the GPa-catalyzed arsenolitic glycogen breakdown reaction (Helmreich and Cori, 1964), the AMP-induced enhancement of As(III) formation could most likely be due to a similar increase in affinity of GPa for As(V), when this enzyme catalyzes reduction of As(V). Furthermore, in the light of the very high affinity of AMP for the allosteric site ($K_M$: 2$\mu$M, Sprang et al., 1988), which is created upon binding of Ser14 phosphate to its acceptor site, it is not surprising that AMP produced maximal stimulatory effect on As(V) reduction even at a concentration as low as 25$\mu$M.

As to the much lower As(V)-reducing activity of GPb than of GPa, there are two important differences between the two isoforms of this enzyme with respect to their glycogenolytic and, possibly, As(V) reductase activities. First, although AMP is obligatory for the glycogenolytic activity of GPb, it binds to GPb with approximately two orders of magnitude lower affinity than to GPa (Sprang et al., 1988); thus, its stimulatory effect on GPb at physiological AMP concentrations falls away from that on GPa. Second, the $K_M$ values of $P_i$ and As(V) even in the presence of AMP are substantially higher for GPb than for GPa (Helmreich and Cori, 1964), indicating that the phosphorylated enzyme exhibits greater affinity for As(V) than the dephosphorylated form. Taken together, the much lower affinity of As(V) for GPb than for GPa and the weaker stimulation of GPb than of GPa by submillimolar AMP could readily account for the low As(V) reductase activity of GPb compared to GPa.

Reduction of As(V) to As(III) by GPb in presence of GSH is apparently coupled to the arsenolitic glycogen breakdown. The following pieces of evidence support this conclusion: (1) presence of glycogen is indispensable for As(V) reduction, and with increase in the concentration of glycogen, As(V) reduction shows saturation kinetics; (2) $P_i$, which competes with As(V) for the active site of GPa and inhibits arsenolitic glycogen breakdown, diminishes As(V) reduction; (3) glucose-1-phosphate, which promotes the reverse reaction (i.e., glycogen synthesis) by the enzyme and counters glycogenolysis by GPa, also inhibits As(V) reduction; (4) AMP, which stimulates the glycogenolytic activity of GPb (Johnson, 1992), enhances As(V) reduction; (5) phosphorylase b kinase, which activates GPb by phosphorylating it at Ser14 into GPb (Johnson, 1992), and sulfate, which mimics the effect of Ser14 phosphate, increase As(III) formation by GPb.

The initially slow and later accelerating As(III) formation from As(V) by GPa may be attributed to similar changes in the glycogenolytic activity of GPa during incubations. GPa is known to be fully active in homodimeric state (Barford and Johnson, 1992). However, the purified enzyme associates into inactive tetramers (Metzger et al., 1967; Wang, 1999), which dissociate into glycogenolytically active dimers only after addition of substrates (i.e., glycerin and $P_i$ or As(V)). Gradual evolution of this process may explain the low As(V) reduction rates observed in incubations lasting for 15 min. On the other hand, arsenolitic glycogen breakdown produces the purportedly unstable glucose-1-arsenate, which rapidly hydrolyzes, and the released glucose promotes the inactive conformation of GPa (Johnson, 1992). Accumulation of glucose at inhibitory concentrations is likely responsible for the slight decline of As(V) reduction after 90 min.

This work also demonstrates that GSH is indispensable for the GPa-mediated As(V) reduction, as without GSH no As(III) formation was detectable even when GPa was supplied with glycogen and AMP. GSH supported As(V) reduction in a concentration-dependent exponential fashion, a feature also characteristic for the As(V) reductase activity of GAPDH (Gregus and Néméti, 2005). As(V) reduction by PNP is also thiol dependent, although it is supported much more by the physiologically irrelevant dithiol compound DTT than GSH (Gregus and Néméti, 2002). The observation that GPa-catalyzed As(V) reduction is GSH dependent supports the potential role of GPa in reduction of As(V) in vivo because recent studies on rats pretreated with the GSH synthesis inhibitor buthionine sulfoximine clearly demonstrate that reduction of As(V) to As(III) is also GSH dependent in vivo (Csánaky and Gregus, 2005). Furthermore, the finding that $P_i$ at physiological intracellular concentration (0.5mM; Iles et al., 1985) exerted only a weak inhibitory effect on As(V) reduction by GPb suggests that reduction of As(V) by GPb, unlike As(V) reduction by PNP (Gregus and Néméti, 2002), would probably be little impaired by $P_i$ in vivo.

In summary, the present work demonstrates that glycogen phosphorylase, yet another cytosolic enzyme catalyzing a phosphorolytic/arsenolitic cleavage reaction, can reduce As(V) in a GSH-dependent manner to the more toxic As(III) and that As(V) reduction apparently takes place in the course, or as a result, of the arsenolitic glycogen breakdown. Of the two forms of GP, the phosphorylated GPb exhibits much higher activity than the dephosphorylated GPb; however, conditions known to activate GPb (e.g., phosphorylase b kinase, high concentrations of sulfate) facilitate As(III) formation. For As(V) reduction by GPb, glycogen and GSH are indispensable, whereas AMP, though not obligatory, amplifies the As(V) reductase activity of GPb. While the in vivo significance of these findings remains to be investigated, characterization of the chemical responsiveness of As(V) reduction mediated by the muscle and liver forms of GPa is presented in the adjoining paper (Gregus and Néméti, 2007).

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