ARSENIC METHYLATION IN PERSPECTIVE

by

Sheila Marie Healy

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H. Vasken Aposhian	Date 11/9/01
Dean E. Carter	Date 11/6/01
Roy R. Parker	Date 11/09/01
Richard R. Vaillancourt	Date 11/08/01
Ted A. Weinert	Date 11-8-01

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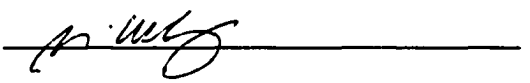
SIGNED: 

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ABSTRACT

The following arsenite methyltransferase activities (U/mg) were measured in untreated mice: liver, 1.42 ± 0.17 (mean ± SEM); kidney, 0.62 ± 0.18; lung, 0.33 ± 0.08; testis, 1.21 ± 0.01. Arsenite methyltransferase metabolites were not detectable using guinea pig liver, kidney, lung or testis cytosol as the source of enzyme. A twofold increase in liver arsenite methyltransferase activity was observed in mice exposed to 28.6 mg sodium arsenite/L drinking water after 24 hr compared to control.

Trivalent arsenic species were separated from pentavalent arsenicals in liver homogenates of hamsters injected 15 hr priorly with [^{73}As]arsenate by (CCl_4)-20 mM DDDC extraction and both phases analyzed by HPLC. Metabolites of inorganic arsenate were observed in the following concentrations (ng/g liver ± SEM); MMA^{III}, 38.5 ± 2.9; DMA^{III}, 49.9 ± 10.2; arsenite, 35.5 ± 3.0; arsenate, 118.2 ± 8.7; MMA^{V}, 31.4 ± 2.8; and DMA^{V}, 83.5 ± 6.7.

Neither *in vitro* nor *in vivo* arsenite methyltransferase activity could be detected in *S. cerevisiae*. Attempting to induce enzyme activity, yeast were grown in 0 - 100 mM, 8 μCi [^{73}As]Na_2HAsO_4. No methylated metabolites were detected in cell lysate or media under these experimental conditions.

The dissociation constants for guinea pig and rabbit cytosolic arsenite binding proteins were determined to be 59.62 ± 11.97 and 120.4 μM As^{III}, respectively, and the respective specificities, 53.83 ± 3.67% and 59%. Guinea pig arsenite binding protein was fractionated using bioaffinity and size exclusion chromatography to yield partially purified protein(s) with an approximate molecular weight of 115 kDa.

Arsenite methyltransferase activity was purified > 7,000-fold from rabbit liver and identified as 1 cys peroxiredoxin, a conserved family of antioxidant proteins characterized by non-selenium dependent glutathione peroxidase and calcium-independent phospholipase A_2 activities. Murine 1 cys peroxiredoxin was cloned and expressed in *E. coli* and *S. cerevisiae* and expressed in reticulocyte lysate. Recombinant proteins had neither arsenite methyltransferase nor glutathione peroxidase activities. Antibodies directed against 1 cys peroxiredoxin immunoprecipitated a ~29 kDa protein from guinea pig kidney cytosol which was identified as 1 cys peroxiredoxin by LC-MS/MS. Under these experimental conditions, guinea pig kidney cytosol did not catalyze the reduction of peroxides

1.

INTRODUCTION

Arsenic is the 18th most abundant element in the universe. It is also the 12th most abundant element in the human body, present in levels comparable to manganese although As is not an essential trace element (Schroeder and Balassa, 1966). Arsenic is a ubiquitous, natural toxicant. Humans are exposed to arsenic from the air, soil, food and water. Naturally contaminated drinking water is a public health concern. High levels of As have been reported in the USA (Oregon, Whanger *et al.*. 1977; Alaska, Kreiss *et al.*, 1983; Wyoming, Goldstein *et al.*, 2001; California, Meng *et al.*, 2001; Utah, Dye *et al.*, 2001) and globally. Chronic arsenic toxicity is evident in populations of Mexico (Cebrian *et al.*, 1983), India (Chatterjee *et al.*, 1995; Chowdhury *et al.*, 2000; Ahmad *et al.*, 2001), Taiwan (Chen *et al.*, 1985; Tseng *et al.*, 1996), China (Aposhian *et al.*, 2000a; Le *et al.*, 2000) and Romania (Aposhian *et al.*, 2000b) where water concentrations far exceed the maximum contaminant level of 10 μg As/L set by the World Health Organization (1993).

Input of arsenic into the global cycle also occurs through industrial pollution. The end-use distribution of arsenic in 1990 was 70% in wood preservatives (chromated copper arsenate, arsenic trioxide, arsenic pentoxide, calcium arsenate, lead arsenate and

sodium arsenate), 22% in agricultural chemicals (principally calcium arsenate and sodium arsenite as herbicides and desiccants), 4% in glass (arsenic trioxide) and 2% in nonferrous alloys (high purity metallic arsenic as semiconductor material) (NTP, 1998). The Toxic Chemical Release Inventory reported releases of arsenic by US industrial facilities to the environment which were estimated to be a total of 192,000 lb. (NTP, 1998).

Arsenic *tops* the 1999 CERCLA (Comprehensive Environmental Response, Compensation and Liability Act) National Priorities List which is defined as "EPA's listing of sites that have undergone preliminary assessment and site inspection to determine which locations pose immediate threat [based on occurrence, toxicity and exposure] to persons living or working near release. These sites are in most need of cleanup." Each substance on the CERCLA list of Priority Hazardous Substances is a candidate to become the subject of a toxicological profile prepared by the Agency for Toxic Substances and Disease Registry (ATSDR) and for the identification of priority data needs. The mode of arsenic toxicity is not completely understood.

Since ancient time, arsenicals have been characterized by actions both beneficial and harmful. Very early in arsenic's history (ca 900 AD), it was found that some arsenic compounds were convenient, scentless and tasteless instruments of murder; arsenic was

widely used as a homicidal agent in Europe during the 1800's ranking 2nd only to opium in poisoning deaths (Polson and Tattersall, 1969). But arsenic has also played notable roles in medicine. Hippocrates is said to have used arsenic sulfide as a remedy for ulcers and similar disorders. Fowler's tonic (1% potassium arsenite) was developed in 1786 and used to treat agues, fevers, headaches and dermatitis (Winship, 1984). In the 19th century, Styrian villagers habitually ingested 130 to 325 mg doses of arsenic trisulfide and trioxide to enhance general health (Roscoe, 1862; Maclagan, 1864). Organic derivatives of trivalent arsenicals ushered in the chemotherapeutic age; Salvarsan (arsphenamine) was used in the treatment of syphilis during the first half of this century (Thorburn, 1983) and Melarsoprol is a current, potent treatment for trypanosomiasis (Tracy and Webster, 1996; Fairlamb *et al.*, 1989). Recently, arsenic trioxide has been successfully employed in the treatment of acute promyelocytic leukemia (Shen *et al.*, 1997; Soignet *et al.*, 1998) reportedly because of the induction of apoptosis (Chen *et al.*, 1996; Larochette *et al.*, 1999; Perkins *et al.*, 2000).

The toxicity of arsenic varies according to its oxidation state, solubility and the animal species exposed (Venugopal and Luckey, 1978; Marafante and Vahter, 1987; Wildfang *et al.*, 2000; Mitchell *et al.*, 2000). Arsenic is prevalent in the environment as pentavalent arsenate (As^{V}) and trivalent arsenite (As^{III}). Both forms of inorganic arsenic

are natural toxicants that can inhibit cellular respiration although the observed effects may not be mediated through similar mechanisms of action. Arsenate may disrupt phosphorylation reactions by substitution for phosphate and consequent formation of unstable arsenyl compounds (Doudoroff *et al.*, 1947; Dixon, 1996): Arsenate competes with phosphate for specific binding at F_1/F_0 ATPase (Cortés *et al.*, 2000) and indiscriminate binding at glyceraldehyde-3-PO_4 dehydrogenase (Ter Welle and Slater, 1967). Arsenite coordinately interacts with vicinal sulfhydryls thereby inhibiting essential enzymes (Webb, 1966) with the highest sensitivity reported for pyruvate and α-ketoglutarate dehydrogenases (Peters, 1955), DNA ligase and glutathione peroxidase (Snow *et al.*, 1999). Specifically, trivalent arsenic can bind the reduced internal disulfide of 6,8-dithiooctanoic acid, forming a stable six-membered ring which prevents lipoate acetyltransferase activity of the E2 PDH subunit (Adamson *et al.*, 1984). Arsenite is 2 – 10 more toxic than arsenate (Kosnett, 1994; Szinicz and Forth, 1988). Acute ingestion of as little as 70 mg $NaAsO_2$ may cause death (Abernathy and Ohanian, 1992). This value would give an LD_{50} of approximately 1 mg/kg and suggests that humans are much more sensitive to acute lethal effects of arsenic than are experimental animals.

Chronic ingestion of 20-60 μg/kg·day of inorganic arsenic has been associated with signs of arsenicism in humans (Kosnett, 1994). Chronic exposure may lead to

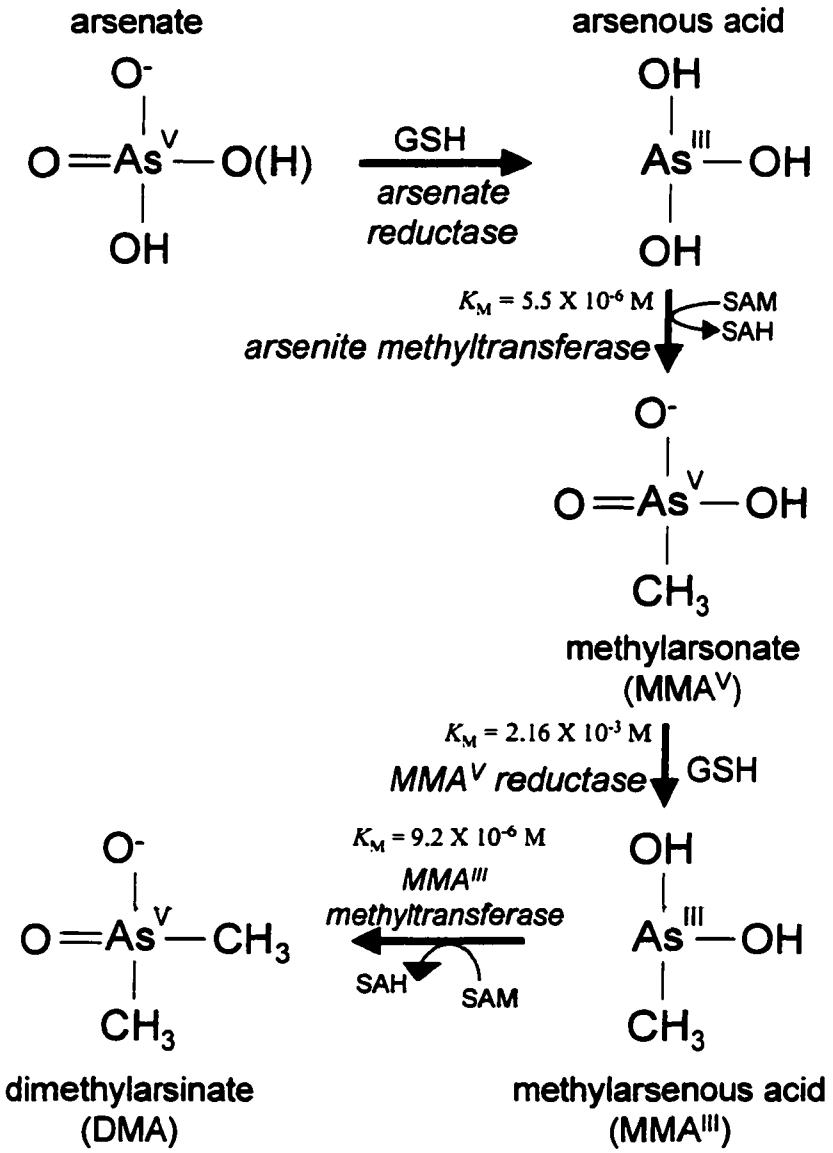
neurotoxicity of peripheral and central nervous systems, liver injury (jaundice, cirrhosis, ascites, Wildfang *et al.*, 2000), and peripheral vascular lesions such as Blackfoot's Disease which is endemic to southwest Taiwan where well water concentrations are 1 mg As/L (Tseng, 1977). Both inorganic and organic arsenic are teratogens (Ferm *et al.*, 1971; Rogers *et al.*, 1981). Inorganic arsenic is correlated convincingly with increased risk of skin, lung, liver and bladder cancer in the human (Neubauer, 1947; IARC, 1987; Chen *et al.*, 1992; NRC, 1999; NTP, 2000). The EPA (1987) and IARC (1987) both classify arsenic as a human carcinogen for which there is sufficient epidemiological evidence to support a causal association between exposure to arsenic via drinking water and cancer of internal organs and skin (Chen *et al.*, 1985). However, in contrast to most other human carcinogens, arsenic carcinogenicity has been difficult to confirm in experimental animals (review Huff, 2000). Mice (C57B1/6J and MT$^-$) exposed to 500 μg As+5/day for 26 months had 41% and 26% incidence of tumors, respectively (Ng *et al.*, 1999). However, it is not known whether the parent or metabolite or both are the ultimate carcinogen(s). Moreover, arsenic may not be a direct carcinogen but rather, an epigenetic carcinogen (Mass and Wang, 1997; Zhou *et al.*, 1997).

The modes of arsenic toxicity and carcinogenicity are not completely understood. Nor is the mode of arsenic detoxification obvious. The putative route of arsenite

detoxification is oxidative methylation (figure 1.1). Methylation converts arsenite to compounds of supposed lower affinity for tissue constituents. At equimolar concentrations, dimethylarsinate, DMA (LD_{50} = 2600 mg/kg) is approximately 50 times less toxic than arsenite (LD_{50} = 41 mg/kg) in the rat (Aposhian, 1989). Biotransformation of inorganic arsenic in most animals is catalyzed by at least four serial enzymes, arsenate reductase (Radabaugh and Aposhian, 2000) arsenite methyltransferase (Buchet and Lauwerys, 1985; Zakharyan *et al.*, 1995; Styblo *et al.*, 1996), monomethylarsonate (MMA^V) reductase (Zakharyan and Aposhian, 1999; Zakharyan *et al.*, 2001) and monomethylarsenous acid (MMA^{III}) methyltransferase (Zakharyan *et al.*, 1999). Arsenate may also be chemically reduced using reducing equivalents from glutathione (GSH, Winski and Carter, 1995). MMA^V reductase activity has recently been assigned to glutathione s-transferase, GSTΩ1-1 (Zakharyan *et al.*, 2001; Board *et al.*, 2000) and arsenate reductase to purine nucleotide phosphorylase (Radabaugh, personal communication). DMA is the major arsenic metabolite excreted in the urine of many mammalian species insofar as it is not demethylated and only 4% is further methylated in man (Marafante *et al.*, 1987; Yamauchi and Yamamura, 1985). Pentavalent DMA may be reduced to DMA^{III} and taken up by erythrocytes (where it may form species-specific protein complexes) but is ultimately effluxed as DMA^V (Shiobara *et al.*, 2001).

figure 1.1

The arsenic biotransformation pathway. SAM, S-adenosyl-L-methionine; SAHC, S-adenosyl-L-homocysteine; arsenate, p*K*a = 2.2, 6.97, 11.53; arsenite, p*K*a = 9.1, 12.13, 13.4; MMA^V, p*K*a = 4.1, 8.7; DMA^V, p*K*a = 6.2. Michaelis constants were determined in rabbit liver (Wildfang *et al.*, 1998; Zakharyan and Aposhian, 1999; Zakharyan *et al.*, 1999).


arsenate
arsenous acid
GSH
arsenate reductase
K_M = 5.5 X 10⁻⁶ M
SAM
SAH
arsenite methyltransferase
methylarsonate
(MMA^V)
K_M = 2.16 X 10⁻³ M
MMA^V reductase
GSH
K_M = 9.2 X 10⁻⁶ M
MMA^{III} methyltransferase
SAH
SAM
dimethylarsinate
(DMA)
methylarsenous acid
(MMA^{III})

Exceptions to this generally accepted detoxification pathway are the lack of methylation of arsenic compounds in the marmoset monkey (Vahter *et al.*, 1982; Zakharyan *et al.*, 1996), chimpanzee (Vahter *et al.*, 1995b) and guinea pig (Healy *et al.*, 1997). The putative detoxification route has also become exceptionable with growing evidence that methylated metabolites are carcinogenic (review Huff, 2000). Dimethylarsinic acid causes DNA damage (Yamanaka *et al.*, 1989), chromosomal aberrations (Moore *et al.*, 1994) and tumor promotion in rats (Yamanaka *et al.*, 1996; Wanibuchi *et al.*, 1996; Yamamoto *et al.*, 1995). Furthermore, methylated trivalent arsenicals are more potent inhibitors of eukaryotic GSH reductase (Styblo *et al.*, 1997), thioredoxin reductase (Lin *et al.*, 1999) and PDH (Petrick *et al.*, 2001) than is inorganic trivalent arsenic. MMA^{III} is more toxic than arsenite in Chang human hepatocytes (Petrick *et al.*, 2000) and more genotoxic (Mass *et al.*, 2001). MMA^{III} may simply be taken up by the cell and (into the mitochondria) more rapidly than arsenite without indication of a different mechanism of action (Snow *et al.*, 2001) but there is growing speculation that methylation may be a bioactivation pathway.

Various mechanisms by which arsenic may induce cancer at the cellular/molecular level have been proposed. There is no unified hypothesis which can account for all of these observations nor the enigmatic behavior of arsenic (Goering *et al.*,

1999). The lack of mechanistic details about arsenic toxicity and carcinogenicity presents a major challenge in health risk assessment.

The objective of this thesis intends to characterize the role of methylation in arsenic toxicity with the following specific aims: 1.) Evaluate *in vitro* arsenite methyltransferase activity in tissues of mice (+ methylators) and guinea pigs (- methylators); 2.) Determine *in vivo* metabolites present in hamsters (an appropriate human model) 15h after a single *i.p.* injection if radiolabeled arsenate; 3.) Establish a tractable model for arsenic metabolism in yeast; 4.) Investigate an alternate mode of arsenite detoxification in the guinea pig (- methylator) and 5.) Identify the protein(s) responsible for arsenite methyltransferase activity and express the recombinant gene product in *E. coli* with the ambition of founding a tractable model for arsenic metabolism in mammals.

2.

ENDOGENOUS *IN VITRO* ARSENITE METHYLTRANSFERASE

INTRODUCTION

Understanding the role of methylation is pertinent to the study of arsenic detoxication as well as intoxication. Our laboratory has isolated and partially purified the rabbit liver enzyme responsible for the oxidative transfer of an activated methyl group from S-adenosylmethionine (SAM) to arsenite and to MMA^{III} (Zakharyan *et al.*, 1995; Zakharyan *et al.*, 1999). The arsenite and MMA ^{III} methyltransferase(s) have been characterized as being on a single 60 kDa protein with 2 activities: 1.) transferring a methyl moiety to arsenite to yield MMA^{V} and 2.) to MMA^{III} yielding DMA^{V} (figure 1.1). To elucidate arsenic metabolism in mammals and to understand the differences in biotransformation among species, exploratory enzyme assays of tissue on many mammals have been carried out by this laboratory (Zakharyan *et al.*, 1996; Aposhian *et al.*, 1997b).

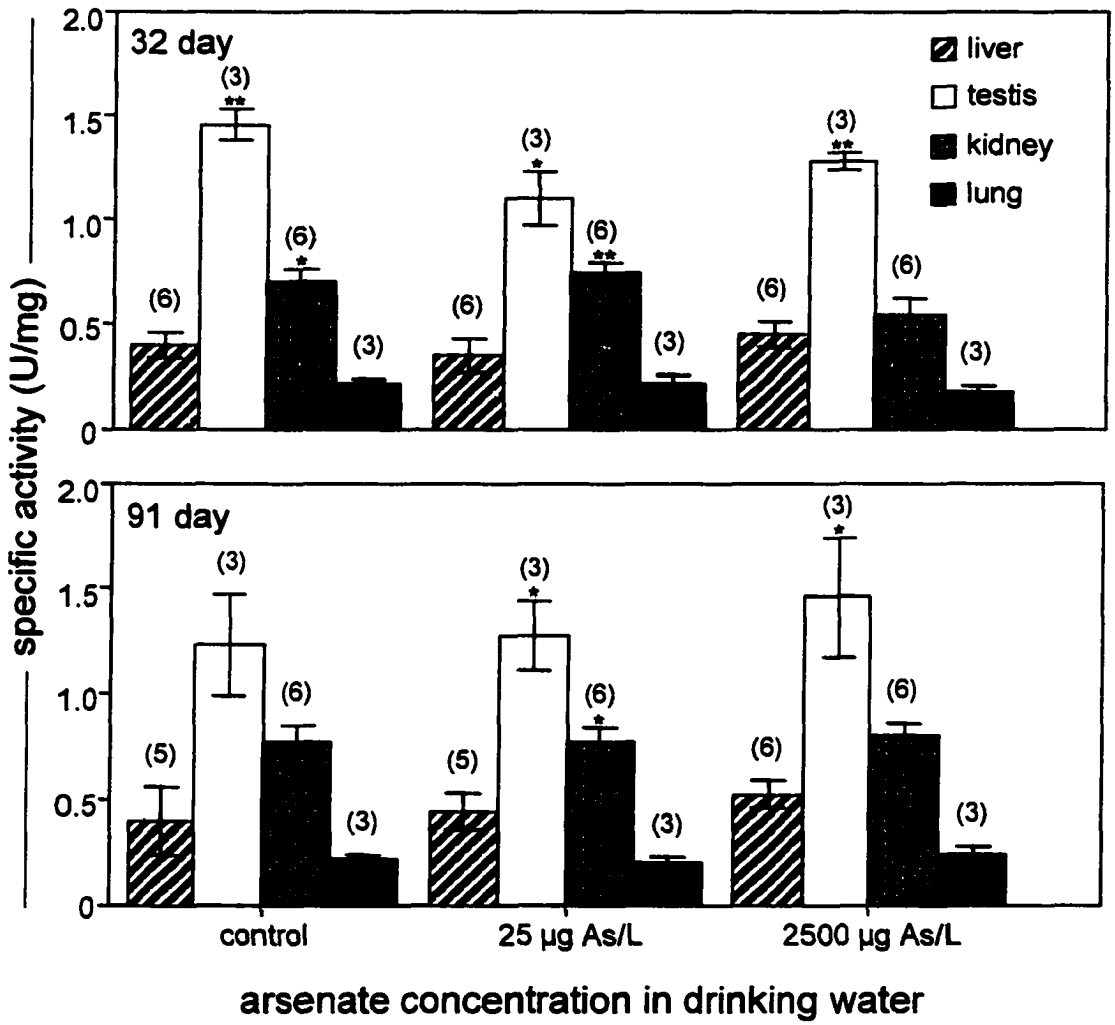
Two founding questions for this chapter are 1.) does the guinea pig methylate arsenic and 2.) can arsenite methyltransferase activity be induced by exposure to arsenic in the drinking water? Partially purified guinea pig cytosol was found to be extremely deficient in *in vitro* arsenite and MMA methyltransferase activity. Additionally, the

products of such *in vivo* enzyme activity were not detected in the urine of most guinea pigs when challenged with sodium arsenate (400 μg, 25 μCi/kg, *i.p.*; Healy *et al.*, 1997). Guinea pig arsenate reductase activity was evident *in vivo* by speciating urinary arsenic (Healy *et al.*, 1997) and *in vitro* by measuring enzyme activity in liver cytosol (Aposhian, 1997). Presently, guinea pig arsenite methyltransferase was sought in the principle organs of metabolism, the liver, kidney and lung and in the testis which was found to have arsenite methyltransferase activity far exceeding that of the liver in mouse (Healy *et al.*, 1998).

Previously, male 76 day old $B_6C_3F_1$ mice were given sodium arsenate in the drinking water at concentrations of 25 or 2500 μg As/L for 32 or 91 days (Healy *et al.*, 1998). By measuring water consumption, it was found that mice drinking water containing 25 μg As/L for 32 or 91 days received an average dose of approximately 6.5 - 8.5 μg As/kg·day, respectively. Those drinking water containing 2500 μg As/L for 32 or 91 days ingested approximately 750 - 827 μg As/kg·day, respectively. If enzyme induction occurred with subchronic exposure, increased arsenite methyltransferase activity would be expected with increased arsenate challenge. Contrariwise, no evidence for the induction of arsenite methyltransferase was found under these subchronic experimental conditions (figure 2.1). When exposure groups were compared, no

figure 2.1

Arsenite methyltransferase activity of mouse liver, testis, kidney and lung cytosol after subchronic exposure to low levels of arsenate. Indicated values were significantly different as compared to control using two-tailed paired Student's t-test (* $p < 0.05$, ** $p < 0.01$; Healy *et al.*, 1998)


32 day
91 day
liver
testis
kidney
lung
specific activity (U/mg)
2.0
1.5
1.0
0.5
0
control
25 µg As/L
2500 µg As/L
arsenate concentration in drinking water

significant differences among the three exposure groups as far as their liver, testis, kidney or lung arsenite methyltransferase activities were observed. Exposure (28 days) to arsenate in drinking water (2.5 ppm) did not increase the amount of methylated arsenic species in the urine of female $B_6C_3F_1$ mice (Hughes and Thompson, 1996). Arsenate is less dependent on methylation for its excretion than arsenite (Vahter, 1981) and hepatic metallothionein is induced by arsenite but not arsenate (Albores *et al.*, 1992). Both of these factors may contribute to arsenite methyltransferase not being substrate inducible under these experimental conditions of subchronic exposure to arsenate. Presently, arsenite methyltransferase induction was attempted with acute exposure to sodium arsenite in drinking water.

MATERIALS AND METHODS

Reagents. S-[methyl-3H]Adenosyl -L-methionine ([^{3}H]SAM, 56 - 71 Ci/mmol) was purchased from Dupont-NEN (Boston, MA) and [^{14}C]monomethylarsonic acid, disodium salt (MMA^V, 4.5 mCi/mmol) from American Radiolabeled Chemicals Inc. (St. Louis, MO). [^{14}C]Dimethylarsinic acid (DMA^V, 11.2 mCi/mmol) was a generous gift from Management Technology (Research Triangle Park, NC). ACS reagent grade sodium arsenite and sodium arsenate were purchased from MCB Reagents (Cincinnati, OH). HCl for trace metal analysis (As < 0.003) was obtained from J.T.Baker, Inc. (Phillipsburg,

NJ). All other chemicals were analytical reagent grade or of the highest quality obtainable. All water was doubly deionized, distilled and filtered (ddiH_2O).

Animals. Male 76-day-old $B_6C_3F_1$ mice were purchased from Taconic Farms Inc. (Germantown, NY) and male 500 - 600g Hartley guinea pigs from Harlan Sprague Dawley (Indianapolis, IN). Animals were maintained in an environmentally controlled animal facility operating at 22°C with a 12 h/12 h light/dark cycle. Mice were provided Teklad (Indianapolis, IN) Mouse/Rat Diet No. 7001 and water *ad libitum*. Guinea pigs were provided Teklad Guinea Pig Diet No. 7006 and water *ad libitum*. Mice were caged separately and exposed to 28.6 mg $NaAsO_2$/L drinking water for 0 (control), 12, 24 or 48 hours. Drinking water was refreshed at t = 24 hr. Greater that 95% of original arsenite concentration remains reduced under these conditions ≤ 24 hr (Buchet and Lauwerys, 1980). This concentration of arsenite was chosen because it was estimated from our previous study that mice with an average body weight of 28 g would cumulatively consume approximately 10 ml water or 75 μmol/kg body weight within 24 hours (Healy *et al.*, 1998). An increase in hepatic metallothionein has been observed 24 hours after a single subcutaneous 75 μmol/kg injection of sodium arsenite in rats (Albores *et al.*, 1992).

Preparation of cytosol. Animals were euthanized with carbon dioxide. The liver, kidneys, lung and testes were carefully excised, immersed in ice-cold 0.9% saline, blotted on filter paper, weighed and immediately stored at -70°C. Liver, kidney, lung or testis homogenates were prepared from each organ in 2 vol (v/w) of homogenization buffer using a glass homogenizer equipped with a motor-driven Teflon-coated stainless steel pestle. The homogenization buffer consisted of 10 mM Tris-HCl pH 7.6 @ 4°C, 250 mM sucrose, 0.5 mM reduced glutathione (GSH) and 0.2mM phenylmethylsulfonyl fluoride (PMSF). Guinea pig homogenate was spun at 14,600*g* for 15 min at 4°C in a Beckman (Fullerton, CA) Model J2-21M induction drive centrifuge and the supernatant further spun at 105,000*g* for 90 min in a Beckman model L2-65B ultracentrifuge at 4°C to obtain cytosol. Because of small volume, mouse homogenate was spun at 15,800*g* for 15 min at 4°C in an Eppendorf (Westbury, NY) Model 5415C microcentrifuge and the supernatant further spun at 105,000g for 90 min in a Beckman TL-100 Tabletop ultracentrifuge at 4°C to obtain cytosol. Cytosol is the supernatant fraction which contains soluble protein and in which microsomes (105,000*g*) and nuclei, mitochondria, lysosomes and cell membrane (9,000*g*) have been removed. All cytosol preparations were stored at -70°C. The protein content was determined by the method of Bradford (1976) using bovine serum albumin standards.

Enzyme assay. The arsenite methyltransferase activity of each cytosol was measured using essentially the method described previously (Zakharyan *et al.*, 1995). Cytosol was incubated with 30 µl 1M Tris-HCl (pH 8.0), 10 µl 0.10 M GSH, 5 µl 0.05 M $MgCl_2$, 2 µl 2.5 mM NaAsO2 and 1 µl 9.8 µM [^{3}H]SAM. These conditions are optimum for all mouse cytosols. The reaction was initiated by the addition of cytosol. The mixture (V_f = 250 µl) was incubated for 60 min in a 37°C water bath, terminated by the addition of 750 µl 12 M HCl and extracted immediately or stored at -15°C. One unit of arsenite methyltransferase (U) was defined as the amount necessary for the catalysis of the formation of 1 pmol [^{3}H]MMA in 60 min at 37°C. [^{3}H]SAM was either purchased at the desired specific activity or diluted with S-adenosyl-L-methionine in the form of *p*-toluenesulfonate salt (Sigma) to yield 56 Ci/mmol. The specific activity of all isotopic solutions used experimentally was 4.5×10^4 cpm/pmol.

Determination of methylated metabolite, [^{3}H]MMA. Formation of the methylated metabolite was measured using the standard extraction procedure described previously (Zakharyan *et al.*, 1995). Briefly, [^{3}H]MMA was separated from [^{3}H]SAM by adding chloroform, potassium dichromate, and potassium iodide (KI) to the acid-terminated reaction mixture. In the presence of HCl and KI, arsenicals exist as halides, which are soluble in $CHCl_3$ (Fitchett *et al.*, 1975; Chappell *et al.*, 1995) while [^{3}H]SAM remains in

the aqueous phase. The organic phase was washed twice with an equal volume of 9.3 M HCl, 1.2 μM KI and then the arsenicals were back extracted into a final aqueous phase absent strong acid and iodide. The aqueous back extract was transferred to a vial and mixed with 5 ml Monoflow 3 liquid scintillator (National Diagnostics). Radioactivity was measured in a Beckman Model LS 7800 liquid scintillation counter. This method for the extraction of methylated arsenic compounds is simple, rapid and reproducible compared to the alternative method of detection which uses hydride generation atomic absorption spectroscopy (AAS, Buchet and Lauwerys, 1985; Smith *at al.*, 1992; Shirachi *et al.*, 1992). With respect to the method of detection, liquid scintillation counting is approximately 100-fold more sensitive than graphite furnace AAS.

For this method of separation, [^{3}H]SAM is the preferred radiolabeled reactant in the reaction mix rather than radioactive arsenite because in the presence of HCl, As^{III} exists as covalent arsenic trichloride, which would also partition into the organic phase and be back-extracted into water (Chappell *et al.*, 1995). Also, prior efforts to develop an enzyme assay utilizing [^{73}As]arsenite revealed that arsenite formed peptide complexes which mimicked MMA or DMA on ion exchange, thin layer and liquid chromatograms (Bogdan *et al.*, 1994). Using [^{73}As]arsenite in an *in vitro* assay of rat cytosol, Styblo and Thomas (1997) reported that approximately 54% of the radiolabel recovered was protein

bound. Arsenic can also form a variety of complexes with GSH (Scott *et al.*, 1993; Delnomdedieu *et al.*, 1994) which influences the metalloid's chemistry.

Confirmation of methylated metabolite. Polyprep chromatography columns (10 ml, Bio Rad) were packed with 2 ml AG 50W-X4 cation exchange resin (100 - 200 mesh hydrogen form, Bio Rad) prepared in 0.5 M HCl. The resin was washed successively with 30 ml 0.5 M HCl, 50 ml H_2O, 30 ml 0.5 M HCl, and 50 ml 0.05 M HCl. The radiolabel in the aqueous back extract or standard solution was applied to the column. MMA was eluted with 6 ml 0.05 M HCl and DMA with 10 ml 0.5 M NaOH (Tam *et al.*, 1978). Fractions (1 ml) were collected, mixed with liquid scintillator and counted for radioactivity.

Statistics. One-way ANOVA and the Bonferroni multiple comparisons test were used when mean differences between the exposure groups were evaluated. The paired Student's *t* test was used when mean differences among the four organs were evaluated. For all comparisons, *p* values less than 0.05 were considered significant.

RESULTS

Using cytosol as the source of enzyme, neither guinea pig liver, kidney, lung nor testis had the capacity to methylate arsenic (figure 2.2b). Without the advantage of

figure 2.2a

Arsenite methyltransferase activity of mouse liver, testis, kidney and lung cytosols after acute exposure to high levels of arsenite in drinking water. Cytosols were prepared from animals exposed to 28.6 mg $NaAsO_2$/L drinking water for 0 (control), 12, 24 or 48 hour. Values are the mean ± SEM specific activities using 50 μg protein per assay. The number of animals used to determine the mean is in parenthesis. Indicated values were significantly different as compared to control using ANOVA (** $p < 0.01$).

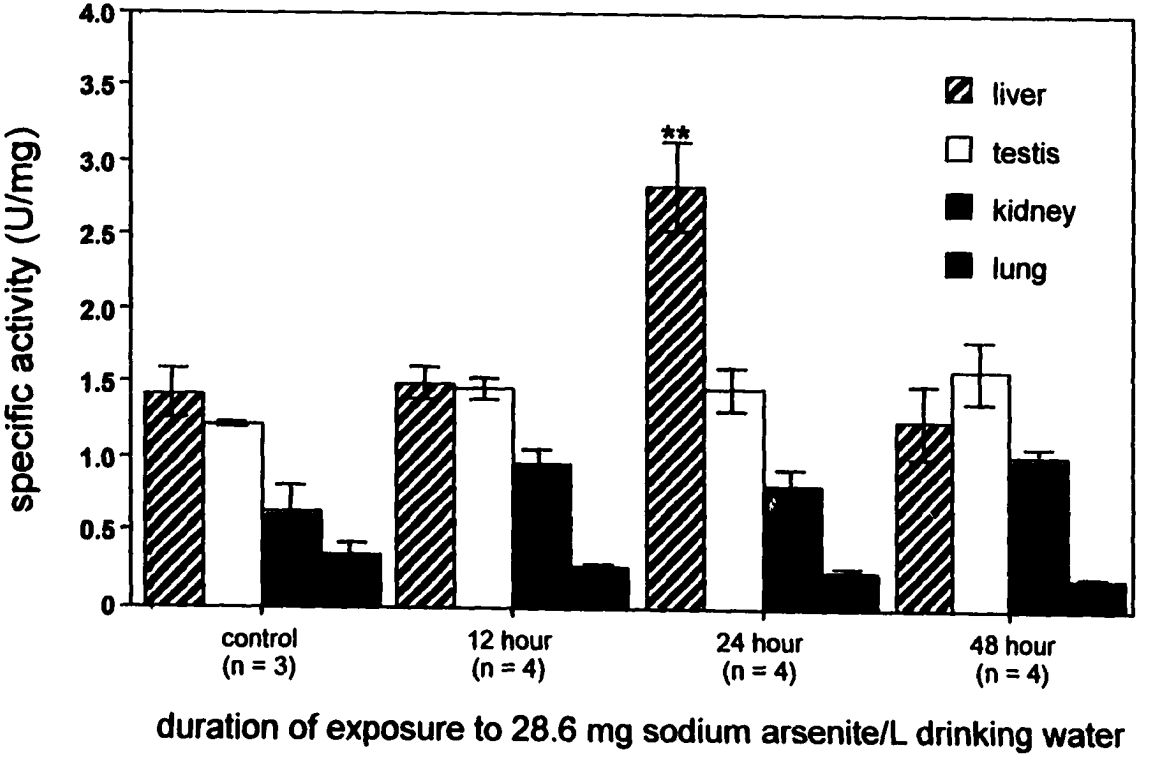
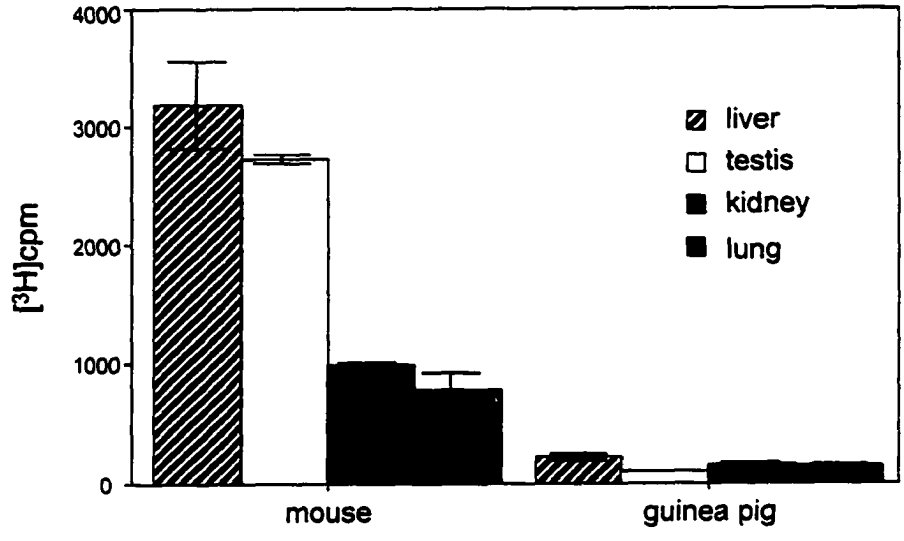

specific activity (U/mg)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
**
liver
testis
kidney
lung
control
(n = 3)
12 hour
(n = 4)
24 hour
(n = 4)
48 hour
(n = 4)
duration of exposure to 28.6 mg sodium arsenite/L drinking water

figure 2.2b

Arsenite methyltransferase activity of guinea pig liver, testis, kidney and lung cytosol compared to mouse. Values are the mean ± SEM [^{3}H]counts using 50 μg mouse or guinea pig cytosolic protein/assay, n = 3 animals.


4000
3000
2000
1000
0
[3H]cpm
liver
testis
kidney
lung
mouse
guinea pig

molecular probes, it is unknown at what level - DNA, RNA or protein - the enzyme is nullified.

A twofold increase ($p < 0.01$) in liver arsenite methyltransferase activity was observed in mice exposed to arsenite in the drinking water at concentrations of 28.6 mg/L after 24 hours compared to control (figure 2.2a). The mean arsenite methyltransferase activity measured in the liver after 24 hours of exposure was 2.84 ± 0.36 U/mg where the mean arsenite methyltransferase activity measured in control mice was 1.42 ± 0.17 U/mg.

The specific activity measured in the liver of control mice of the acute study was 3 times greater than the specific activity of control liver measured in the 91 day study (figure 2.1). The marked increase in mean hepatic arsenite methyltransferase activity of the 48 hour control animals as compared to the control animals of the 32 and 91 day study was unexpected and is, as yet, not understood. Testis, kidney and lung methyltransferase specific activity values were reproducible. The reports that a 12-fold interindividual variability was observed in the urinary excretion of MMA by male c57 BL/6J mice (Morel *et al.*, 1995) may be relevant to the markedly different arsenite methyltransferase activities observed in the control livers of the subchronic and acute exposure studies.

Another point of interest is that induced arsenite methyltransferase activity of the liver was reduced to control levels after 48 hours despite continued exposure (figure 2.2a). Perhaps after 48 hours, and approximately 0.6 mg of cumulatively consumed sodium arsenite, the substrate is inhibiting the enzymes which catalyze its methylation. An *in vitro* inhibition of arsenic methyltransferases with increased substrate concentration has been reported (Buchet and Lauwerys, 1985)

DISCUSSION

Most animals methylate inorganic arsenite and excrete mostly DMA and very little, if any, MMA via the kidney (Vahter, 1994). Methylation has generally been accepted as the mechanism of arsenic detoxification in mammals. But methylation for this purpose has been questioned; first in 1982 when neither MMA nor DMA was found in the urine of the marmoset monkeys challenged with arsenic (Vahter *et al.*, 1982) and more recently, when a lack of methylated arsenic species was observed in chimpanzee urine (Vahter *et al.*, 1995b). Additionally, our laboratory has shown that marmoset and tamarin monkey livers have very little, if any, arsenite and MMA methyltransferase activities (Zakharyan *et al.*, 1996; Aposhian *et al.*, 1997b).

The arsenic burden in various organs of the guinea pig is similar to that of the rabbit, with the greatest concentration (μg As^{III}/g tissue) detected in the liver (Hunter *et*

al., 1942; Lanz *et al.*, 1950). The guinea pig does not methylate arsenite: No catalytic activity has been observed in crude or purified fractions of liver cytosol, nor have methylated metabolites been observed in urine (Healy *et al.*, 1997). Yet, the guinea pig is not uniquely susceptible to acute inorganic arsenic toxicity compared to the rabbit: median lethal doses (LD_{50}) of oral and subcutaneous administrations of arsenic trioxide are 30 and 13 mg/kg, respectively, in the guinea pig and 15 and 8 mg/kg, respectively, in the rabbit (Venugopal and Luckey, 1978). The *in vitro* and *in vivo* deficiencies of the methylation of arsenite and MMA in the guinea pig suggest an alternate mechanism or metabolic pathway for the detoxification of inorganic arsenic. Protein binding (*i.e.* sequestration) of arsenic may be a mechanism of detoxification. While the predominant route of soluble arsenic excretion is urinary, 8% of a 0.02 mmol As_2O_3/kg dose was observed in guinea pig bile 24 hr after a single *s.c.* administration (Reichl *et al.*, 1994).

Arsenic tolerance may be a result of increased methylation, perhaps arsenite methyltransferase induction. In the 19th century, Styrian villagers habitually ingested 130 - 325 mg doses of arsenic trisulfide and trioxide as alteratives, ascribing improved appearance, increased strength and endurance and general prophylaxis to arsenic consumption (Roscoe, 1862; Maclagan, 1864). From this consumption of lethal quantities of arsenic without untoward effect, emerged the theory of arsenic tolerance.

Furthermore, when mice were chronically exposed to arsenic trioxide in drinking water (50 ppm), an initial phase of accumulation followed by a rapid decrease in liver arsenic concentration was observed despite continued exposure (Bencko and Symon, 1969). Increased tissue clearance was subsequently attributed to increased excretion (Bencko *et al.*, 1973). From the knowledge of the fate of arsenite in mice (Vahter, 1981), these latter observations suggest that arsenic tolerance is the result of increased methylation, perhaps arsenite methyltransferase induction. But subchronic exposure to arsenate in drinking water (2.5 ppm) did not increase the amount of methylated species in the urine of mice (Hughes and Thompson, 1996) nor the amount of arsenite methyltransferase activity in the principle organs of metabolism (figure 2.1). The present results show a modest 2-fold increase ($p < 0.01$) in $B_6C_3F_1$ mouse liver arsenite methyltransferase activity after 24 hr exposure to arsenite in drinking water (28.6 ppm).

One of the putative sites of oxidative methylation is the liver. The first indication that inorganic arsenic was metabolized in the liver was that less methylated metabolites, MMA and DMA, were observed in the urine of mice and dogs after subcutaneous and intravenous administration of inorganic arsenic than after oral administration of the same doses. (Vahter, 1981; Charbonneau *et al.*, 1978; Charbonneau *et al.*, 1979). The liver as the main site of methylation was also supported by the observation in rabbits that

[^{74}As]DMA was present only in the liver 1 hour after intravenous administration of [^{74}As]arsenate (Marafante *et al.*, 1985). At four hours, relatively high concentrations of DMA were also observed in the kidney but attributed to rapid excretion of plasma DMA. The subsequent high concentration of [^{74}As]DMA in the lungs was ascribed to a rapid uptake of the methylated metabolite rather than *in situ* synthesis (Marafante *et al.*, 1985). *In vitro* enzyme studies by some investigators have further localized arsenite metabolism to the liver. Compared to the liver, the methylation capacity of rat erythrocyte, brain, lung, intestine and kidney crude homogenates were stated to be insignificant (Buchet and Lauwerys, 1985). Hirata *et al.* (1989) also observed MMA formation by liver homogenates to be greater than that of kidney homogenates in both the rat and mouse. And, when rat liver, kidney or lung slices were incubated in the presence of arsenite, the formation of MMA by the liver was approximately twice that of the lung and 4 times that of the kidney (Georis *et al.*, 1990).

A variety of animal tissues accumulate arsenic (Hunter *et al.*, 1942; Lowry *et al.*, 1942; Lanz *et al.*, 1950; Lindgren *et al.*, 1982) and are, therefore, potential target organs. Although hepatic metabolism may play the central role in arsenic biotransformation, extrahepatic methylation may have important toxicological implications for the particular tissue. Arsenite inhibits DNA ligase activity in Chinese hamster V79 lung fibroblasts (Li

and Rossman, 1989; Hu *et al.*, 1998) and cytotoxic mediator production in rabbit pulmonary alveolar macrophages (Lantz *et al.*, 1994). When inhalation is the route of exposure, inorganic arsenic is a pulmonary as well as an inhalation toxicant since the lung receives all of the cardiac output thus ensuring a rapid distribution of soluble arsenic. The arsenite methyltransferase of the lung may play a very important role in the biotransformation of inorganic arsenic prior to systemic absorption.

When mice received a single oral dose of arsenite, 8% of the dose was excreted unmethylated in the urine after 48 hours (Vahter, 1981). The kidney is not only burdened with unmetabolized arsenite which is rapidly cleared from the blood but also with arsenate which is not readily taken up by the hepatocyte (Lerman *et al.*, 1983). The arsenic concentration was greater in the kidneys of mice than in the liver from 0.5 to 72 hours following a single intravenous dose of arsenate (Lindgren *et al.*, 1982). Additionally, when liver or kidney slices were incubated with arsenate, approximately 3 times more arsenic was taken up by the kidney slices and five times more DMA was found in the kidney slice medium than in the liver slice medium (Lerman and Clarkson, 1983). The kidney may have a greater capacity to reduce arsenate to arsenite and therefore may be the primary site of arsenate metabolism but it also has the substantial capacity to methylate arsenite. (figures 2.1 and 2.2a).

When the disposition of [^{74}As]-arsenite was studied *in vivo* in the marmoset monkey, radioactivity was observed in the testes, particularly localized in the seminiferous tubules (Vahter *et al.*, 1982). The seminiferous tubules contain differentiating germ cells and Sertoli cells whose junctions form the blood-testis barrier. Arsenous acid (lower pK_a = 9.1) will diffuse across the permeability barrier while arsenate (lower pK_a = 2.2) may compete with phosphate for passive transport sites as it does in the blood-brain barrier (Dalliare and Beliveau, 1992). Moreover, while 90% of the arsenic was protein bound in marmoset liver cytosol, only 50% was protein bound in testis cytosol (Vahter *et al.*, 1982). Sequestration via a specific arsenite binding protein may be an alternative and/or complimentary mechanism of arsenic detoxification (Bogdan *et al.*, 1994). It is apparent that the testis is vulnerable to inorganic arsenic and also that the testis is a target organ: Testicular necrosis accompanied by complete destruction of spermatozoa was observed in rats after a single 4.6 mg/kg intratesticular dose of arsenic pentoxide (Kamboj and Kar, 1964) and less than 10% survival was observed when rat Sertoli cells were grown in the presence of 10 μM arsenic trioxide (Espevik *et al.*, 1982). A significant decrease in testicular weight was observed in mice drinking water containing 25 and 2500 μg As/L for 91 days (Healy *et al.*, 1998). Inorganic arsenic may influence the integrity of germ cell DNA since arsenate inhibits

the incorporation of thymidine, uridine, adenosine and their corresponding nucleotides into the nucleic acid of human lymphocytes (Baron *et al.*, 1975). Additionally, repeated arsenate exposure was associated with proliferative, preneoplastic lesions in testes of mice (Waalkes *et al.*, 2000). Testis arsenite methyltransferase specific activity is ≥ to that of the liver (figures 2.1, 2.2a). The liver receives and extracts or chemically modifies all foreign compounds absorbed by the gastrointestinal tract before they are stored, secreted into bile or released into general circulation. Extrahepatic tissues are limited with respect to the diversity of chemicals they can handle, and thus their contribution to overall biotransformation of xenobiotics is limited. However, biotransformation of a chemical within an extrahepatic tissue may have an important toxicological implication for that particular tissue. The relative arsenic methyltransferase activity in germinal tissue may weigh in favor of arsenic methylation as route of detoxification.

3.

ENDOGENOUS *IN VIVO* ARSENIC METABOLITES

INTRODUCTION

The mechanisms of inorganic arsenic toxicity and carcinogenicity are not understood. Nor is the specific mechanism of arsenic detoxification obvious. It is known, however, that trivalent arsenic is a reactive electrophile with an affinity for proximate sulfhydryls and that oxidized, methylated arsenic species are not reactive electrophiles. The putative route of arsenite detoxification has been oxidative methylation. The biotransformation of inorganic arsenic is catalyzed (figure 1.1) by arsenate reductase (Radabaugh and Aposhian, 2000), arsenite methyltransferase (Zakharyan *et al.*, 1995; Buchet and Lauwerys, 1985; Styblo *et al.*, 1996), monomethylarsonate (MMA^{V}) reductase (Zakharyan and Aposhian, 1999; Zakharyan *et al.*, 2001) and monomethylarsenous acid (MMA^{III}) methyltransferase (Zakharyan *et al.*, 1999). S-adenosyl-L-methionine is a methyl donor for purified arsenite and MMA^{III} methyltransferases (Zakharyan *et al.*, 1999). Humans excrete a significant amount of MMA (10-20% of total urinary arsenic) upon exposure to inorganic arsenic (Crecelius, 1977). DMA is the major arsenic metabolite excreted in the urine of mammals that employ this pathway and is considered to be the endpoint of arsenic metabolism insofar

as it is rarely further methylated (Marafante *et al.*, 1987; Yamauchi and Yamamura, 1985; Yoshida *et al.*, 1998). DMA does not appear to be demethylated by mammals (Chen *et al.*, 1996; Yoshida *et al.*, 1998). However, demethylation of DMA may be associated with intestinal bacteria (Chen *et al.*, 1996).

This methylation process has become questionable as a detoxification route with increasing evidence that methylated metabolites are carcinogenic and acutely toxic. DMA^V causes DNA damage (Yamanaka *et al.*, 1989), chromosomal aberrations (Moore *et al.*, 1994) and tumor promotion in mice and rats (Yamanaka *et al.*, 1996; Wanibuchi *et al.*, 1996; Yamamoto *et al.*, 1995). Increased urinary bladder tumors occur in rats with long-term exposure to 50 ppm DMA^V in drinking water (Wei *et al.*, 1999). MMA is also a carcinogen, causing thyroid tumors in rats at 200 ppm in a chronic bioassay (EPA, 1981). Furthermore, MMA^{III} has been implicated as an acutely toxic biotransformant: based on *in vitro* cell viability, $MMA^{III}I_2$ is approximately 26 times more toxic than arsenite in Chang human hepatocytes (Petrick *et al.*, 2000); the minimum inhibitory concentration of methylarsine oxide is one tenth that of arsenite in *Candida humolica* (Cullen *et al.*, 1989) and methylated trivalent arsenicals are more inhibitory toward *S. cerevisiae* GSH reductase (Styblo *et al.*, 1997), glutathione reductase, glutathione s-transferase (Chouchane and Snow, 2001), pyruvate dehydrogenase (Petrick *et al.*, 2001)

and thioredoxin reductase (Lin *et al.* 1999) than is the inorganic trivalent arsenic species, arsenite). Methylation may be a pathway of bioactivation. To wit, the intermediate chemical species may be more reactive than either the parent compound or its excreted metabolites.

MMA^{V} is a relatively less toxic metabolite of inorganic arsenic methylation (Petrick *et al.*, 2000; Moore *et al.*, 1997) but it must be reduced to monomethylarsonous acid (MMA^{III}) *in vitro* before it can be metabolized to DMA^{V} (Zakharyan *et al.*, 1999; Cullen *et al.*, 1984; Styblo *et al.*, 1995). MMA^{III} has recently been detected for the first time in human urine (Aposhian *et al.*, 2000a; Aposhian *et al.*, 2000b; Le *et al.*, 2000; Mandal *et al.*, 2001) demonstrating that it may no longer be dismissed as a transient, rapidly biotransformed intermediate in the study of arsenic toxicity and/or carcinogenicity. The primary objective of this work was to determine whether MMA^{III} is present and persistent in hamster liver after a single *i.p.* injection of arsenate. MMA^{III} represented approximately 10% of the [^{73}As] dose retained in the liver 15 hr after arsenate injection.

MATERIALS AND METHODS

Reagents. Carrier-free [^{73}As]arsenate (2.46 - 5.61 μCi/ml) was purchased from Los Alamos National Laboratory (Los Alamos, NM). [^{14}C]MMA^{V} (4.5 mCi/mmol), was

purchased from American Radiolabeled Chemicals (St. Louis, MO). [^{14}C]DMA^{V} (11.2 mCi/mmol) was a generous gift from Management Technology (Research Triangle Park, NC). [^{73}As]Arsenate, [^{14}C]MMA^{V} or [^{14}C]DMA^{V} was reduced to its trivalent species in the presence of 0.2 N H_2SO_4, 85 mM sodium metabisulfite, 1% sodium thiosulfate (Reay and Asher, 1977). The reduction was monitored by HPLC and all solutions were found to be > 98% reduced at the time of experimentation. Sodium arsenate (ACS grade) was purchased from MCB Reagents (Cincinnati, OH). All other chemicals were analytical reagent grade or of the highest quality obtainable. Water was doubly deionized, distilled and filtered.

Animals. Male Golden Syrian Hamsters (130 - 180 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN) and acclimated prior to experimentation for at least 2 weeks in an environmentally controlled animal facility operating on a 12/12 hr dark/light cycle and at 20 - 22°C. Animals were provided with Teklad (Indianapolis, IN) 4% Mouse/Rat Diet #7001 and water, *ad libitum*, throughout acclimation and experimentation.

Preparation of homogenate for analysis of arsenic species. Hamsters (n = 3) were each administered a single intraperitoneal dose of [^{73}As]arsenate (145 μCi/animal, 2mg As/kg body weight). After 15 hr, animals were euthanized by CO_2 inhalation. The livers were

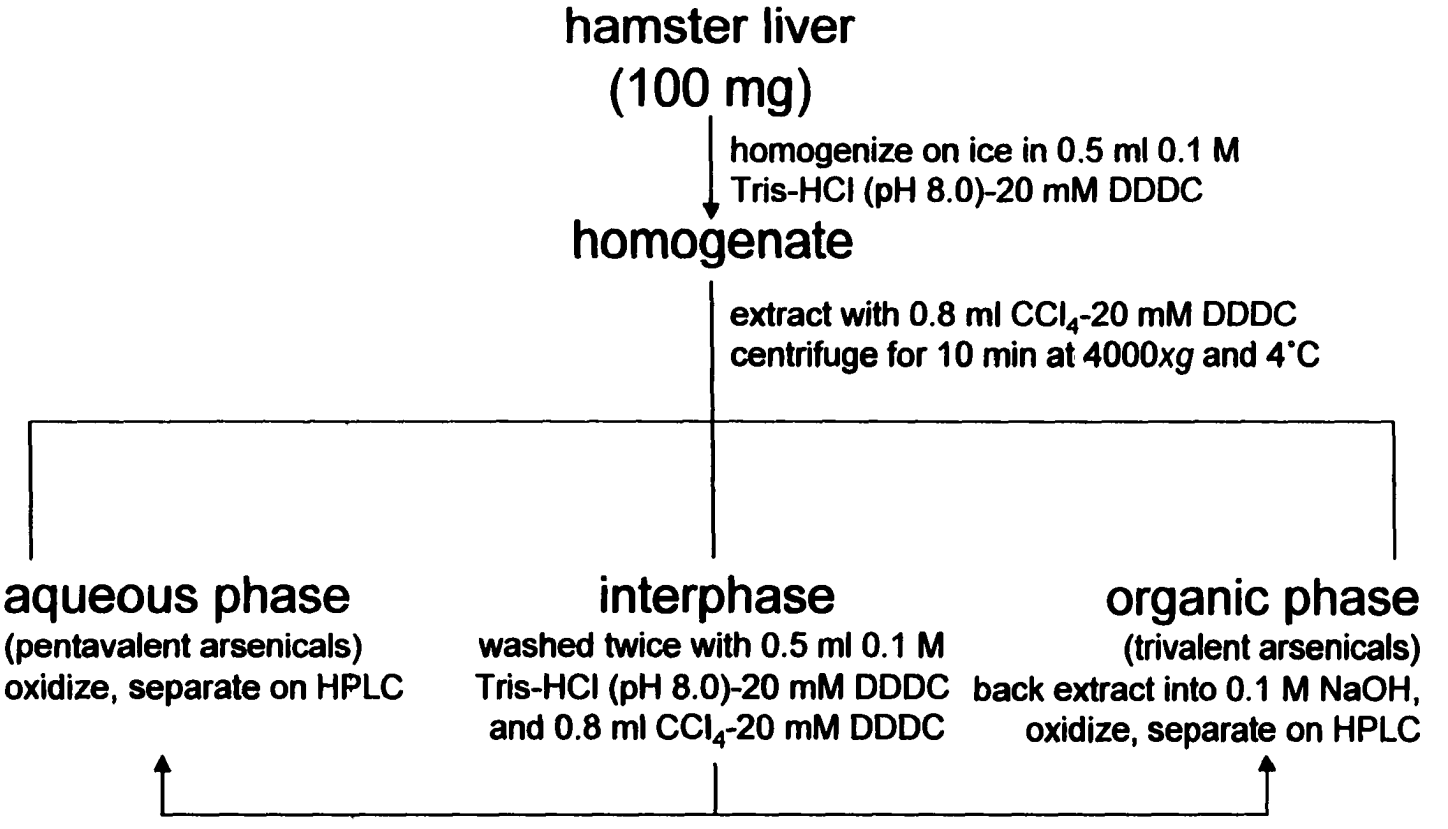
excised, immediately rinsed in ice-cold 0.9% NaCl, blotted on filter paper and weighed. They were homogenized immediately or stored at -70 °C. Liver homogenates were prepared in 4 volumes (v/w) of 0.020 M Tris-HCl pH 8.0-0.010 M diethyldithiocarbamic acid (DDDC) at 0°C using a glass homogenizer and pestle and analyzed immediately.

Extraction of arsenic species. Trivalent arsenic species of tissue homogenates in 5 ml metal-free polypropylene tubes were extracted into 2 volumes (v/v) of CCl_4-0.020 M DDDC by continuous vortex mixing for 30 min at room temperature. After centrifugation, organic, aqueous and interphases were obtained (figure 3.1). The organic phase was transferred to another tube and back extracted into 160 µl 0.1 M NaOH by vortex mixing for 15 min (Hasegawa *et al.*, 1994). To the interphase, 500 µl 0.1 M Tris-HCl-20 mM DDDC (N_2 saturated) was added and then extracted with 800 µl CCl_4-20 mM DDDC. Trivalent arsenicals were oxidized by the addition of H_2O_2 to a final concentration of 3%, mixing and incubation for 60 min at room temperature. The pooled aqueous phase containing pentavalent arsenicals was incubated in the presence of 10% H_2O_2 for 2 hr at room temperature (figure 3.1).

HPLC separation of arsenic species. Samples of extracts spiked with [^{73}As]arsenite, [^{73}As]arsenate, [^{14}C]MMA^{III}, [^{14}C]MMA^{V}, [^{14}C]DMA^{III} or [^{14}C]DMA^{V} or from [^{73}As] arsenate exposed animals were boiled for 5 min (in an open Eppendorf tube), centrifuged

figure 3.1

Extraction method for trivalent and pentavalent arsenicals from hamster liver.


hamster liver
(100 mg)
homogenize on ice in 0.5 ml 0.1 M
Tris-HCl (pH 8.0)-20 mM DDDC
homogenate
extract with 0.8 ml CCl_4-20 mM DDDC
centrifuge for 10 min at 4000*xg* and 4°C
aqueous phase
(pentavalent arsenicals)
oxidize, separate on HPLC
interphase
washed twice with 0.5 ml 0.1 M
Tris-HCl (pH 8.0)-20 mM DDDC
and 0.8 ml CCl_4-20 mM DDDC
organic phase
(trivalent arsenicals)
back extract into 0.1 M NaOH,
oxidize, separate on HPLC

for 5 min at 15,000*g* and 4°C and then 100 µl was injected onto a PRP-X100 inorganic anion exchange HPLC column, 250 x 4.1 mm (Hamilton, Reno, NV). Liquid chromatography was performed using 30 mM Na-phosphate pH 5: MeOH::8:2 (v/v) as the mobile phase at a flow rate of 0.8 mL/min (Gailer *et al.*, 1999). A post-column, in-line ß-Ram Model 2 Flow-Through β–/soft γ– detector (IN/US Systems, Tampa, FL) was used to detect radioactivity of the effluent mixed with Monoflow III scintillator (National Diagnostics, Atlanta, GA) in a 1:3 ratio.

RESULTS

The following standardizations validate this method (Hasegawa *et al.*, 1994) for the extraction and complete separation of trivalent and pentavalent metabolites of inorganic arsenate in hamster liver: Naïve hamster liver homogenate (500 µl) was incubated with 3 µL (7.6 nCi) [^{73}As]arsenite, 0.5 µL (7.7 nCi) [^{14}C]MMA^{III} or 2 µL (10.7 nCi) [^{14}C]DMA^{III} for 10 min at 0°C. Trivalent arsenic from each of the spiked homogenates was extracted into CCl_4-20 mM DDDC and back extracted into 160 µL 0.1 M NaOH. The back extract was immediately injected onto the HPLC column without prior oxidation. The following recoveries were observed: DMA^{III}, 97%; MMA^{III}, 102%; arsenite, 76%. Untreated hamster liver homogenate was also incubated with 2 µL (7.1 nCi) [^{73}As]arsenate, 2 µL (50.5 nCi) [^{14}C]MMA^{V} or 1 µL (7.1 nCi) [^{14}C]DMA^{V} and each

organic phase back extracted and analyzed by HPLC (figure 3.2a). Only 0.9% [^{73}As]-arsenate, 1.8% [^{14}C]-MMA^{V} or 0.4% [^{14}C]-DMA^{V} radioactivities were detected in the organic phase. A large fraction of arsenate, 90%, partitioned into the aqueous phase and approximately 7% remained within the interphase after 3 washes. MMA^{V} (97%) and DMA^{V} (105%) partitioned into the aqueous phases. This extraction procedure allows the separation of all trivalent from pentavalent arsenic species into CCl_4-DDDC over a pH range of 2 - 10. However, both Hasegawa *et al* (1994) and we observed that, once separated from the pentavalent species, limited oxidation of trivalent arsenic may occur in a solution of 0.1 M NaOH. For this reason, trivalent arsenicals of the back extract of the organic phase were henceforth oxidized to As^{V}, MMA^{V} and DMA^{V} prior to HPLC analysis in order to obtain a more accurate determination of As^{III}, MMA^{III} and DMA^{III}, respectively.

Trivalent arsenicals were extracted into the organic phase (figure 3.1) from liver homogenates of hamsters 15 hours after an injection of [^{73}As]arsenate. After back extraction into 0.1 M NaOH, the trivalent arsenicals were oxidized and analyzed by HPLC (figures 3.1 and 3.2b). The pooled aqueous phase (0.1 M Tris-HCl-20 mM DDDC) containing pentavalent arsenicals was also analyzed by HPLC (figures 3.1 and 3.2c). The presence of trivalent methylated arsenicals in hamster liver was confirmed

figure 3.2a

HPL Chromatogram of pentavalent arsenic species. A 250 × 4.1 mm PRP-X100 inorganic anion exchange HPLC column was used with 80% 30 mM Na-phosphate (pH 5)-20% MeOH mobile phase at a flow rate of 0.8 mL/min. A mixture of pentavalent standards gave the following retention times: [^{14}C]-DMA^{V} (4.52 nCi), 3:48 min; [^{14}C]-MMA^{V} (1.55 nCi), 6:48 min; [^{73}As]-arsenate (0.2 nCi), 10:04 min. Retention times correspond to individual retention times for arsenate, MMA^{V} or DMA^{V}.

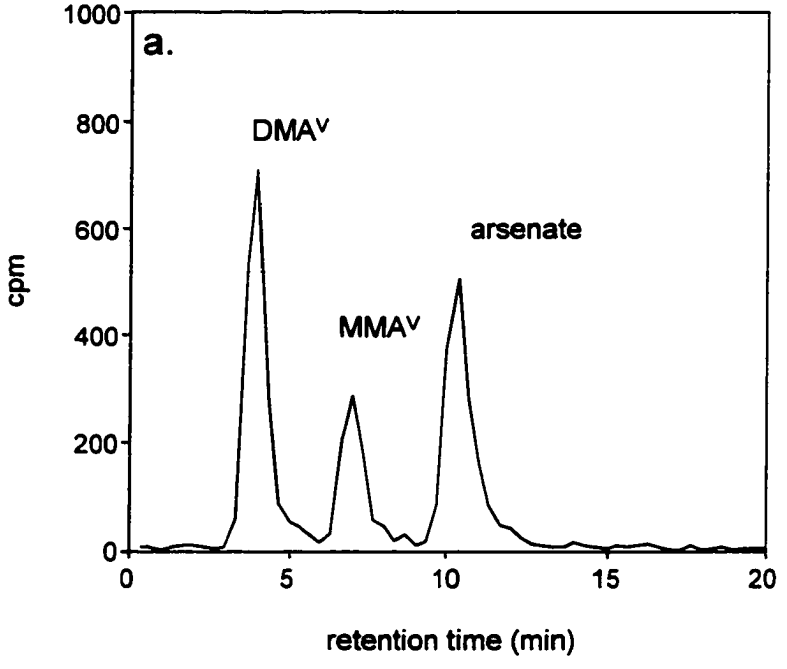
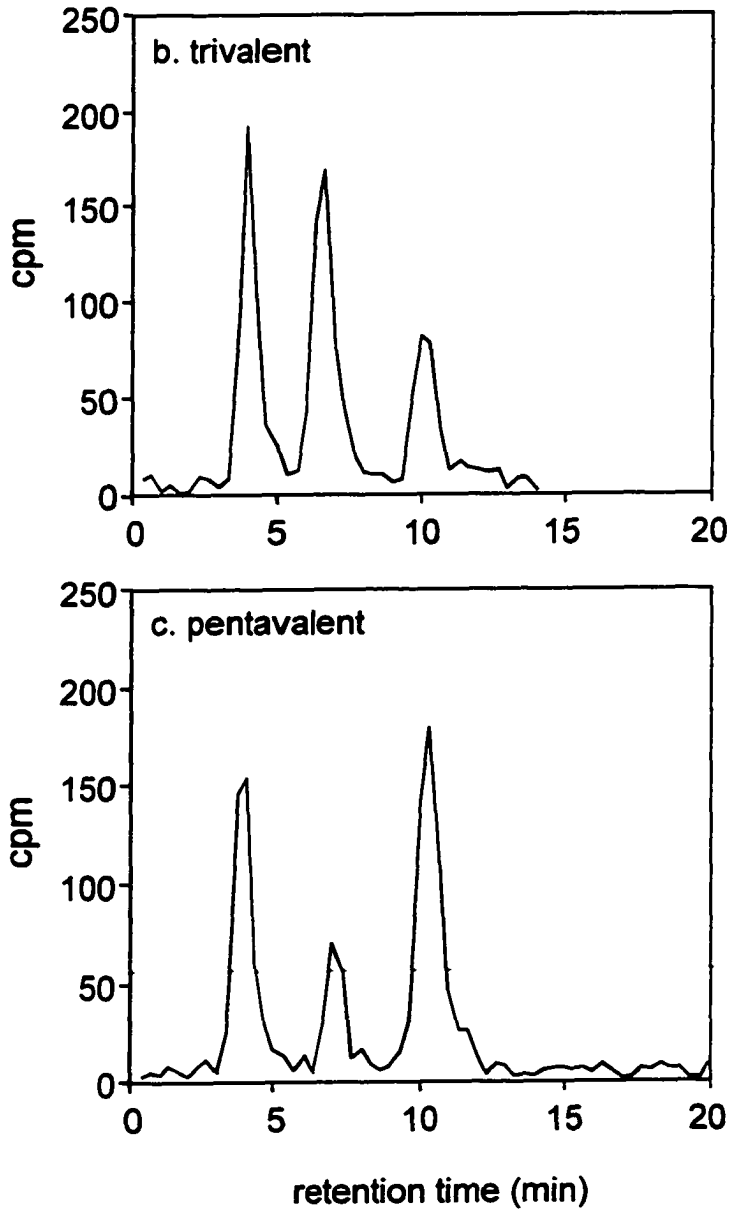

a.
DMA^V
MMA^V
arsenate
cpm
1000
800
600
400
200
0
0
5
10
15
20
retention time (min)

figure 3.2b

Extraction of trivalent arsenic species from hamster liver. Animals were administered 145 μCi [^{73}As]-arsenate, 2 mg As/kg. After 15 hr exposure, arsenicals were extracted from liver as described in MATERIALS AND METHODS. Representative chromatogram of organic phase containing trivalent arsenic species after 1st wash of liver homogenate and oxidation. Peaks correspond to DMA^V (1299 integral cpm), MMA^V (1444 integral cpm) and arsenate (716 integral cpm), oxidation products of the trivalent arsenic species extracted into CCl_4-DDDC.

figure 3.2c

Extraction of pentavalent arsenic species from hamster liver. Animals were administered 145 μCi [^{73}As]-arsenate, 2 mg As/kg. After 15 hr exposure, arsenicals were extracted from liver as described in MATERIALS AND METHODS. Representative chromatogram of pooled aqueous washes containing pentavalent arsenic species of liver homogenate. Peaks correspond to DMA^V (1250 integral cpm), MMA^V (463 integral cpm) and arsenate (1643 integral cpm).


b. trivalent
c. pentavalent
cpm
250
200
150
100
50
0
0
5
10
15
20
retention time (min)

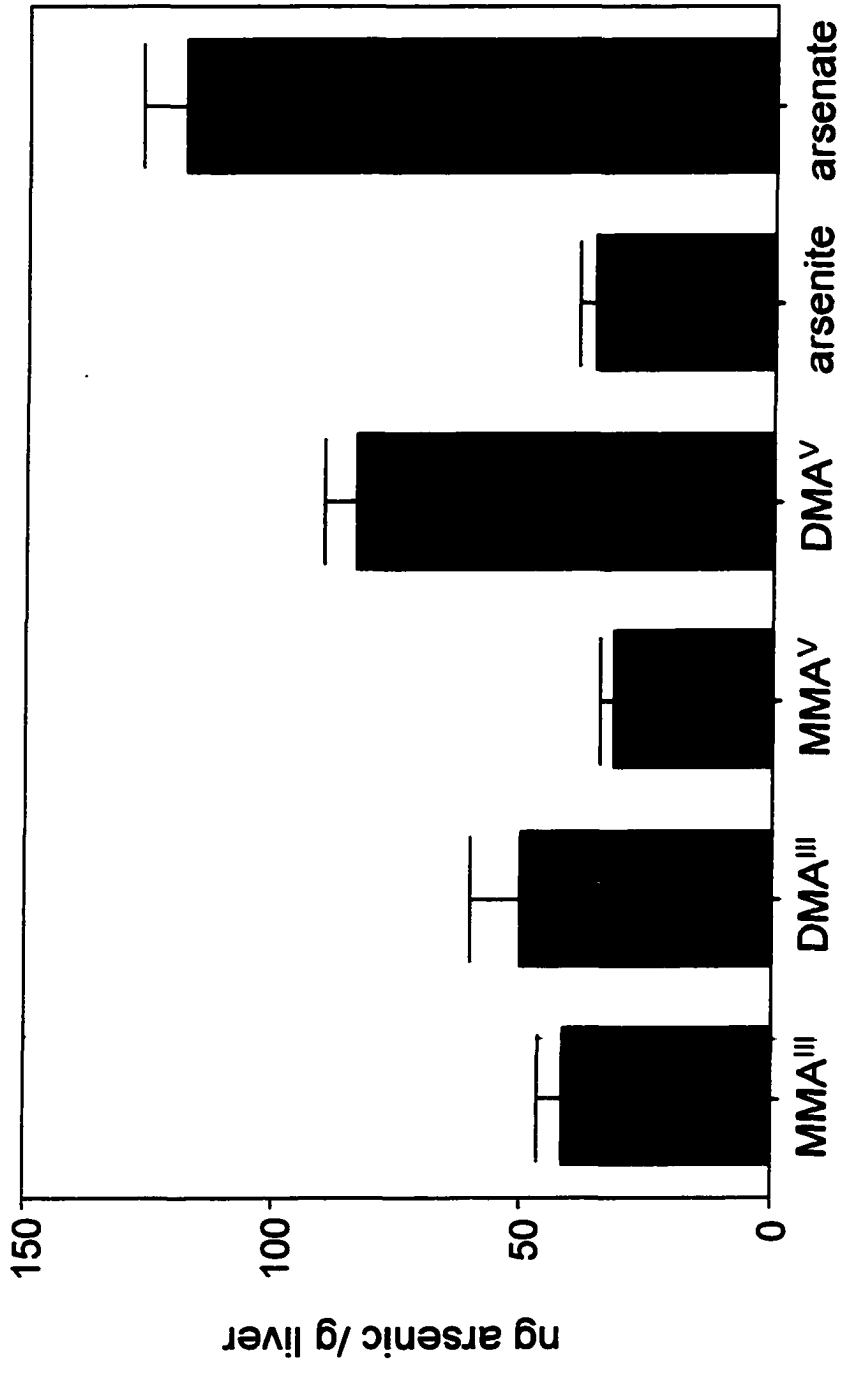
using anion exchange chromatography (Tam *et al.*, 1978). By transforming integral counts into ng As/g liver, it was determined that 0.75% of the [^{73}As] dose remained in the liver after 15 hours. Of this hepatic retention, approximately 11% of [^{73}As] was present as MMA^{III}, 14% as DMA^{III}, 9% as MMA^{V}, 23% as DMA^{V}, 10% as arsenite and 33% as arsenate (figure 3.3). Arsenate and DMA^{V} were present in the greatest amount per gram tissue. This is the first demonstration of the heretofore elusive trivalent MMA^{III} and DMA^{III} in animal tissue.

Discussion

Based on MMA excretion, the hamster is an appropriate model for human arsenic metabolism; approximately 17% of total arsenic is excreted as MMA 24-48 hr following arsenite ingestion (Hirata *et al.*, 1990). Arsenite is methylated in hamster liver by arsenite methyltransferase to MMA^{V} (Wildfang *et al.*, 1998), which is a relatively innocuous metabolite of arsenic methylation (Moore *et al.*, 1997; Petrick *et al.*, 2000). But MMA^{V} must be reduced to MMA^{III} before a second methylation can take place to yield the even less acutely toxic compound, DMA^{V}. Once sufficient levels of MMA^{V} are produced by arsenite methyltransferase, MMA^{V} reductase can produce highly toxic MMA^{III}, which can then be methylated by MMA^{III} methyltransferase to DMA^{V} (Zakharyan *et al.*, 1999). MMA^{V} reductase is the rate limiting reaction in the

figure 3.3

Concentrations of arsenic species in hamster liver 15 hr after a single *i.p.* dose of 145 μCi [^{73}As]arsenate, 2 mg As/kg body weight. Values represent the mean ± SEM of 3 hamsters. The following differences were significant using ANOVA with the Tukey-Kramer multiple comparisons post-test: $p < 0.001$ for DMA^{V} vs. MMA^{III}, arsenate vs. arsenite, arsenate vs. MMA^{V}, arsenate vs. DMA^{III}; $p < 0.01$ for DMA^{V} vs. arsenite, DMA^{V} vs. MMA^{V}; $p < 0.05$ for DMA^{V} vs. DMA^{III}, arsenate vs. DMA^{V}. All other differences were considered insignificant ($p > 0.05$).


ng arsenic /g liver
150
100
50
0
MMA^{III}
DMA^{III}
MMA^{V}
DMA^{V}
arsenite
arsenate

biotransformation of inorganic arsenic in the rabbit (Zakharyan and Aposhian, 1999) and MMA^{III} methyltransferase (Zakharyan *et al.*, 1999) appears to prevent toxic levels of MMA^{III} from accumulating in the cell. Many tissues have the enzymatic capacity to produce the highly toxic intermediate, MMA^{III}, and each may therefore be a potential target organ for arsenic toxicity and/or carcinogenicity (Sampayo-Reyes *et al.*, 2000).

In 1970, Horiguchi claimed that DMA^{III} and MMA^{III} were more toxic than arsenite in mammals (Horiguchi, 1970). Cullen *et al* (1989) later reported minimum inhibitory concentrations of 0.8 mmol $(MeAsO)_x/dm^3$ and 8 mmol arsenite/dm^3 in fungus. Since then, trivalent methylarsenicals have received little attention. Styblo *et al* (1997), using yeast GSH reductase *in vitro*, found inhibitory constants in the millimolar range for $NaAsO_2$, CH_3AsI_2 and $(CH_3)_2AsI$ to be 5.7 mM, 0.074 mM and 0.056 mM, respectively. MMA^{III} was the most potent competitive inhibitor of thioredoxin reductase with a K_i of 100 nM (Lin *et al.*, 1999). Petrick *et al* (2000) demonstrated the following relative toxicities of MMA^{III} (LC_{50} = 6 μM) > arsenite (LC_{50} = 68 μM) > arsenate (LC_{50} = 1628 μM) > MMA^{V} (LC_{50} = 8235 μM) ≈ DMA^{V} (LC_{50} = 9125 μM) in Chang human liver cells. Thus, it has become evident that MMA^{III} and possibly DMA^{III} are the most toxic of intermediates in the methylation pathway of inorganic arsenic. Since DMA^{V} is less toxic and MMA^{III} is more toxic than inorganic arsenite, the possible benefit of arsenic

methylation as a detoxification pathway must be evaluated with respect to the accumulation of toxic intermediates.

The use of HPLC separation prior to hydride generation atomic fluorescence spectrometry (Le and Ma, 1998; Le *et al.*, 2000) has allowed the recent detection of MMA^{III} in human urine (Aposhian *et al.*, 2000a; Aposhian *et al.*, 2000b). MMA^{III} is chelated *in vivo* by DMPS (Aposhian *et al.*, 2000a) and also excreted without DMPS challenge (Aposhian *et al.*, 2000b) implicating MMA^{III} as a stable and persistent metabolite in humans.

Now, we have demonstrated MMA^{III} stability and retention by isolating it from hamster liver 15 hours after a single intraperitoneal dose of sodium arsenate. According to Yamauchi and Yamamura (1985), the concentration of MMA in the liver is greatest between 12-24 hr following oral administration. The concentration of MMA^{III} presently detected in hamster liver at 15 hours may be biologically significant inasmuch as it is comparable to that of arsenite, yet the former is 3 - 26 times more acutely toxic than the latter (Petrick *et al.*, 2000). Recently, MMA^{III} has also been detected in rat bile (Gregus *et al.*, 2000).

The abundance of injected parent compound in the liver was expected since Yamauchi and Yamamura (1985) report that inorganic arsenic was 80% of the total

arsenic excreted one day after oral administration. The rate constant of arsenate reductase is not known, but the abundance of arsenate in a principle organ of arsenic metabolism may reflect another rate limiting step. Nor is the abundance of DMA^{V} unexpected. DMA is the major metabolite excreted in urine of hamsters (Marafante *et al.*, 1987; Yamamuchi and Yamamura, 1985; Yamauchi *et al.*, 1992). If not for the potent toxicity of MMA^{III} and, perhaps DMA^{III}, the relative concentrations of As metabolites shown in figure 3.3 would support the methylation of arsenic as a route of detoxification; DMA^{V} is the least acutely toxic and most abundant metabolite of arsenate in hamster liver.

The mechanism of arsenite toxicity is the formation of a labile covalent coordinate complex with spatially close sulfhydryls. Presumably, the mechanism of MMA^{III} toxicity is similar. However, the methyl moiety of MMA^{III} may hinder ligand exchange so that the coordination complex becomes kinetically inert. Assuming a high concentration of free thiol groups in the biological system, MMA^{III} is likely to be bound to macromolecules as methylarsenic(III) sulfide (Cullen *et al.*, 1984). If arsenite has a greater ligand exchange rate than MMA^{III}, the inorganic arsenic-sulfur bond is more labile and reversible while the MMA^{III}-sulfur bond is expected to be less labile and less reversible. This may explain the greater toxicity of MMA^{III}. This may also explain why

a DMPS challenge yields approximately 2.5 times as much MMA as inorganic arsenic in the urine (Aposhian *et al.*, 2000a).

The present concentration of DMA^{III} in hamster liver is surprisingly high considering that only 6.4% of [^{74}As]DMA, given *p.o.* is metabolized to trimethylarsine oxide (TMAO) in the hamster (Marafante *et al.*, 1987). Yamauchi and Yamamura (1985) could not detect TMAO in the urine or blood but could detect a small amount in the liver of hamsters after a single oral dose of As_2O_3. The trivalent DMA-S bond is expected to be far more labile since dimethylated arsenic may complex with only one thiol. DMA has been implicated primarily as a carcinogen (Moore *et al.*, 1994; Yamanaka *et al.*, 1996; Wanibuchi *et al.*, 1996; Yamamoto *et al.*, 1995; Wei *et al.*, 1999) rather than an acute toxicant (NIOSH, 1986; Sakurai *et al.*, 1998; Moore *et al.*, 1997; Petrick *et al.*, 2000). The presence of DMA^{III} in hamster liver 15 hr after arsenate exposure points out the importance of the oxidation state of DMA as a tumor promoter (Yamamoto *et al.*, 1995; Yamanaka *et al.*, 1996; Wei *et al.*, 1999). DMA^{III} may be the active carcinogenic species.

Trivalent methylarsenicals must be considered when evaluating the toxicity and carcinogenicity of arsenic. This first-time identification of MMA^{III} and DMA^{III} in liver after arsenate exposure indicates that the significance of arsenic species in mammalian

tissue must evaluated and considered when defining the role of methylation in arsenic biotransformation.

4.

ARSENIC METABOLISM IN *SACCHAROMYCES CEREVISIAE*

INTRODUCTION

Candida humolica metabolize arsenate to arsenite, MMA, DMA and trimethylarsine (Cullen *et al.*, 1979a). Moreover, growing cells were made to increase production of trimethylarsine from arsenate by pretreatment with arsenic (Cullen *et al.*, 1979b). Anticipating a tractable model system for arsenic metabolism, methylated metabolites were sought in eukaryotic *S. cerevisiae*. Budding yeast are a convenient candidate to deconstruct cellular processes because of the relatively small genome (~ 3×10^7 vs. ~ 2×10^9 mammalian genome), the entire sequence of which is known, and because cells can carry either a haploid or diploid set of chromosomes. If the *S. cerevisiae* model system is found to methylate arsenic, then the arsenic biotransformation pathway may be manipulated metabolically and genetically to interpret the consequence of arsenic methylation.

MATERIALS AND METHODS

Reagents. Carrier-free [^{73}As]arsenate (2.46 - 5.61 μCi/ml) was purchased from Los Alamos National Laboratory (Los Alamos, NM). [^{14}C]MMA^{V} (4.5 mCi/mmol), was purchased from American Radiolabeled Chemicals (St. Louis, MO). [^{14}C]DMA^{V} (11.2

mCi/mmol), was a generous gift from Management Technology (Research Triangle Park, NC). [^{73}As]Arsenate was reduced to [^{73}As]arsenite according to the method of Reay and Asher (1977). *S. cerevisiae* strain w303 (DLY62 genotype MATa, bar::hisg ade2-1, trp1-1, can1-100, leu2-3, 112, his3-11, 15, ura3, GAL+, psi^{-}, ssd1-d2) was obtained from the laboratory of Ted Weinert.

Preparation of yeast extract. Yeast were grown to saturation phase in 500 ml complete medium, YEPD (2% yeast extract (DIFCO), 2% peptone, 2% dextrose, 0.5% adenine hemisulfate) at 30°C. Cells (1.05×10^8 cells/ml) were pelleted at 4225*g* in a Sorvall Superspeed RC2-B centrifuge (Wilmington, DE) at 4°C for 10 min. The yeast pellet was washed twice with 20 ml PBS (0.14 M NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.2 at 25°C) and yielded a packed cell volume of approximately 2 ml. Next, the pellet was resuspended in 8 ml lysis buffer and fractionated into 4 15 ml polypropylene conical tubes. Lysis buffer was 50 mM Tris-HCl, 2 mM EDTA, 100 mM NaCl (pH 7.5 @ 4°C), 0.1% Nonidet P-40 (Igepal CA-630), 1.3 µg aprotinin/ml, 1.3 µg leupeptin/ml and 1 µg pepstatin/ml. Another 2 volumes of lysis buffer and 4 volumes of acid-washed glass beads (425 - 600 µm) were added to each and each suspension vortexed at high speed in a VWRbrand multitube vortexer for 5 60 sec intervals at 4°C with intermittent reprieve on ice. The crude lysate was centrifuged at 12,000*g* and the

supernatants, cleared lysates, were pooled. Cleared lysate was either stored at -70°C or desalted on a Sephadex G-25 size exclusion column (PD-10, Pharmacia, Sweden) primarily to exclude NP-40 (~ 625 g/mol). Six 9.1 ml PD-10 columns were each equilibrated with 25 ml 50 mM Tris-HCl pH 7.6. Cleared lysate (2.5 ml) was applied and allowed to settle into the column. The high molecular weight components (≥ 5,000 Da) were finally eluted with 3.5 ml equilibration buffer. Increasing amounts of cleared lysate ± G-25 size exclusion (10 - 1,000 μg) were incubated in the standard arsenite methyltransferase assay (Zakharyan *et al.*, 1995) using 120 mM Tris-HCl pH 6 - 8. All enzyme assays were corrected for non-specific methylation defined as the control value obtained by incubating the reaction mixture in the absence of arsenite. The pellet (1.6 g) remaining after 12,000*g* was solubilized by adding 2 volumes (v/w) of lysis buffer containing 2% TritonX. The paste was mechanically disrupted using a mortar and pestle on ice and assayed for arsenite methyltransferase activity.

Stepwise purification of yeast arsenite methyltransferase. Diethylaminoethyl (DEAE) cellulose was prepared in 0.1 M HCl, poured onto a 1.5 × 3 cm column and washed successively with 80 ml 0.1 M HCl, 80 ml H_2O, 80 ml 0.1 M NaOH, 80 ml H2O, 120 ml 0.5 M NaCl at room temperature. The column was equilibrated overnight at 4°C with 10 mM Tris-HCl pH 7.6 at 4°C and a flow rate of approximately 5 ml/hr. Mouse cytosol

(34.5 mg) or yeast cleared lysate (37.6 mg) was applied to the column, each in volume of 1.2 ml. The sample was allowed to settle into the column before it was washed with 3 bed volumes of 10 mM Tris-HCl pH 7.6. To elute the enzyme, 10 mM Tris-HCl pH 7.6 with increasing concentrations of NaCl (0.1 - 0.5M) was applied stepwise in 1 ml increments. Each fraction (1 ml) was assayed for protein and arsenite methyltransferase activity.

Microcolony assay. DLY62 YEPD cultures (2ml, 2×10^6 cells) were grown in the presence of 1 μM, 10 μM, 100 μM, 1 mM or 10 mM sodium arsenite for 0, 3, 6 or 16 hr at 34°C. At each time point, 500 μl aliquots of control or experimental cultures were washed twice with sterile water, resuspended in 500μl sterile water and 100 μl applied to YEPD agar plates. After 2hr at 34°C, plates were scored for viability (≥ 5 cells/colony).

In vivo arsenic metabolism. Radiolabeled standards, 0.018 μCi [^{73}As]arsenite, 0.018 μCi [^{73}As]arsenate, 0.2 μCi [^{3}H]MMA and 0.042 μCi [^{3}H]DMA were prepared in YEPD 6% dextrose pH 4.5, 50 mM phosphate buffer, or crude lysate, boiled 3 min, centrifuged 15,000*g* 10 min and injected (100 μl) onto a PRPX-100 inorganic anion exchange HPLC column (250 × 4.1 MM (Hamilton, Reno, NV) with a 50 mM phoshate pH 8 mobile phase running at 2.5 ml/min. A post-column, in-line Beckman 171 γ detector (Fullerton, CA) was used to detect the radioactivity of the effluent mixed with Monoflow III

scintillator (National Diagnostics, Atlanta, GA) in a 1:3 ratio. YEPD (6% dextrose) cultures (25 ml) were inoculated with 0 or 50 µl stationary phase culture (to give a starting cell density of ~ 6×10^5 cells/ml), 20.83 µM arsenate (to yield C_f = 0.80 µM $Na_2HAsO_4 \cdot 7H_2O$) and 8 µCi [^{73}As]arsenate and grown overnight at 30°C in a shaking water bath. After 12 hr, the suspension was centrifuged 3020*g* to separate the media from the cells. Each was analyzed for extracellular and intracellular arsenic metabolites, respectively.

Yeast (~ 8×10^5 cells/ml) were also grown in the presence of 0 - 100 mM, 8 µCi [^{73}As]Na_2HAsO_4. After 15 hr at 30°C (~ 4×10^7 cells/ml), 1 ml suspension was counted in an LKB (Gaithersburg, MD) Compugamma counter. The suspension was centrifuged at 3020*g* and the supernatant also counted for gamma irradiation to estimate the amount of radioactivity associated with the cell. The supernatant, media was injected onto an HPLC column for speciation (As^V, As^{III}, MMA and DMA) of *in vivo* arsenic metabolites. In search of intracellular metabolites, 4 volumes of acid-washed glass beads and 1 volume of lysis buffer were added to the 3020*g* pellet and the mix vortexed 6 min at room temperature. The lysate (500 µl) was aspirated from the glass beads using a gel-loading pipet and cleared at 10,000*g* and 4°C. The cleared lysate was boiled 3 min, centrifuged 12 min at 14,000*g* and injected onto the HPLC column.

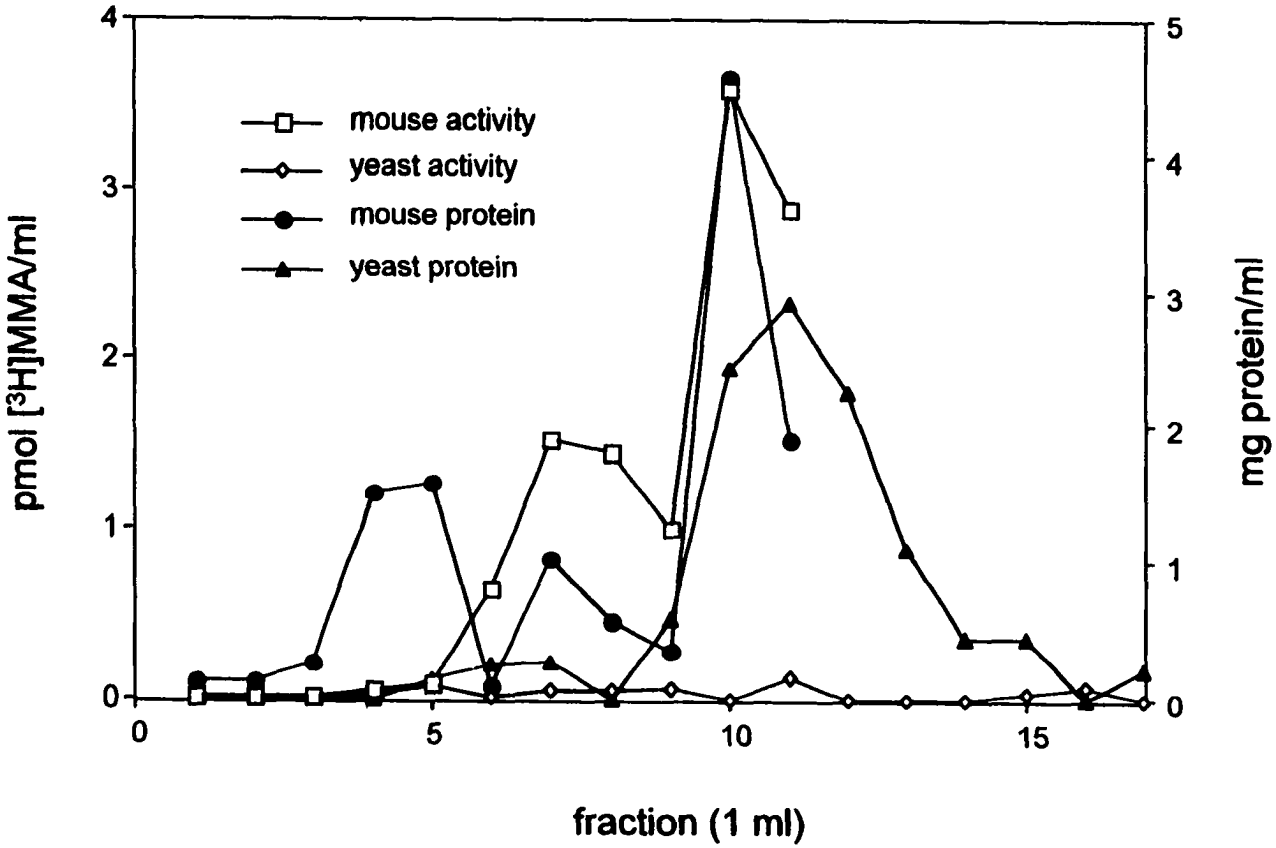
RESULTS

In vitro arsenite methyltransferase activity. Using a protein range of 10 μg to 1 mg, pH range 6 to 8.4 and substrate range 5 to 100 μM, yeast lysate prepared from cultures grown to both midlog and saturation phases did not have detectable *in vitro* arsenite methyltransferase activity as measured by the formation of [^{3}H]MMA. Detergents are not likely to affect activities of soluble enzymes and mouse liver arsenite methyltransferase is not influenced by 0.5 - 4% TritonX or 0.1% Igepal. Withdrawal of detergent from yeast lysate was inconsequential. Chromatography of rabbit liver cytosol on DEAE cellulose increased arsenite methyltransferase activity 1600% (table 6.1). Arsenite methlytransferase activity could not be elicited from budding yeast extract after anion exchange chromatography (figure 4.1).

In vivo arsenite methyltransferase activity. The uptake of arsenic by yeast was considered: Differential uptake mechanisms of arsenite and arsenate were observed in KB oral epidermoid carcinoma cells. Arsenate and phosphate share a common transport system and arsenite uptake is probably accomplished through simple diffusion (Huang and Lee, 1996). In growing yeast, the active uptake of phosphate requires the presence of sugar which suggests gylcolysis supplies the energy for phosphate uptake (Goodman and Rothstein, 1957). The rate of phosphate assimilation is little effected by varying pH

figure 4.1

DEAE elution profile of yeast lysate. Mouse cytosol (34.5 mg) or yeast 12,000*g* lysate (37.6 mg) was applied to a 1.5 × 3 cm equilibrated DEAE cellulose anion exchange column at 4°C. Protein was successively, stepwise eluted with 0.1 M increments of NaCl, 10 mM Tris-HCl pH 7.6 at 4°C. Fractions (1ml) were assayed for arsenite methyltransferase activity at pH 7.6 and protein (Bradford, 1976).


mouse activity
yeast activity
mouse protein
yeast protein
pmol [3H]MMA/ml
mg protein/ml
fraction (1 ml)
0
1
2
3
4
5
10
15

values of medium between 4 and 7.5 or by varying phosphate concentration between 0.002 and 0.1 M (Schmidt *et al.*, 1949).

When yeast cultures were initially grown in the presence of 0.5 μM arsenite, metabolism was not obvious (data not shown). When yeast cultures were grown in the presence of 0.8 μM arsenate, it became apparent the *S. cerevisiae* uptake arsenate, reduce it and efflux arsenite (figure 4.2c). The *ars* operon of *E. coli* R773 plasmid confers resistance to a variety of heavy metals including arsenate and arsenite in a similar fashion (Mobley and Rosen, 1982; Chen *et al.*, 1985). Cullen *et al.* (1979b) suggested that increased methylation may increase arsenic tolerance in the yeast *C. humolica*. Yeast were grown in arsenate concentrations increasing into toxic levels (figure 4.3). After 15 hr in excess of 10 μM As^{V}, cells were not able to tolerate arsenate challenge (figures 4.4a and 4.4b). There was no evidence of methylation in *S. cerevisiae* under these experimental conditions.

Discussion

S. cerevisiae is not a good model system for arsenic metabolism in mammals: Budding yeast do not methylate arsenic.

Resistance to arsenicals and antimonials in *E. coli* is conferred by an arsenic resistance (*ars*) operon. The *ArsA* gene product is the catalytic component and *ArsB*, the

figure 4.2a

Standard retention times in YEPD. Radiolabeled arsenite ([^{73}As], 0.018 μCi), arsenate ([^{73}As], 0.018 μCi), MMA ([^{3}H], 0.2 μCi) and DMA ([^{3}H], 0.042 μCi) in a total volume of 200 μl YEPD, 6% glucose, pH 4.5 were injected onto an anion exchange HPLC column. Under the conditions described in MATERIALS AND METHODS, the following retention times were observed: arsenite, 1.6 min; DMA, 2.2 min; MMA, 2.8 min; arsenate, 5.6 min.

figure 4.2b

Arsenate is not chemically reduced in YEPD, 6% glucose pH 4.5. Media (50 ml) absent yeast was inoculated with 8 μCi, 20.83 μM [^{73}As]arsenate, incubated 12 hr at 30°C and chromatographed on a PRPX-100 anion exchange column.

figure 4.2c

In vivo metabolism of arsenate in *S. cerevisiae*. Yeast were grown in the presence of YEPD 6% glucose pH 4.5 and 8 μCi, 20.83 μM [^{73}As]arsenate for 12 hr at 30°C with constant aeration. At t = 12, the supernatant was separated from cells (4×10^7 cells/ml) and analyzed by HPLC. Cultures inoculated with equal cell density and grown in the absence of arsenite also yielded 4×10^7 cells/ml after 12 hr at 30°C.

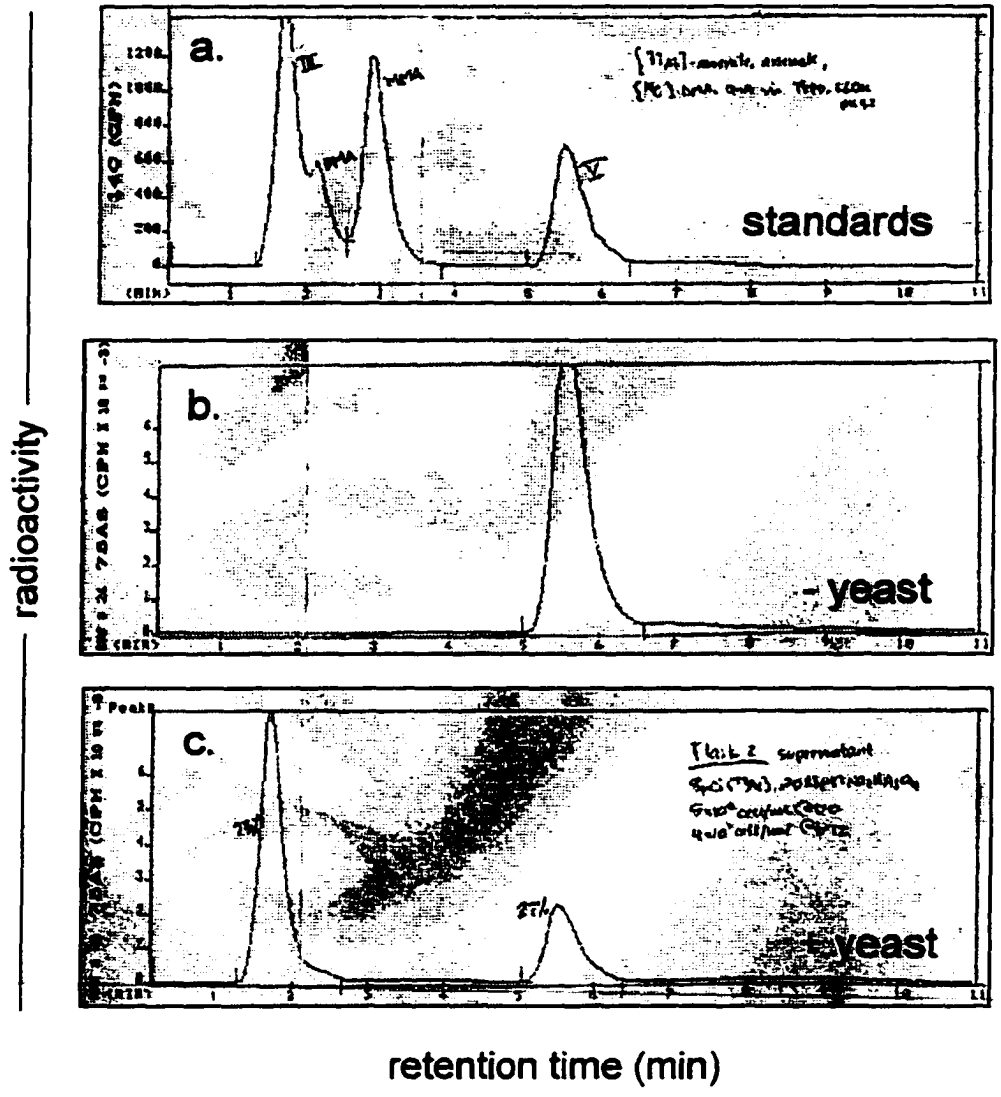

a.
standards
b.
- yeast
c.
+ yeast
radioactivity
retention time (min)

figure 4.3

Cell viability assay of yeast grown in the presence of arsenite. Midlog *S. cerevisiae* (W303, DLY62) were diluted 20-fold in 2 ml YEPD and incubated at 34°C. After 4 hr, sodium arsenite was added to a final concentration of 1 μM, 10 μM, 100 μM, 1 mM or 10 mM. At 3, 6 and 16 hr, 500 μl of each suspension was washed twice and resuspended in an equal volume of YEPD. The washed suspension (100 μl) was spread on YEPD agar and incubated 2.5 hr @ 34°C. Viability was defined as colonies comprised of ≥ 5 cells. Values represent the fraction of total (untreated) colonies.

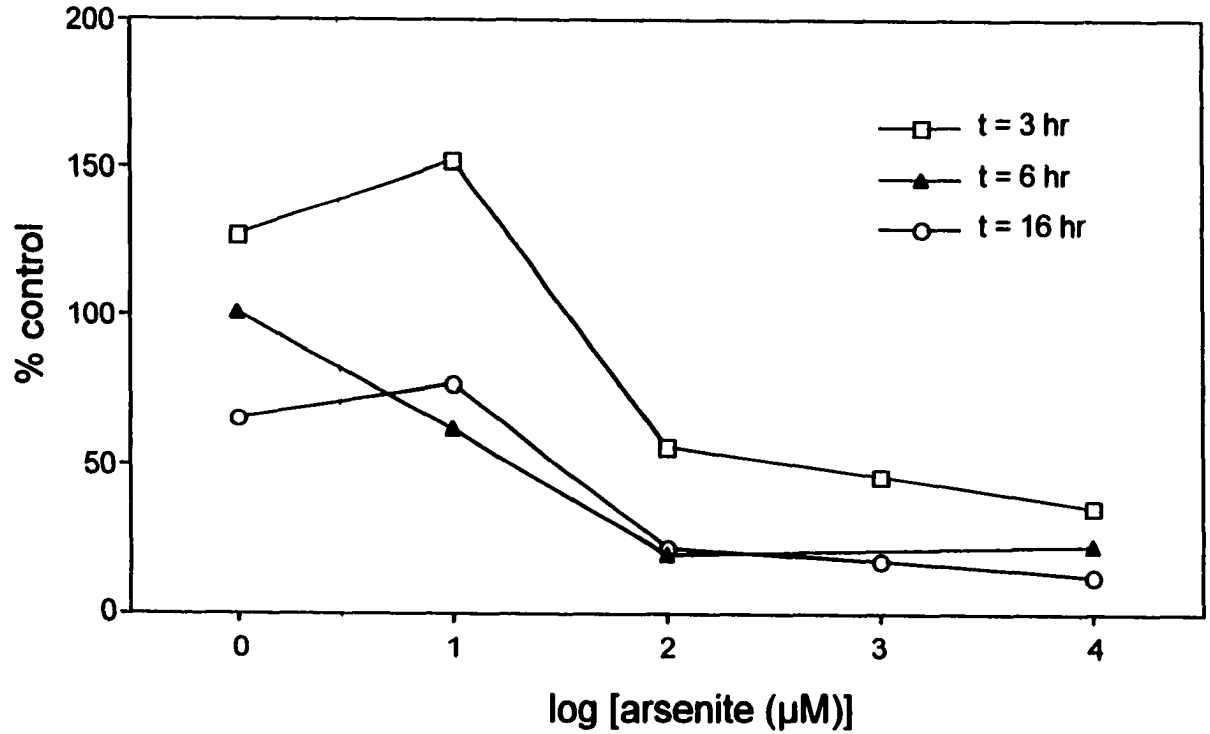
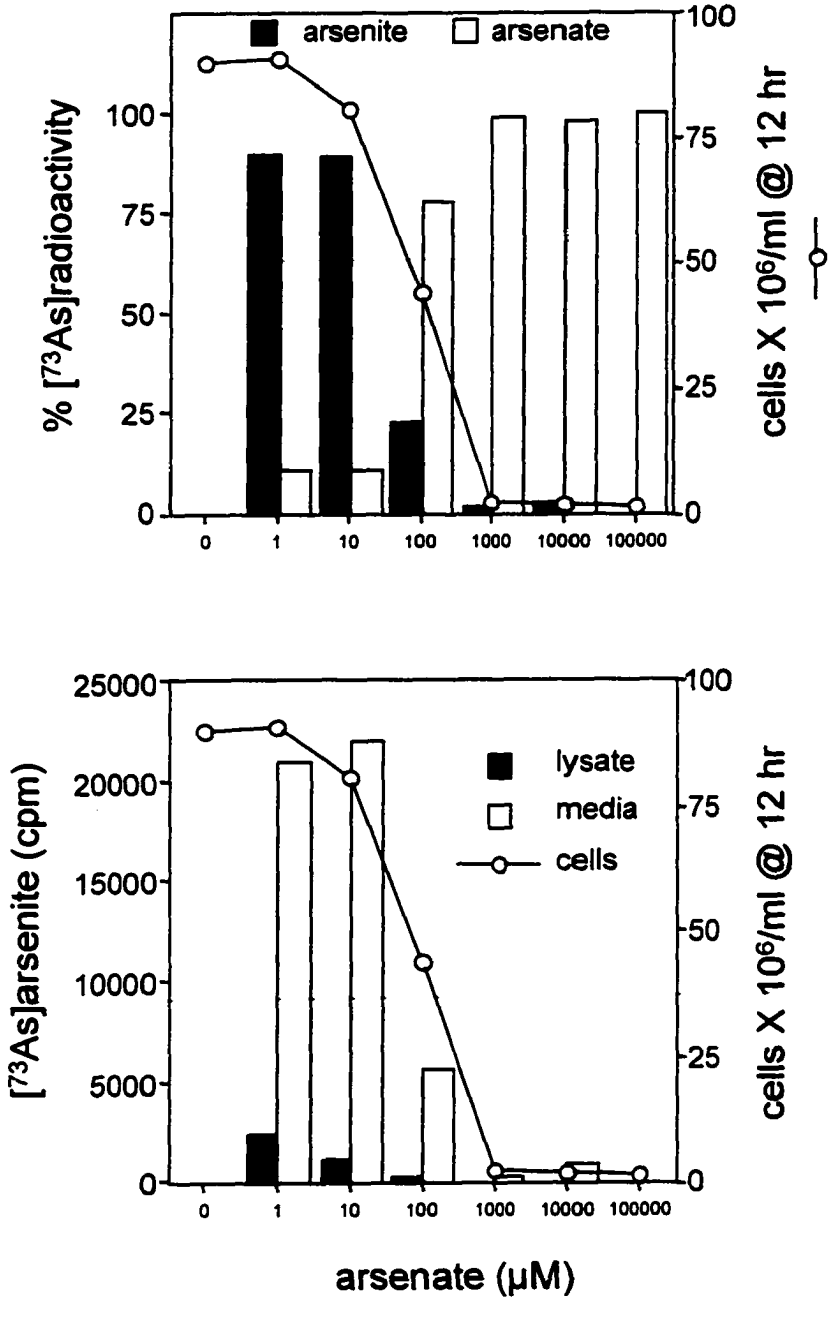

200
150
100
50
0
% control
0
1
2
3
4
log [arsenite (μM)]
t = 3 hr
t = 6 hr
t = 16 hr

figure 4.4a

Extracellular *in vivo* metabolites of arsenic. Yeast (~ 8×10^5 cells/ml) were grown in the presence on increasing arsenate, 8 μCi [^{73}As] at 30°C for 15 hours in 2.5 ml YEPD 6% glucose. Media (supernatant was analyzed for effluxed metabolite by HPLC. Ordinate values represent the fraction of total area (radioactivity) under each peak. Peaks corresponding to MMA and DMA retention times were not detected.

figure 4.4b

Intracellular metabolites after 12 hr treatment with arsenate. Lysate of yeast (~ 8×10^5 cells/ml) treated with 8 μCi [$^{73}As^{V}$], 1 μM - 100 mM arsenate was prepared as described in MATERIALS AND METHODS and analyzed for metabolites by HPLC. While cells are viable, arsenite comprises 100% intracellular [^{73}As]radioactivity. Media counts are displayed to show that yeast do not accumulate arsenic under these experimental conditions


arsenite
arsenate
% [73As]radioactivity
cells X 10^6/ml @ 12 hr
100
75
50
25
0
0
1
10
100
1000
10000
100000
lysate
media
cells
[73As]arsenite (cpm)
25000
20000
15000
10000
5000
arsenate (μM)

membrane anchor and anion channel of an arsenical ATPase efflux pump (Rosen *et al.*, 1988; Wu *et al.*, 1992). *ArsC* encodes an arsenate reductase (Ji and Silver, 1992) which uses GSH with glutaredoxin as electron donors (Shi *et al.*, 1999).

Resistance in *S. cerevisiae* has since been investigated. Using a yeast genomic library, a 4.2 kb region of chromosome XVI was isolated as a fragment conferring resistance to 7 mM $NaAsO_2$ when put on a multicopy plasmid. Three new open reading frames (ORFs) *ACR1* (*a*rsenic *c*ompound *r*esistance), *ACR2* and *ACR3* hypothetically encode products similar to transcriptional regulatory proteins (Yap1 and Yap2), ArsCp arsenate reductase and ArsBp membrane protein (Bobrowicz *et al.*, 1997), respectively. *ACR2* confers arsenate resistance and *ACR3*, arsenite resistance. Δ*acr1* is hypersensitive to both arsenite and arsenate.

Ghosh *et al.* (1999) have further characterized Acr3p as one of two independent transport systems for the removal of toxic arsenite from yeast cytosol. Disruption of *ACR3* results in increased sensitivity and increased arsenite accumulation. The other pathway is vacuolar sequestration of As^{III} in a reaction catalyzed by *YCF1* (yeast cadmium factor). Ycf1p is a homologue of the human multidrug resistance protein (MRP, 63% similarity) and has been shown to be a vacuolar glutathione S-conjugate pump with a broad range of substrate specificity (Li *et al.*, 1996; Szcypka *et al.*, 1994).

This GS-X pump is a MgATP-dependent transporter that catalyzes the efflux of GS-conjugates from cytosol via the plasma- and/or endomembranes. This may be regarded as the terminal phase of xenobiotic detoxification (Phase III). Yap1p transcriptionally activates both *YCF1* and *GSH1*. *GSH1* encodes γ-glutamylcysteine synthetase (Wu and Moye-Rowley, 1994).

In Chinese Hamster Ovary cells, resistance is correlated with active efflux of arsenite from the cell (Wang *et al.*, 1996). But extrusion may only be a temporary solution in a multicellular organism since arsenite is a covalent molecule and is nearly completely absorbed (Crecelius *et al.*, 1977; Hughes *et al.*, 1994). Ycf1p provides a 2nd independent pathway to remove arsenite from cytosol by sequestering it in the vacuole as a glutathione conjugate. Ycp1p may prove coincident with arsenite binding protein(s), arsenotriglutathione ($As(GS)_3$) as a likely biotransformation intermediate (Delnomdedieu *et al.*, 1993; Gailer and Lindner, 1998), biliary excretion of an arsenic glutathione complex via MRP2 (Kala *et al.*, 2000) or glutathione S-transferase Ω as MMA^V reductase (Zakharyan *et al.*, 2001)

5.

ARSENITE BINDING PROTEIN(S)

INTRODUCTION

Interactions between trivalent arsenic and thiol containing residues in proteins have generally been regarded as the basis of acute arsenic toxicity. For example, As^{III} binds critical cysteine residues of the glucocorticoid receptor (GR) and occludes steroid binding (Simon *et al.*, 1990) which selectively inhibits GR-mediated transcription (Kaltreider *et al.*, 2001). However, the protein binding of metals may also be a mechanism of detoxification. DMPS (2,3-dimercaptopropanol, meso-2,3-dimercaptosuccinic acid) chelates, mobilizes and hence, detoxifies inorganic arsenic (Aposhian *et al.*, 1984). Low molecular weight Pb binding proteins in the brain and kidney reverse lead-inhibition of δ-aminolevulinic acid dehydratase activity in rats (Goering *et al.*, 1986). Tellurium is strongly bound to soluble serum, liver and kidney protein sulfhydryls (Agnew and Cheng, 1971). Accumulation of cadmium in the kidney without apparent toxic effect is possible because of the formation of a cadmium-metallothionein complex (6.5 kDa) (Suzuki, 1982). Approximately 30% of metallothionein is cysteine.

Arsenic bound proteins have been observed in bovine kidney cytosol (38 kDa; Chatt and Jayawickreme, 1989), rat liver cytosol (> 1,000, 135 and 38 kDa; Styblo and Thomas, 1997) and rabbit liver cytosol (>2,000, 450 and 100 kDa; Bogdan *et al.*, 1994) but none have been identified by amino acid sequence. Nor have these proteins been shown to be a mechanism of detoxification. The objective of this research project was to isolate and characterize an arsenic binding protein(s) from guinea pig cytosol. While the effect of methylation on arsenic toxicity is uncertain, it is heretofore generally believed that methylation is the primary route of detoxification. The experiments detailed below are driven by the following hypothesis: An arsenic binding protein(s) plays the paramount role in regulating the acute intracellular toxicity of arsenic by sequestering arsenic and thus making it less available to interact with sensitive organelles or enzyme systems: Methylation is secondary to protein binding. The guinea pig is chosen as the animal model because it does not methylate arsenic. Therefore, metabolites will not be variables. Using an *in vitro* methyltransferase system, inorganic as well as mono- and dimethylated arsenic all associate with rat cytosolic proteins (Styblo and Thomas, 1997).

MATERIALS AND METHODS

Reagents. Carrier-free [^{73}As]arsenate (2.46 - 5.61 μCi/ml) was purchased from Los Alamos National Laboratory (Los Alamos, NM). ACS reagent grade sodium arsenite

($NaAsO_2$) and sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$) were purchased from Sigma Chemical Co. (St. Louis, MO) and MCB Reagents (Cincinnati, OH), respectively. Bovine Fraction V albumin and *p*-arsanilic acid were purchased from Sigma; Sephacryl S300-HR and molecular weight gel filtration calibration standards from Pharmacia Biotech (Piscataway, NJ); Novarose Act^{High}-10,000/40 from Inovata (Broma, Sweden) and DMPS from Heyl Co. Chem. - Pharm. (Berlin, Germany). All other chemicals were analytical reagent grade or of the highest quality obtainable. All water was doubly deionized, distilled and filtered ($ddiH_2O$).

Preparation of cytosol. Male Hartley guinea pigs (500-600 g) from Harlan Sprague Dawley (Indianapolis, IN) were acclimated in an environmentally controlled animal facility operating on a 12 h light/dark cycle at 22°C prior to experimentation. Animals were provided Teklad (Indianapolis, IN) Guinea Pig Diet # 7006 and water *ad libitum.* Guinea pigs were sacrificed by CO_2 asphyxiation. Their livers, removed immediately, were rinsed in ice-cold 0.9% NaCl, blotted on filter paper, cut into approximately 5 g pieces and homogenized at 4°C in a 30 ml glass mortar equipped with a motor-driven Teflon-coated stainless steel pestle. The homogenization buffer was comprised of 50 mM HEPES pH 6.8 at 25°C, 500 μM PMSF and 146 μM pepstatin A (Sigma, St. Louis, MO) and added to yield a 30% (1:2 w/v) homogenate. The homogenate was spun at

14,600*g* for 15 min at 4°C in a Beckman (Fullerton, CA) Model J2-21M induction drive centrifuge yielding the S9 (post-mitochondrial) fraction which was further centrifuged at 105,000*g* for 90 min in a Beckman Model L2-65B ultracentrifuge at 4°C yielding cytosol (soluble protein).

Protein binding assay. In order to quantitate inorganic arsenic binding to cellular proteins, an *in vitro* incubation system was optimized (figure 5.1; Bogdan *et al.*, 1994). The saturation of arsenic binding sites on cytosolic arsenic binding protein(s) was determined by increasing the amount of unlabeled ligand in the following *in vitro* binding assay: guinea pig or rabbit liver cytosol or BSA (2 mg) was incubated in a 10 mM HEPES pH 7.4 buffered solution at 37°C with a constant amount of [^{73}As]arsenite reduction solution (0.294 μCi/assay) and cold $NaAsO_2$ at final concentrations of 0.5 nM, 5 nM, 25 nM, 150 nM, 1.5 μM, 15 μM, 150 μM. 1.5 mM, 3.0 mM in a total volume of 250 μl. After 30 min, the reaction mixture was precipitated with 65% ice-cold acetone and centrifuged at 10,500*g* for 10 min. The pellet was washed twice with 65% acetone and the supernatants pooled. Protein pellets and corresponding supernatants were counted in an LKB (Gaithersburg, MD) Compugamma counter. Bound ligand was expressed as % total radioactivity in the protein pellet and plotted against the log of unlabeled arsenite concentration to determine the dissociation constant, K_D.

Specific binding of arsenite to protein was discriminated from non-specific adsorption. Briefly, arsenite binding protein(s) (2 mg) were incubated at two concentrations of radioinert arsenite, approximately $(0.1)(K_D)$ and $(10)(K_D)$, containing the same amount of [^{73}As]arsenite (0.294 μCi). Bound ligand was expressed as the percent of total radioactivity in the precipitated pellet. At arsenite concentration $(10)(K_D)$, the specific activity of [^{73}As]-arsenite will be 1% that of the lower arsenite concentration. Specific arsenite binding is saturable but non-specific adsorption is not and is, therefore, independent of excess arsenite. At high concentrations of unlabeled arsenite and low specific radioactivity, [^{73}As]-ligand is out-competed by radioinert ligand at specific binding sites so only non-specific sites will be labeled. The difference between [^{73}As]-arsenite bound at $(10)(K_D)$ arsenite (non-specific adsorption) and $(0.1)(K_D)$ arsenite (total binding sites) represents saturable or specific binding (Titeler, 1983).

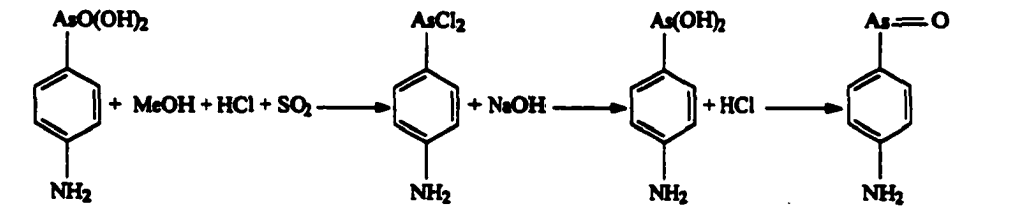
Preparation of arylarsine oxide. *p*-Amino phenylarsine oxide (PAPAO) was prepared according to Blicke and Smith (1929). Briefly, *p*-arsanilic acid (16.35 g) was added to a solution of 45 ml methanol, 36 ml concentrated HCl and 0.15g of potassium iodide (Stevenson *et al.*, 1978). The amber solution was infused with sulfur dioxide for approximately 15 min or until a light yellow precipitate formed. The solution was cooled

on ice. The precipitate was collected on filter paper using a Büchner funnel, washed thrice with excess diethyl ether and the vacuum-dried solid completely dissolved in 10% NaOH. Next, the basic solution neutralized with 1M HCl and gently stirred on ice whereupon PAPAO precipitated (scheme 5.1). PAPAO was collected on filter paper, washed thoroughly with ddiH_2O, diethyl ether and dried. The melting point was measured at 50 V for n = 3 batches of PAPAO using a MEL-TEMP apparatus. The reported melting point of PAPAO is 93 - 95°C but varies upon recrystallization conditions (Blicke and Smith, 1929).

Preparation of PAPAO affinity media. Novarose (10,000/40) Act^{High} resin was washed with an excess of ddiH_2O and suspended in 27% dimethylsufoxide (DMSO), 0.2 M carbonate buffer pH 11 containing 700 mg PAPAO/10 g resin. PAPAO, or ligand, was coupled via the bromohydrine method (Hannestad *et al.*, 1982) by gentle agitation for approximately 60 hr at ambient temperature. After incubation, the gel was washed with an excess of 20% DMSO, excess of 50% ethanol. PAPAO-coupled agarose was then suspended in 0.1 M NaOH for 30 min to facilitate oxidation of the arsine oxide group. Next, the matrix was collected on a coarse glass filter, resuspended in an excesss of 1M ethanolamine pH 9 and incubated at ambient temperature for 4 hr to block the unreacted

scheme 5.1

Synthesis of PAPAO



bromohydrine groups of Novarose (10,000/40) Act^{High}. The final resin was collected on a sintered filter, washed with water and stored in 20% DMSO at 4°C.

The total arsenic content of the PAPAO resin was determined by hydride generation atomic fluorescence spectroscopy (HGAFS) (Millenium Excaliber 10.055, P.S. Analytical, Kent, U.K.). The ligand-bound resin was collected on a coarse glass filter and dried 48 hr on filter paper. Dried samples (n = 3) were digested with 15.4 M nitric acid in a microwave. Using a standard curve generated with sodium arsenite in reagent blank solution (30% (v/v) HCl, 1% (w/v) KI, 0.2% (w/v) ascorbic acid), the concentration of bound ligand was determined.

Arsenic bioaffinity chromatography. Arsenic bioaffinity chromatography operates principally through the biospecific interaction of the natural ligand, trivalent arsenite, and the binding site, vicinal sulfhydryl groups, of the arsenic binding protein(s). This is in contrast to immobilized metal affinity chromatography (IMAC) which does not, in general, act biospecifically. Rather, IMAC operates through coordinate bonds between metal ions (Zn, Cu, Nu, Ca, Fe) immobilized on a column and basic groups on proteins, mainly histidine, cysteine and rarely tryptophan residues.

Affinity media was washed on a sintered glass filter with an excess of starting buffer (TB, 100 mM Tris-HCl pH 7.5, 500 mM NaCl, 1 mM EDTA). Vacuum-dried

resin was resuspended in starting buffer and incubated with approximately 80 mg guinea pig liver cytosolic protein in a water bath set at 80 oscillations/min and 37°C. After 30 min, the resin incubation mix was put on a filter flask and vacuumed to damp dryness at 4°C and the flowthrough collected. In the presence of the high ionic strength of the starting buffer, protein will not be bound to the stationary phase via nonspecific electrostatic interactions. Nor should basic residues bind with As, a group III transition metal, in the presence of EDTA. After the resin was completely washed of unbound protein, it was batchwise eluted (gentle agitation for 15 min at 4°C) with 2 ml of the following buffers: starting buffer/30% ethylene glycol (TBEG) to nullify nonspecific hydrophobic interactions; 10 mM cysteine (pK_a = 8.5)/0.1 M sodium acetate pH 4.6/30% ethylene glycol (CEG) to remove proteins bound to phenylarsine oxide via monothiol interactions; 10 mM DMPS and 100 mM DMPS, both in starting buffer/30% ethylene glycol (DEG), to remove protein(s) of interest bound to ligand through vicinal sulfhydryl moieties; 1% $NaBH_4$/0.1 M Tris-HCl pH 8 to reduce any protein still bound to column by a sulfur bond; and 10 mM PAPAO in starting buffer/30%ethylene glycol to compete with resin functional groups for protein binding. Eluates were desalted on Sephadex G-25 gel filtration columns and concentrated in centrifugal filters with a 10 kDa MWCO (YM-10).

Eluates were assayed for protein content by the method of Bradford (1976) or Smith *et al.*, (1985) according to eluant compatibility and using bovine serum albumin standards.

Protein eluted from the PAPAO resin was labeled with [^{73}As]arsenite. Two parts [^{73}As]arsenate were reduced to arsenite in one part solution of 0.2N H_2SO_4, 85 mM Na-metabisulfite, 0.1% w/v Na-thiosulfate (1977). The reduction was monitored by injecting 20 µl sample onto a Hamilton PRP-X100 inorganic anion exchange HPLC column, 250 x 4.1 mm, with a 50 mM phoshate pH 8 mobile phase running at 2.5 ml/min. A post-column, inline ß-RAM, Model 2 Flow-Through System (IN/US Systems, Inc (Tampa, FL) was used to detect radioactivity of effluent mixed with Monoflow III scintillator (National Diagnostics, Atlanta, GA) at a flow rate of 7.5 ml/min. Only reduction solutions yielding greater than 90% [^{73}As]arsenite were used for binding assays. In a final volume of 10 µl, 20 µg of fractionated proteins were incubated with approximately 7 µCi [^{73}As]arsenite in buffer at 37°C. After 30 min, 2X Laemmli buffer (1970) was added to the binding reaction and proteins resolved by SDS-PAGE

Size exclusion chromatography (SEC). Sephacryl S300-HR (Pharmacia, Uppsala, Sweden) was packed into a 50 x 1.5 cm column and washed with running buffer (50 mM HEPES, 150 mM NaCl pH 6.8 @ 25°C) in excess of 3 bed volumes. In running buffer, the void volume was determined by chromatographing Blue Dextran 2,000 (4.7 mg) and

the column was calibrated @ 4°C with approximately 10 mg of the following standards (Pharmacia): α-chymotrypsinogen A (25 kDa) and thyroglobulin (669 kDa) simultaneously, and a mixture of ribonuclease A (13.7 kDa), aldolase (158 kDa) and ferritin (440 kDa). Desalted protein selectively eluted from the PAPAO resin with DMPS was labeled with 50 μCi [^{73}As]arsenite by incubation for 30 min @ 37°C, pH 7.4. The radiolabeled sample was gravity loaded and then eluted from the SEC column with running buffer at a flow rate of approximately 17 ml/hr @ 4°C. Fractions (1 ml) were collected and assayed for radioactivity in an LKB (Gaithersburg, MD) Compugamma counter, and protein (Bradford, 1976). The fractions with peak radioactivity (47 - 52) were pooled and concentrated 2.5X with YM-10 filters. Protein purity was assessed by SDS-PAGE.

RESULTS

Optimization of the in vitro binding assay. The pH of 10 mM buffer added to the incubation mix had essentially no effect on the arsenite binding activity of guinea pig or rabbit liver cytosolic proteins within the range assayed (figure 5.1a). Arsenic protein complexes in bovine kidney were observed to be stable over a pH range of 4.5 to 10.5 with maximum stability at pH 5.5 (Chatt and Jayawickreme, 1989).

Proteins may be fractionated by their solubility in acetone. Soluble protein could not be detected when final concentrations in excess of 50% acetone terminated the *in vitro* binding assay. Figure 5.1b shows the increasing final concentrations of acetone used to precipitate proteins and the percentage of protein remaining soluble in those supernatants. Since Bogdan *et al.* (1994) reported a that ≥ 60% acetone (C_f) was required to fully precipitate arsenite binding proteins in rabbit cytosol, a final concentration of 65% was chosen for subsequent precipitation experiments in order to compare rabbit and guinea pig binding proteins.

The length of time required for optimal binding of arsenite to cytosolic proteins was examined in figure 5.1c. After 30 min, 85% of arsenite was protein bound and after 90 min, 91%: at 30 min, 93% of arsenite binding occurred in 1/3 time. Significant binding occurs within 15 min (83%) but Bogdan *et al.* (1994) reports only ~ 50% binding under similar conditions using rabbit liver cytosol as the source of arsenite binding proteins.

Arsenic binding protein ligand. In order to confirm that the predicted trivalent arsenic species was preferentially bound to cytosolic proteins, both oxidized arsenate and reduced arsenite were incubated separately with equal amounts of cytosolic protein. Increasing amounts of protein in the binding assay increased arsenite but not arsenate

figure 5.1a

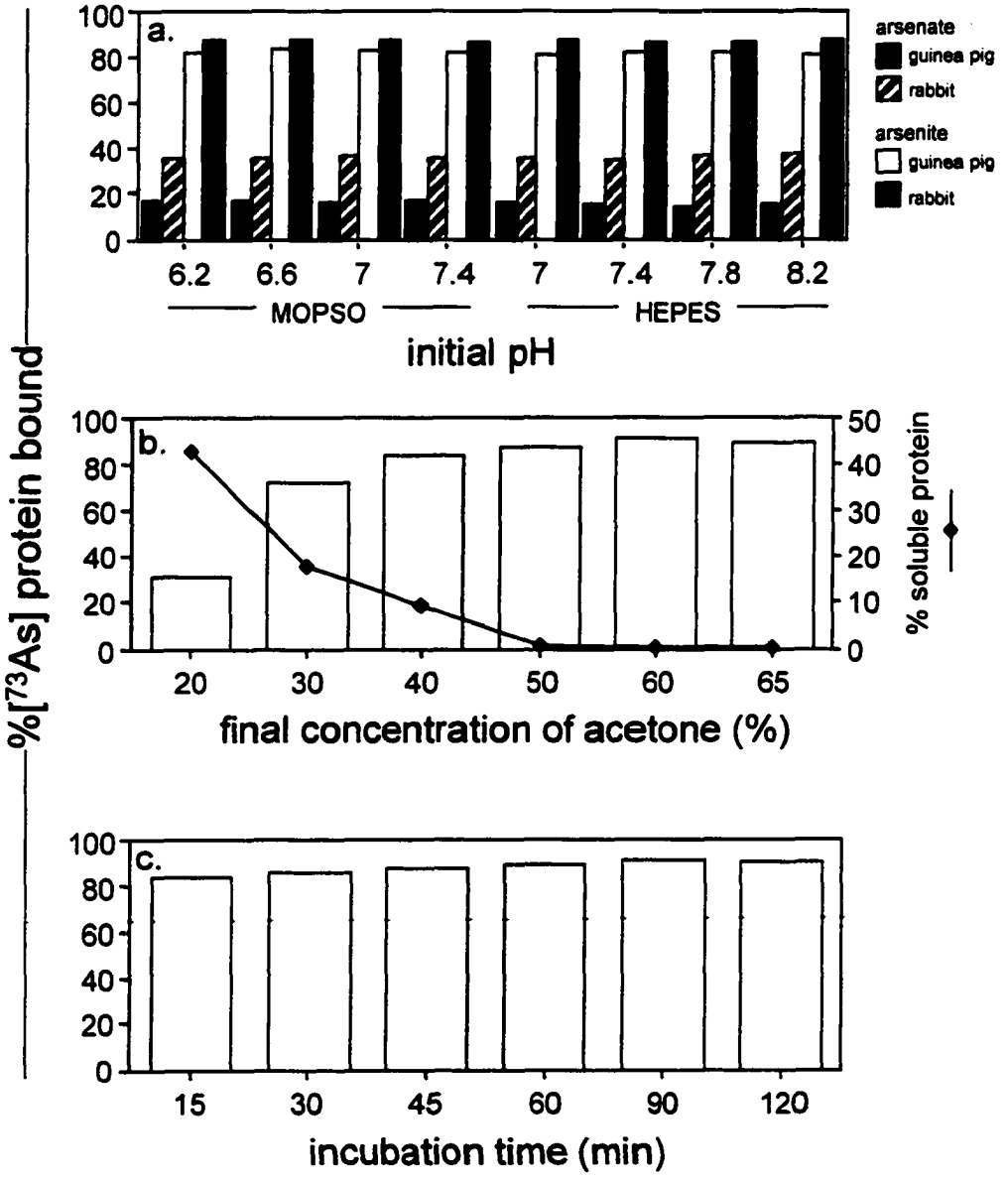
pH optima of arsenite binding protein(s). The pH-dependence of arsenite binding was determined by incubating 5 mg rabbit or guinea pig liver cytosolic protein in the presence of 0.294 μCi [^{73}As]arsenite and 10 mM MOPSO or HEPES buffer with initial pH range of 6.2 - 8.2.

figure 5.1b

Determination of acetone concentration required to fully precipitate arsenite binding protein(s). Guinea pig liver cytosolic protein (5 mg) was assayed for arsenic binding in the presence of 10 mM HEPES pH 7.4, 0.294 μCi [73As]arsenite for 30 min. The assay was terminated and protein precipitated by the addition of acetone to give a final concentration of 20 - 65%. Samples were centrifuged at 10500*g* for 10 min, pellets counted for γ-radioactivity and supernatants assayed for protein according to Bradford (1976).

figure 5.1c

Time course of [^{73}As]arsenite binding by cytosolic protein. Guinea pig liver cytosol (2 mg) was incubated @ 37°C for 15 -120 min in a 500 μL reaction mix containing 10 mM HEPES pH 7.4 and 0.294 μCi [$^{73}As^{III}$]. At assay termination, radiolabeled, precipitated protein was quantified.


a.
100
80
60
40
20
0
arsenate
guinea pig
rabbit
arsenite
guinea pig
rabbit
6.2
6.6
7
7.4
7
7.4
7.8
8.2
MOPSO
HEPES
initial pH
b.
50
40
30
20
10
0
% soluble protein
20
30
40
50
60
65
final concentration of acetone (%)
c.
15
30
45
60
90
120
incubation time (min)
%[73As] protein bound

binding (figures 5.2a, 5.2b). Relatively minimal binding of arsenate to protein occurred even amid 10 mg liver cytosol. Contrariwise, arsenite binding increased within the linear range of 0.5 - 5 mg rabbit or guinea pig cytosolic protein. Albumin contains 35 cysteinyl residues and all but one are involved in disulfide bridges (Brown, 1976). Since arsenite has a high affinity for protein sulfhydryl groups, its affinity for the single free thiol in BSA was examined. Neither oxidized nor reduced inorganic arsenic bound to BSA markedly. Within the linear range of arsenite binding, [^{73}As]arsenite counts associated with BSA were essentially undetected (< twice background).

Figure 5.2c shows the effect of increasing concentrations of unlabeled arsenite on the fractional occupancy of binding sites by radioactive ligand. At the concentration of arsenite which yields 50% fractional occupancy, the dissociation constant (K_D) can be assigned (figure 5.2d).

The specificity of arsenite binding in guinea pig and rabbit liver cytosol was determined according to Titeler (1983). Arsenite was incubated with cytosolic proteins at concentrations far above and far below K_D for guinea pig cytosol, 600 - 1,000 μM and 6 - 10 μM $NaAsO_2$, respectively. Equivalent amounts of [^{73}As]arsenite were used for each assay so that the specific activity of the former reaction was ≤ 1% of the latter. Arsenite-specific binding sites on guinea pig cytosolic protein(s) were determined to be 54 ± 4%

figure 5.2a

Arsenite is the preferred substrate for arsenite binding protein(s). Under optimum conditions, 0.5, 1, 5 or 10 mg of rabbit or guinea pig liver cytosol or BSA were reacted with [^{73}As]arsenite and assayed for arsenite-protein binding. Ordinate values represent the fraction of total radioactivity (0.294 μCi) detected in the pellet. Values were corrected for non-specific adsorption of [^{73}As] to reaction vessels (assay in absence of protein).

figure 5.2b

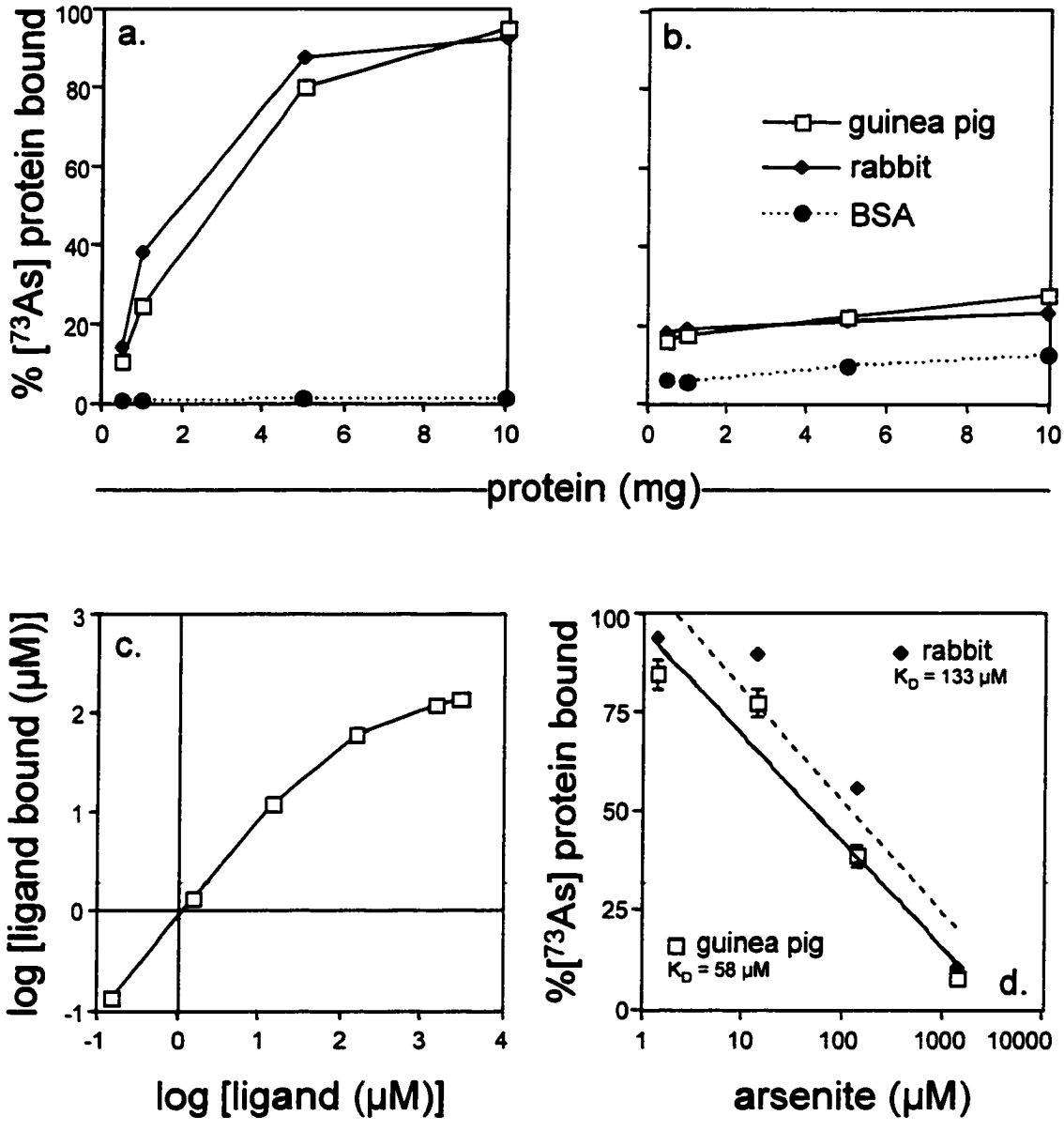
Arsenate does not bind well to arsenite binding protein(s). Increasing amounts of rabbit or guinea pig liver cytosol or BSA were incubated with 0.294 μCi [^{73}As]arsenate in an assay optimized for arsenite-protein binding.

figure 5.2c

Competition assay. Guinea pig liver cytosolic protein (2 mg) was assayed for arsenite-protein complex in the presence of 0.294 μCi [^{73}As]arsenite and increasing concentrations of cold $NaAsO_2$. Bound and free ligand ([^{73}As]arsenite) were separated by 65% acetone precipitation. This saturation plot is modeled after the binding of a ligand to its cognate membrane receptor.

figure 5.2d

K_D plot of guinea pig (n = 3) and rabbit (n = 1) arsenite binding protein(s). Guinea pig or rabbit liver cytosolic protein (2 mg) was assayed for arsenite-protein complex in the presence of 0.294 μCi [^{73}As]arsenite and increasing concentrations of cold $NaAsO_2$. Regression coefficients (r^2) of 0.944 and 0.900 were observed for guinea pig and rabbit cytosol, respectively.


a.
b.
% [73As] protein bound
guinea pig
rabbit
BSA
protein (mg)
c.
log [ligand bound (μM)]
log [ligand (μM)]
%[73As] protein bound
rabbit
KD = 133 μM
guinea pig
KD = 58 μM
d.
arsenite (μM)

of the total (specific + non-specific) number of possible binding sites. Similarly, arsenite-specific sites on rabbit liver cytosolic protein(s) accounted for 59% of the total binding sites (data not shown). The previously reported value for rabbit liver arsenite protein binding specificity is 67% (Bogdan *et al.*, 1994). Guinea pig liver cytosol specific binding is represented in figure 5.2c. Modeled on the very specific ligand-receptor interaction, specific binding is hyperbolic and shows saturation. Guinea pig liver cytosol arsenite binding activity reached saturation at 150 μM (figure 5.2c).

Purification of arsenite binding protein(s). Bioaffinity resin exploits a unique property of an extremely specific biological interaction to achieve separation and purification. Theoretically, bioaffinity chromatography is capable of giving absolute purification even from complex mixtures in a single process. However, this method requires detailed preliminary knowledge of structure and biological specificity of the compound to be purified. Guinea pig liver cytosolic arsenite binding proteins are not monothiols, have an intermediate affinity for trivalent arsenite and are relatively specific for ligand binding (figures 5.2a through d).

Blicke and Smith (1929) synthesis of *p*-amino phenylarsine oxide yielded ~ 3g. The melting point for n = 3 batches was determined to be 84 - 86 °C. Approximately 1.3 g of yield was solubilized in 27% DMSO and reacted with approximately 20 g Novarose

(10,000/40) Act^{High} for 60 hr. The total arsenic content of the PAPAO resin was determined by HGAFS to be 22.8 ± 0.4 mg As/g resin or ~ 90 μmol ligand/ml resin.

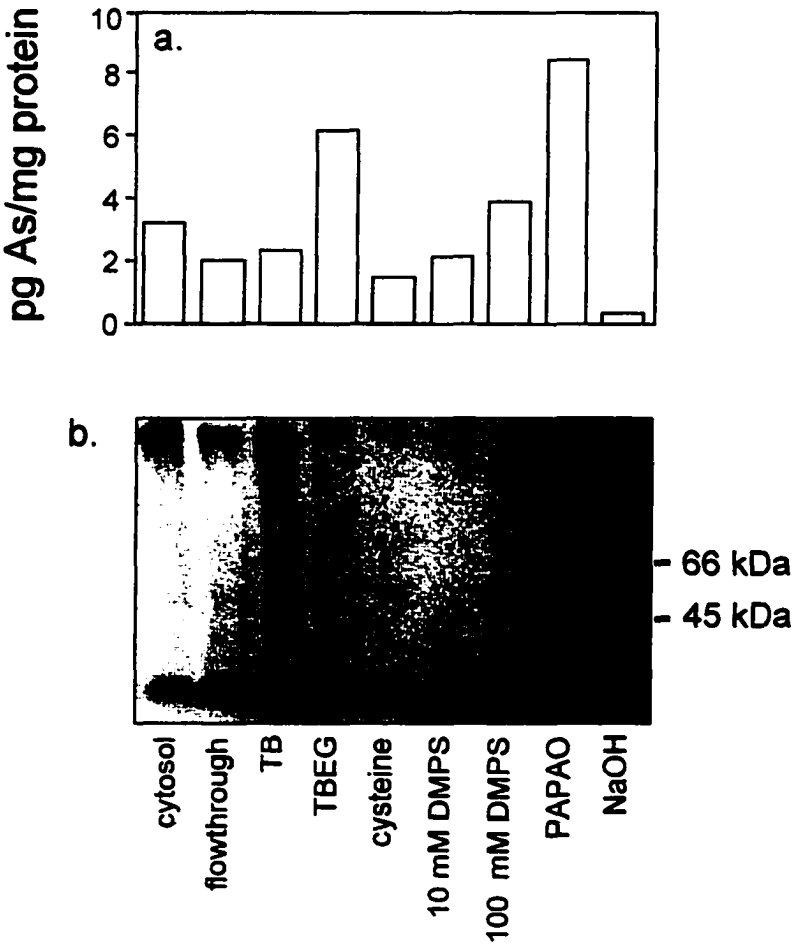
Batchwise elution of guinea pig liver cytosol from PAPAO resin yielded 46% total protein recovery. Of the protein eluted, the majority of protein was eluted with ligand (~ 20%) or under denaturing conditions (also ~ 20%). Approximately 17% of the successfully eluted protein was displaced by DMPS. Each eluate was desalted, concentrated and assayed for binding activity. The fraction displaced by DMPS bound 3.87 pg As/mg protein (figure 5.3a) and yielded a single [^{73}As]arsenite labeled band when resolved on SDS-PAGE and autoradiographed (figure 5.3b). The protein eluted by PAPAO bound 8.4 pg As/mg protein in an *in vitro* binding assay (figure 5.3a). However, it is not apparent that this *in vitro* binding was specific since [^{73}As]arsenite labeled protein was not as persistant through SDS-PAGE as was the DMPS displacement (figure 5.3b). To wit, PAPAO may have shifted the equilibrium of vicinal thiols of two proximate molecules (*i.e.* 2 non-specific monothiols) as well as specific dithiols to the mobile phase. Purportedly, DMPS displaces molecules bound to trivalent arsenic via 2 coordinate As-S complexes with 1:1 stoichiometry. Because the 100 mM DMPS fraction also yielded several Coomassie stained bands after 12% SDS-PAGE, arsenite binding protein(s) eluted with 100 mM DMPS were further purified on a

figure 5.3a

Arsenite binding activity after arsenic bioaffinity chromatography. Guinea pig cytosol was purified by batch adsorption, selectively eluting arsenite binding protein(s) with the dithiol, DMPS as described in MATERIALS AND METHODS. Fractions (2 ml) were collected and 500 μg of each concentrated eluate was assayed for arsenite binding in 250 μl 10 mM HEPES pH 7.4 containing 0.294 μCi [^{73}As]arsenite. The specific activity of the reduced arsenite solution was 11.4 pg As/66,000 cpm.

figure 5.3b

Autoradiogram of partially purified arsenic binding protein(s). Guinea pig cytosol was reacted with PAPAO-agarose and successively eluted with monothiol, dithiol and PAPAO. Fractions (2 ml) were collected, concentrated and 10 μg each assayed for arsenite binding in a 10 μl buffered reaction. After 30 min at 37°C, 10 μl 2X Laemmli (-βME) buffer was added to each reaction mix which were then resolved on SDS-PAGE (4-100%). The fixed gel was allowed to expose X-omat film for 72 hr.


pg As/mg protein
10
8
6
4
2
0
a.
b.
66 kDa
45 kDa
cytosol
flowthrough
TB
TBEG
cysteine
10 mM DMPS
100 mM DMPS
PAPAO
NaOH

calibrated S300-HR column with a broad fractionation range of 10 - 1500 kDa. One broad peak of protein and three peaks of radioactivity were observed after 88 ml elution. The first peak of radiation represented globular protein larger than the exclusion limit of the column, the second represented protein with an approximate molecular weight of 115 kDa and the third represented unbound [^{73}As]arsenite (figure 5.4c). Fractions constituting the 115 kDa peak were pooled and 39 μg resolved on 12% SDS-PAGE (figure 5.4d). Radioactivity could neither be detected in fixed gels by autoradiography nor Phosphor-imaging. After silver staining, one band was detected on a native gel and two prominent bands on a denaturing gel (figure 5.4d). Comparing figures 5.3b and 5.4d, it appears that a ≥ 50 kDa band which may dimerize (figure 5.4c) is suspect for arsenite binding. SEC-purified protein precipitated in 2-D isoelectric focusing rehydration buffer (8 M urea. 0.5% CHAPS, 20 mM DTT, 0.1 - 0.2% Biolytes; Bio Rad).

DISCUSSION

Arsenic binding protein(s) (AsBP) were sought in guinea pig liver cytosol because 1.) the guinea pig does not methylate arsenic, 2.) the liver is the principle organ of detoxification (first pass elimination) and 3.) twice as much arsenite bound to proteins in rabbit liver cytosol than in any other subcellular fraction (nuclei, mitochondria,

figure 5.4a

Determination of void volume (V_o) of a 50 × 1.5 cm Sephacryl-300HR column. Blue Dextran 2,000 (4.7 mg) was gravity loaded and chromatographed at ~ 17 ml/hr in 50 mM HEPES pH 7.4, 150 mM NaCl and detected by absorbance at 620 nm.

figure 5.4b

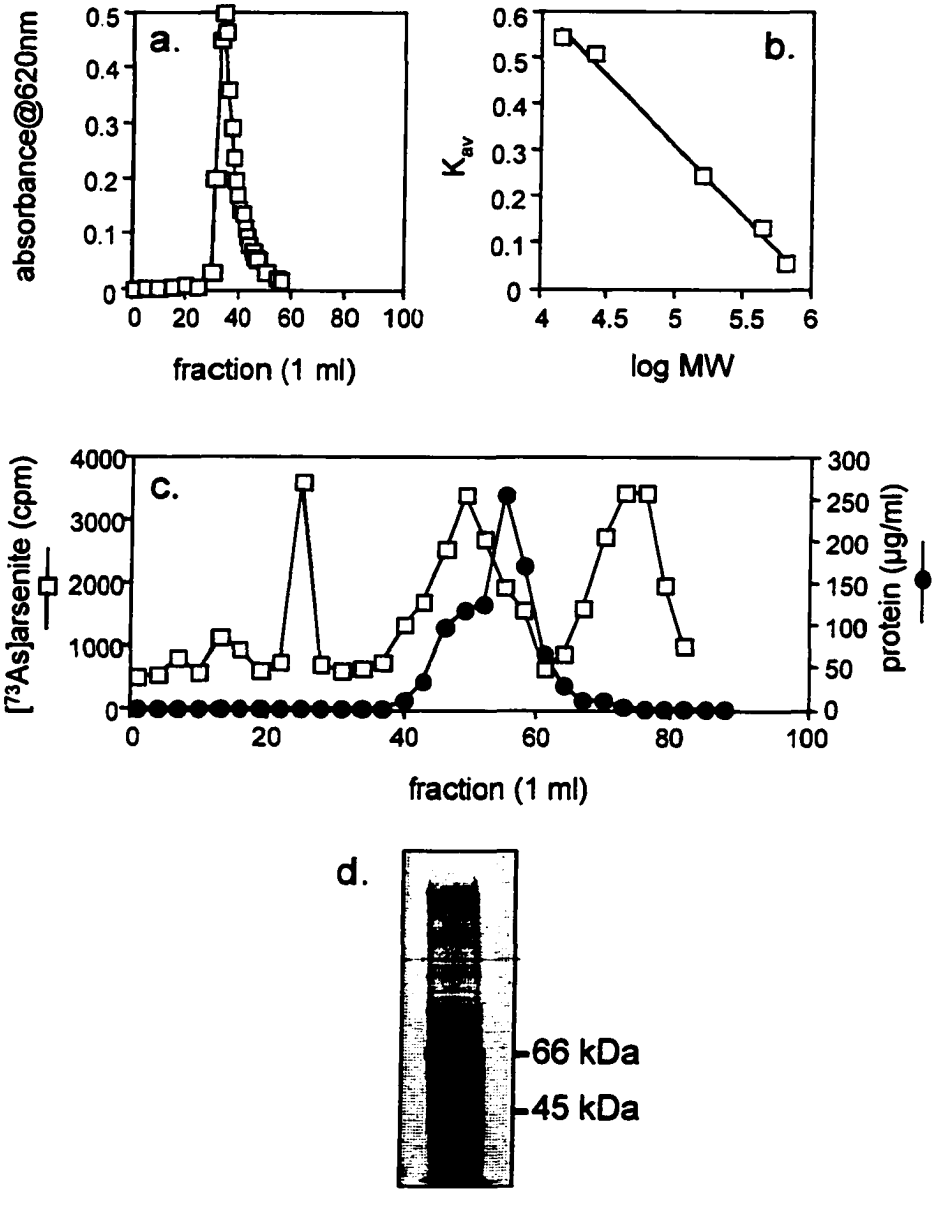
Calibration of 87 ml (V_t) S-300HR. Column was calibrated with 10.9 mg a-chymotrypsinogen (25 kDa), 10.4 mg thyroglobulin (669 kDa), 11.3 ribonuclease A (13.7 kDa), 10.1 mg aldolase (158 kDa) and 9.5 mg ferritin (440 kDa) in 50 mM HEPES, 150 mM NaCl pH 7.4 at 4°C and ~ 17 ml/hr . A regression coefficient of 0.995 was observed.

figure 5.4c

Size exclusion chromatogram of [$^{73}As^{III}$]-labeled protein(s). Protein eluted with 100 mM DMPS was labeled with 50 μCi [^{73}As]arsenite in 50 mM HEPES, 150 mM NaCl pH 7.4 for 30 min at 37°C with gentle agitation. Labeled protein was gravity loaded onto the calibrated column in 2ml running buffer and chromatographed at ~ 17 ml/hr. Fractions (1 ml) were assayed for radioactivity and protein.

figure 5.4d

PAG Electrophoretogram of SEC-purified arsenite binding protein(s). Fractions with peak radioactivity (47-52) were pooled, concentrated, resolved on SDS-PAGE (7.5%) and visualized with silver nitrate.


a.
absorbance@620nm
0.5
0.4
0.3
0.2
0.1
0
0 20 40 60 80 100
fraction (1 ml)
b.
K_{av}
0.6
0.5
0.4
0.3
0.2
0.1
0
4 4.5 5 5.5 6
log MW
c.
[^{73}As]arsenite (cpm)
4000
3000
2000
1000
0
0 20 40 60 80 100
fraction (1 ml)
protein (µg/ml)
300
250
200
150
100
50
0
d.
66 kDa
45 kDa

lysosome, endoplasmic reticulum, Bogdan *et al.*, 1994). Previous reports have ascribed 50-65% arsenite binding protein(s) to cytosol (Klaassen, 1974; Marafante *et al.*, 1981, Vahter and Marafante, 1983).

If, as hypothesized, the guinea pig compensates for an arsenite methyltransferase deficiency with keen arsenic sequestration, the results reported here do not evince arsenite binding as the primary mode of arsenic detoxification: In liver cytosol, arsenite binding in the guinea pig parallels that of the rabbit which is a human model of methylation (Vahter and Marafante, 1988; Maiorino and Aposhian, 1985). It is not apparent that guinea pig cellular thiols serve more effectively as a sink for As^{III} species.

However, it is pertinent and prudent to note that cytosol is not the ideal source with which to characterize arsenite binding kinetics. Arsenite binding activity of guinea pig liver cytosol was not sufficiently purified. Arsenic bound proteins have been observed in bovine kidney cytosol (38 kDa; Chatt and Jayawickreme, 1989), rat liver cytosol (> 1,000, 135 and 38 kDa; Styblo and Thomas, 1997) and rabbit liver cytosol (>2,000, 450 and 100 kDa; Bogdan *et al.*, 1994) but none have been identified by amino acid sequence. Once the secondary structures of these proteins with the homologous function of sequestering arsenic are known, comparison may elucidate the guinea pig's tolerance of inorganic arsenic despite his lack of putative, model arsenic detoxification.

Nor have these proteins been shown to be a mechanism of detoxification (Chatt and Jayawickreme, 1989; Styblo and Thomas, 1997; Bogdan *et al.*, 1994). Future experiments in characterizing AsBP(s) may hypothesize the attenuation of arsenite-inhibition of pyruvate dehydrogenase (PDH; Peters 1955) by AsBP. It would also be of interest to compare the ability of purified AsBP to prevent PDH inhibition to that of purified arsenite methyltransferase.

AsBP(s) is expected to have a greater effect in preventing inhibition of PDH sulfhydryls than in reactivating them. DMPS was found to completely prevent inhibition of PDH complex activity at a DMPS:As ratio of 2:1. DMPS completely reversed (via reactivation of sulfhydryls) arsenic inhibition of PDH at concentrations 3 times greater than arsenite (Hsu *et al.*, 1983). Administration of DMPS increased urinary excretion of inorganic arsenic and MMA but decreased urinary excretion of DMA (Aposhian *et al.*, 1997a). DMPS-As chelates may be present in the urine of As-exposed humans treated with DMPS (Aposhian *et al.*, 1997a). This suggests that arsenite is very tightly bound to DMPS. AsBP is expected to have a lower affinity for arsenite than DMPS which would allow for the slow release of inorganic arsenic into the methylation pathway and the subsequent yield of dimethylated arsenic. Since urinary excretion is the principal elimination pathway, biliary/fecal excretion of an AsBP-As complex is not likely. For

this reason, proteins strategically displaced from the PAPAO bioaffinity column with DMPS were regarded as the arsenite binding protein(s) for further purification and characterization. Despite 46% protein recovery, this bioaffinity elution strategy may prove veritable with further investigation. Batchwise elution proved more convenient than chromatography on a column where binding activity could not be maintained likely because of oxidation.

6.

IS ARSENITE METHYLTRANSFERASE 1 CYS PEROXIREDOXIN?

STATEMENT BY THE AUTHOR

The author wishes to acknowledge Rhett Michelson's extremely generous, patient and insightful assistance with the cloning and expression of recombinant 1 cys peroxiredoxin in *E. coli*. Likewise, the help of Deanna Adams with transient expression experiments in COS 7 cells is recognized with immense gratitude.

INTRODUCTION

By measuring the formation of [^{3}H]MMA or [^{3}H]DMA, arsenite and MMA methyltransferase activities have been biochemically purified from rabbit (Zakharyan *et al.*, 1995), Chang human hepatocytes (Zakharyan *et al.*, 1999) rhesus monkey (Zakharyan *et al.*, 1996) and hamster liver (Wildfang *et al.*, 1998). The molecular masses of both methyltransferases of rabbit liver were determined to be ~ 60 kDa (Zakharyan *et al.*, 1995). The identical chromatographic elution profiles of arsenite and MMA methyltransferase activities in each of these animal species suggest dual activities of a single protein which catalyze the methylation of both arsenite and methylarsenite. There is no evidence for these enzyme activities being on different protein molecules. Phosphatidylethanolamine N-methyltransferase and other mammalian small molecule

methyltransferases perform successive methylations of their substrate (Ridgway and Vance, 1987). With partially purified hamster and 2,000-fold rabbit liver arsenite and MMA methyltransferase enzyme preparations, the major requirements for enzyme activity appear to be arsenite or MMA^{III}, a thiol and S-adenosyl methionine (Zakharyan *et al.*, 1995; Zakharyan *et al.*, 1999).

Arsenite is the substrate for arsenite methyltransferase. At concentrations of 20, 50 and 100 μM, arsenite is approximately 140, 60 and 258 times more active, respectively, than arsenate as a substrate of arsenite methyltransferase in the rabbit (Zakharyan *et al.*, 1995). Only 10% arsenite methyltransferase activity was observed when arsenite was replaced with arsenate using partially purified hamster liver cytosol as the source of enzyme (Wildfang *et al.*, 1998).

A monothiol was necessary for optimal results of both arsenic methyltransferase activities using the standard assay conditions. However, there was not a specific GSH requirement for these methyltransferases. Arsenite methyltransferase activity was greatest when L-cysteine was present. Even though GSH is the major thiol in the liver, it is likely not a cofactor of these methyltransferase enzymes since results with 2,000-fold purified rabbit enzymes indicate that arsenite and MMA methyltransferases do not have an absolute requirement for GSH. The enzymes appear only to require a reducing

environment which can be supplied by GSH and other thiol or dithiol compounds (Zakharyan *et al.*, 1995; Wildfang *et al.*, 1998; Zakharyan *et al.*, 1999). It has been reported that GSH at concentrations greater than 1.0 mM inhibits MMA production and concentrations greater than 3.0 mM inhibit DMA formation in rat liver cytosol (Buchet and Lauwerys, 1988). But both hamster and rabbit purified methyltransferase activities were stimulated by GSH, with arsenite methyltransferase being more greatly affected than the MMA methyltransferase (Zakharyan *et al.*, 1995; Wildfang *et al.*, 1998).

Rabbit arsenite and MMA^{III} methyltransferases display Michaelis-Menton kinetics with substrate saturation occurring at 20 and 70 μM, respectively (Zakharyan *et al.*, 1995; Zakharyan *et al.*, 1999). Rabbit arsenite and MMA methyltransferase V_{max} values are considerably larger than the hamster enzyme velocities (Wildfang *et al.*, 1998) which may explain why the rabbit excretes a greater amount of the total arsenic dose as compared to the hamster in a given period of time (Hirata *et al.*, 1990; Vahter and Marafante, 1985). Comparison of the kinetics of purified hamster and rabbit arsenite and MMA methyltransferase(s) suggests that the rate of methylation of inorganic arsenite may be a major factor in determining the urinary arsenic metabolite profiles observed in these species following inorganic arsenic exposure.

The rate of methylation and consequent excretion of oxidized metabolites likely also factors into toxicity of arsenic. Undoubtedly, oxidative methylation renders arsenite innocuous. MMA^V may be excreted but the majority is committed through dimethylation and thus reduced (Vahter and Marafante, 1985). MMA^{III} is the most toxic intermediate but the reduction of of MMA^V is checked by MMA^V reductase ($K_M = 2 \times 10^{-3}$ M), the rate limiting enzyme in this pathway. Purified arsenite methyltransferase has been extensively characterized by this laboratory but because of low abundance and activity loss, the protein sequence has been elusive. The objective of this research project was to identify the protein sequence of arsenite methyltransferase and confirm the sequence with recombinant protein expression. Once the protein responsible for arsenic methylation is identified, it may be manipulated to decipher the effect of methylation on arsenic toxicity.

MATERIALS AND METHODS

Reagents. S[methyl-^{3}H]Adenosyl-L-methionine ([^{3}H]SAM, 55 - 85 Ci/mmol) was purchased from Dupont-NEN (Boston, MA). $As(GS)_3$ was prepared according to Scott *et al.* (1993). ACS reagent grade sodium arsenite, H_2O_2 and cumene peroxide, ebselen, glutathione reductase from bakers yeast, ANTI-FLAG M2 monoclonal antibody and a FLAG-tagged protein immunoprecipitation kit were purchased from Sigma Chemical Co.(St. Louis, MO); *Pwo* DNA polymerase, CIAP, T4 DNA ligase, *Eco*RI, *Xba*I, *Not*I

and *Cla*I from Roche Molecular Biochemicals (Indianapolis, IN); *Nde*I, *Eag*I and *Bgl*II from New England Biolabs (Beverly, MA) and pET-21b from Novagen (Madison, WI). pET-16b and *E. coli* BL21 and DH5α were generously provided by the Weinert Lab; pESC-URA and *S. cerevisiae* YHP strains by the Parker Lab; pcDNA3.1/HisC and COS 7 cells by the Vaillancourt Lab; monoclonal antibodies mAb16 and mAb17 from Aron B. Fisher (Chen *et al.*, 2000) and polyclonal antibodies pAb35 from Hitoshi Shichi (Peshenko *et al.*, 2001). Spectra/Por MWCO 12-14,000 dialysis tubing was obtained from VWR Scientific (Phoenix, AZ). All other chemicals were analytical reagent grade or of the highest quality obtainable. All water was doubly deionized, distilled and filtered (ddiH_2O).

Preparative scale purification of arsenite methyltransferase activity. Male New Zealand white rabbits (2.5 kg) were purchased from Myrtle's Rabbitry (Thompson Station, TN) and acclimated prior to experimentation for at least 2 weeks in an environmentally controlled animal facility operating on a 12:12 light cycle and at 22°C. Animals were provided Teklad (Indianapolis, IN) 0533 Rabbit Diet #7008 and water *ad libitum*. Five rabbits were used for the preparative scale purification of arsenite methyltransferase.

Animals were euthanized with CO_2. Livers were removed immediately, rinsed in ice-cold 0.9% NaCl, cut into approximately 5 g pieces and homogenized in a 30 ml glass mortar equipped with a motor-driven Teflon-coated stainless steel pestle with 2 volumes of 10 mM Tris-HCl pH 7.6 at 4°C, 250 mM sucrose, 0.5 mM GSH, and protease inhibitors at concentrations of 10 μg leupeptin, 10 μg aprotinin, 5 μg pepstatin A, 25 μg trypsin-chymotrypsin inhibitor and 35 μg PMSF per ml homogenization buffer. The homogenate was spun at 14,600*g* for 15 min at 4°C in a Beckman (Fullerton, CA) Model J2-21M induction drive centrifuge. The supernatant was further spun at 105,000*g* for 90 min in a Beckman Model L2-65B ultracentrifuge at 4°C and the supernatant yield designated as cytosol.

Arsenite methyltransferase activity was purified following essentially the scheme of Zakharyan *et al.* (1995) (table 6.1). Cytosol (500 ml, 14.0 g, 2010 U) was gravity-loaded onto a DEAE-cellulose (Bio Rad, Hercules, CA) anion exchange column (9 x 17 cm) (Elmo Maiolatesi, Baltimore, MD) which had been pre-equilibrated with 10 mM Tris-HCl pH 7.6 at 4°C. The column was washed until the absorbance (280 nm) returned to baseline and was then eluted at 4°C and at a rate of 240 ml/hr with a linear gradient of 1.8 L 30 mM Tris-HCl (pH 7.6) and 1.8 L 30 mM Tris-HCl, 0.5 M NaCl (pH 7.6). Fractions (16 ml) were collected and every fifth assayed for arsenite

table 6.1
prep-scale purification of arsenite methyltransferase from rabbit liver

preparation	(ml)	(mg/ml)	(mg)	(pmol/ml)	(pmol/mg)	(pmol)	(x-fold)
cytosol	500	29	14,500	3.77	0.13	1885	
DEAE cellulose	1136	1.9	2192	4.3	2.22	4877	17
50-85% $(NH_4)_2SO_4$	40	27.4	1099				
Sephadex G-200	216	3.5	749.5	48.1	13.8	9803.7	106
100% $(NH_4)_2SO_4$	20	30.4	608				
Sephadex G-100	128	1.6	200.4	81.2	48.7	9749.2	374
Q-support	72.5	0.39	27.9	38.4	99.7	2781	767
phenyl sepharose	98	0.02	2	4.6	224	452	1725
Red-120	10	0.005	0.053	4.9	926.7	48.7	7129

methyltransferase activity and protein and NaCl concentration. Fractions with peak arsenite methyltransferase activity, eluted with 0.15 – 0.17 M NaCl, were combined.

Pooled DEAE fractions (1152 ml, 2193 mg, 4877 U) were precipitated at 50 – 85% $(NH_4)_2SO_4$ saturation. The solution was spun at 14,600*g* for 15 min at 4°C and the consequent pellet resuspended in 32 ml 30 mM Tris-HCl, 100 mM NaCl pH 7.6 at 4°C to yield 1099 mg protein in a total volume of 40 ml.

The 50-85% $(NH_4)_2SO_4$ fractionated protein (40 ml) was gravity-loaded onto a Sephadex G200 (Sigma, St. Louis, MO) size exclusion column (5 x 67 cm) which had been pre-equilibrated with 30 mM Tris-HCl, 100 mM NaCl pH 7.6 at 4°C. The column was eluted at 4°C with equilibration buffer at 30 ml/hr. All fractions (12 ml) were assayed for protein concentration. Each fraction with $V_e \geq 680$ ml was assayed for enzyme activity. Fractions with peak arsenite methyltransferase activity, eluted with 696 - 900 ml 30 mM Tris-HCl, 100 mM NaCl pH 7.6 at 4°C, were combined.

Pooled G200 fractions (216 ml, 750 mg, 9803 U) were concentrated by adding 210 g $(NH_4)_2SO_4$ (> 100% saturation), centrifuging (14,600*g* for 15 min at 4°C) and resuspending the precipitate in 30 mM Tris-HCl, 50 mM NaCl pH 7.6 at 4°C. The concentrated protein (20 ml, 608 mg) was then gravity-loaded onto a Sephadex G100 (Pharmacia, Uppsala, Sweden) size exclusion column (5 x 67 cm) which had been pre-

equilibrated with 30 mM Tris-HCl, 50 mM NaCl pH 7.6 at 4°C. The column was eluted at 4°C with equilibration buffer and with a flow rate of 24 ml/hr. Fractions (8 ml) were collected and assayed for protein content and arsenite methyltransferase activity. Peak arsenite methyltransferase fractions, those eluting with 216 – 336 ml 30 mM Tris-HCl, 50 mM NaCl pH 7.6 at 4°C, were combined.

Pooled G100 fractions (104 ml, 169 mg and 9749 U) were loaded (140 ml/hr) onto a MacroPrep High Q-support (Bio Rad, Hercules, CA) strong anion exchange column (1.5 x 45 cm) which had been pre-equilibrated with 10 mM Tris-HCl pH 7.6 at 4°C. The column was eluted at 4°C and at a rate of 140 ml/hr with a linear gradient of 400 ml 10 mM Tris-HCl pH 7.6 and 400 ml 10 mM Tris-HCl, 0.3 M NaCl pH 7.6. Fractions (14.5 ml) were collected and assayed for arsenite methyltransferase activity and protein and NaCl concentration. Peak arsenite methyltransferase activity was observed in fractions 23 - 27 which eluted with approximately 0.095 - 0.115 M NaCl and were combined.

Pooled fractions (73 ml, 28 mg, 2781 U) were saturated 30% with ammonium sulfate and then gravity-loaded onto a Phenyl Sepharose 6 Fast Flow (40 μmol phenyl/ml gel, Pharmacia, Uppsala, Sweden) column (1 x 13.8 cm) which had been pre-equilibrated with 600 ml 1.5 M $(NH_4)_2SO_4$, 30 mM Tris-HCl, 50 mM NaCl pH 7.6 at 4°C. The

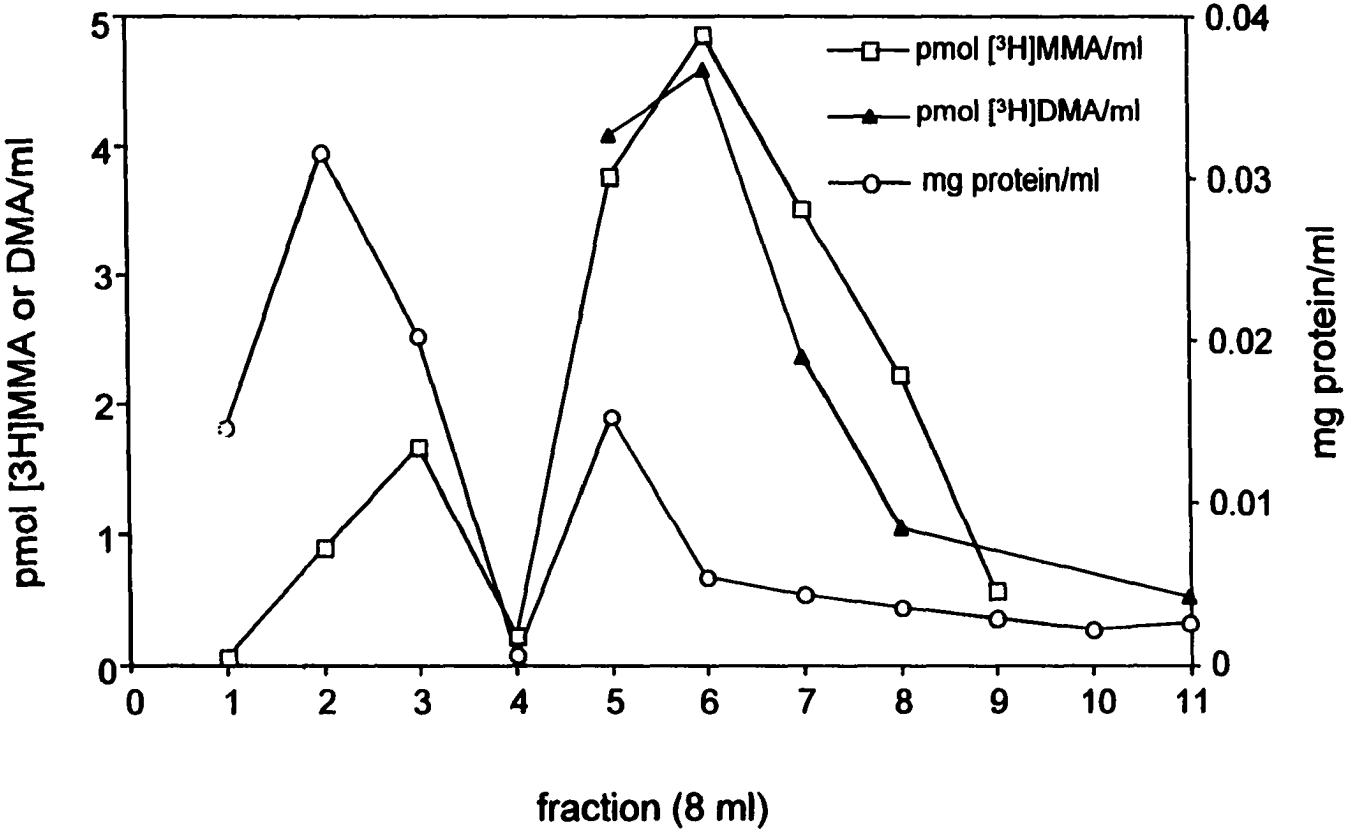
column was eluted at 4°C with a 360 ml linear gradient of 30 mM Tris-HCl, 50 mM NaCl, 1.5 M $(NH_4)_2SO_4$ pH 7.6 at 4°C and 30 mM Tris-HCl, 50 mM NaCl pH 7.6 at 4°C. Fractions (15 ml) were collected and assayed for arsenite methyltransferase activity and protein and ammonium sulfate concentration. Peak arsenite methyltransferase activity was observed in fractions 24 and 25 which eluted with 0.242 – 0.197 M $(NH_4)_2SO_4$.

Fractions 24 and 25 were combined to yield 30 ml, 1.5 mg and 289 U and gravity loaded onto a Reactive Red 120 (Sigma, St. Louis, MO) immobilized dye column (1.5 x 1.7 cm) which had been preequilibrated with 20 mM Tris-HCl, 50 mM NaCl pH 7.6 @ 4°C. Arsenite methyltransferase was eluted stepwise with 20 mM Tris-HCl, 0.8 M NaCl (pH 7.6 @ 4°C) at a flow rate of 45 ml/hr. Fractions (10 ml) were collected and assayed for arsenite methyltransferase activity and protein concentration. Upon final chromatography and after desalting and 5X concentration to 210 µl (2ml centricon 10, Amicon, Beverly) Fraction 6 from Reactive Red 120 (figure 6.1) was assayed for arsenite methyltransferase activity, submitted to the LPSA, Arizona Research Laboratories at the University of Arizona for amino acid sequencing and resolved by 12% PAGE.

Bacterial expression of 1 cys peroxiredoxin (1 cys Prx). The EST (AI116776) encoding AOP2_MOUSE (accession no. O08709) was obtained from The IMAGE Consortium of Lawrence Livermore National Laboratory in *E. coli* harboring pME18s-FL3 (Amp^R).

figure 6.1

Reactive Red 120 chromatogram of arsenite methyltransferase activity. Rabbit liver cytosolic protein was purified for arsenite methyltransferase activity according to table 6.1. Peak enzyme activity eluted from the prior Phenyl Sepharose column was loaded onto 1.5 × 1.7 cm column and eluted with 20 mM Tris-HCl, 0.8 M NaCl pH 7.6 at 4°C with a flow rate of 45 ml/hr. Fractions (8 ml) were collected and assayed for protein content (Bradford, 1976) and arsenite methyltransferase activity (Zakharyan *et al.*, 1995) using either $NaAsO_2$ or MMA^V as the substrate.


pmol [3H]MMA/ml
pmol [3H]DMA/ml
mg protein/ml
pmol [3H]MMA or DMA/ml
mg protein/ml
fraction (8 ml)
0
1
2
3
4
5
6
7
8
9
10
11
0
0.01
0.02
0.03
0.04

The source of this clone was Sugano mouse kidney. Plasmid DNA was isolated using essentially the alkaline lysis method (Maniatis *et al.*, 1989). The full length expressed sequenced tag (EST) was amplified by PCR in a 50 µl buffered reaction containing 2.5 U *Pwo* DNA polymerase, 0.2 mM dNTPs, 1 µM each forward and reverse primers and pME18s-FL4 as the template. The reaction was initiated by 2 min at 94°C followed by 20 cycles of: 94°C, 1 min; 55°C, 1 min; 72°C, 2 min and a 7 min extension at 72°C in a Gene PCR System 400 thermocycler (Perkin Elmer, Norwalk, CT). The oligomer 5'-GGG AAT TCC CAT atg ccc gga ggg ttg ctt ctc ggg g-3' was used as the forward primer. Reverse primers either encoded the stop codon UAA (5'-GGG AAT TCC ATA TGt taa ggc tgg ggt gta taa cgg agg tat ttt ttg c) or did not (5'-GGG AAT TCC ATA TGa ggc tgg ggt gta taa cgg agg tat ttt ttg c). PCR products were digested with excess (40 U) *Nde*1 endonuclease and resolved on a 0.8% agarose/TBE gel. The digested inserts (~ 672 bp) were visualized by ethidium bromide (EtBr), excised from the gel and purified according to Maniatis *et al.* (1989). Expression vectors pET-16b and pET-21b were also digested for 60 min at 37°C with excess (60 U) *Nde*1 and their 5' phosphoryl termini eliminated with successive addition of 2 U alkaline phosphatase from calf intestine (CIAP) to decrease the background of non-recombinants due to self-ligation of the vector. Vectors were agarose gel-purified to remove residual nicked and supercoiled plasmid which

transform very efficiently relative to the desired ligation product. In separate 30 µl buffered reactions, pET-16b (which contains a N-term $(His)_{10}$ tag) was ligated to the PCR insert containing a stop codon and pET-21b (which contains a C-term $(His)_6$ tag) was ligated to the PCR insert without a stop codon by the action of T4 DNA ligase (1 U).

pET-16b and –21b are both translation vectors which contain the highly efficient ribosome binding site from phage T7 major capsid protein; pET vectors are under the control of strong bacteriophage T7 transcription and translational signals. Host RNA polymerases do not initiate from T7 promoters and the cloning sites in pET plasmids are in regions weakly transcribed (if at all) by read-through activity of bacterial RNA polymerase. T7 RNA polymerase is specific for its own promoter, a conserved 23 bp sequence immediately upstream of the multiple cloning site (MCS) (Studier and Moffatt, 1986). Both vectors contain the *bla* (β-lactamase) coding sequence which confers ampicillin resistance and a relaxed bacterial origin of replication which ensures that the plasmid will be replicated; replication activity is not tightly linked to cell division so plasmid replication will be initiated far more frequently than chromosomal replication.

DH5α *E. coli*, the non-expression host does not contain T7 RNA polymerase gene which eliminates plasmid instability due to the production of proteins potentially toxic to the host cell. Additionally, the use of DH5α bacteria is prudent for maintaining plasmids

because they are *recA*$^-$ (recombination) and *endA*$^-$ (endonuclease) (Woodcock *et al.*, 1989). DH5α cells were chemically made competent with 10 mM PIPES, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, pH 6.7 (RJ Michelson, personal communication; Maniatis *et al.*, 1989). Competent bacteria were transfected by incubating 100 µl cells with 15 µl ligation mix on ice for 30 min, subsequent heat shock at 42°C for 90 s and 60 min recovery in Luria Bertani (LB) broth at 37°C. Transformed cells were plated on LB/Amp (50 µg/ml) agar and compared to negative (ligated vector absent insert) and positive (undigested empty vector) controls after 12-16 hours at 37°C. Plasmid DNA was isolated from 2 ml LB containing 1µg Amp/ml (LB/Amp) cultures and the forward orientation of the insert in pET-16b was determined with the restriction map generated by *Eag*1 and *Bgl*II endonucleases and the insert in pET-21b, with *Eag*1 and *Nde*1 (figure 6.4a).

Target protein expression was initiated by transferring the plasmid containing the 672 bp insert into an expression host, BL21(DE3) harboring a chromosomal copy of T7 RNA polymerase under *lacUV5* control: BL21 hosts are lysogens of bacteriophage DE3. DE3 is a λ derivative that has the immunity region of phage 21 and carries a DNA fragment containing *lacI* gene and *lacUV5* promoter and the gene for T7 polymerase. BL21 *int* prevents DE3 from integrating into or excising from the chromosome without

the input of helper phage. BL21 is also null for *ompT* which encodes an outer membrane trypsin-like protease which cleaves T7 RNA polymerase (Grodberg and Dunn, 1988). T7 polymerase is stable in *E. coli* but is very susceptible to proteolytic cleavage after cell lysis. Once DE3 lysogen is formed, the only promoter to direct transcription of the T7 polymerase gene is *lacUV5* promoter which is inducible by isopropyl β-D thiogalactopyranoside (IPTG), a β-galactosidase resistant galactoside which inactivates the *lac* operon repressor. The addition of IPTG to growing cultures of lysogen turns on transcription of T7 polymerase which is translated and subsequently transcribes target DNA. Recombinant 1 cys Prx is a T7 polymerase transcript.

Purification of recombinant 1 cys peroxiredoxin in E. coli. BL21(DE3) cells were chemically made competent and transformed as described for the non-expression host. One colony was grown up in LB/Amp overnight at 37°C. This culture was transferred to 100 ml LB/Amp and grown until OD_{600} was between 0.8 and 1.0. Protein expression was induced by the addition of 1 mM IPTG and incubating cells for 1 - 6 additional hr at 37°C. After a time of induction, cells were centrifuged at 4,000*g* for 20 min in a 50 ml conical tube. Pellets were washed once and resuspended in PBS, aliquoted to 1.7 ml Eppendorf tubes and centrifuged 3,000*g*. Homogenization buffer (1 ml, 10 mM Tris-HCl pH 7.6 at 4°C, 250 mM sucrose, 0.5 mM GSH, 200 μM PMSF, 5 mM imidazole) was

added to each Eppendorf tube, the bacterial pellets were sonicated (six 15 s bursts at ~ 75W with intermittent 30 s cooling periods) and the lysates were cleared by 10,000*g* for 20 min at 4°C and pooled. Ni-NTA superflow agarose (1 ml, Qiagen, Valencia, CA) was added to the cleared lysate and the resin allowed to react with His-tagged protein at 4°C with gentle rotation. After 90 min, the resin was poured onto a 3 x 1cm column and successively eluted with 15 ml 5 mM imidazole, 500mM NaCl, 20 mM Tris-HCl pH 7.9, 10 ml 60 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl pH 7.9 and finally, 4 ml 1 M imidazole, 500mM NaCl, 20 mM Tris-HCl pH 7.9. Fractions (5 ml) were collected stepwise. Protein eluted with imidazole was dialyzed into 20 mM Tris-HCl, 50 mM NaCl pH 7.6 @ 4°C overnight to yield a final imidazole concentration < 1 μM. The protein content was determined by the method of Bradford (1976) using bovine serum albumin standards.

Immunoprecipitation and Western analysis. Mouse and guinea pig cytosol were each pretreated with 30 μl rabbit IgG agarose and 30 μl rabbit serum agarose in a 300 μl reaction for 1 hr at 4°C on an orbital shaker to remove proteins which may bind non-specifically to agarose. IgG and serum agarose were removed by 200*g*, 1 min @ 4°C. Pooled monoclonal antibodies (5 μl each, mAb17 and mAb16) were added to mouse pre-cleared kidney cytosol (5.6 mg) and guinea pig pre-cleared kidney cytosol (6.4 mg). The

volume was made to be 1 ml with dilution buffer (TBS (50 mM Tris-HCl, 0.138 M NaCl, 2.7 mM KCl pH 8 @ 25°C), 1% Igepal, 1 mg BSA/ml). The antibody antigen reaction was allowed to incubate for 2 hr on ice. Protein G-sepharose 4 Fast Flow (100µl 40% slurry, 80 µg, Pharmacia) was added and the immunoprecipitation (Ip) reaction incubated overnight at 4°C with gentle rotation. Protein G is a streptococcal cell wall protein which has the unique property of binding to the IgG molecule, preferentially the F_c portion (Akerstrom and Bjorck, 1986). At 4°C, IgG bound sepharose was washed twice with 1 ml dilution buffer, 1 ml TBS and finally, 50 mM Tris-HCl pH 6.8. Ip reactions were monitored by non-reducing SDS-PAGE and Western analysis

Antigenic proteins resolved on polyacrylamide gel were transferred to PVDF membrane (0.2 µm, Bio Rad) by constant 40 V for 150 min in the presence of 24.8 mM Tris-HCl, 192 mM glycine pH 8.3. The membrane was washed thrice with TBS (20 mM Tris-HCl, 500 mM NaCl pH 7.5), 0.05% Tween 20 (polyoxyethylene sorbitan monolaurate, Sigma), and thrice with TBS. The membrane was then blocked with 2% (w/v) nonfat dry milk in a Tris-buffered 0.9% NaCl solution for at least 1 hr at 4°C. Blocked membranes were probed with pAb35, mAb16 or mAb17 at a titer of 1:2,000 in blocking solution. Hybridized membranes were either probed with rabbit anti-mouse IgG + IgA + IgM (Zymed, San Francisco, CA) 1:750 or HRP (horseradish peroxidase)-goat

anti mouse IgG (Zymed) 1:2,000 in the presence of 2% milk. Membranes probed with rabbit anti-mouse IgG + IgA + IgM were washed and then probed 1:15,000 with an additional, tertiary antibody, AP (alkaline phosphatase)-goat anti-rabbit IgG (Sigma). Polyclonal 1° antibodies were probed with AP-goat anti-rabbit IgG, 1:15,000. Antigenic protein reacted with an AP conjugate was visualized by brief incubation with the phosphatase substrate, 5-bromo4-chloro-3-indolyl phosphate/nitro-blue tetrazolium (Sigma). Membranes of target protein probed with the HRP conjugate was incubated with 5 mM H_2O_2 in the presence of 1.25 mM luminol and 200 μM *p*-coumaric acid and the Tris-buffered reaction allowed to proceed for 1 min (N.A. Sachs, personal communication) before exposure of X-Omat FS-1 Imaging Film (Fisher Scientific, Tustin, CA).

PCR-mediated site-directed mutagenesis. Mouse kidney full length cDNA (AI116776) putatively encoding 1 cys Prx was sequenced by the University of Arizona ARL DNA Sequencing Service and found to have a worrisome G → A point mutation that is translated as glutamate rather than lysine at amino acid position 63 of AOP2_MOUSE. Using both pET-16b and –21b translation vectors as templates, QuikChange (Stratagene, La Jolla, CA) was used to elicit PCR-mediated, site-directed mutagenesis with the following internal primers: E63K forward, 5'-ggg cca gag ttt gcc aag agg aat gtt aag atg

aat gtt aag; E63K reverse, 5'-ctt aac att cat ctt aac att cct ctt ggc aaa ctc tgg cgc. The PCR reaction was carried out under the following conditions in a 50 µl reaction: 2 mM dNTPs, 0.01 µg primer/ml, 2.5 U *Pfu* Turbo DNA polymerase; 95°C for 1:00, 18 cycles of 95°C for :50, 60°C for :50 and 68°C for 10:54 and 68°C for a final 7:00. After temperature cycling, the products were digested with 10 U *Dpn*I which exclusively cleaves *dam*-methylated parental plasmid DNA. Digestion reactions (2 µl) were the source of nicked vector DNA for the transformation of XL10-Gold Ultracompetent cells (Stratagene) 42°C for 90 s. E63K 1 cys Prx recombinants were ultimately expressed in BL21(DE3).

Transient expression of 1 cys Prx in mammalian cells. The cDNA insert encoding mouse kidney 1 cys Prx was subcloned from plasmid DNA isolated from XL-10-Gold cells harboring pET-21b, E63K. The following primers were used to put *EcoR*I and *Kpn*I restriction sites 5' and a *Xba*I site 3' of the 672 bp insert: forward, 5'-GGG AAT TCG GTA CC**a tgc** ccg gag ggt tgc ttc tcg ggg; reverse, 5'-GCG GTC TAG A**uu** aag gct ggg gtg tat aac gga ggt att ttt tgc. The PCR reaction was carried out precisely as it was when AI116776 was originally subcloned from pME18s-FL4. pCNA3.1HisC is a N-term $(His)_6$ tagged high level mammalian expression vector under the control of the constitutive cytomegalovirus (CMV) promoter. A bovine growth hormone (BGH) polyA

signal and transcription termination sequence are present immediately downstream of the MCS. This 5.5 kb vector also harbors SV40 origin for episomal replication in COS 7 and is neomycin resistant for selection (figure 6.4b). Albeit, mouse kidney 1 cys Prx was only transiently expressed. Both vector and PCR products were successively digested with 30 U *Eco*RI and 30 U *Xba*I. CIAP (2 U) was added to the vector for an additional 60 min at 37°C. Both digest reactions were resolved on a 0.8 %/TAE agarose gel. Bands visualized by EtBr and corresponding to vector, insert DNA were excised and purified using the Ultraclean 15 DNA purification kit (MoBio, Solana Beach, CA). Components were ligated overnight at room temperature. Chemically competent DH5α cells were transformed with the 1 cys Prx-wielding construct at 42°C for 90 s. A single transformed colony was propagated into 1,000 ml LB/Amp, and closed, circular plasmid DNA isolated by alkaline lysis and purified in a CsCl gradient (D.G. Adams, personal communication; Maniatis *et al.*, 1989) to yield 5 μg DNA/μl.

COS 7 cells are African green monkey kidney cells transformed with origin defective SV40. SV40 T-antigen mediated replication may amplify copy number plasmids containing SV40 origin of replication to 100,000/cell which results in high expression levels from the transfected DNA (Gluzman, 1981). For our initial purpose, however, cells were transiently transfected and the vector translated from the CMV

promoter. Transient transfection may allow the vector to be introduced into ~ 30% of the mammalian cells. If protein expression is not great enough with this efficiency, it may be necessary to select transfected cells with an antibiotic (neomycin) so that 100% of the cells harbor the expression vector. COS 7 cells were grown at 37°C, 5% CO_2 to 40 – 60% confluency in DMEM (Gibco, Rockville, MD), 10% fetal bovine serum (FBS, Hyclone, Logan, UT), 50 U penicillin (pen)/ml and 50 μg streptomycin (strep)/ml (Gibco). CsCl purified pCNA3.1HisC + insert construct DNA (2 μg, 4 μl) or controls were added to 275 μl DEAE-dextran chloroquine solution (10 mg 500 kDa dextran, 52 mg chloroquine diphosphate/ml 0.137 M NaCl, 5.1 mM KCl, 1.41 mM Na_2HPO_4, 1.35 mM $CaCl_2$, 490 μM $MgCl_2$, 24.8 mM Tris-HCl pH 7.4) and vortexed. DNA/dextran solution was added dropwise with swirling motion to cells that were washed and cultured with DMEM, 1% pen/strep, 10% NuSerum. After 4 hr at 37°C, 5% CO_2, media was removed and 4 ml 10% dimethyl sulfoxide (DMSO, in PBS) was added for 2 min at room temperature. Finally, cells were washed and cultured in DMEM, 10% FBS, 1% pen/strep (D.G. Adams, personal communication). After 24 – 72 hr, cells were harvested in 10 mM Tris-HCl pH 7.8, 100 mM NaCl, 0.5% Triton X, 2 μg aprotinin/ml, 200 μM PMSF, 0.5 mM GSH, 5 mM imidazole and sonicated. Protein content was determined by the

method of Bradford (1976) using bovine serum albumin standards. Expression of recombinant protein was monitored by Western analysis.

Cloning and expression in S. cerevisiae. . The cDNA insert encoding mouse kidney 1 cys Prx was subcloned from plasmid DNA isolated from XL-10 Gold harboring pET-21b, E63K. The following primers were used to put *EcoR*I and *Not*I restriction sites 5' and a *Cla*I site 3' of the 672 bp insert: forward, 5'-GGG AAT TCG CGG CCG **C**at gcc cgg agg gtt gct tct cgg gg; reverse, 5'-GCG GAT CGA Tag gct ggg gtg tat aac gga ggt att ttt tgc. The PCR reaction was carried out precisely as it was when AI116776 was originally subcloned from pME18s-FL4. pESC-URA (6635 bp) is a yeast episomal plasmid derived from pBluescript II SK(+) which allows the expression of eukaryotic genes in yeast. Albeit, two different genes may be expressed simultaneously in this vector, only the MCS under the control of GAL10 activator and encoding the T3 promoter and FLAG epitope (DYKDDDDK) was utilized for our purpose (figure 6.4c). There are no consensus sequences in yeast mRNA untranslated leader regions analogous to bacterial ribosome binding sites so the only consideration in subcloning was that the C-term FLAG-tag be in frame with the start site of the insert. Both vector and PCR products were successively digested with 30 U *Cla*I and 30 U *Not*I. CIAP (2 U) was added to the vector for an additional 60 min at 37°C. Both digest reactions were resolved on a 0.8

%/TAE agarose gel. Bands visualized by EtBr and corresponding to vector, insert DNA were excised and gel-purified (Maniatis *et al.*, 1989). Components were ligated overnight at room temperature. Chemically competent DH5α cells were transformed with the pESC-URA + insert DNA construct and with controls at 42°C for 90 s. A single transformed colony was propagated into 1,000 ml LB/Amp and the plasmid DNA isolated with Qiagen Plasmid Maxi Kit (Qiagen, Valencia, CA) to yield 3.3 μg DNA/μl.

Yeast strains YPH499, (MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200, leu 2-Δ1);YPH500, MATα (same genotype as YPH499); and YPH501 MATa/α (same genotype as YPH499) were made competent by 50 ml YEPD incubation at 30°C until $OD_{600} \approx 1.0$, centrifugation at 1,000*g* for 5 min, resuspension in 10 ml 0.1 M LiOAc, 10 mM Tris-HCl pH 7.5, 1 mM EDTA (LTE), centrifugation at 1,000g for 5 min and final suspension in 500 μl LTE. Competent yeast cell suspensions (50 μl) were transformed with 3 μg pESC-URA + insert (and controls) in the presence of 300 μl 40% polyethylene glycol 3350 in LTE. Transformants were plated on SD-URA dropout plates with dextrose as the carbon source and incubated 2.5 day at 30°C. Synthetic dextrose minimal medium (SD dropout media) was comprised by 0.67% yeast nitrogen base without amino acids, 2% dextrose, 0.2% yeast synthetic dropout media supplement without uracil (-URA, Sigma), 0.5% ammonium sulfate, adenine hemisulfate (12 μg/L) and 2% Bacto

agar for SD-URA dropout plates. SG dropout media was prepared as was SD dropout media substituting galactose for dextrose. Protein expression was induced by the presence of galactose (Schultz *et al.*, 1987). Yeast activator GAL10 controls the transcription of genes whose products are responsible for metabolizing galactose. GAL80, a negative regulatory protein, binds GAL activator in the presence of dextrose and prevents transcription. Recombinant 1 cys Prx was expressed and purified from a 200 ml SG-URA culture as follows: Two volumes (v/w wet pellet) of lysis buffer A (10mM Tris-HCl pH 7.6, 100 mM KOAc, 2 mM MgOAc and 2mM βME), 200 μM PMSF, 2 μg aprotinin/ml, 1 μM EDTA was added to the cell pellet. An equal volume of acid-washed glass beads (425 - 600 mm) was added and the suspension vortexed for 8 1 min intervals @ 4°C with intermittent 1 min intervals on ice. FLAG M2 monoclonal Ab immunoaffinity agarose (500 μl) was added to the aspirated lysate and allowed to react for 2 hr @ 4°C with gentle rotation. The reacted resin was poured onto a 3 x 1cm column and successively eluted with 15 bed volumes each of buffer AN (buffer A, 0.05% Igepal), buffer ANS (buffer AN, 0.7M KOAc), buffer AN and finally, 2.5 ml buffer AN containing 150 μg FLAG peptide/ml (Tharun and Parker, 1999). FLAG-tagged proteins were sought in representative fractions (0.5 ml) by Western analysis.

In vitro translation. pET-21b E63K and pcDNA3.1HisC + insert plasmid DNA both harbor a T7 promoter region and were both substrates for rabbit reticulocyte lysate *in vitro* translation (Pelham and Jackson, 1976; Krieg and Melton, 1984). Using the TNT/T7 polymerase Quick Coupled Transcription/Translation System (Promega, Madison, WI), 1 μg DNA was incubated with 40 μl Master Mix and 20 μM methionine in a nuclease-free 50 μl reaction. Transcripts were detected by Western analysis.

Glutathione peroxidase (GPx) assay. Unless otherwise noted, glutathione peroxidase activity, using either hydrogen peroxide (0.25 mM) or cumene hydroperoxide (0.25 mM) as the substrate, was indirectly assayed in a 1 ml reaction mix containing 1 mM GSH, 250 μM NADPH, 25 mM potassium phosphate, 250 μM EDTA and 250 μM NaN_3 (Wendel, 1981; Chen *et al.*, 2000). Ebselen was used as a positive control (Sies, 1993; Noguchi *et al.*, 1994; Schewe *et al.*, 1994).

RESULTS

Purification of arsenite methyltransferase. Upon final chromatography and after desalting and 5X concentration to 210 μl, fraction 6 from Reactive Red 120 (figure 6.1) was assayed for arsenite methyltransferase activity, submitted to the LPSA, Arizona Research Laboratories (ARL) at the University of Arizona for amino acid sequencing and resolved by 12% PAGE. Fraction 6 arsenite methyltransferase activity was 7,129-fold

purified and catalyzed the formation of 926.7 pmol [^{3}H]MMA/mg protein (figure 6.1, table 6.1). Approximately 300 and 100 ng purified protein was run on 12% native and SDS-PAGE, respectively and silver stained to reveal one band with an approximate molecular weight of ~ 30 kDa (figure 6.2a). The N-terminal sequence of the putative endogenous protein catalyzing arsenic methylation was Pro-Gly-Gly-Leu-Leu-Leu-Gly-Asp-Glu-Ala-Pro-Asn-Phe-Glu-Ala-Asn-Thr-Thr-Ile-Gly-Arg-Ile. A homology search in BLAST nr databases revealed a conserved family of antioxidant proteins (1 cys Prx) characterized by non-selenium dependent glutathione peroxidase (NSGPx) and calcium-independent phospholipase A2 (aiPLA$_2$) activities (Singh and Shichi, 1998; Iakoubova *et al.*, 1997; Kim *et al.*, 1997; Choi *et al.*, 1997; Choi *et al.*, 1998; Frank *et al.*, 1997). These full-length proteins share ≥ 93% homology (figure 6.3)

Expression of recombinant 1 cys Prx in E. coli. Murine non-selenium dependent glutathione peroxidase, (AOP2_MOUSE, assession number O08709; Iakoubova *et al.*, 1997) was chosen for further characterization because endogenous arsenite methyltransferase has been well characterized in the mouse. Humans excrete methylated arsenic metabolites in their urine but human *in vitro* arsenite methyltransferase activity has been elusive. Arsenic toxicity may be contingent upon nutritional status (Peraza *et al.*, 1998). Unlike most mammals, humans are unable to synthesize vitamin C but the

figure 6.2a

Native PAGE analysis of 7,129-fold purified arsenite methyltransferase. Peak activity eluted from Reactive Red 120 column was concentrated 5X by 10 kDa MWCO ultrafiltration and assayed for arsenite and MMA methyltransferase activity, resolved on a 12% non-denaturing gel and submitted to LPSA, Arizona Research Laboratories at the University of Arizona for amino acid.

figure 6.2b

SDS-PAGE analysis of 7,129-fold purified arsenite methyltransferase. Peak activity eluted from Reactive Red 120 column was concentrated 5X by 10 kDa MWCO ultrafiltration and assayed for arsenite and MMA methyltransferase activity, resolved on a 12% fully denaturing gel and submitted for sequencing by Edman degradation.

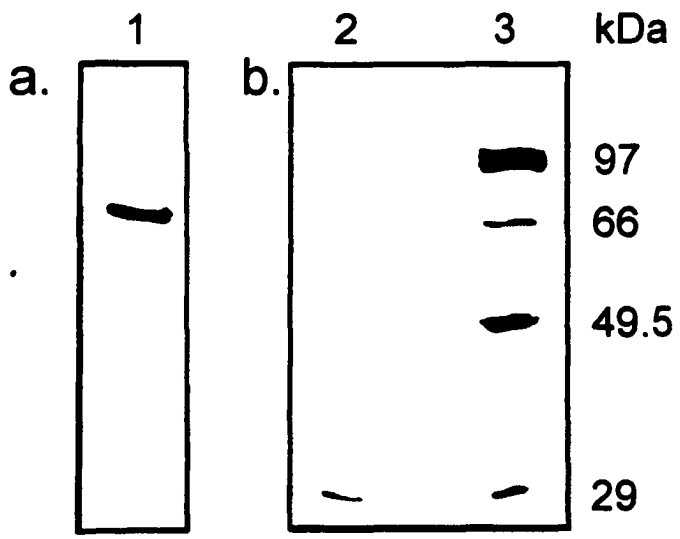

1
2
3
kDa
a.
b.
97
66
49.5
29

figure 6.3

N-terminal 22 amino acids of biochemically purified arsenite methyltransferase match 4 mammalian antioxidant proteins with 100% identity. Full length proteins share 93% homology.

mouse mpgglllgde apnfeantti grirfhdflg dswgilfshp rdftpvctte lgraaklape fakrnvklia
bovine mpgglllgde apnfeantti grirfhdylg dswgilfshp rdftpvctte lgraaklape fakrnvkmia
rat mpgglllgde apnfeantti ghirfhdflg dswgilfshp rdftpvctte lgraaklape fakrnvklia
human mpgglllgdv apnfeantti grirfhdflg dswgilfshp rdftpvxtte lgraaklape fakrnvklia

mouse lsidsvedhl awskdinayn getpteklpf piiddkgrdl aillgmldpv ekddnnmpvt arvvfifgpd
bovine lsidsvedhl awskdinayn geepteklpf piiddknrdl aiqlgmldpa ekdekgmpvt arvvfifgpd
rat lsidsvedhf awskdinayn gaapteklpf piiddkdrdl aillgmldpa ekdekgmpvt arvvfifgpd
human lsidsvedhl awskdinayn seepteklpf piiddrnrel aillgmldpa ekdekgmpvt arvvfvfgpd

mouse kklklsilyp attgrnfdei lrvvdslqlt gtkpvatpvd wkkgesvmvv ptlseeeakq cfpkgvftke
bovine kklklsilyp attgrnfdei lrviislqlt aekrvatpvd wkngdsvmvl ptipeeeakk lfpkgvftke
rat kklklsilyp attgrnfdei lrvvdslqlt asnpvatpvd wkkgesvmvl ptlpeeeakq lfpkgvftke
human kklklsilyp attgrnfdei lrvvislqlt aekrvatpvd wkdgdsvmvl ptipeeeakk lfpkgvftke

mouse lpsgkkylry tpqp
bovine lpsgkkylry tpqp
rat lpsgkkylry tpqp
human lpsgkkylry tpqp

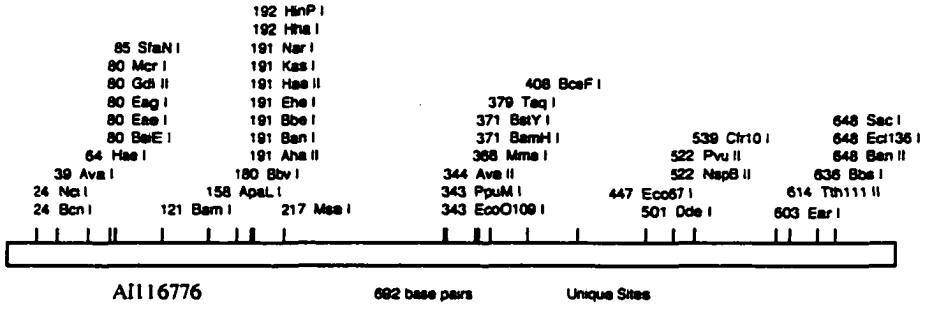
recommended daily allowance (RDA) provides a total body store of 600 - 1,000 mg (Marcus and Coulston, 1996). Glutathione peroxidase activities are increased in guinea pigs fed megadoses of ascorbic acid (Cadenas *et al.*, 1994; Surèsh *et al.*, 1999). It may be that exogenous ascorbic acid supports human arsenic methylation *in vivo* but not *in vitro*.

A 692 bp mRNA full-length EST (assession number AI116776; Marra *et al.*, 1998) encoding mouse NSGPx, 1 cys Prx (and putatively arsenite methyltransferase) was successfully subcloned from pME18s-FL3 and ligated into the *Nde*I site of both pET vectors. After a restriction digest of pET-16b with *Eag*I and *Bgl*II, plasmid DNA yielding 4036 bp fragments was chosen for BL21 transformation (figure 6.4a). Likewise, pET-21b plasmid DNA yielding 684 and 5431 bp fragments after *Eag*I and *Nde*I cleavage was chosen for subsequent transformation into the expression host (figure 6.4a). Vector constructs and protein products were confirmed by the UA ARL LMSE DNA sequencing core and the UA SWEHSC Analytical Core Facility (LC MS/MS), respectively.

1 cys Prx was initially expressed with a His-tag at the N-terminus. However, recent experiments demonstrated greater GPx and PLA_2 activity in constructs with C-terminally tagged human 1 cys Prx clone HA0683 so AI116776 was also inserted into pET-21b (Chen *et al.*, 2000). Both vectors expressed abundant soluble protein that was

figure 6.4a

Bacterial expression vectors. pET-16b provides an N-terminal $(His)_{10}$ tag and harbors *BglI* and *Eag*I restriction sites (chosen for restriction analysis) outside of the MCS. pET-21b provides a $(His)_6$ tag at the carboxyl terminus. The full length EST, AI116776, was inserted into *Nde*I in both vectors.


192 HinP I
192 Hha I
85 SfaN I
191 Nar I
80 Mcr I
191 Kas I
80 Gdi II
191 Hae II
408 BceF I
80 Eag I
191 Ehe I
379 Taq I
80 Ese I
191 Bbe I
371 BstY I
648 Sac I
80 BstE I
191 Ban I
371 BamH I
539 Ctr10 I
648 Ecl136 I
64 Hae I
191 Aha II
368 Mme I
522 Pvu II
648 Ban II
39 Ava I
180 Bbv I
344 Ava II
522 Nsp8 II
636 Bbs I
24 Nci I
158 ApaL I
343 PpuM I
447 Eco57 I
614 Tth111 II
24 Bcn I
121 Bam I
217 Mse I
343 EcoO109 I
501 Ode I
603 Ear I
AI116776
692 base pairs
Unique Sites

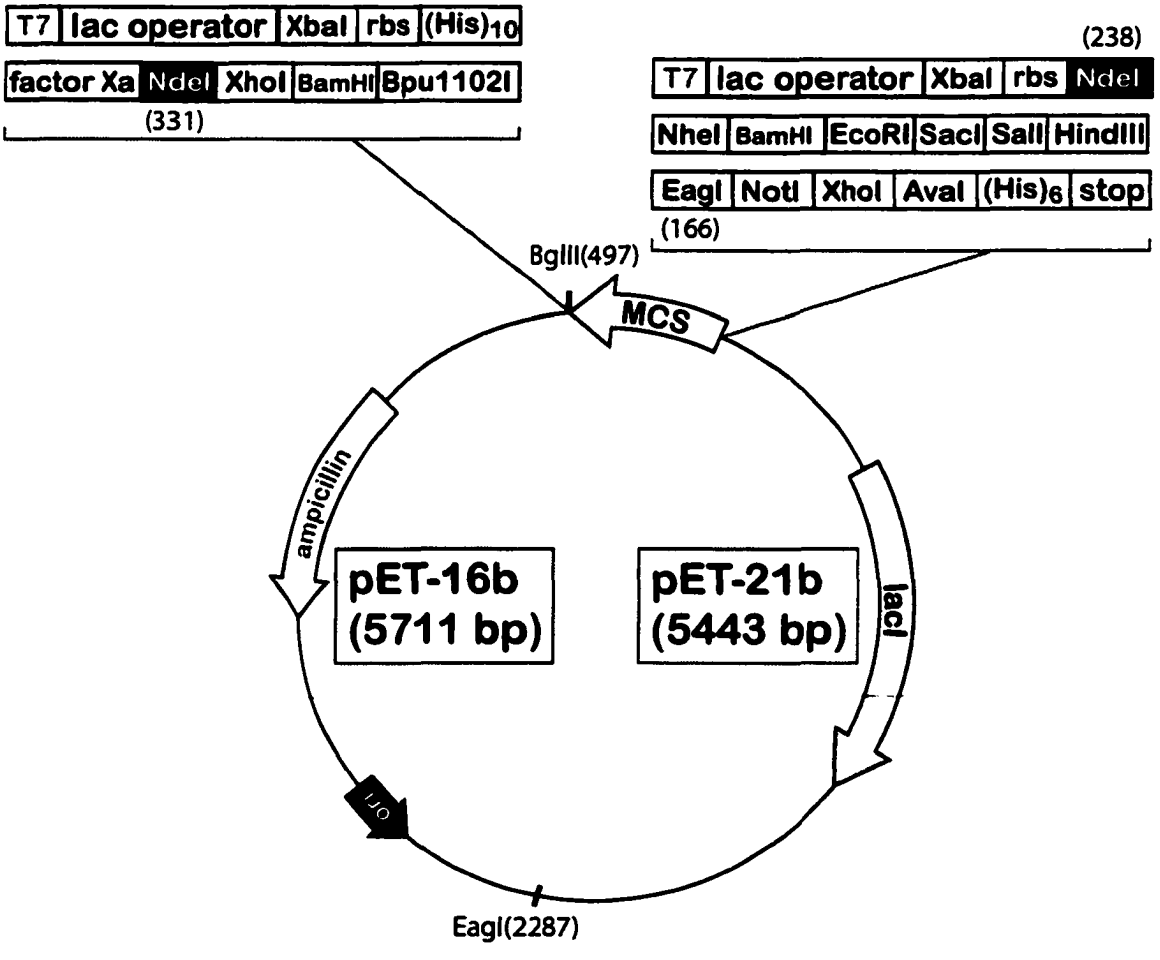

T7
lac operator
XbaI
rbs
(His)10
factor Xa
NdeI
XhoI
BamHI
Bpu1102I
(331)
(238)
T7
lac operator
XbaI
rbs
NdeI
NheI
BamHI
EcoRI
SacI
SalI
HindIII
EagI
NotI
XhoI
AvaI
(His)6
stop
(166)
BglII(497)
MCS
ampicillin
pET-16b
(5711 bp)
pET-21b
(5443 bp)
lacI
ori
EagI(2287)

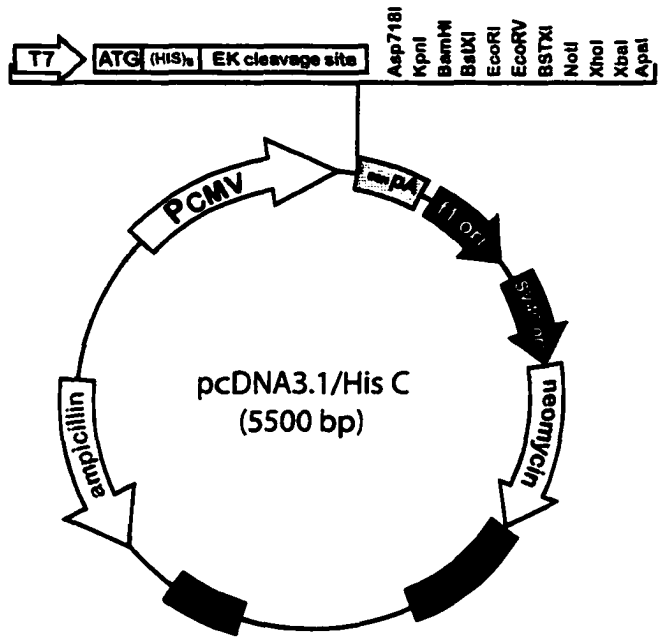
figure 6.4b

Mammalian expression vector. AI116776 was ligated into *Eco*RI and *Xba*I sites in pcDNA3.1/HisC, translated by T7 polymerase and proceeded by $(His)_6$. This construct was used to transiently transfect COS 7 cells.

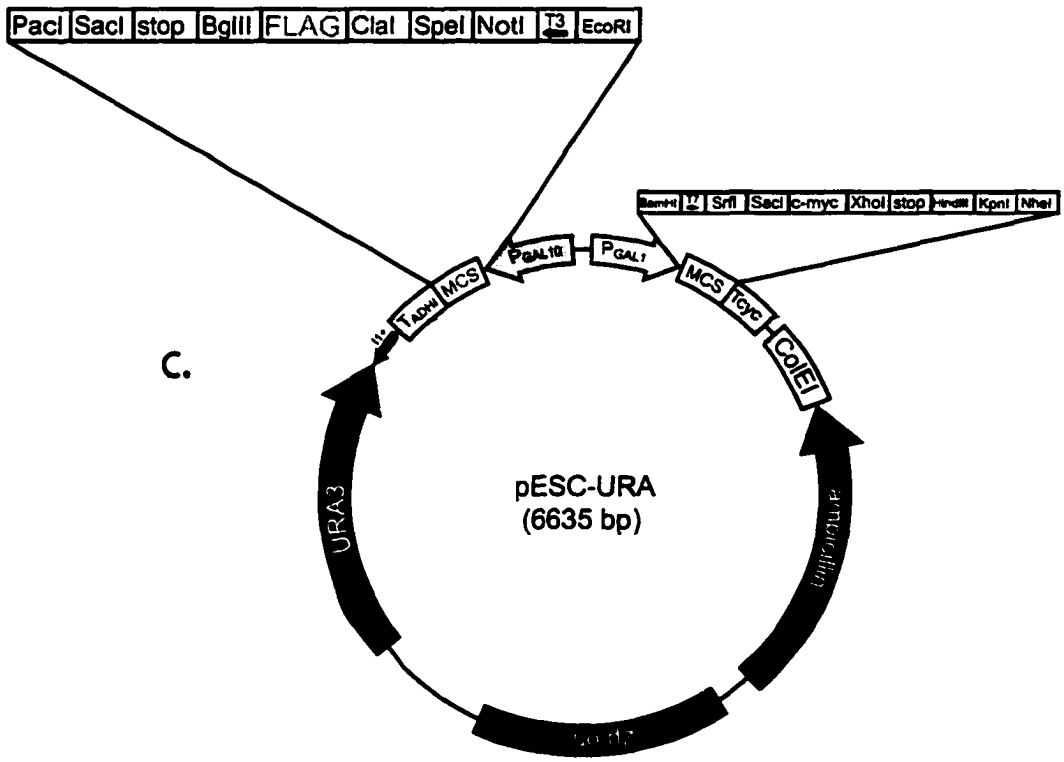
figure 6.4c

Yeast expression vector. 1 Cys Prx cDNA was inserted between *Not*I and *Cla*I upstream of FLAG and translated from the P_{GAL10} operator.

b.


T7
ATG
(HIS)6
EK cleavage site
Asp718I
KpnI
BamHI
BstXI
EcoRI
EcoRV
BSTXI
NotI
XhoI
XbaI
ApaI
PCMV
pcDNA3.1/His C
(5500 bp)
ampicillin
neomycin

c.


PacI
SacI
stop
BglII
FLAG
ClaI
SpeI
NotI
T3
EcoRI
BamHI
T7
SrfI
SacI
c-myc
XhoI
stop
HindIII
KpnI
NheI
MCS
MCS
TCYC
ColE1
URA3
pESC-URA
(6635 bp)

amenable to purification (figures 6.5a and 6.5c). However, crude and cleared induced BL21 lysates and purified N- or C-term His-tagged recombinant proteins did not catalyze the methylation of $NaAsO_2$ or the reduction of H_2O_2.

In order to elicit enzyme activity, the following strategies were employed: the pET-16b expression product was cleaved with Factor Xa protease (figure 6.4a); protein was purified in the presence or absence of dithiothreitol (DTT); both vectors were grown at 23°C to slow the rate of protein folding; and an erroneous point mutation on AI116776 cDNA was corrected with PCR-mediated site-directed mutagenesis and subsequently expressed from pET-16b and pET-21b. Protein encoded by NSGPx cDNA AI116776 and expressed in *E. coli* did not have arsenite methyltransferase or glutathione peroxidase activity under any of these experimental conditions.

Further, *in vitro* assays were varied. Recombinant 1 cys Prx was assayed for arsenite methyltransferase activity in the presence of 20 - 200 μM $NaAsO_2$, 120 mM Tris-HCl pH 7 - 8, 10 mM acetate pH 4, DTT, 1 - 10 mM GSH, NADPH and GSSG reductase, ATP, ascorbic acid and finally mouse cytosol. When cleared lysates from cells induced with IPTG were added to mouse cytosol, no activation or inhibition of arsenite methyltransferase activity was observed (figure 6.6): The *in vitro* environment optimized for mouse kidney cytosolic arsenite methyltransferase activity could not evoke enzyme

figure 6.5a

Representative expression of soluble 1 cys Prx in BL21(DE3). Recombinant protein was grown at 37°C to OD_{600} ~ 0.8 and expression was induced by the addition of 1 mM IPTG for 3 hr. Pellets were washed and sonicated in PBS. After 15,000*g*, 10 min at 4°C, the supernatant (soluble protein) was separated from the pellet (inclusion bodies). The following samples were loaded onto 12% SDS-PAGE: lane 1, 5 µl uninduced pellet (-IPTG); lane 2, 2 µl induced pellet (+IPTG); lane 3, MW standards; lane 4, 25 µl uninduced supernatant; lane 5, induced supernatant.

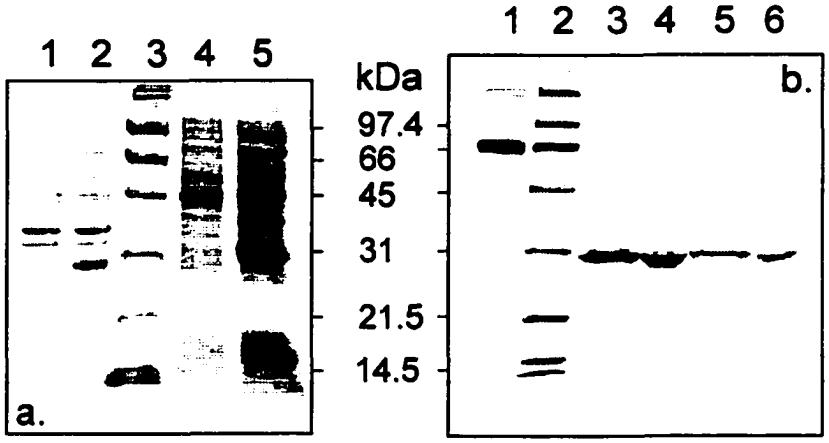
figure 6.5b

Recombinant 1 cys Prx does not have apparent disulfides. The following samples were loaded onto 12% SDS-PAGE: lane 1, 5 µg BSA; lane 2, MW standards; lane 3, 1 cys Prx in pET-16b + βME; lane 4, 1 cys Prx in pET-16b - βME; lane 5, 1 cys Prx in pET-21b + βME; lane 6, 1 cys Prx in pET-21b - βME.

figure 6.5c

Representative purification of 1 cys Prx from bacteria. Expression of E63K in pET-21b was induced with 1 mM IPTG for 3 hr in BL21(DE3) at 37°C. Cells were sonicated in homogenization buffer and the cleared lysate allowed to react with Ni-agarose for 60 min at 4°C with gentle agitation. His-tagged proteins were purified according to MATERIALS

AND METHODS. The following samples were resolved on 12% SDS-PAGE: lane 1, cleared lysate; lane 2, flowthrough; lane 3, 5 mM imidazole wash; lane 4, 60 mM imidazole wash; lane 5, 1 M imidazole elution; lane 6, dialyzed 1 M imidazole eluate; lane 7, dialyzed 60 mM imidazole eluate; lane 8, MW standards.


1 2 3 4 5
kDa
97.4
66
45
31
21.5
14.5
a.
1 2 3 4 5 6
b.

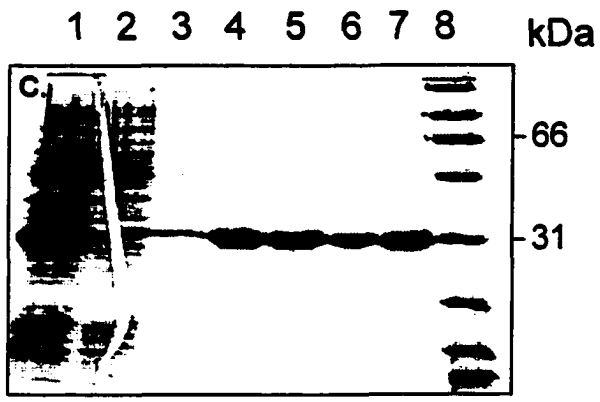
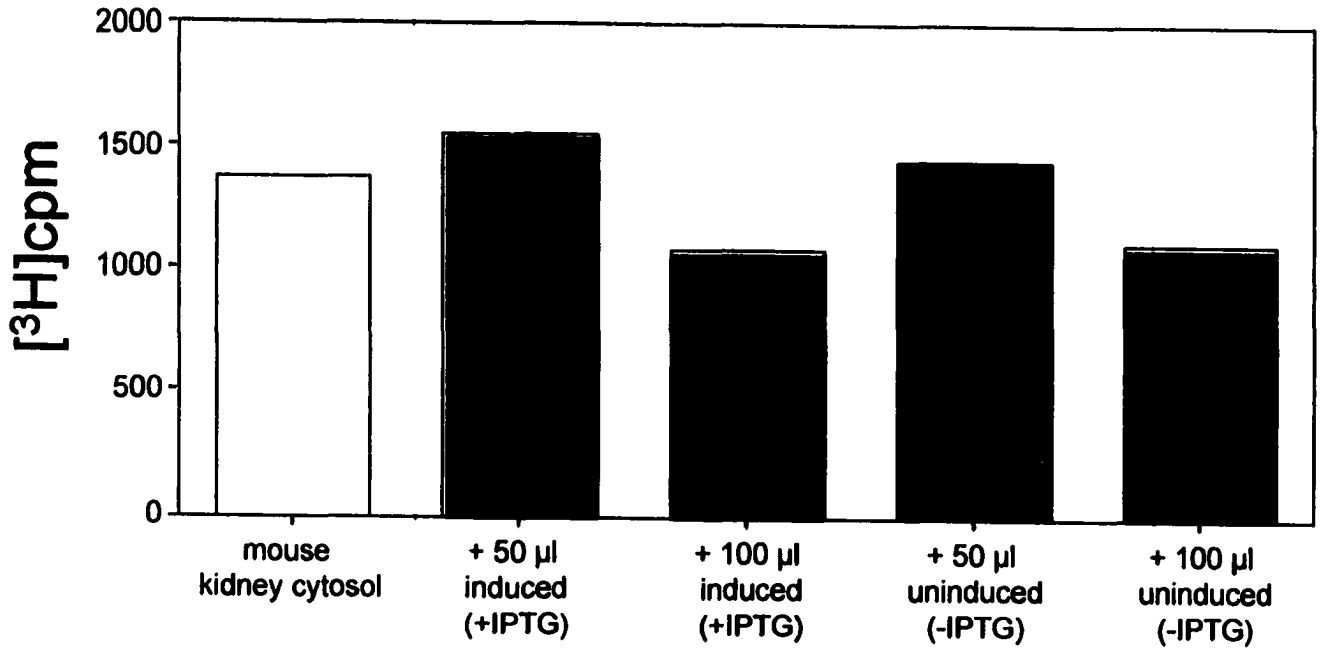

1 2 3 4 5 6 7 8
kDa
c.
66
31

figure 6.6

Cofactors for putative recombinant arsenite methyltransferase activity are not present in mouse kidney cytosol. Mouse kidney cytsol (100 μg) was assayed for arsenite methyltransferase as described in (Zakharyan *et al.*, 1995) alone or in the presence of 50 or 100 μl induced (E63K in pET-21b) cleared lysate or 50 or 100 μl uninduced cleared lysate.


[3H]cpm
2000
1500
1000
500
0
mouse kidney cytosol
+ 50 µl induced (+IPTG)
+ 100 µl induced (+IPTG)
+ 50 µl uninduced (-IPTG)
+ 100 µl uninduced (-IPTG)

activity in bacterially expressed proteins. Similarly, endogenous mouse kidney glutathione peroxidase activity was not effected by the addition of 1 - 100 μg purified 1 cys Prx-$(His)_6$ (data not shown). Increasing GSH, including DTT or varying pH did not increase the rate of NADPH disappearance compared to buffer controls with rNSGPx as the source of enzyme. Finally, recombinant proteins did not act on alternate substrates: 1 cys Prx expressed in pET-21b did not catalyze the formation of [^{3}H]MMA from $As(GS)_3$ or [^{3}H]DMA from MMA. Nor did it catalyze the reduction of cumene hydroperoxide.

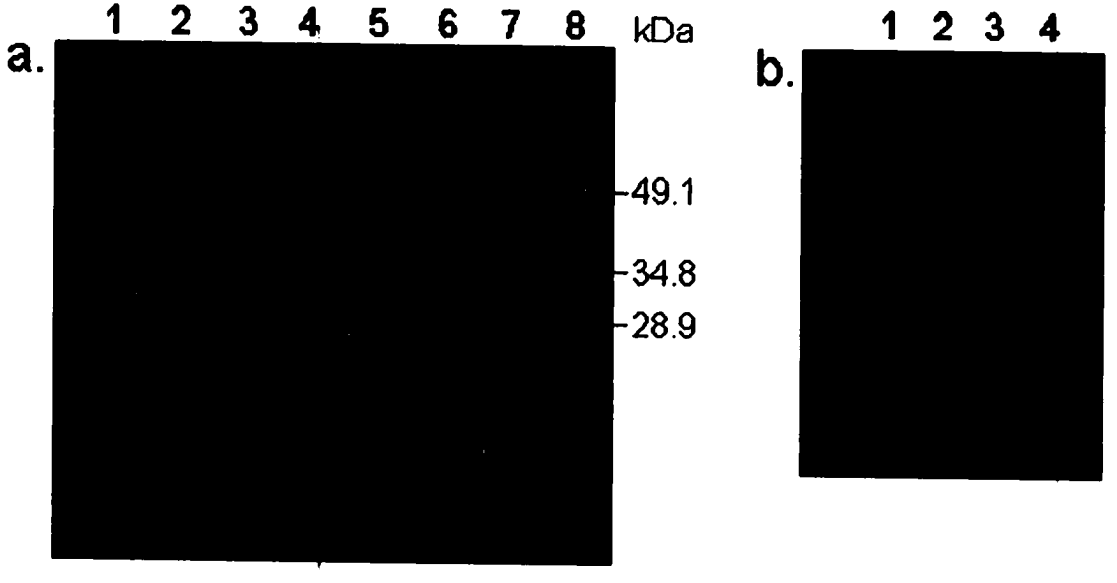
Immunoprecipitation of endogenous 1 cys peroxiredoxin. Immunoprecipitation reactions of 1 cys Prx from guinea pig and mouse kidney cytosol are represented in figure 6.7a. When endogenous 1 Cys Prxs were reacted with pooled monoclonal antibodies (mAb16 + mAb17) directed against human ORF6 (Chen *et al.*, 2000), and precipitated with protein G Sepharose, 1 cys Prx was very unexpectedly not detected in mouse and surprisingly detected guinea pig kidney with mAb16. However, 1 cys Prx was detected in mouse kidney cytosol, as well as rabbit liver and guinea pig kidney cytosol, using mAb17 (figure 6.7b) but not with pAb35 (data not shown). pAb35 is an anti-peptide directed against the 16 C-most terminal amino acids (figure 6.3; Peshenko *et al.*, 2001). Since the mouse methylates arsenic and the guinea pig does not and 1 cys Prx is the putative catalytic protein in question, it may be that arsenite methyltransferase and

figure 6.7a

Immunoprecipitation of 1 cys Prx from guinea pig (6.38 mg) and mouse (5.6 mg) cytosol. Endogenous 1 cys Prxs were reacted with pooled monoclonal antibodies, mAb16 and mAb17, directed against human ORF6 (Chen *et al.*, 2000), and precipitated with protein G Sepharose. Proteins were resolved by 12 % non-reducing SDS-PAGE and detected by Western analysis using mAb16 (1°) and AP-goat anti-rabbit IgG (3°). Lane 1, mock/buffer Ip; lane 2, guinea pig cytosol (6.4 mg); lane 3, guinea pig Ip supernatant; lane 4, guinea pig protein G pellet; lane 5, MW standards; lane 6, mouse protein G pellet; lane 7, mouse Ip supernatant; lane 8, mouse cytosol (5.6 mg).

figure 6.7b

One (1) cys Prx is detected in mouse cytosol with mAb17. The following samples were loaded onto 12% fully denaturing SDS-PAGE: lane 1, purified recombinant mouse 1 cys Prx (E63K); lane 2, 20 μg rabbit liver cytosol; lane 3, 20 μg guinea pig kidney cytosol; lane 4, 20 μg mouse kidney cytosol.


a.
1 2 3 4 5 6 7 8
kDa
49.1
34.8
28.9
b.
1 2 3 4

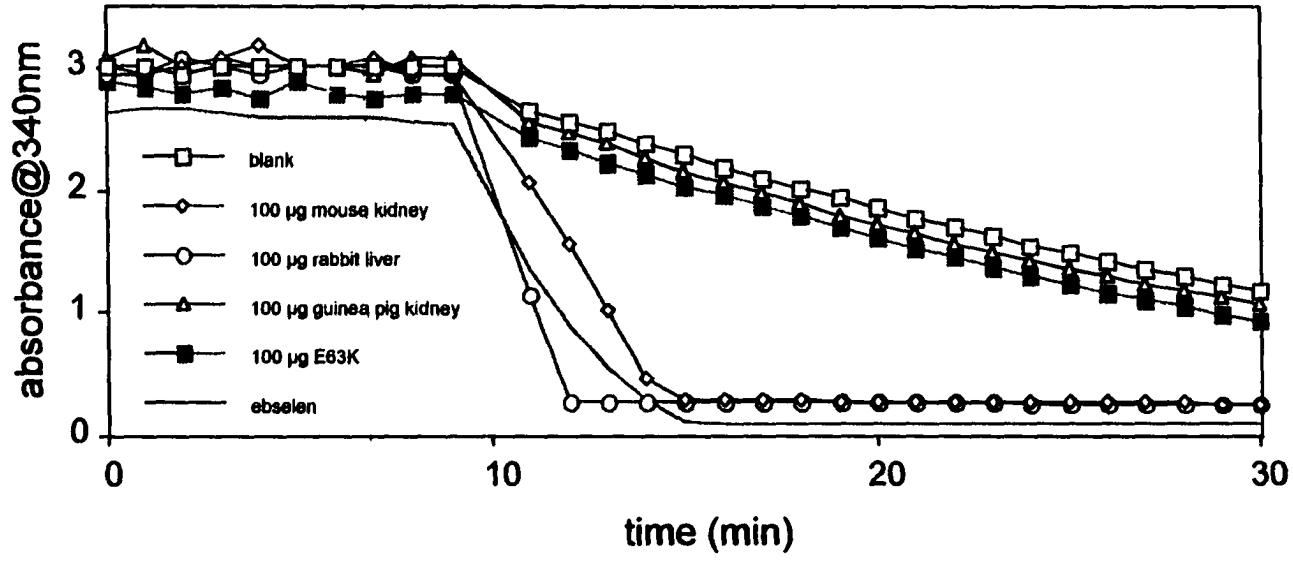
associated activities must be cleaved to an active form. Cleavage of iPLA2 at Asp^{183} by caspase-3 gave rise to a truncated enzyme with increased function (Atsumi *et al.*, 2000). Regardless, arsenite methyltransferase was not successfully immunoprecipitated from mouse kidney cytosol: Neither arsenite methyltransferase nor GPx could be detected in the protein G or A sepharose pellets. Nor did the remaining supernatants have decreased enzyme activities.

Two immunoprecipitation reactions were carried out in parallel and the 12% non-denaturing gels were either Western blotted or reverse Zn-imidazole stained (Castellanos-Serra *et al.*, 1999). Bands corresponding to anti-1 cys Prx in the Zn-imidazole gel were cut out and submitted for LC MS/MS. After trypsin digestion, guinea pig cytosolic protein was identified as AOP2_MOUSE, O08709.

Guinea pig enzyme deficiencies. The guinea pig is deficient in arsenite methyltransferase(s) (Healy *et al.*, 1997), selenium-dependent GPx (Himeno *et al.*, 1993), nonselenium-dependent (NS) GPx (Lauterman *et al.*, 1997) and L-gulonolactone oxidase (Sato and Udenfriend, 1978) activities. Using an enzyme assay for total glutathione peroxidase activity (GPx + NSGPx), GPx activity was not observed in guinea pig kidney cytosol (figure 6.8). L-gulonolactone oxidase catalyzes the synthesis of ascorbic acid (Vit C) and humans are also unable to synthesize Vit C. Ascorbic acid (AA) functions as

figure 6.8

Guinea pig kidney cytosol is deficient in glutathione peroxidase activity. Mouse, rabbit or guinea pig cytosol or purified recombinant 1 cys Prx (100 μg each) were assayed for glutathione peroxidase activity (Wendel, 1981). Substrate (0.25 mM H_2O_2) was added at 10 min. Glutathione peroxidase activity was indirectly observed and quantified as the disappearance of NADPH at 340 nm. Glutathione peroxidase activities of 473 nmol/min/mg and 7.7 nmol/min/mg were observed for mouse and guinea pig cytosol, respectively.


absorbance@340nm
3
2
1
0
0
10
20
30
time (min)
blank
100 µg mouse kidney
100 µg rabbit liver
100 µg guinea pig kidney
100 µg E63K
ebselen

a cofactor in a number of reactions by transferring electrons to enzymes that provide reducing equivalents and the human RDA maintains a 45 μM intracellar concentration of Vit C (Marcus and Coulston, 1996). Glutathione peroxidase activities are increased in guinea pigs fed megadoses of ascorbic acid (Cadenas *et al.*, 1994; Suresh *et al.*, 1999). When 100 μM or 2mM L-ascorbic acid was added to both *in vitro* arsenite methyltransferase and GPx assays, guinea pig kidney cytosol was still unable to catalyze reactions (data not shown). AA did not effect mouse kidney arsenite methyltransferase activity.

Transient expression of 1 cys Prx in mammalian cells. COS 7 or HEK 293 cells were respectively DEAE dextran or calcium phosphate transfected with pcDNA3.1/HisC + insert to no avail. After 24, 48 or 72 hr, 1 cys Prx could not be detected by Western analysis in COS 7 cell lysate or pellet. The positive control, MEKK3 in pcDNA3.1/His A (D.G. Adams) was expressed abundantly at all time points (data not shown). The pcDNA3.1/HisC + insert construct was validated by restriction analysis and the DNA sequence from the T7 promoter confirmed that the insert was in frame. The experimental construct was then used as a template for *in vitro* translation. The 672 bp AI116776 insert encoding 1 cys Prx could not be translated on pcDNA3.1/HisC (figure 6.9).

figure 6.9

In vitro translation of 1 cys Prx. Template DNA (1 μg) was transcribed/translated and the resulting product resolved by 12% SDS-PAGE. The following plasmids were used as template DNA in the TNT/T7 polymerase Quick Coupled Transcription/Translation kit (Promega): lane 1, empty pcDNA3.1/HisC; lane 2, 1 in cys Prx (E63K) cDNA pET-21b; lane 3, 1 cys Prx (E63K) cDNA in pcDNA3.1/HisC. Lane 4 contains 2 μg recombinant E63K. *In vitro* translated proteins were detected by Western analysis using mAb16.


1
2
3
4

However, 1 cys Prx (E63K) cDNA pET-21b was transcribed/translated by reticulocyte lysate (figure 6.9) but did not have arsenite methyltransferase or GPx activity.

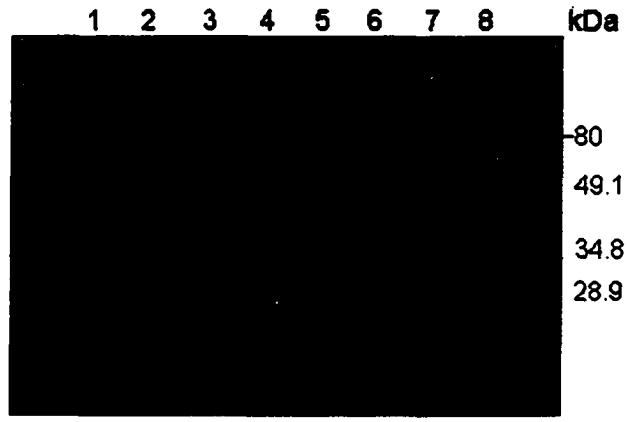
Cloning and expression in S. cerevisiae. The expression of FLAG-tagged protein was induced by galactose in all yeast strains with the greatest expression in haploids (figure 6.10). *In vitro* arsenite methyltransferase or GPx activity was not observed in induced lysates nor in fractions eluted from anti-FLAG M2 with the FLAG peptide.

DISCUSSION

The effect of a single enzyme on chemical toxicity can be precisely determined by either disrupting expression or introducing/increasing expression in genetically manipulated organisms. Xenobiotic metabolizing enzymes are responsible for either mediating the toxicity of a chemical through metabolic activation of chemicals and procarcinogens or protecting an organism by rapidly processing chemicals to inert derivatives that can be easily eliminated. It is not clear whether methylation is a detoxification or bioactivation pathway for inorganic arsenic. Animals in which arsenite methyltransferase is nullified or overexpressed will be valuable *intact* models with which to evaluate whether the parent or metabolite(s) is the ultimate toxicant/carcinogen. The National Institutes of Health (NCI, NIEHS) encourage the development of transgenic mice and carcinogen bioassay protocols that may be more predictive for identifying

figure 6.10

Expression of recombinant FLAG-protein in yeast. Dropout (SD or SG) 5 ml cultures were inoculated with a, α or a/α YPH strains, 1 μg/ml ampicillin and grown overnight at 30°C. Pellets were lysed according to MATERIALS AND METHODS and the following samples resolved by 12% SDS-PAGE: lane 1, 79 kDa positive FLAG control (N.A. Sachs); lane 2, 20 μl 499a grown in galactose; lane 3, 20 μl 499a grown in dextrose; lane 4, 20 μl 500α grown in galactose; lane 5, 20 μl 500α grown in dextrose; lane 6, 20 μl 501a/α grown in galactose; lane 7, 20 μl 501a/α grown in dextrose, lane 8, MW standards. Expressed proteins were detected by Western analysis using FLAG M2 mAb.


1 2 3 4 5 6 7 8
kDa
80
49.1
34.8
28.9

human carcinogens and validate the molecular epidemiology studies ongoing in humans that seek to establish a role for polymorphisms in cancer risk. (Gonzalez and Kimura, 1999). Epidemiological and animal studies imply that arsenite methyltransferase may be polymorphic (Vahter *et al.*, 1995a; Morel *et al.*, 1995; Healy *et al.*, 1997); a population in NE Chile shows no signs of chronic arsenic toxicity despite 720 μg As/L well water concentrations (Aposhian *et al.*, 1997a). The answer to the decisive research question of whether or not arsenic methylation is beneficial lies within the verification of the protein and consequent identification of the gene responsible for the formation of MMA and DMA.

Arsenite methyltransferase activity was purified from rabbit liver. The protein associated with enzyme activity was identified as a 1 cys peroxiredoxin. Peroxiredoxin was first identified in budding yeast as soluble 25 kDa thiol specific antioxidant (TSA) which reduced peroxides using thioredoxin (Trx) as the immediate electron donor. TSA confers protection against inactivation of glutamine synthetase (GS) by a thiol/Fe3+/mixed function oxygenase (MFO) system (Jin and Jeang, 1999) but does not explicitly destroy O_2^- or H_2O_2 (Kim *et al.*, 1998).

TSA has no detectable absorption in the range of 320-600nm indicating that peroxiredoxins do not contain prosthetic groups such as heme and flavin (Kim *et al.*,

1988; Choi *et al.*, 1998) and distinguishing it from other peroxidases. When Chae *et al.*, (1993) isolated and sequenced the yeast genomic DNA fragment that encodes TSA and compared it to the predicted amino acid sequences of conventional antioxidant enzymes (catalase, GPx, SOD), no sequence homology was observed. TSA resides on chromosome XIII and encodes a 195 amino acid cytosolic protein with 2 conserved cysteine residues.

Salmonella typhimurium and *E. coli* both possess alkylhydroperoxide reductase (AhpC) which converts alkyl hydroperoxides to corresponding alcohols. The catalytic activity is the reduction of peroxides with subsequent reduction of AhpC by AhpF coupled to either NADH or NADPH oxidation. AhpC/F is likely to comprise an operon with a promoter upstream of AhpC gene regulating levels of both gene products. AhpC activity requires the presence of 2 separable components having subunit molecular weights of 22 and 57 kDa (Jacobsen *et al.*, 1989). AhpF has considerable homology to *E. coli* Trx (Taritaglia *et al.*, 1990) and AhpC has TSA activity. AhpC and TSA define a large family of related proteins which play a major role as cellular antioxidant enzymes. AhpC/TSA homology searches identified 23 additional proteins from a variety of organisms including human (ORF6). Similarity among family members extends over entire sequence and ranges between 23 and 98% identity (Chae *et al.*, 1994a; Jin and

Jeang, 1999). One cysteine (which corresponds to Cys 47 in yeast Prx) is conserved in all family members and a majority of proteins also have a 2nd conserved cysteine (~ Cys 170) residue. The invariant N-terminal cysteine is more likely to be critical in catalysis (Chae *et al.*, 1994b). Peroxiredoxins can be divided into 2 groups – one containing Cys 47 (1 cys Prx) only and another containing an additional conserved cysteine near the C-terminus (2 cys Prx). With the exception of AhpC, no other family members are associated with known biochemical reactions.

The crystal structure of 1 cys Prx hORF6 was determined at 2 Å resolution by Choi *et al.*, (1998). The subunit contains two discrete domains and forms a homodimer. Within the N-term is a Trx fold and the C-term is necessary for dimerization. The Trx fold is a an 80 amino acid motif found in various proteins such as Trx, glutaredoxin, GPx and GST which are all involved in redox reactions. But, Trx is not the immediate electron donor for 1 Cys Prx (Kang *et al.*, 1998b)

At concentrations higher than 1mg/ml, purified hORF6 exists as a dimer that can be detected by gel filtration (Choi *et al.*, 1998). However, the activity of Prx can be modulated by subunit assortment through either homo- or heterodimerization with other members of the family. Prx AOE372 (2 cys Prx) forms a heterodimer with another human Prx, proliferation associated protein (PAG). This AOE372-PAG heterodimer is

shown to have increased activity as compared to its homodimeric form (Jin *et al.*, 1997). Therefore, dimerization might be an important determinant in regulation of enzyme activity and substrate specificity.

There is also an indication that hORF6 has alternative monomeric forms in certain conditions (Choi *et al.*, 1998). If hORF6 exists as a monomer in the cell, it may adopt a different conformation. Otherwise, hydrophobic residues at the dimer interface would be exposed to solvent. Some dimeric proteins are known to make a conformation transition to a monomeric form. Diphtheria toxin uses such transition between monomeric and dimeric forms for regulation of its activity; only the monomeric from is toxic (Bennet and Eisenberg, 1994). Monomer $\leftrightarrow$ dimer transition may occur in hORF6 as a mechanism of activity regulation, including substrate specificity and, as yet unknown, other functions (Choi *et al.*, 1998).

Reduction of H_2O_2 by 1 Cys Prx is through an, as yet, unidentified physiological electron donor (Kang *et al.*, 1998a). Presently, 1 cys Prx was unable to reduce H_2O_2 in the presence of GSH, DTT, NADPH or ascorbic acid. Under these experimental conditions many of the possible *in vivo* variables such as monomer $\leftrightarrow$ dimer transition, heterodimerization or the necessity of an AhpF homologous function are unknown and uncontrolled. Many of the recent reports describing recombinant 1 cys Prx characterize

glutathione peroxidase activity with the GS protection assay (Chen *et al.*, 2000; Fisher *et al.*, 1999; Kang *et al.*, 1999a; Kang *et al.*, 1999b). Catalase and superoxide dismutase also inhibit inactivation of yeast GS by the thiol/Fe3+/MFO system. Catalase and superoxide dismutase (SOD) demonstrate IC_{50}'s of 6 and 2 μg/ml, respectively where 70 μg 2 cys Prx (TSA)/ml is required for 50% inhibition of GS inactivation. Cys peroxiredoxins do not explicitly destroy $O_2^{\cdot}$ or H_2O_2 (Kim *et al.*, 1998). Interestingly, NSGPx activity described in Chen *et al.*, (2000) was not reproducible (A.B. Fisher, personal communication). While this cytosolic homodimer without a prosthetic group seems amenable to bacterial expression, it is not surprising that the recombinant enzyme was inactive.

While arsenite methyltransferase activity could not be verified as 1 cys Prx, NSGPx, correlations may exist: The major mechanism postulated for arsenic's biotransformation by mammals and many other organisms involve GSH-dependent methylation (Aposhian, 1997); GSH is likely not a cofactor of arsenic methyltransferase enzymes since results with 2,000-fold purified rabbit enzymes indicate that arsenite and MMA methyltransferases do not have an absolute requirement for GSH. The enzymes appear only to require a reducing environment which can be supplied by GSH and other thiol or dithiol compounds (Zakharyan *et al.*, 1995; Wildfang *et al.*, 1998; Zakharyan *et*

al., 1999); The $As(GS)_3$ complex has been postulated as a transition intermediate in process of arsenic methylation (Styblo *et al.*, 1997); MMA^V reductase is a glutathione s-transferase (Zakharyan *et al.*, 2001). The activity of glutathione peroxidase was 5.4-fold lower in *xrs-5* CHO cells which were also 7.1-fold more sensitive to arsenite compared to wildtype (Wang *et al.*, 1997). As^{III} increases binding of nuclear transcription factor Sp1 2.5 fold (compare figure 2.2a; Kaltreider *et al.*, 1999) and there are several potential Sp1 binding sites -60 through -96 bp from the putative transcriptional start site of murine 1 cys Prx (Lee *et al.*, 1999). One (1) cys Prx shares the 3 regions of sequence similarity in most mammalian non-nucleic acid methyltransferases (Hamahata *et al.*, 1996; Schluckebier *et al.*, 1995; Kagan and Clarke, 1994). Presently, guinea pig cytosol could neither catalyze arsenite methylation nor peroxide reduction.

It is not clear whether methylation is a detoxification or bioactivation pathway for inorganic arsenic. With the identification of arsenate reductase (Radabaugh, personal communication) and MMA^V reductase (Zakharyan *et al.*, 2001), it appears as if arsenic competes with endogenous substrates; so far, arsenic biotransformation enzymes are not novel proteins. Rather, figure 1.1 describes novel functions. This enzyme system may not be perfectly suited for arsenic metabolism; MMA^{III} is the most toxic known intermediate in arsenic biotransformation (Petrick *et al.*, 2000; Mass *et al.*, 2001) and

MMA^{III} is persistent (chapter 3). The *in vivo* synthesis of trivalent mono- and, perhaps, dimethylated arsenic is apparently costly to the organism (Styblo *et al.*, 1997; Chouchane and Snow, 2001; Lin *et al.* 1999; Petrick *et al.*, 2001). However, MMA^{V} reductase is the rate limiting enzyme (Zakharyan and Aposhian, 1999) and the majority (35%) of arsenate shunted into the methylation pathway is conveyed through oxidative dimethylation, the endpoint (chapter 3; Marafante *et al.*, 1987). The ultimate effect of methylation cannot be perceived until a tractable model (*e.g.* transgenic mice, Ho *et al.*, 1997) for arsenic biotransformation exists. The objective of this research project was to identify the protein sequence of arsenite methyltransferase and confirm the sequence with recombinant protein expression. The answer to the decisive research question of whether or not arsenic methylation is beneficial lies within the verification of the protein(s) and identification of the gene(s) responsible for the formation of MMA^{V} and DMA^{V}.

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