REDUCTION OF ARSENATE TO ARSENITE – BIOCHEMICAL BACKGROUND OF A TOXIFICATION PROCESS

Ph.D. THESIS

Balázs Németi, MD

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Supervisor: Prof. Zoltán Gregus, MD, PhD, DSc, DABT

University of Sciences of Pécs,

Faculty of Medicine

Department of Pharmacology and Pharmacotherapy

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INTRODUCTION

The toxicity of arsenicals and their fate in the body

Arsenic has been known as a chemical element since ancient times. It became notorious due to the high acute toxicity of its compound, arsenic trioxide (As_2O_3). Acute arsenic intoxication is very rare nowadays. However, besides cyanide, arsenic trioxide had been the most frequently used suicide and homicide agent from ancient times till the second half of the 19th century (Jolliffe, 1993). Chronic arsenic poisoning is seen more often. The primary source of arsenic exposure concerning large human populations is the contaminated drinking water, in which the predominant form of inorganic arsenic is arsenate (As^V), though arsenite (As^{III}) may also be present. Chronic exposure can cause skin lesions, nervous system disorders, peripheral vascular disease (Chen *et al.*, 1985; Goyer and Clarkson, 2001). In addition, arsenic has been recognized as carcinogen in human (IARC, 2002; Gebel, 2001; Goering *et al.*, 1999). In medical therapy, arsenic compounds have been used since antiquity, too, although their use has greatly rolled back, owing to the carcinogenicity of arsenic. Interestingly, despite the definite carcinogenic effect of arsenic, arsenic trioxide has recently been shown to induce complete remission in acute promyelocytic leukemia (APL) patients (Soignet *et al.*, 1998). As^{III} has been found to induce apoptosis in human ovarian and cervical carcinoma, esophageal carcinoma, gastric cancer, neuroblastoma (Wang, 2001), and myeloma (Rousselot *et al.*, 1999) cells, suggesting that As^{III} may also be effective in the treatment of other malignancies (Bachleitner-Hofmann *et al.*, 2002). However, As_2O_3 treatment of patients with APL causes many unwanted effects related to arsenic toxicity.

Chemical reactivity and mechanism of toxicity of arsenate and arsenite. The structure of As^V closely resembles to that of inorganic phosphate (P_i). Due to this structural similarity, As^V is taken up by living organisms and cells via their P_i transport system (Csanaky and Gregus, 2001; Dixon, 1997; Rosen, 2002). In the cell, As^V may replace P_i in enzymatic reactions leading to the formation of arsenate esters and anhydrides (Chan *et al.*, 1969; Dixon, 1997; Hughes, 2002). However, while esters and anhydrides of P_i are aptly stable in aqueous environment, those of As^V are labile, because the longer As–O bond is more readily accessible to water molecules in order to hydrolyze this bond (Dixon, 1997). However, such mechanism could underlie the toxicity of As^V itself at very high As^V concentrations (several mM). Therefore, it is thought that biotransformation of As^V into much more toxic trivalent derivatives is responsible largely for the toxicity of As^V.

Trivalent arsenicals are much more toxic than their pentavalent counterparts, because they exhibit facile covalent reactivity with thiols. As^{III} complexes formed with monothiols (e.g., glutathione, cysteine) are relatively labile, whereas those formed with dithiols (e.g., dihydrolipoic acid, dithiothreitol, dimercaprol) are fairly stable (Knowles, 1985; Knowles and Benson, 1983). The trivalent methylated metabolites of As^{III} (see later) are also thiol-reactive but they form relatively stable complexes even with monothiols (Knowles and Benson, 1983). The high affinity of trivalent arsenicals to thiols makes many proteins targets, thereby impairing the cellular metabolism at many different points. Moreover, As^{III} at higher concentrations (1-10 μM) induces oxidative stress in the cells that may lead to the formation of DNA mutations. Below this range, it still can enhance the mutagenic and carcinogenic effects of other agents, such as ultraviolet radiation or alkylating compounds (Bernstam and Nriagu, 2000; Gebel, 2001; Rossman, 2003; Rossman *et al.*, 2002; Shi *et al.*, 2004; Wang *et al.*, 2001).

Fate of arsenate and arsenite in the body. In human and most of the mammalian species, As^{III} and As^{V} are absorbed well from the gastrointestinal tract (Vahter, 1983). As^{V} enters cells via their inorganic phosphate (P_i) transport system (Csanaky and Gregus, 2001; Dixon, 1997), whereas As^{III} likely through aquaporin channels or equilibrative glucose transporters (Liu *et al.*, 2004a, 2004b).

In the cells, inorganic arsenic undergoes extensive metabolism, which includes alternation of reduction and in many, but not all, mammalian species oxidative methylation (Aposhian, 1997; Ishinishi *et al.*, 1986; Vahter, 1999). First, As^{V} is reduced by hitherto unidentified enzymes using glutathione (GSH) to As^{III} (Thomas *et al.*, 2001). As^{III} thus formed is methylated yielding monomethylarsonic acid ($MMAs^{V}$). $MMAs^{V}$ is then reduced to monomethylarsonous acid ($MMAs^{III}$), which is further methylated to dimethylarsinic acid ($DMAs^{V}$). $DMAs^{V}$ may then be reduced to dimethylarsinous acid ($DMAs^{III}$). Nevertheless, some researchers doubt the validity of this metabolic scheme. They claim that arsenic remains in its trivalent state during the methylation reactions, which are not oxidative, and the pentavalent methylated metabolites represent dead ends of arsenic metabolism (Hayakawa *et al.*, 2005).

The pentavalent arsenicals are exclusively excreted in the urine, whereas the trivalent ones can be excreted in either the bile or urine (Csanaky and Gregus, 2002; Gregus *et al.*, 2000). Following inorganic arsenic exposure, the primary elimination route is the urinary excretion. Besides the inorganic forms (i.e., As^{III} and As^{V}), $MMAs^{V}$ and $DMAs^{V}$ are the main urinary metabolites of As^{V}. The fecal arsenic content originates partly from

the ingested and not absorbed amount, and partly from the biliary excretion of trivalent arsenicals. Despite its significant biliary excretion, however, the fecal elimination of arsenic is markedly limited, because trivalent arsenicals can be reabsorbed from the intestines, as their GSH conjugates are relatively labile and decompose (Klaassen, 1974).

Importantly, reduction of As^{V} and the methylated pentavalent arsenicals to the corresponding trivalent species is considered toxification of high significance, because trivalent arsenic species are highly toxic, whereas the pentavalent ones are relatively atoxic (Thomas *et al.*, 2001). On the same token, oxidative methylation of trivalent arsenicals into pentavalent ones is considered detoxication. The primary step in the disposition of the environmentally often-prevalent As^{V} is its reduction to As^{III}. This is not only a major toxification process, but also must precede the formation of methylated metabolites. Despite the toxicological importance of As^{V} reduction, its biochemical background was explored only in microorganisms, and there was no mammalian enzyme identified possessing As^{V} reductase activity prior to our work.

Reduction of arsenate

Reduction of arsenate by microorganisms. In prokaryotes, As^{V} is reduced to As^{III}, which is then extruded from the cell. Several different enzymes have been identified in bacteria that carry out reduction of As^{V}. The peculiar bacterium *Chrysiogenes arsenatis* is unique in that this species can use As^{V} during respiration instead of oxygen while producing As^{III} (Krafft and Macy, 1998). In the presence of As^{V}, *C. arsenatis* cells can grow under anoxic conditions.

The *Escherichia coli* plasmid R773 encodes a complete operon responsible for reduction of As^{V} and extrusion of the formed As^{III}. The arsenic resistance operon called "*ars* operon" encodes four proteins, one regulatory (ArsR) and three structural (ArsA, ArsB, and ArsC). Of these proteins, ArsC is the As^{V} reductase enzyme, whereas ArsA and ArsB work coupled as an ATP-dependent As^{III} exporter (Rosen *et al.*, 1991) that, besides As^{III}, can also export antimonite. The ArsC-catalyzed As^{V} reduction depends on the presence of glutathione (GSH) and glutaredoxin (Grx), and produces oxidized glutathione (GSSG) (Gladysheva *et al.*, 1994).

The *ars* operon of the *Staphylococcus aureus* plasmid pI258 encodes a structurally different family of arsenic resistance proteins that consists of only three genes, namely arsR, arsB, and arsC. Similarly to *E. coli*, the ArsR is the regulatory protein, and ArsC is the reducing enzyme. However, ArsB is not an ATP-dependent but rather a membrane

potential-driven As^{III} exporter (Guangyong *et al.*, 1994). The catalytic mechanism of pI258 ArsC is termed “dynamic disulfide cascade” (Messens *et al.*, 2002) and is essentially different from that observed in ArsC of *E. coli* plasmid R773. With the *S. aureus* enzyme, the three thiol groups contributing to the reduction of As^{V} belong to the enzyme protein, and the disulfide formed in the protein during reduction of As^{V} is reduced by thioredoxin. In addition, the enzyme contains a specific anion-binding signature motif (P-loop) consisting of Cys-X_5-Arg, which is also the catalytic motif of low molecular weight tyrosine phosphatases.

In the genome of *Saccharomyces cerevisiae*, a gene cluster consisting of *acr1*, *acr2*, and *acr3*, has been shown to confer resistance to inorganic arsenic (i.e., As^{V} and As^{III}). Acr1p appears to be the regulator of this gene cluster, whereas Acr2p and Acr3p are structural proteins with As^{V} reductase and As^{III} exporter roles, respectively (Bobrowicz *et al.*, 1997). Interestingly, the Acr2p protein, the first identified eukaryotic As^{V} reductase, exhibits structural similarity to the *S. aureus* (pI258) ArsC arsenate reductase, as it contains the tyrosine phosphatase Cys-X_5-Arg motif at its active site. On the other hand, Acr2p is functionally analogous to the *E. coli* (R773) ArsC As^{V} reductase, because it exhibits reductase activity only when both GSH and Grx are present as electron donors (Mukhopadhyay *et al.*, 2000).

It is important to note that all these microbial non-respiratory As^{V} reductase enzymes require the contribution of three thiol groups for proper function. One such group always belongs to the enzyme, whereas the other two thiols may also belong to the enzyme (as seen with the *S. aureus* ArsC) or may be brought by GSH and Grx (as with the *E. coli* ArsC or the yeast Acr2p).

Reduction of arsenate in mammals. In mammalian organisms, As^{V} is rapidly reduced to the much more toxic As^{III}, as after administration of As^{V} to laboratory animals, As^{III} rapidly (within 5 min) appears in the blood, bile, urine, and tissues (Csanaky and Gregus, 2005; Thomas *et al.*, 2001). However, there had been no enzyme identified contributing to this process before our work. It has been shown that GSH is able to reduce As^{V} chemically (Delnomdedieu *et al.*, 1994a), although the concentrations of the two reactants were physiologically irrelevant (300 mM and 150 mM, respectively). Reduction of As^{V} is apparently GSH-dependent in mouse embryo cells (Bertolero *et al.*, 1987), and disposition of As^{V} is GSH-dependent in rats *in vivo* (Gyurasics *et al.*, 1991). Direct evidence has recently been presented that As^{V} reduction in rats indeed depends on GSH availability (Csanaky and Gregus, 2005).

Reduction of As^V, the first step in its biotransformation, is of great toxicological importance not only in As^V metabolism but also in determining its toxicity and carcinogenicity, as the product of this biochemical reaction is As^{III} with high toxic potential. The importance of this process and its uncleared mechanisms prompted us to initiate research on the biochemical background of As^V reduction with the final goal to identify subcellular fractions or even specific enzymes that can catalyze the conversion of As^V to As^{III}.

RESEARCH OBJECTIVES

The primary goal of our research has been to find and identify mammalian enzymes that can catalyze the reduction of As^V. For this purpose, we have determined what cell fractions can catalyze reduction of As^V, and have characterized the observed As^V reductase activities biochemically in order to draw conclusions on the properties of the contributing enzyme. Then we have attempted to directly prove the role of the implicated enzyme by using specific inhibitors and purified enzymes. Our questions have been as follows:

1. Do mitochondria isolated from rat liver and incubated with As^V reduce As^V to As^{III}? Mitochondria take up As^V (Chan *et al.*, 1969; Wohlrab, 1986), and because they are similar to bacterial cells, which reduce As^V and export the formed As^{III}, these organelles may also carry out As^V reduction. If they do, how does the functional state of mitochondria (as influenced by respiratory substrates as well as anions structurally similar to As^V, GSH content, inhibitors and uncouplers of oxidative phosphorylation) affect formation of As^{III} from As^V? Do mitochondria export the formed As^{III}? Can solubilized mitochondria preserve As^V-reducing activity, thereby permitting purification and identification of the mitochondrial As^V reductase?
2. Are extramitochondrial enzymes involved in reduction of As^V? Do the postmitochondrial cell fractions (i.e., microsomes and cytosol) of the rat liver as well as human red blood cells (devoid of mitochondria) reduce As^V to As^{III}? What are the biochemical characteristics (thiol dependence, effects of inorganic phosphate, nucleotides, thiol reactive compounds, substrates and inhibitors of enzymes and metabolic pathways) of the observed As^V-reducing activities? Based on these biochemical properties, what can the catalyzing enzyme be?
3. What is the significance of the enzymes, had they been found to carry out reduction of As^V *in vitro*, in the disposition of As^V *in vivo*? This question is important to answer

because it is not sufficient to demonstrate that an enzyme can reduce As^{V} to As^{III} *in vitro* but its role in the *in vivo* As^{V} reduction should also be assessed.

METHODS

Animals

Usually, male Wistar rats (*Rattus norvegicus Wistar)* weighing 250-300 g were used. However, when testing interspecies differences in As^{V} reductase activities, we used CFLP mice (*Mus musculus CFLP*, 33-36 g), English shorthair guinea pigs (*Cavia porcellus*, 400-450 g), Syrian golden hamsters (*Mesocricetus auratus*, 80-100 g), and New Zealand white rabbits (*Oryctolagus cuniculus N. Z. white*, 1.8-2.5 kg). The animals were kept at room temperature, at 55-65% relative air humidity, and on a 12-hour light/dark cycle and provided with tap water and rodent or rabbit lab chow *ad libitum*. All procedures were carried out according to the Hungarian Animals Act, and the studies were in agreement with the rules of the Ethics Committee on Animal Research of the University of Pécs, Center for Medical and Health Sciences.

Treatments

Treatments with glutathione depletors. Some rats were injected intraperitoneally with GSH depletors, i.e., buthionine-sulfoximine (BSO, 5 mmol/kg), diethylmaleate (DEM, 6 mmol/kg), or phorone (2 mmol/kg) at 6, 3, and 3 hours before sacrifice, respectively. Mitochondria were isolated from the livers of the thus-pretreated rats.

Treatments with BCX-1777. In order to test the *in vivo* effectiveness of BCX-1777 as an inhibitor of purine nucleoside phosphorylase in liver, rats were anesthetized with urethane (1.2 g/kg) injected ip (5 ml/kg). Through a median abdominal incision, the renal pedicles were ligated to prevent the rapid urinary elimination of BCX-1777, and the animals were injected with BCX-1777 (50 μmol/kg, iv) in saline (2 ml/kg) through their left saphenous vein. 15 min after injection of BCX-1777, the liver was perfused through the portal vein with ice-cold isotonic saline then quickly removed and homogenized, and its cytosolic fraction was isolated.

To determine the *in vivo* effect of BCX-1777 on disposition of As^{V}, rats were anesthetized with urethane and injected with BCX-1777, after ligation of their renal pedicles, as described above. 15 min after administration of BCX-1777, As^{V} was injected (50 μmol/kg, iv). DTT (300 μmol/kg, iv) was given to some rats 2 min before As^{V}.

Treatments with (S)-α-chlorohydrin. In order to test the effect of (S)-α-chlorohydrin (ACH) on glyceraldehyde-3-phosphate dehydrogenase and As^V reductase activities in tissues, rats were injected with ACH (100 or 200 mg/kg, ip) or saline (3 ml/kg, ip). Three hours later liver, kidney, and muscle samples were removed, and their cytosolic fractions were prepared.

In order to test the effect of ACH on reduction of As^V *in vivo*, two different experiments were carried out. In both, the rats were pretreated ip with ACH (100 mg/kg or 200 mg/kg) or saline (3 ml/kg), 3 hours before As^V administration. Immediately before injection of As^V, the rats in the first experiment were subjected to bile duct ligation (BDL-rats), whereas the rats in the second experiment underwent both bile duct and renal pedicle ligation (BDRPL-rats).

Preparation of cell fractions and red blood cells for assaying As^V reductase activity

Isolation of subcellular fractions. Isolation of subcellular fractions (e.g., mitochondria, cytosol) was carried out using differential centrifugation, as described previously (Hobgeboom, 1955). All steps were carried out at 0-4 °C. The protein concentration of the thus prepared fractions was determined using the biuret method (Gornall *et al.*, 1949) or the bicinchoninic acid method (Brown *et al.*, 1989).

Preparation of human red blood cell suspension. These studies were approved by the Regional Scientific Research Ethics Committee of the University of Pécs, Center for Medical and Health Sciences. Blood (approximately 5 ml) was collected from healthy adult volunteers after informed consent. For experiments with red blood cells (RBC), the preparation and the incubations were carried out in a chloride- and phosphate-free buffer because chloride and phosphate inhibit the uptake of As^V by the erythrocytes.

Enzymatic assays

The prepared cell fractions or RBC were incubated at 37°C with As^V in the presence of test compounds. The incubation was started with addition of As^V and was stopped by protein precipitation.

In order to determine the respiratory control ratio, we measured mitochondrial oxygen consumption polarographically at 25 °C using a Clark-type oxygen electrode. Respiratory control ratio was calculated by dividing State III rate by State IV rate.

GSH levels in tissue samples and mitochondria were determined by the method of Tietze (1969), while non-protein thiol (NPSH) levels by Sedlak and Lindsay (1968).

Purine nucleoside phosphorylase was assayed by the spectrophotometric method of Kalckar (1947). The activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was assayed spectrophotometrically based on the decrease of NADH concentration (0.25 mM) during the GAPDH-limited conversion of 3-phosphoglycerate (5 mM) to glyceraldehyde-3-phosphate in the presence of excess phosphoglycerate kinase and ATP.

Studying the disposition of As^V on rats

In order to assess the contribution of enzymes to the *in vivo* reduction of As^V that catalyzed this process effectively *in vitro*, we performed experiments on anesthetized rats. The anesthesia and surgical preparation of rats was adapted to the experimental objectives (specific details of these experiments are given in the Methods sections in the respective publications). Thereafter, the rats were injected with As^V (50 μmol/kg body weight) intravenously (3 ml/kg b.w.), and bile and/or urine were collected in 20-min periods for 60 minutes then tissue samples were removed and immediately processed for arsenic analysis.

Arsenic analysis

Sample preparation. The incubates originating from the As^V reductase assays and subjected to protein precipitation were centrifuged. The resultant supernatant was used for speciation and quantification of arsenic. Bile, urine, and tissue samples from experiments on As^V-injected rats were prepared for arsenic analysis immediately after collection. The bile and urine samples were deproteinized and diluted appropriately, and applied to the arsenic speciation. The blood samples from the *in vivo* experiments were mixed with Triton X-100 then aqueous $HgCl_2$ solution was added to displace thiol-bound arsenic. Finally, perchloric acid was added, mixed thoroughly, and centrifuged. The resultant supernatant was applied to arsenic speciation. The weighed tissue samples were homogenized in perchloric acid. Thereafter, 0.5 ml homogenate was mixed with $HgCl_2$, and then centrifuged. The resultant supernatant was applied to arsenic speciation.

Speciation of arsenic by HPLC–HG–AFS. Arsenicals from the As^V reductase assays and biological samples obtained from As^V injected rats were separated and quantified by HPLC–hydride generation–atomic fluorescence spectrometry (HPLC–HG–AFS) based on the procedure of Gomez-Ariza *et al.* (1998), as described in detail by Gregus *et al.* (2000).

RESULTS

Mitochondrial reduction of arsenate

Background

The environmentally prevalent As^{V} undergoes reduction to As^{III} in living organisms as the first step of its metabolism (Thomas *et al.*, 2001). However, neither the contributing enzymes nor the cellular localization of this important toxification process has been known. Reduction of As^{V} in cultured cells (Bertolero *et al.*, 1987; Huang and Lee, 1996), erythrocytes (Delnomdedieu *et al.*, 1995), and human liver cytosol (Radabaugh and Aposhian, 2000) has been observed, but the participating enzyme remained unknown. Bacterial or yeast cells can take up As^{V} from the environment, and then reduce it to As^{III} in a GSH- and Grx-dependent or thioredoxin-dependent manner. The formed As^{III} is then extruded from these cells via ATP-driven or membrane potential-dependent transporters.

Mitochondria also take up As^{V} through their P_i-moving transport proteins, i.e., the phosphate transporter (Chan *et al.*, 1969; Wohlrab, 1986) and the dicarboxylate carrier (Indiveri *et al.*, 1989). Mitochondrial accumulation of arsenic has been found in the kidneys of As^{V}-injected rabbits (Vahter and Marafante, 1989). At high concentrations, As^{V} uncouples the oxidative phosphorylation in a P_i-free medium (Crane and Lipmann, 1953), most likely because ATP synthase forms ADP-arsenate, which rapidly hydrolyses to ADP and As^{V}. This uncoupling mechanism could be prevented by oligomycin (Estabrook, 1961), which inhibits the ATP synthase. Mitochondria are believed to have arisen from aerobic bacteria and resemble bacterial cells in many aspects; therefore, they may be able to carry out reduction of As^{V} to As^{III}. We tested this hypothesis to determine if mitochondria isolated from rat liver reduced As^{V} to As^{III}, and if they did so, what the characteristics of this process were.

Results and Conclusions

Isolated rat liver mitochondria rapidly reduced As^{V} to As^{III}. Among the respiratory substrates supporting the citric acid cycle, glutamate enhanced reduction of As^{V} the most, whereas succinate and malate exerted inhibitory effect of on As^{V} reduction that may originate, at least partly, from countering the mitochondrial As^{V} uptake through the dicarboxylate carrier. Inorganic substrates of this transporter (e.g., sulfate, sulfite, thiosulfate) also decreased mitochondrial As^{V} reduction. In the absence of dicarboxylates, both the dicarboxylate carrier and the mitochondrial P_i transporter can mediate As^{V} uptake

(Chan *et al.*, 1969; Wohlrab, 1986). Inhibitors of these proteins (e.g., *N*-ethylmaleimide, mersalyl, and butylmalonate) abolished mitochondrial formation of As^{III} most likely by preventing the entry of As^{V}. P_i inhibited As^{V} reduction in a concentration-dependent manner most likely not only by competing with As^{V} uptake but also by directly interfering with the reducing enzyme, owing to their structural similarity. ADP increased, whereas ATP and AMP decreased, As^{III} formation, and their effects could be prevented by atractyloside. Electron transport inhibitors and uncouplers abolished As^{V} reduction, whereas ATP-synthase inhibitors almost completely inhibited it. As^{III} was recovered completely from the supernatant of the mitochondrial incubate, suggesting that mitochondria exported the formed As^{III}. Testing the effects on As^{V} reduction of chemicals that interfere with thioredoxin reductase failed to support the role of this enzyme in reduction of As^{V}. Depletion of mitochondrial GSH impaired mitochondrial As^{V}-reducing activity but also diminished the respiratory control ratio. Upon solubilization of mitochondria, their reducing activity was lost and was not recovered by addition of GSH and NADH or NADPH. In summary, we have demonstrated for the first time that mitochondria are capable of reducing As^{V} to the more toxic As^{III}. Like some microbial cells, mitochondria take up As^{V}, reduce it, and export the formed As^{III}. In this process, these organelles work as integrated units, like chemical reactors, requiring both structural and functional integrity. Further research is deemed necessary to clarify the molecular mechanisms of mitochondrial As^{V} reduction as well as the role of mitochondria in the conversion of As^{V} to As^{III} *in vivo*.

Purine nucleoside phosphorylase as a cytosolic arsenate reductase

Background

After finding that mitochondria isolated from rat liver reduce As^{V} to the much more toxic As^{III}, it was of interest to know if other cell fractions could also carry out this important toxification reaction. It was found that the cytosolic fraction of the human liver exhibits As^{V} reductase activity, which requires a heat-stable cofactor of less than 3000-dalton molecular mass (Radabaugh and Aposhian, 2000). Therefore, we intended to determine whether the postmitochondrial cell fractions of rat liver also posses As^{V} reductase activity, and if they do, to characterize this activity with the ultimate goal of identifying the enzyme(s) involved.

Results and Conclusions

Incubations of rat liver postmitochondrial supernatant (PMSN) with As^{V} revealed that PMSN reduced As^{V} to As^{III} only in the presence of a thiol. GSH supported the PMSN-catalyzed As^{III} formation poorly, whereas dithiothreitol (DTT) enhanced it markedly. After separating the microsomal and cytosolic fractions and testing their As^{V}-reducing activities, we demonstrated that the microsomes did not contain any As^{V} reductase activity, but the cytosol catalyzed the formation of As^{III} from As^{V} effectively, indicating that the catalyzing enzyme resides in the cytosol. Oxyanions related to As^{V} structurally (such as P_i or *o*-vanadate) as well as mercurial thiol-reagents inhibited As^{V} reduction, indicating the involvement of an SH-enzyme, which possesses a P_i-binding site that can accommodate As^{V} too.

On searching for a reduction partner, we surprisingly found that oxidized pyridine nucleotides (i.e., NAD and NADP) but not their reduced counterparts, markedly enhanced the cytosolic reduction of As^{V}. This observation prompted us to test a number of other nucleotides as well. These experiments revealed that some other purine nucleotide derivatives (e.g., AMP, GMP, *S*-adenosylhomocysteine), but not pyrimidine nucleotides, enhanced the conversion of As^{V} to As^{III}. Because these nucleotides can readily be transformed into purine nucleosides during the incubations, we tested the effect of nucleosides and nucleobases on cytosolic As^{V} reduction. Pyrimidine nucleosides did not affect As^{III} formation. In contrast, purine nucleosides, especially the 6-oxopurine ones (i.e., inosine and guanosine), increased it dramatically (80-100 fold). Adenosine (6-aminopurine nucleoside) was much less potent, suggesting that it may be converted into inosine by adenosine deaminase. In contrast, the 6-oxopurine bases strongly inhibited As^{III} formation from As^{V} by rat liver cytosol (by 80-90%). Moreover, ultrafiltration of rat liver cytosol yielded a retentate lacking As^{V} reductase activity almost completely. The As^{V}-reducing activity of the retentate was restored by adding the filtrate, inosine, or guanosine to it, indicating that endogenous purine nucleosides are essential for the cytosolic As^{V} reduction. In addition, this 6-oxopurine nucleoside-stimulated As^{V} reductase activity was demonstrated to be present in the hepatic cytosol of other common laboratory animals, such as mice, hamsters, guinea pigs, and rabbits.

These findings allowed us to make the following tentative conclusions: (1) The cytosolic As^{V} reductase requires the presence of an appropriate thiol and is inhibited by thiol-reagents; therefore, the enzyme most likely possesses functionally critical SH-groups. (2) P_i inhibits the As^{V}-reducing activity of this enzyme; therefore, it possesses a P_i-binding

site and probably utilizes P_i as a substrate. (3) 6-oxopurine nucleosides strongly increase the As^V reductase activity; therefore, the enzyme may accept 6-oxopurine nucleosides as substrates. (4) 6-oxopurine nucleobases markedly decrease the reducing activity; therefore, the catalyzing enzyme may form 6-oxopurine nucleobases.

The enzyme that fits these deduced characteristics is known as purine nucleoside phosphorylase (PNP). PNP is soluble cytosolic enzyme with important thiol groups (Bzowska *et al.*, 2000; Parks and Agarwal, 1972). The enzyme, while utilizing P_i, catalyzes the phosphorolytic cleavage of 6-oxopurine nucleosides to the corresponding nucleobase and ribose-1-phosphate. Like many P_i-utilizing enzymes, PNP also accepts As^V instead of P_i and produces the purportedly unstable ribose-1-arsenate (Kline and Schramm, 1993). Therefore, we tested the hypothesis that PNP is responsible for the thiol- and purine nucleoside-dependent reduction of As^V by hepatic cytosol. The following pieces of evidence supporting this hypothesis that PNP can function as As^V reductase were obtained: (1) Specific and highly potent inhibitors of PNP (i.e., BCX-1777 and CI-1000) decreased the As^V reductase activity of rat liver cytosol in a concentration-dependent manner, causing complete inhibition at a concentration as low as 1 μM in the hepatic cytosol. (2) The As^V reductase activity consistently and perfectly co-eluted with the PNP activity during the anion exchange chromatography of rat liver cytosol, representing circumstantial evidence that both activities belong to the same protein. (3) Purified PNP effectively catalyzed the reduction of As^V, provided its nucleoside substrate and appropriate thiol were present simultaneously, proving directly that PNP can indeed function as an As^V reductase. (4) Various chemicals similarly influenced the reduction of As^V by rat liver cytosol and by purified PNP, as both were activated by 6-oxopurine nucleosides and DTT, and both were inhibited by P_i, mercurial thiol reagents, and specific PNP inhibitors. These observations constitute compelling evidence that the DTT-supported As^V reductase activity in the hepatic cytosol of rats and other species can be ascribed to PNP. Our observations indicated that the PNP-catalyzed As^V reduction takes place during, or as a consequence of, the arsenolytic cleavage of 6-oxopurine nucleosides.

Our results presented so far clearly indicated that PNP is an efficient As^V reductase *in vitro.* It was therefore of high interest whether this ubiquitous enzyme contributes to the reduction of As^V *in vivo.* To assess such a role of PNP, we used two experimental approaches. First, we tested if compounds influenced As^V reduction by intact human red blood cells (RBC) similarly to that by purified PNP. The erythrocytes reduced As^V at a considerable rate, which could be enhanced by inosine or inosine plus DTT. These

stimulated As^{III} formation rates were PNP-dependent, as PNP inhibitors strongly inhibited them. In contrast, PNP inhibitors had little if any inhibitory effect on As^{III} formation in the absence of exogenous inosine, indicating that this basal rate of As^{V} reduction is PNP-independent. Second, we assessed the role of PNP in reduction of As^{V} *in vivo* by investigating the effect of the specific and potent PNP inhibitor BCX-1777 on the biotransformation of As^{V} in control and DTT-treated rats. Although it abolished hepatic PNP activity, BCX-1777 influenced neither the biliary excretion of As^{III} and $MMAs^{III}$ nor the tissue concentrations of As^{V} and its metabolites in either group of As^{V}-injected rats. Thus, despite its *in vitro* activity, PNP does not appear to play a significant role in AsV reduction in human erythrocytes and in rats *in vivo*.

Glyceraldehyde-3-phosphate dehydrogenase as a cytosolic arsenate reductase

Background

Despite the promising findings that PNP reduces As^{V} to the much more toxic As^{III}, provided its nucleoside substrate and an appropriate dithiol are present simultaneously, we found that PNP does not contribute significantly to the reduction of As^{V} either in intact human erythrocytes or in rats *in vivo*. Intact human RBC retained most of their As^{V}-reducing activity even in the presence of high concentrations of BCX-1777. Moreover, complete inhibition of PNP in rats by administration of BCX-1777, a highly potent transition state analogue inhibitor of the enzyme, did not delay the elimination of As^{V} and the formation of As^{V} metabolites. In addition, the observation that the As^{V} reductase activity of PNP is poorly supported by GSH, whereas As^{V} reduction is apparently GSH-dependent in cells (Bertolero *et al.*, 1987) and in rats (Csanaky and Gregus, 2005), also contradicts to the role of PNP as an *in vivo* relevant As^{V} reductase.

As demonstrated, intact human erythrocytes reduce As^{V} to As^{III} in a manner mostly independent of PNP. To characterize this PNP-independent As^{V} reductase activity, we started another series of experiments with the final aim of identifying the enzyme responsible for the PNP-independent reduction of As^{V}.

Results and Conclusions

Intact human red blood cells reduce As^{V} to As^{III} intracellularly, because both the natural substrate chloride and an irreversible inhibitor (diisothiocyanatostylbene-disulfonic acid) of the chloride-bicarbonate exchanger (which mediates erythrocytic P_i and As^{V} uptake) inhibited As^{V} reduction by intact, but not lysed, RBC. The basal (i.e., PNP-

independent) As^{V}-reducing activity of RBC requires GSH, because the GSH depletor diethylmaleate strongly diminished As^{III} formation. The erythrocytic As^{V} reduction apparently depends on NAD and/or NADP supply, because oxidants of NAD(P)H (e.g., pyruvate, ferricyanide, dehydroascobate, 4-dimethylaminophenol) enhanced As^{III} formation. The oxidant-stimulated As^{V} reduction is PNP-independent, because BCX-1777 failed to influence it, but is GSH-dependent because the GSH-depleting diethylmaleate impaired it. Pyruvate-induced glucose-depletion, which causes NAD enrichment at the expense of NADH, enhanced As^{V} reduction. This suggests that the erythrocytic As^{V} reduction requires both NAD supply and operation of the lower part of the glycolytic pathway starting from glyceraldehyde-3-phoshate dehydrogenase (GAPDH) that, unlike the upper part, remains fed with substrates originating from the degradation of the RBC-specific compound 2,3-bisphosphoglycerate. These substrates cannot go above GAPDH because this would require NADH, which is depleted in RBC pretreated with pyruvate. Fluoride, which arrests glycolysis at enolase, inhibited As^{V} reduction in glucose-sufficient RBC, but increased it in glucose-deficient (NAD-enriched) cells, suggesting that the section of glycolysis coupled to As^{V} reduction lies between GAPDH and enolase (i.e., one or more of GAPDH, phosphoglycerate kinase, and phosphoglycerate mutase).

In order to characterize this PNP-independent As^{V} reductase activity further, we examined the effects of GSH, inorganic phosphate, some inhibitors of glucose metabolism, glycolytic substrates, and pyridine as well as adenine nucleotides on As^{V} reduction in lysed RBC and rat liver cytosol in the presence of BCX-1777, a PNP inhibitor. In hemolysate, GSH enhanced As^{V} reduction in a concentration dependent manner, whereas phosphate inhibited it. Glycolytic substrates, especially fructose-1,6-bisphosphate and phosphoglyceric acids, improved As^{V} reductase activity. NAD, especially together with these substrates, strongly increased As^{III} formation, whereas NADH strongly inhibited it. NADP and adenine nucleotides diminished, while 2-phosphoglycollate, which increases the breakdown of the RBC-specific compound 2,3-bisphosphoglycerate to 3-phosphoglycerate, doubled the As^{V} reductase activity. Although As^{V} reduction by the liver cytosol responded similarly to GSH, NAD, and glycolytic substrates as in the hemolysate, it was barely influenced by NADH, was diminished by 2-phosphoglycollate and stimulated by NADP. Collectively, hemolysate and rat liver cytosol possess a PNP-independent As^{V} reductase activity, which requires GSH, NAD, and glycolytic substrates. The need for GSH and the sensitivity to mercurial thiol reagents indicate the presence of critical thiol groups. The lack of such groups in phosphoglycerate mutase excludes this enzyme as a candidate

As^V reductase. In contrast, the two functionally linked glycolytic enzymes, GAPDH and phosphoglycerate kinase (PGK) came into view, as possible As^V reductases, because they contain functionally important thiol groups, and their substrates enhanced reduction of As^V the most.

In testing the hypothesis that one or both of GAPDH and PGK can reduce As^V to As^{III}, we found that, if supplied with glutathione (GSH), NAD, and glycolytic substrate, the mixture of purified GAPDH and PGK indeed catalyzed the reduction of As^V. Further analysis revealed that GAPDH is endowed with As^V reductase activity, whereas PGK serves as an auxiliary enzyme, when 3-phosphoglycerate is the glycolytic substrate. The GAPDH-catalyzed As^V reduction required GSH, NAD, and glyceraldehyde-3-phosphate. ADP and ATP moderately, whereas NADH strongly inhibited the As^V reductase activity of the enzyme even in the presence of NAD. Koningic acid (KA), a specific and irreversible inhibitor of GAPDH, inhibited both the classical enzymatic and the As^V-reducing activities of the enzyme in a concentration-dependent fashion. To assess the contribution of GAPDH to the reduction of As^V carried out by hemolysate, rat liver cytosol, or intact erythrocytes, we determined the concentration-dependent effect of KA on As^V reduction by these cells and extracts. Inactivation of GAPDH by KA abolished As^V reduction in intact RBC as well as in the hemolysate and the liver cytosol, when GAPDH in the latter extracts was abundantly supplied with exogenous NAD and glycolytic substrate. However, despite complete inactivation of GAPDH by KA, the hepatic cytosol exhibited significant residual As^V-reducing activity in the absence of exogenous NAD and glycolytic substrate, supporting our finding that besides GAPDH, other cytosolic enzyme(s) may contribute to As^V reduction in the liver. In conclusion, the key glycolytic enzyme GAPDH can fortuitously catalyze the reduction of As^V to As^{III}, if GSH, NAD, and glycolytic substrate are available. As^V reduction may take place during, or as a consequence of, the arsenolytic cleavage of the thioester bond formed between the enzyme's Cys149 and the 3-phosphoglyceroyl moiety of the substrate.

An important further question is whether or not GAPDH significantly contributes to reduction of As^V *in vivo*. The relevance of this question can be appreciated by the example of PNP, which works very efficiently as an As^V reductase *in vitro* but not *in vivo*. To test this hypothesis that GAPDH significantly contributes to the disposition of As^V *in vivo*, we examined the effect of (S)-α-chlorohydrin (ACH) – which *in vivo* likely forms a GAPDH-inhibitory metabolite mainly in the liver – on the reduction of As^V in rats. These studies confirmed the *in vitro* role of GAPDH as an As^V reductase, inasmuch as 3 hours after

administration of ACH (100 or 200 mg/kg, ip) to rats both the cytosolic GAPDH activity and the As^{V}-reducing activity dramatically fell in the liver, moderately decreased in the kidneys, and remained unchanged in the muscle. Moreover, the As^{V}-reducing activity closely correlated with the GAPDH activity in the hepatic cytosols of control and ACH-treated rats. Two confounding effects of ACH (i.e., a slight fall in hepatic glutathione levels and a rise in urinary As^{V} excretion) prompted us to examine its influence on the disposition of injected As^{V} in rats with ligated bile duct as well as in rats with ligated bile duct and renal pedicles. These experiments demonstrated that the hepatic retention of As^{V} significantly increased and the combined levels of As^{V} metabolites (i.e., As^{III} plus methylated arsenicals) in the liver decreased in response to ACH; however, ACH failed to delay the disappearance of As^{V} from the blood of rats with blocked excretory routes. Thus, the GAPDH inactivator ACH inhibits As^{V} reduction by the liver, but not by the whole body, probably because the impaired hepatic reduction is compensated for by hepatic and extrahepatic As^{V}-reducing mechanisms spared by ACH. It is most likely that ACH inhibits hepatic As^{V} reduction predominantly by inactivating GAPDH in the liver; however, a slight ACH-induced glutathione depletion may also contribute. While these results seem to support the conclusion that GAPDH in the liver is involved in As^{V} reduction in rats, confirmation of the *in vivo* role of GAPDH as an AsV reductase calls for further research.

NEW RESULTS

1. We have demonstrated for the first time that mitochondria isolated from rat liver can take up As^{V}, reduce it to the much more toxic As^{III}, and export the formed As^{III}. Mitochondrial reduction of As^{V} requires both the structural and the functional integrity of these organelles. Inorganic phosphate at physiologically relevant concentrations markedly diminished mitochondrial As^{V} reduction, suggesting that the contribution of these organelles to the reduction of As^{V} *in vivo* might not be significant.

2. Not only hepatic mitochondria but also rat liver cytosol can reduce As^{V} to As^{III} in a thiol-dependent fashion. One cytosolic As^{V} reductase activity is supported by the physiologically irrelevant thiol compound DTT as well as by purine nucleosides, especially the 6-oxopurine ones, and is inhibited 6-oxopurine bases and mercurial thiol reagents. We found that the purine nucleoside- and DTT-supported cytosolic As^{V}-reducing activity is ascribable to purine nucleoside phosphorylase (PNP). It is indicated

by (1) coelution of As^{V} reductase and PNP activities during anion exchange chromatography of cytosolic proteins, (2) sensitivity of the As^{V}-reducing activity to PNP inhibitors (i.e., BCX-1777 and CI-1000), and (3) the reduction of As^{V} by purified PNP, provided an appropriate thiol (e.g., DTT, dimercaprol) and its substrate (inosine or guanosine) are present simultaneously. This DTT-stimulated As^{V} reductase activity inhibitable by PNP inhibitors could be detected in the hepatic cytosol of rats, mice, hamsters, guinea pigs, and rabbits. Inhibitors of the enzyme inhibited not only its classical biochemical activity but also its As^{V} reductase activity.

3. In spite of the fact that under appropriate conditions PNP rapidly reduces As^{V} to As^{III} *in vitro*, PNP is apparently not involved in the reduction of As^{V} *in vivo*. This conclusion is supported by the observation that BCX-1777 administered to rats completely inhibited the hepatic PNP activity, but failed to alter the *in vivo* disposition of As^{V} either the animals were or were not injected with DTT, the activator of the PNP-catalyzed As^{V} reduction.

4. Intact human red blood cells can take up As^{V} via the chloride-bicarbonate exchanger, and reduce As^{V} to As^{III}. The erythrocytic As^{V} reductase activity is increased by inosine and/or DTT. This increment is PNP-dependent, as it is abolished by the PNP inhibitor BCX-1777. The basal erythrocytic As^{V} reduction, however, is PNP-independent, as it is not affected by PNP inhibition.

5. The PNP-independent As^{V} reductase activity of human RBC apparently depends on intraerythrocytic availabilities of GSH and NAD, as the GSH depletor DEM inhibits it, whereas chemicals that promote oxidation of NADH to NAD enhance the reduction of As^{V} by intact RBC. This GSH- and NAD-dependent As^{V} reductase activity is also present in RBC lysate and in rat liver cytosol. This activity is augmented by glycolytic substrates, especially together with NAD, suggesting involvement of a glycolytic enzyme.

6. The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase reduces As^{V} to As^{III}, provided GSH, NAD, and glycolytic substrate are present. Koningic acid inhibits both the glycolytic activity and the As^{V} reductase activity of GAPDH. Using the GAPDH-inhibiting koningic acid, it can be observed that GAPDH is exclusively

responsible for the PNP-independent As^V reductase activity of human RBC and significantly contributes to that in rat liver cytosol.

7. Apparently, GAPDH contributes to the reduction of As^V *in vivo*, at least in the rat liver. This conclusion is supported by the observation that pretreatment of rats with ACH (which forms a GAPDH inhibitory metabolite) decreases both GAPDH and As^V reductase activities in hepatic cytosol and also decreases the As^V metabolite-to-As^V ratio in the liver of As^V-injected rats.

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ABBREVIATIONS

As^{V}	arsenate
As^{III}	arsenite
$MMAs^{III}$	monomethylarsonous acid
$MMAs^{V}$	monomethylarsonic acid
$DMAs^{V}$	dimethylarsinic acid (cacodylic acid)
ACH	(S)-α-chlorohydrin
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BDL	bile duct-ligated (rat)
BDRPL	bile duct- and renal pedicle ligated (rat)
BSO	D,L-buthionine-S,R-sulfoximine
DEM	diethyl maleate
DTT	dithiothreitol
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Grx	glutaredoxin
GSH	reduced glutathione
GSSG	oxidized glutathione
HPLC-HG-AFS	high performance liquid chromatography – hydride generation – atomic fluorescence spectrometry
KA	koningic acid
NAD(P)	nicotinamide adenine dinucleotide (phosphate)
NAD(P)H	reduced nicotinamide adenine dinucleotide (phosphate)
NPSH	non-protein thiol
PGK	phosphoglycerate kinase
P_i	inorganic orthophosphate
PMSN	postmitochondrial supernatant
PNP	purine nucleoside phosphorylase
RBC	red blood cell
Trx	thioredoxin
TRR	thioredoxin reuctase

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