OXIDATION AND REDUCTION OF INORGANIC ARSENIC IN MAMMALIAN SYSTEMS

By

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Timothy Robert Radabaugh on the topic of OXIDATION AND REDUCTION OF INORGANIC ARSENIC IN MAMMALIAN SYSTEMS and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

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DEDICATION

I would like to dedicate the following work to the millions of people in the world who are suffering from arsenic related illnesses. Hopefully, in the not so distant future a better understanding of the mechanisms of arsenic toxicity and detoxification will lead to a reduction in arsenic related human suffering.
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ABSTRACT

Arsenic is a toxic metalloid and is ubiquitous in our environment. In ancient cultures it was valued as a poison and today is becoming an increasing public health problem. Chronic arsenic exposure has a broad range of toxic effects including cancer. Currently millions of people are exposed to higher levels of arsenic in their food and drinking water than is considered safe by the World Health Organization. Although arsenic metabolism is not completely understood, it is known that inorganic arsenate is reduced to arsenite which can then be methylated and excreted in the urine. It is also known that some arsenic is retained in the body, presumably by binding to cellular proteins. To better understand how arsenic is metabolized, our approach was to identify and characterize proteins that are involved in arsenic metabolism.

Using biochemical approaches we demonstrated that arsenate reductase activity from human liver was purine nucleoside phosphorylase (PNP). We were able to demonstrate that calf spleen PNP has arsenate reductase activity in vitro in the presence of inosine and dihydrolipoic acid, and that the reaction exhibits Michaelis-Menten kinetics. This identifies an enzymatic route for arsenate reduction.

We also demonstrate that ferritin, an iron storage protein containing phosphate, can bind arsenic both in vitro and in vivo. In addition, we demonstrate that ferritin can oxidize arsenite to arsenate, and then interact with arsenate as it does with phosphate. We also establish that arsenate can inhibit ferritin’s ability to store iron in vitro. Our results combined with data reported by others, suggest that DNA damage and enzyme inactivation associated with arsenic challenge may occur via reactive oxygen species.
generated by arsenic-iron redox reactions in ferritin, and that iron may augment arsenic toxicity.

The interaction between ferritin and arsenate has two important implications. First, it suggests that iron exposure may be an important parameter to examine in epidemiological studies of arsenic sensitivity. Second, it suggests that iron chelation therapy might be beneficial in conjunction with arsenic chelation therapy for patients suffering from acute arsenic poisoning.
CHAPTER 1

INTRODUCTION

Inorganic arsenic is ubiquitous in the environment and is carcinogetic to man (1). It poses a threat to public health thereby necessitating a better understanding of its metabolism. Arsenic, valued as a poison for thousands of years, is the 33rd element in the periodic table. Everyday, every human in the world is exposed to inorganic arsenic through air, water, and food. Arsenic is associated with a wide range of health problems affecting the nervous, hematopoietic, and vascular systems, as well as organs like the lung, skin, and kidney (2). In addition arsenic has been classified as a human carcinogen (3) and is associated with a number or organ related cancers (4, 5). For most people in developed countries, chronic exposure is not a problem. However, thousands if not millions of people are suffering from arsenic related illnesses in under-developed countries such as India (6), China (7), Chile (8), Taiwan (9), and Mexico (10) to name a few. There is little doubt that arsenic as a public health problem will become more prominent with the increases in agriculture, well drilling, mining, ore processing, and industrial manufacturing, which must occur to sustain Earth’s rapidly growing population.

The mechanisms for arsenic toxicity are not well understood. One reason for this is that arsenic exists in a number of oxidation states (0, -3, +3, -5, and +5) in both inorganic and organic forms. Each arsenical can have different mechanisms of toxicity, and different symptoms of toxicity are presented depending on what tissue or animal model is being studied (11). Inorganic arsenate (As(V)) is the predominant arsenical in the
environment which humans are exposed to on a daily basis. Once in the body it is chemically or enzymatically reduced to arsenite and subsequently biotransformed into a number of methylated metabolites of varying oxidation states \(I2\), which can then exert their toxic effects. Although the mechanisms of arsenic toxicity are poorly understood, it is generally accepted that much of the toxicity associated with arsenic challenge is due to the inhibition of an extensive number of key metabolic enzymes \(I3\) and arsenic-induced DNA damage \(I4\). The putative pathway for the biotransformation of inorganic arsenic (Figure 1.1) suggests that pentavalent inorganic arsenate \(\text{As}^V\) is chemically reduced in the blood by glutathione \(I5, I6\) to trivalent arsenite \(\text{As}^{III}\) – a more toxic form \(I7, I8\). After reduction arsenite can be methylated \(I9\). The two methylated forms, monomethylarsonic acid and dimethylarsinic acid are the primary urinary metabolites in mammals \(I2\). Since the methylated forms are more water-soluble and more easily excreted into the urine it has been a popular view that the methylation pathway represents a route of detoxification. However, recent findings suggest that the trivalent arsenic intermediates, which are formed during methylation, are much more toxic than the inorganic species \(I20\) and suggest that methylation may not represent a pathway for detoxification.

Experimental data from a number of mammalian species demonstrate that a significant amount of arsenic is retained by the body and does not appear in the urine for a considerable time after administration \(I21, I22, I23\). It has been suggested that arsenite “storage” proteins (Figure 1.1) may protect the cell by sequestering arsenite \(I23, I24\) thereby preventing the inactivation of key enzymes \(I3\).
This work focuses on two aspects of arsenic metabolism. First, we are interested in seeing if there is an enzymatic route for arsenate reduction in the cell. Although it has been demonstrated that arsenate can be reduced by glutathione to arsenite \((\text{As}^{III})\) in the bloodstream before entering the tissues \((15, 16)\), most chemical reactions in the cell are enzymatic. There is also a strong precedent for the hypothesis that an enzymatic route of arsenate reduction exists. In *Escherichia coli* and *Staphylococcus aureus*, plasmids containing the *arsC* gene encode an arsenate reductase that reduces arsenate to arsenite in the cell and then actively effluxes it via an ATP-dependant pump \((25)\). The ATPase activity of the ArsA protein is not activated by arsenate, hence the need for reduction \((26)\). In addition, a novel arsenate reductase *Arr*, which plays an essential role in the growth of *Chrysiogenes arsenatis* has been purified and characterized \((27)\) which differs from the *E. coli* and *S. aureus* arsenate reductases. In the case of *C. arsenatis*, the *Arr* protein catalyzes the reduction of arsenate by accepting an electron from acetate to complete the anaerobic respiratory chain. In addition to the bacterial reductases, the ARC2 gene in *Saccharomyces cerevisiae* has been demonstrated to encode and arsenate reductase \((28, 29)\).

Initial efforts in our laboratory have detected a cytosolic protein in human liver which can reduce arsenate to arsenite in the presence of an unknown cofactor and a dithiol \((30)\). Since there is little similarity in the known arsenate reductases (only 18\% sequence similarity between *E. coli* and *S. aureus* enzymes) I have chosen to use standard enzyme purification as my approach versus gene cloning to identify the mammalian arsenate reductase. This is the focus of the first part of this dissertation.
The second issue I am addressing involves arsenic binding proteins. Previous work on the tissue distribution of arsenic after administration has shown a significant localization of arsenic in the liver (23, 24, 31, 32, 33). Although work has been done to identify proteins which bind arsenic with high affinity (34, 35), to date an arsenic "storage" protein has not yet been identified. Of interest to this work is a previously described 450 kDa arsenite binding protein partially purified from rabbit liver (36).

Three indications suggest this partially purified 450 kDa arsenite binding protein may be ferritin, and the species of arsenic bound to it is arsenate, not arsenite. 1) The partially purified 450 kDa arsenite binding protein is the same size as ferritin - a 450 kDa iron storage protein highly concentrated in liver. Ferritin oxidizes ferrous iron (Fe$^{II}$) in the cytosolic free iron pool to the insoluble ferric form (Fe$^{III}$), and safely sequesters it from the cytosol in a microcrystalline hydrous ferric-oxide phosphate core within a protein shell typically composed of 24 subunits (37). 2) The $^{73}$As-arsenite (As$^{III}$) used to label the arsenic-binding protein in the previously mentioned work (36) would be oxidized by ferritin’s ferric iron (Fe$^{III}$) to $^{73}$As-arsenate (As$^{V}$), a well characterized redox reaction (13, 38). Since biochemically, arsenate has been demonstrated to act as a phosphate analog (39), it can be expected to associate with the ferric-oxide hydrate core of ferritin in a manner similar to phosphate. 3) Arsenic has been shown to stimulate the synthesis of ferritin which is known to occur when Fe$^{II}$ is increased (40, 41).

These three pieces of evidence suggest a hypothetical model of arsenic-ferritin binding, where arsenite (As$^{III}$) in the cytosol enters ferritin and is oxidized by ferric iron (Fe$^{III}$) to arsenate (As$^{V}$). The arsenate produced by the Fe$^{III}$/As$^{III}$ redox reaction would
bind the ferric iron core like its analog phosphate whereas the ferrous iron (Fe$^{II}$) produced via reduction by arsenite (As$^{III}$) would be liberated from ferritin simultaneously. Evidence for this latter step already exists by the demonstration that iron is released from ferritin in the presence of arsenite (42). Another component of the model is that arsenate may inhibit ferritin iron uptake like its chemical analog phosphate has been demonstrated to do (37).

Recent work demonstrated that DNA damage and enzyme inactivation associated with arsenic challenge may be from reactive oxygen species (ROS) induced by arsenic versus direct interaction with arsenic (43, 44, 45, 46, 47). Because ferritin is very abundant in tissues that retain arsenic, and is the major source of cellular iron, the primary ROS generating metal (48, 49), it is critical to better understand arsenic-ferritin interactions.

The objective of the second part of this dissertation is to test my model of arsenic-ferritin binding and inhibition of ferritin iron uptake by arsenite using both in vitro and in vivo experiments.
Figure 1.1 Putative Pathway for Arsenic Biotransformation.
CHAPTER 2
PARTIAL PURIFICATION AND CHARACTERIZATION OF A HUMAN LIVER ARSENATE REDUCTASE

Statement by Author

This chapter is a modified version of an article to Chemical Research in Toxicology that was published in 2002 (50). My specific contributions to this work are. 1) I partially purified an arsenate reductase activity from human liver. 2) I isolated it and identified it by amino acid sequencing to be purine nucleoside phosphorylase. 3) I demonstrated that calf spleen PNP had arsenate reductase activity and had the same requirements for reductase activity (an unknown cofactor and a dithiol) as the partially purified enzyme. 4) I demonstrated that inosine could replace the unknown cofactor as a necessary requirement for arsenate reductase activity for the purified enzyme as well as PNP. 5) I demonstrated that DHLP could replace DTT for the thiol requirement for the partially purified enzyme.

The enzyme kinetics, the comparison of different thiols, and the experiments to determine if PNP showed specificity for arsenate reduction with respect to other phosphorylases were done by Dr. Robert A. Zakharyan and Dr. Adriana Sampayo-Reyes under the direction of Dr. H. Vasken Aposhian.

EXPERIMENTAL PROCEDURES

Caution: Arsenic has been classified as a human carcinogen by the International Agency for Research on Cancer (3).
**Reagents.** Sodium arsenate was ACS reagent grade and was purchased from MCB Reagents (Cincinnati, OH); carrier-free $^{73}\text{As}$-arsenate was purchased from Los Alamos National Laboratory (Los Alamos, NM); DEAE Sepharose Fast Flow, Sephacryl S-200 HR, and molecular mass standards were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). PMSF (phenylmethylsulfonyl fluoride), inosine, EDTA (ethylenediaminetetraacetic acid), sodium $m$-arsenite, hypoxanthine, 5’AMP, DTT ($\alpha$-dithiothreitol), GSH (glutathione), BAL (2,3-dimercaptopropanol), ascorbic acid, $\beta$-mercaptoethanol, and lipoic acid (DHLP, the $\text{DL}-6,8$-thioctic acid reduced form), calf spleen purine nucleoside phosphorylase (PNP) - 2 units/mg with bovine serum albumin as a stabilizer, rabbit muscle phosphorylase $b$ (21 units/mg), *Escherichia coli* thymidine phosphorylase (EC 2.4.2.4) -10.7 units/mg with bovine serum albumin as a stabilizer, thioredoxin from spirulina, and glycogen type IX from bovine liver were purchased from Sigma Chemical Co. (St. Louis, MO).

Stock solutions of DHLP were prepared in ethanol as instructed by the technical support department at Sigma Chemical Co. The final concentration of ethanol in incubation mixtures was 1% or less.

Cow milk xanthine oxidase, 20 units/mL was purchased from Roche Diagnostics (Basil, Switzerland), DMPS (sodium 2,3-dimercapto-1-propanesulfonate) was from Heyl (Berlin, Germany). Monoflow III scintillation cocktail was purchased from National Diagnostics (Atlanta, GA). All other reagents were analytical reagents or of the highest quality available.
**Tissue.** Human liver from a male was purchased from the Association of Human Tissue Users (Tucson, AZ).

**Arsenate Reductase Assay Used during Enzyme Purification Procedures.** The assay used during purification was the same as described previously (30). Arsenite and arsenate were separated using a modification of the Gailer method (51) on a 4.9 x 250 mm Hamilton PRP X-100 anion exchange HPLC column using 50 mM sodium phosphate buffer (pH 8.0) with a flow rate of 2.5 mL/min. Arsenicals were detected and quantified with a postcolumn inline β-RAM radioisotope detector (INUS Systems Inc., Tampa, FL). Monoflow III scintillation cocktail (National Diagnostics, Atlanta, GA) was used at a flow rate of 7.5 mL/min.

**Partial Purification of Arsenate Reductase**

**Cytosol Preparation.** Human liver (225 g) was divided into 5 g pieces, minced with scissors, and homogenized with 10 mL of homogenization buffer in a glass Dounce homogenizer. Homogenization buffer contained 20 mM Tris-HCl (pH 8.0), 5 mM EDTA, 1 mM DTT, and 0.2 mM PMSF. The homogenate was centrifuged at 12,000 x g for 15 min at 4 °C. Lipids were skimmed from the supernatant by spatula, and the supernatant was then centrifuged at 105,000 x g for 90 min at 4 °C. Lipids were removed from the supernatant by filtration through glass wool, and the resulting cytosol was designated as Fraction I and stored at -70 °C.

**DEAE Chromatography.** Cytosol (250 mL, containing 8350 mg of protein) was thawed and diluted with 500 mL of 10 mM Tris-HCl buffer (pH 7.5), and loaded onto a 2.5 x 18 cm DEAE Sepharose Fast Flow (Amersham Pharmacia Biotech) column
equilibrated with 30 mM Tris-HCl, (pH 7.5). The sample was loaded at 2 mL/min, and the column was washed with 1 column volume of equilibration buffer. Elution was carried out with a gradient of 250 mL of 30 mM Tris-HCl (pH 7.5) and 250 mL of 30 mM Tris-HCl/300 mM NaCl (pH 7.5) at a flow rate of 300 mL/h. Fractions containing the highest arsenate reductase activity were pooled and concentrated by ultrafiltration using a 30 kDa cutoff membrane. Fractions were not stable unless concentrated before freezing. A series of these columns were run and the partially purified enzymes from all the columns were combined before the next step in purification. The concentrate (pooled active fractions from all DEAE columns) was designated Fraction II and stored at -70 °C.

**Hydroxyapatite Chromatography.** A portion of Fraction I (87.5 mg of protein) was thawed and transferred into 10 mM potassium phosphate buffer (pH 6.8) using PD-10 desalting columns (Amersham Pharmacia Biotech). The protein was then applied to a 1.5 x 17 cm column of 80 μm Type I ceramic hydroxyapatite (BioRad Laboratories, Hercules, CA) and equilibrated with 10 mM potassium phosphate buffer (pH 6.8). The column was washed with 2 column volumes of the equilibration buffer and then eluted with a continuous gradient of 150 mL of 10 mM potassium phosphate (pH 6.8) and 240 mM potassium phosphate (pH 6.8) at a flow rate of 2 mL/min. Collected fractions were transferred into 30 mM Tris-HCl buffer (pH 7.35), using PD-10 columns and then assayed for arsenate reductase activity and protein concentration. This chromatography was repeated several times to collect enough enzyme to proceed with purification. Fractions containing the highest activity were pooled, concentrated, designated as Fraction III and stored at -70 °C.
Sephacryl S-200 Chromatography. A portion of Fraction III (52.5 mg) from the hydroxyapatite column was applied to a 2.5 x 62 cm Sephacryl S-200 HR column equilibrated with 30 mM Tris-HCl/50 mM NaCl (pH 7.5). Fractions with the highest activity were pooled, concentrated, designated as Fraction IV and stored at -70 °C.

Preparative Isoelectric Focusing. Protein from Fraction IV (3.44 mg in 280 μL) was loaded onto a 60 mL Rotofor Cell (BioRad Laboratories). The cell was pre-focused with Bio-Lyte ampholytes pH 3-10 (BioRad Laboratories) according to the manufacturer’s instructions. After loading, the sample was focused for 2.5 h at 12 W constant power. Fractions were collected in tubes containing 200 μL 1.0 M MOPS buffer pH 7.0 (some fractions required centrifugation to remove precipitated proteins) and assayed for arsenate reductase activity and protein concentration. Active fractions were pooled, concentrated, designated as Fraction V and stored at 4 °C overnight.

Preparative Electrophoresis. Concentrated protein (1.8 mg, in 20% buffered glycerol pH 7.35) from the Rotofor Cell (Fraction V) was applied to a Mini Prep Cell (BioRad Laboratories, Hercules, CA) for non-denaturing preparative electrophoresis. Electrophoresis using a 6% native PAGE column gel was carried out at 100 V at 4 °C, using 30 mM Tris-HCl buffer (pH 7.35) for the elution buffer at a flow rate of 100 μL/min. Fractions were collected (1 mL) and assayed for arsenate reductase activity and protein concentration. Active fractions were combined into seven pools – factions 9 – 13, 14 – 18, 19 – 23, 24 – 28, 29 – 33, 34 – 38, and 39 – 43 and designated A - G respectively. The fractions were concentrated by ultrafiltration and stored at -70 °C. The pooled fractions (A – G) were assayed for arsenate reductase activity and protein
concentration. Fraction D had the highest specific activity and was designated Fraction VI.

**Mass Spectrometry of Partially Purified Arsenate Reductase.** Electrophoresis of equal amounts of protein (2 μg) from samples A, B, C, D, E, F, and G was performed using a 7.5% SDS-polyacrylamide gel and then stained with Coomassie blue. The gel was photographed wet, and three major bands were excised and delivered to the Analytical Core Facility of the Southwest Environmental Health Science Center at The University of Arizona for analysis. The gel slices were digested with trypsin (52), and the extracted peptides were analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) using a quadrupole ion trap Finnigan LCQ Classic mass spectrometer equipped with a Finnigan MAT Spectra System quaternary pump P4000 HPLC and a Finnigan electrospray ion source (Thermoquest, San Jose, CA). Peptides were eluted from a reversed-phase C18 Vydac 250 mm x 1 mm micro-column, (Hesperia, CA) with a gradient of 3-95% acetonitrile in 0.5% formic acid, 0.01% trifluoroacetic acid over 150 min at a flow rate of 15 μL/min. Tandem MS spectra of peptides were analyzed with the Sequest program (v. C1) to assign peptide sequences to the spectra (52). Sequest analyses were performed against the OWL database SWISS-PROT with PIR.

**Arsenate Reductase Assay Using PNP/HPLC.** The reaction mixture (100 μL) containing 0.1 M MOPS buffer (pH 7.5), 1.0 mM EDTA, 10 mM inosine, 0.37-6 mM sodium arsenate, 0.5 μCi of carrier-free [73As]-sodium arsenate, 0.5-5 mM DHLP, and PNP or human liver arsenate reductase was incubated for 30 min at 37 °C. After incubation, 100 μL of 0.1 M sodium arsenite was rapidly added to the reaction, and the
tubes were immersed in a boiling water bath for 2 min, and cooled on ice for 5 min. The reactions were then centrifuged at 12,000 rpm for 6 min. A 100 μL sample of the supernatant was injected onto a 4.9 mm x 250 mm Hamilton PRP-X100 anion exchange column using 30 mM sodium phosphate (pH 5.0) with a flow rate of 1.45 mL/min. The arsenite and arsenate were quantified using a postcolumn inline β-RAM radioisotope detector. Monoflow III was used as the scintillation cocktail, and a flow rate was used that was 3 times that of the mobile phase.

**Purine Nucleoside Phosphorylase (PNP), Spectrophotometric Assay.** PNP activity with inosine as the substrate as measured by a spectrophotometric assay, which monitors the change in absorption at 293 nm (ε = 1.25 x 10⁴) which is associated with the formation of uric acid by xanthine oxidase (53). The assay was performed in a final volume of 1 mL by incubating 0.5 mM inosine, 5 mM DHLP, 1 μg of PNP, and 0.02 units of xanthine oxidase in 50 mM potassium phosphate buffer (pH 7.5) at 30 °C, for 5 min (53).

The PNP arsenolysis activity with inosine as the substrate was performed as above except potassium phosphate buffer was replaced with 100 mM MOPS buffer (pH 7.5), and 20 mM sodium arsenate and incubated at 30 or 37 °C.

**Thymidine Phosphorylase, HPLC Assay.** The assay (100 μL) was performed as above for PNP, only MOPS buffer was replaced with 20 mM Tris-HCl (pH 7.3), in the presence of 6 mM [⁷³As]-sodium arsenate (pH 7.3), 10 mM thymidine, 1 - 5mM DHLP, and 11 μg of thymidine phosphorylase at 37 °C for 30 min. The arsenicals were quantified as previously described.
Thymidine Phosphorylase, Spectrophotometric Assay. The thymidine phosphorylase activity with thymidine as the substrate was measured by a spectrophotometric assay, which monitors the change in absorption at 300 nm associated with formation of thymine. The assay was performed in a final volume of 1 mL by incubating 5 mM thymidine, 10 mM sodium arsenate in 10 mM Tris-HCl buffer (pH 7.3), with 5.5 or 11 μg of thymidine phosphorylase at 37 °C for 5 min as previously described (54).

Phosphorylase b Assay. The assay (100 μL) was performed with 230 μg of glycogen, 6 mM [\(^{73}\)As]-sodium arsenate, 2 mM EDTA (pH 7.5), 10 mM MgCl₂, 5 mM DHP, 1 mM 5′AMP in 50 mM Tris-acetate buffer (pH 7.5), and 10 μg of rabbit muscle phosphorylase b. The reaction was started by addition of 5′AMP (55) and incubated for 30 min at 37 °C. The arsenite and arsenate were determined by HPLC as previously described.

Other Methods. The protein concentration was determined by the method of Bradford using BSA as a standard (56).
RESULTS

Partial Purification of Human Liver Arsenate Reductase. The results of our purification suggest that a ~34 kDa protein is most likely the arsenate reductase and warrants further characterization.

The elution profile of the arsenate reductase activity from a representative DEAE column can be seen in Figure 2.1. The elution profile of the arsenate reductase activity from a representative hydroxyapatite column can be seen in Figure 2.2. The elution profile of the arsenate reductase activity from the size exclusion column can be seen in Figure 2.3. The distribution of arsenate reductase activity in the fractions collected from the preparative isoelectric focusing step can be seen in Figure 2.4, and the elution profile of arsenate reductase activity from the preparative PAGE column can be seen in Figure 2.5. Fractions D and E of the last purification step had a specific activity of 1705 nmol of arsenite/mg of protein/30 min compared with 2.8 nmol of arsenite/mg of protein/30 min in the cytosol resulting in a 609-fold purification. Fraction D contained 250 μg of protein and 7.3% of the initial activity of 2088 mg of protein. The results from the purification process are summarized in Table 2.1. The values in the table come from each fraction assayed in the linear range for activity. Protein was normalized to reflect only protein carried through to the last step of purification since significant amounts of purified protein were set aside for other purposes after the DEAE and Sephacryl steps.

Of the pooled fractions (A – G) from the last step in purification (preparative PAGE), fractions D and E contained two major bands which can be seen in Figure 2.6A. One of these bands is a ~34 kDa protein which can be seen to correspond in intensity to the
Figure 2.1 DEAE Elution Profile of Arsenate Reductase Activity.
Figure 2.2 Hydroxyapatite Elution Profile of Arsenate Reductase Activity.
Figure 2.3 Sephacryl S-200 Elution Profile of Arsenate Reductase Activity.
Figure 2.4 Preparative Isoelectric Focusing of Arsenate Reductase Activity.
Figure 2.5 Preparative Native PAGE of Arsenate Reductase Activity.
Figure 2.6 SDS-PAGE Analysis of Preparative PAGE Pooled Fractions. (A) SDS-β-mercaptoethanol PAGE of fractions A - G from preparative electrophoresis. Each lane contained 2 μg of protein from fractions A - G. (B) Arsenate reductase activity of fractions A - G from the nondenaturing preparative PAGE column.
### Table 2.1 Partial Purification of Human Liver Arsenate Reductase

<table>
<thead>
<tr>
<th>fraction</th>
<th>step</th>
<th>protein&lt;sup&gt;a&lt;/sup&gt; (mg)</th>
<th>activity&lt;sup&gt;b&lt;/sup&gt; (units)</th>
<th>spec. act. (units/mg)</th>
<th>recovery (%)</th>
<th>purification (x-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Cytosol</td>
<td>2088</td>
<td>5831</td>
<td>2.8</td>
<td>100</td>
<td>n/a</td>
</tr>
<tr>
<td>II</td>
<td>DEAE</td>
<td>291</td>
<td>6479</td>
<td>22.3</td>
<td>111.1</td>
<td>8</td>
</tr>
<tr>
<td>III</td>
<td>Hydroxyapatite</td>
<td>29.8</td>
<td>6576</td>
<td>220.7</td>
<td>112.8</td>
<td>79</td>
</tr>
<tr>
<td>IV</td>
<td>Sephacryl S-200</td>
<td>3.4</td>
<td>3331</td>
<td>979.7</td>
<td>57.1</td>
<td>350</td>
</tr>
<tr>
<td>V</td>
<td>BioRad Rotofor</td>
<td>1.8</td>
<td>2595</td>
<td>1441.7</td>
<td>44.5</td>
<td>515</td>
</tr>
<tr>
<td>VI</td>
<td>BioRad Miniprep Cell</td>
<td>0.250</td>
<td>426</td>
<td>1704.0</td>
<td>7.3</td>
<td>609</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values are adjusted to represent only protein applied to the next step in purification relative to the yield of the prior step.

<sup>b</sup>A Unit is defined as the amount of activity which will reduce 1 nmol of arsenate in 30 minutes.
activity of fractions A – G which can be seen in Figure 2.6B. This correspondence between band intensity and arsenate reductase activity suggests that the ~34 kDa protein is a likely candidate for the arsenate reductase.

**Mass Spectrometry of Partially Purified Human Liver Arsenate Reductase.** Mass spectra analysis using Sequest in conjunction with the SWISS–PROT database matched the ~34 kDa protein of interest (Figure 2.6A) with human purine nucleoside phosphorylase (accession no. AAA36460). The peptide fragments equal to 40.1% of the total protein were 100% identical to the corresponding regions of the human purine nucleoside phosphorylase and are shown in Figure 2.7.

**Dihydrolipoic Acid Is Required by Arsenate Reductase.** Since we could not obtain human PNP, we used calf spleen PNP for these studies. The homology of the calf spleen enzyme with the human enzyme is 87% overall and 100% within the active site (57, 58).

Reduction of arsenate in the purine nucleoside arsenolysis required both PNP and DHLP – see Table 2.2. In the absence of DHLP, the amount of arsenite formed was decreased to about 2% of that found with the complete reaction. If PNP was omitted, the reduction of arsenate was 13% of that found with the complete reaction. Significant activity was observed only in the presence of PNP and DHLP. The reduction of arsenate coupled with the arsenolysis of inosine was linear to at least 1.0 μg. This amount, therefore, was used for the remainder of experiments since it was in the linear range of the assay. Substrate saturation for the arsenate reductase (PNP) occurred at about 6 mM sodium arsenate. The rate of arsenite production depended on the concentration of DHLP
Figure 2.7 Sequence Comparison between Arsenate Reductase and PNP. The sequence of PNP is aligned with arsenate reductase peptide fragments determined by mass spectrometry. PNP is row 1; human liver arsenate reductase is row 2.
Table 2.2 Dihydrolipoic Acid is Required for Reduction of Arsenate by PNP*  

<table>
<thead>
<tr>
<th></th>
<th>arsenite produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>complete reaction mixture</td>
<td>103.6 ± 2.9</td>
</tr>
<tr>
<td>minus PNP</td>
<td>13.6 ± 1.0</td>
</tr>
<tr>
<td>minus DHLP</td>
<td>2.09 ± 0.11</td>
</tr>
</tbody>
</table>

*The complete reaction mixture contained 10 mM inosine, 5 mM DHLP, 6 mM \(^{73}\text{As}\)-sodium arsenate, and 1 μg PNP in 100 mM MOPS buffer (pH 7.5). The reaction was incubated for 30 min at 30 °C. The final reaction volume was 100 μL, n = 3.
Michaelis–Menten kinetics indicated that the arsenate reductase activity of PNP in the presence of 2 and 5 mM DHLP had $K_m = 0.81 \times 10^{-3} \text{ M}$, $V_{\text{max}} = 62.5 \mu\text{mol/mg/30 min}$; $K_m = 1.8 \times 10^{-3} \text{ M}$, $V_{\text{max}} = 166 \mu\text{mol/mg/30 min}$, respectively as can be seen in Figure 2.8.

Hypoxanthine production by either PNP phosphorolysis or arsenolysis of inosine was not influenced by DHLP as seen in Table 2.3. Monomethylarsonate (MMA$^+$) was not reduced by PNP (data not shown).

Among different thiols tested in the reduction of arsenate to arsenite by the arsenolysis reaction, the most active naturally occurring thiol was DHLP. The rate of reduction of arsenate by reducing agents such as GSH or ascorbic acid was negligible compared to that of the endogenous dithiol DHLP and the synthetic DTT as can be seen in Figure 2.9 and 2.10.

Michaelis–Menten kinetics for PNP in the arsenolysis and reduction of arsenate using increasing concentrations of DHLP yielded $K_m = 2.6 \times 10^{-3} \text{ M}$ and $V_{\text{max}} = 143 \mu\text{mol/mg/30 min}$ as seen in Figure 2.11.

**Arsenate and Thymidine Phosphorylase, PNP, or Phosphorylase b.** To test the uniqueness of PNP for arsenate reduction, other phosphorylases were investigated. At different concentrations of DHLP (1, 2.5, or 5 mM), the arsenolysis reaction of thymidine phosphorylase had approximately 5% of the PNP arsenolysis activity as seen in Table 2.4. Rabbit muscle phosphorylase b was inactive in the reduction of arsenate to arsenite.
Figure 2.8 Michaelis-Menten Kinetics for PNP Arsenolysis and Reduction of Arsenate. DHLP was present at 2 or 5 mM in reactions containing 1 μg PNP. For 5 mM DHLP, $K_m = 1.8 \times 10^{-3}$ M and $V_{max} = 166 \mu$mol/mg/30 min. For 2 mM DHLP, $K_m = 0.81 \times 10^{-3}$ M and $V_{max} = 62.5 \mu$mol/mg/30 min. S is the concentration of sodium arsenate.
Table 2.3 Dihydrolipoic Acid Did Not Influence Hypoxanthine Production during Phosphorolysis or Arsenolysis Using PNP (n = 4)

<table>
<thead>
<tr>
<th>hypoxanthine formed, ( \mu \text{mol mg}^{-1} \text{ min}^{-1} \pm \text{SE} )</th>
<th>phosphorolysis(^a)</th>
<th>arsenolysis(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(30^\circ\text{C})</td>
<td>(30^\circ\text{C})</td>
</tr>
<tr>
<td>minus DHLP</td>
<td>2.4 ± 0.16</td>
<td>4.42 ± 0.06</td>
</tr>
<tr>
<td>5 mM DHLP</td>
<td>2.6 ± 0.04</td>
<td>4.16 ± 0.29</td>
</tr>
</tbody>
</table>

\(^a\)PNP activity with inosine as the substrate was determined by the spectrophotometric assay, which measures the change in absorption at 293 nm (\(\varepsilon = 1.25 \times 10^4\)) associated with the formation of uric acid by xanthine oxidase. For the assay, 1 \(\mu\)g PNP, 0.5 mM inosine, 5 mM DHLP, and 0.02 units xanthine oxidase in 50 mM potassium phosphate buffer (pH 7.5) was incubated for 5 min at 30 °C in a final reaction volume of 1.0 mL.

\(^b\)PNP arsenolysis activity was performed as above except potassium phosphate buffer was replaced with 20 mM sodium arsenite in 100 mM MOPS buffer (pH 7.5).
Figure 2.9 Dihydrolipoic Acid and the Reduction of Arsenate by PNP. The assays were performed by incubating 10 mM inosine, 6 mM [\(^{75}\)As]-sodium arsenate, 1 mM EDTA, and 1 μg PNP in 100 mM MOPS buffer (pH 7.5) for 30 min at 37°C. The final reaction volume was 100 μL.
Figure 2.10 Arsenate Reductase Activity of PNP with Different Thiols. Assays were performed by incubating 1 µg PNP, 10 mM inosine, 6 mM [\textsuperscript{75}As]-sodium arsenate, and 0.25 - 5 mM of the thiol being evaluated in 100 mM MOPS buffer (pH 7.5) for 30 min at 37 °C. The final reaction volume was 100 µL.
Figure 2.11 Michaelis-Menten Kinetics for PNP Arsenolysis and Reduction of Arsenate. $S =$ different concentrations of DHLP; the reactions contained 1 μg PNP and 6 mM $[^{75}\text{As}]$-sodium arsenate. $V_\text{max} = 143 \mu\text{mol/mg/30 min}; K_m = 2.6 \times 10^{-4} \text{M}$. 
<table>
<thead>
<tr>
<th>DHLP (mM)</th>
<th>thymidine phosphorylase</th>
<th>PNP</th>
<th>phosphorylase b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.2</td>
<td>37.3</td>
<td>—</td>
</tr>
<tr>
<td>2.5</td>
<td>3.5</td>
<td>85.6</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>4.9</td>
<td>101.4 ± 3.08</td>
<td>&lt;0.3</td>
</tr>
</tbody>
</table>

*Assay conditions for PNP are described under Experimental Procedures. For the thymidine phosphorylase assay, MOPS buffer was replaced with 20 mM Tris-HCl (pH 7.3). The reaction contained 6 mM \[^{77}\text{As}^\]-sodium arsenate, 10 mM thymidine, 5 mM DHLP, and 11 μg of *Escherichia coli* thymidine phosphorylase. The reactions were incubated at 37 °C for 30 min. The arsenicals were separated and quantified by HPLC.*
DISCUSSION

The purification and characterization of the enzymes involved in the biotransformation of inorganic arsenic are crucial for understanding the mechanism of inorganic arsenate and arsenite metabolism. We have determined some of the amino acid sequences of partially purified human liver arsenate reductase. Based on amino acid homology, our evidence indicates that it is identical to human purine nucleoside phosphorylase (PNP).

PNP is an essential enzyme of purine metabolism. Many sophisticated biochemical studies of it are available, for a review, see Bzowska et al. (59). It is specific for purine and purine analogues, ribonucleosides, and deoxyribonucleosides. Pyrimidines or pyridines are not active as substrates. PNP catalyzes the cleavage of the glycosidic bond of ribo- and deoxyribonucleosides in the presence of inorganic orthophosphate to yield the purine base and ribose (deoxyribose) 1-phosphate (60, 61, 62, 63, 64). The arsenolysis reaction catalyzed by PNP is similar to phosphorolysis as can be seen in Figure 2.12, but the product ribose 1-arsenate is unstable and rapidly hydrolyzed to ribose and arsenate (60, 65).

Since we were unable to obtain human PNP, we characterized the properties of arsenate reductase using calf spleen PNP, which belongs to the same group of low molecular mass homotrimers with a molecular mass of 86 kDa. They have substrate specificity for 6-oxopurines, their nucleosides, and some analogues (53, 58). The homology of the calf spleen enzyme with the human enzyme is 87% overall and 100% within the active site (57, 58).
1) Purine nucleoside + $\text{H}_2\text{PO}_4^-$ \(\xrightarrow{\text{PNP}}\) purine base + ribose-1-phosphate

2) Purine nucleoside + arsenate \(\xrightarrow{\text{PNP}}\) purine base + ribose-1-arsenate

\(\xrightarrow{\text{H}_2\text{O}}\) ribose + arsenate

3) Purine nucleoside + arsenate \(\xrightarrow{\text{PNP}}\) purine base + ribose-1-arsenate

\(\xrightarrow{\text{DHLP}}\) ribose + arsenite + lipoic acid (oxidized form)

Figure 2.12 Scheme for PNP Catalyzed Arsenate Reduction with DHLP.
Both PNP and DHLP are required for arsenate reduction. The reduction of arsenate in the absence of DHLP was only 2% compared to the complete reaction and about 13% without PNP. We found that the $K_m$ for arsenate in the presence of 2 and 5 mM DHLP was $0.81 \times 10^{-3}$ or $1.8 \times 10^{-3}$ M, respectively. The $K_m$ of human erythrocyte’s PNP for arsenate is $0.74 \times 10^{-3}$ M (60). These are of the same order of magnitude as the $K_m$ of PNP from various sources for phosphate (60).

Although the formation of arsenite requires both PNP and a thiol, the formation of hypoxanthine from inosine by PNP involving phosphorolysis or arsenolysis was not affected by DHLP. The formation of arsenite during or following the arsenolysis reaction catalyzed by PNP could be the result of a thiol-involved hydrolysis of ribose 1-arsenate. A thiol and enzyme-assisted hydrolysis of ribose-1-arsenate could also occur prior to release from the catalytic site. The hydrolysis of ribose-1-arsenate is believed to occur by hydrolytic attack on the arsenate (65), resulting in an O–As bond loss. The precise mechanism of arsenolysis in the presence of DHLP with the formation of arsenite needs further investigation.

The reduction of arsenate in the cell should not be attributed only to a non-enzymatic reaction as has been done in the past. In our experiments, the rate of reduction of arsenate by the reducing agents GSH or ascorbic acid was negligible compared to that by the naturally occurring dithiol DHLP, and synthetic dithiols such as BAL, DMPS, or DTT. The intracellular form of such a dithiol may be a protein that contains dithiols.
It is of concern to us that the intracellular concentrations of lipoic acid are micromolar and not millimolar (66), but there are systems to synthesize and shuttle lipoic acid to where it is needed in cells (67, 68) as well as maintain its oxidative status.

Although rabbit liver arsenite methyltransferase has a thiol requirement satisfied by L-cysteine, GSH, or DTT, DHLP is inactive (69, 70). The rate-limiting step in the methylation of arsenite is reduction of MMA\textsuperscript{V} by monomethylarsonate reductase, which has an absolute requirement for GSH (71). On the other hand, arsenate reductase (PNP) required and preferred DHLP for full activity.

Thymidine phosphorylase had limited activity and phosphorylase \textit{b} no activity as an arsenate reductase. Since PNP has such activity, other intracellular phosphorylases may also have arsenate reductase activity.

DHLP is the prosthetic group of pyruvate dehydrogenase, \(\alpha\)-ketoglutarase dehydrogenase and \(\alpha\)-keto acid dehydrogenase complexes (68). Evidence is accumulating that free \(\alpha\)-DHLP can act at various levels in biochemical pathways, interacting with various protein systems as a substrate, inhibitor, or effector (68). Cytosolic PNP has long been recognized (57, 58, 60, 64, 72). PNP also has been purified and characterized from mammalian mitochondria (73), suggesting an additional site for the formation of highly toxic, trivalent arsenite. Enzymes most sensitive to arsenite are found in mitochondria (74).

The influence of inorganic phosphate on the reduction of arsenate to arsenite and on the other enzymes of inorganic arsenic biotransformation needs investigation. Arsenate and phosphate use the phosphate carrier system to enter cells. They compete for the
carrier. Arsenate is known also to compete with phosphate for enzymes involved in ATP synthesis (39). Arsenate can replace phosphate in various phosphorolysis reactions including PNP (39). PNP has a $K_m$ of $0.74 \times 10^{-3}$ M for arsenate versus $1.8 \times 10^{-3}$ M for phosphate (60, 75). Preliminary evaluation of the role of PNP in reducing arsenate was obtained using human liver cytosol containing millimolar concentrations of intracellular inorganic phosphate (30). The specific activity of human liver cytosol for the reduction of arsenate was 0.9 nmol/mg/30 min. The range of serum inorganic phosphate is 0.8–1.3 mM (76). In the cell however, most of the phosphate is bound to proteins or other constituents such as ATP (77).

After the publication of this work another laboratory independently verified our results using a completely different approach. The Németi Group was studying arsenic metabolism in mitochondria and discovered that mitochondria reduce arsenate to arsenite (78). By subcellular fractionation they determined that the arsenate reductase activity was localized in the cytosol. They also reported that the activity required a dithiol (DTT) and began looking for an endogenous reducing partner. They found that oxidized purine nucleotides (NAD$^+$ and NADP$^+$) but not the reduced forms stimulated the arsenate reductase activity. These results — a thiol containing enzyme which used purine nucleosides as a substrate (such as PNP) allowed them to determine that PNP reduces arsenate to arsenite in rat liver cytosol (79).

Previous work in our lab compared the arsenate reducing activity of a number of different primate cytosols with their thiol content to see if there was a correlation between reductase activity and thiol content (80). We found none, this suggested that some other
factor must be involved in reduction. The Németi Group did a similar experiment to see if there was a correlation between arsenate reductase and PNP activity but did not find one. They did however demonstrate that anion exchange chromatography of rat liver cytosol resulted in the exact co-elution of arsenate reductase and PNP activity indicating that they are the same enzymes. They also demonstrated that the PNP inhibitor CI-1000 and BCX-1777 completely inhibited cytosolic arsenate reductase activity suggesting that PNP is the only enzyme responsible for arsenate reduction under these conditions (79).

Recently the same group has demonstrated that inosine and DTT stimulate arsenate reductase activity in erythrocytes and that this activity can be repressed by BCX-1777. However, they also demonstrated that BCX-1777 does not repress arsenate reductase activity in erythrocytes that were not treated with inosine and DTT (81). The fact that the PNP inhibitor does not affect the basal rate of arsenate reduction suggests that arsenate reduction may be PNP-independent and brings into question the physiological relevance of PNP as and arsenate reductase.

In summary, our data demonstrate that human liver arsenate reductase and human PNP are the same protein. PNP can reduce arsenate to arsenite \textit{in vitro} in the presence of a purine nucleoside and a dithiol, where DHLP is the most active naturally occurring thiol tested so far. Recent studies by another group have shown that PNP-mediated arsenate reduction occurs \textit{in vitro} and can occur \textit{in vivo} in erythrocytes – but only if they are supplemented with inosine and DTT. Further research is needed to determine the extent of PNP arsenate reduction \textit{in vivo} and its relevance in arsenic metabolism.
CHAPTER 3

Arsenic-Iron Interactions in Ferritin

Experimental Procedures

Caution. Arsenic has been classified a human carcinogen by the International Agency for Research on Cancer (3).

Animals. New Zealand White rabbits were purchased from Myrtle’s Rabbitry Inc. (Thompson Station, TN).

Reagents. Sodium arsenate, sodium arsenite, horse spleen apoferritin, horse spleen ferritin, and ferrous ammonium sulfate were purchased from Sigma Chemical Co. (St. Louis, MO). For some experiments apoferritin was prepared from ferritin by reduction (82). Sephacyrl G-300 was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Rabbit anti-ferritin antibodies were purchased from Roche Diagnostics Corporation (Indianapolis, IN). BCIP/NBT Color Development Solution was purchased from Bio-Rad Laboratories (Hercules, CA). Carrier free $[^{73}\text{As}]$-arsenate was purchased from Los Alamos National Laboratory (Los Alamos, NM). $[^{73}\text{As}]$-arsenite was prepared by the method of Reay and Asher (83) and analyzed for purity by HPLC with slight modification of a previously described method (51). Briefly, arsenate and arsenite were separated using a 4.9 mm x 250 mm Hamilton PRP X-100 anion exchange column equilibrated with 50 mM sodium phosphate buffer (pH 8.0) at a flow rate of 2.5 mL/min. The arsenicals were detected and quantified with a postcolumn inline $\beta$-RAM radioisotope detector (INUS Systems Inc., Tampa, FL) using Monoflow III scintillation cocktail (National Diagnostics, Atlanta, GA) at a flow rate of 7.5 mL/min.
Interaction between arsenic and ferritin during ferritin formation. Ferritin reconstitution reactions were performed in a manner previously described (84). Reactions prepared with and without iron contained 1.0 mg apoferritin, 300 µM KIO₃, 1.2 mM Na₂S₂O₃, and 60 µM [³⁷⁷As]-sodium arsenate in 20 mM imadazole buffer pH 7.4. Reactions containing iron had a final concentration of freshly prepared 300 µM Fe(NH₄)₂(SO₄)₂. In addition, a reaction containing apoferritin without [³⁷⁷As]-sodium arsenate and a reaction containing [³⁷⁷As]-sodium arsenate without apoferritin were prepared in order to determine the retention times of ferritin and unbound [³⁷⁷As]-arsenic. All reactions had a final volume of 1.0 mL and were incubated at 25°C for 30 min. To separate the ferritin and non-ferritin bound [³⁷⁷As]-arsenic, reactions were loaded onto a 1 cm x 44 cm Sephacryl S-300 column equilibrated with 30 mM Tris-HCl pH 7.2. The flow rate was 30 mL/hr and 1.0 mL fractions were collected. The fractions were counted for [³⁷⁷As]-arsenic in a LKB Compugamma counter, and protein concentrations were determined by the Bradford method using apoferritin for the protein standard (56).

Relationship between arsenic-ferritin binding and ferritin-ferric oxide hydrate concentration. To determine the effect of iron concentration on arsenic binding, 1 mL reactions with final iron concentrations of 0 µM, 50 µM, 100 µM, 150 µM, 200 µM, and 250 µM freshly made Fe(NH₄)₂(SO₄)₂ were prepared. In addition, the reactions contained 1.0 mg apoferritin, 300 µM KIO₃, 1.2 mM Na₂S₂O₃, and 60 µM [³⁷⁷As]-sodium arsenate in 20 mM imadazole buffer pH 7.4. Reactions were incubated at 25⁰ C for 30 min. The non-ferritin bound [³⁷⁷As]-arsenic was separated from ferritin by ultrafiltration using Centricon 100 cartridges (Millipore Corporation, Bedford, MA). The retentate
containing the ferritin-bound $^{73}\text{As}$-arsenic was washed 4 times with 2.0 mL volumes of 20 mM imidazole buffer pH 7.4 and counted in a LKB Compugamma Counter. The concentration of iron incorporated into apoferritin during ferritin reconstitution was determined by measuring the absorption of ferric iron hydrate ($\varepsilon = 100$) at 420 nm in a Beckman DU-6 spectrophotometer (84).

**Oxidative status of ferritin-bound arsenicals.** Radiolabeled $^{73}\text{As}$-arsenite prepared by reduction (83) was concentrated under a stream of N$_2$ and injected onto a 4.9 mm x 250 mm Hamilton PRP-X100 column equilibrated with ddH$_2$O at a flow rate of 1 mL/min. The void volume containing the $^{73}\text{As}$-arsenite was collected in a tube containing cold sodium arsenite carrier in 20 mM HEPES buffer pH 7.4, and the volume was adjusted to the desired concentration with 20 mM HEPES buffer pH 7.4. The resulting $^{73}\text{As}$-arsenite was determined to be > 99.9% pure by HPLC (51). This method produces highly purified $^{73}\text{As}$-arsenite as the contaminating $^{73}\text{As}$-arsenate binds the column very tightly. However, even though the radiolabeled arsenate is not irreversibly bound to the column, a spare column should be used as it takes quite some time to wash the $^{73}\text{As}$-arsenate off the column with concentrated sodium phosphate buffer. To examine the oxidation state of ferritin-bound arsenic, 1.0 mL reactions containing 1.0 mg horse spleen ferritin, 60 $\mu$M HPLC purified $^{73}\text{As}$-sodium arsenite, and 5 mM glutathione in HEPES buffer pH 7.4 were incubated at 37$^\circ$ C for 30 min. The non ferritin-bound $^{73}\text{As}$-arsenic was separated from the ferritin-bound $^{73}\text{As}$-arsenic by washing the ferritin with 4 successive 2.0 mL volumes of 20 mM HEPES buffer pH 7.4 using Centricon 100 cartridges. The resulting retentate containing the ferritin-bound
[\textsuperscript{73}As]-arsenic was re-suspended in 200 mM sodium phosphate buffer pH 7.4 containing 100 mM cold sodium arsenate and 100 mM cold sodium arsenite in order to remove the ferritin-bound [\textsuperscript{73}As]-arsenicals by competition and simultaneously protect their oxidation state. This mixture was incubated at 37\degree C for 30 min and the non ferritin-bound [\textsuperscript{73}As]-arsenic was removed from the ferritin by ultrafiltration using Centricon 100 cartridges. The ultrafiltrate containing the non ferritin-bound [\textsuperscript{73}As]-arsenicals were analyzed by HPLC to determine their oxidation state.

**Effect of phosphate on arsenic-ferritin binding.** Ferritin was prepared in the absence of phosphate in a 20 mL reaction volume containing 20.0 mg horse spleen apoferritin, 50 mM KIO\textsubscript{3}, 250 mM freshly prepared Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3}, and 1.3 mM freshly prepared Fe(NH\textsubscript{4})\textsubscript{2}(SO\textsubscript{4})\textsubscript{2} in 20 mM imidazole buffer pH 7.4. The ferritin reconstitution reaction was incubated at 25\degree C for 30 min and concentrated by ultrafiltration and washed with 20 mL 20 mM imidazole buffer pH 7.4 in Centriplus 100 cartridges (Millipore Corporation, Bedford, MA). This “phosphate free” ferritin was re-suspended in 2.0 mL 20 mM imidazole buffer pH 7.4 and stored at -70\degree C. To study the effect of phosphate on arsenic-ferritin binding, 1.0 mL reactions containing 1.0 mg “phosphate free” ferritin, 50 \mu M [\textsuperscript{73}As]-sodium and concentrations of 0 \mu M, 10 \mu M, 20 \mu M, 30 \mu M, 40 \mu M, and 50 \mu M sodium phosphate buffer pH 7.4 were prepared. The reactions buffered with 20 mM imidazole pH 7.4 were incubated at 25\degree C for 30 min. The ferritin was washed 4 times with 2.0 mL imidazole buffer pH 7.4 by centrifugation using Centricon 100 cartridges to remove unbound [\textsuperscript{73}As]-arsenic. To determine ferritin-bound [\textsuperscript{73}As]-arsenic, the retentates were counted in a LKB Compugamma counter.
Effect of arsenic on iron uptake during ferritin formation. To determine the effect of arsenic on iron uptake during ferritin reconstitution, 1.0 mL reactions containing 1.0 mg apoferritin, 300 μM KIO₃, 1.2 mM Na₂S₂O₃, and 0 μM, 10 μM, 30 μM, 50 μM, and 100 μM of sodium arsenate in 20 mM imidazole buffer pH 7.4 were prepared. Lastly, 1.3 mM freshly prepared Fe(NH₄)₂(SO₄)₂ was rapidly added. In addition, reactions without sodium arsenate but containing final concentrations of 0 μM, 10 μM, 30 μM, 50 μM, and 100 μM sodium phosphate were prepared. All reactions were incubated at 25°C for 12 min in a Beckman DU-6 spectrophotometer. After 12 min the ferric iron incorporated into ferritin was determined by measuring the absorption of ferric iron hydrate (ε = 100) at 420 nm.

Arsenic-ferritin binding in vivo. To determine if arsenic-ferritin binding occurs in vivo, 100 μg sodium arsenite containing 1.0 mCi [⁷³As]-arsenite pH 7.4 was administered by intraperitoneal injection to a 1.2 kg male New Zealand White rabbit. After 3.0 hr the rabbit was euthanized with CO₂ and the liver was removed, perfused with cold saline and stored at -70°C. To prepare rabbit liver cytosol, rapidly thawed liver was divided into 5 g pieces, minced with scissors, and homogenized with 10 mL ice cold HEPES buffer pH 7.4 in a Dounce homogenizer. The homogenate was centrifuged at 15,000 x g for 15 min at 4°C. Lipids were removed from the supernatant by aspiration and supernatant was then centrifuged at 42,000 x g for 90 min at 4°C. The supernatant was filtered through glass wool to remove lipids and the resulting cytosol was stored at -70°C. A native 7.5% Tris-HCl Ready Gel (Bio-Rad Laboratories, Hercules, CA) with a 4% stacking gel was loaded with 3 sets (each separated by an empty lane) of two adjacent lanes containing 2
μg horse spleen ferritin next to a lane containing 30 μg of radiolabeled rabbit liver cytosol protein. Electrophoresis was carried out at 12 W constant power and the gel was cut into three pieces, where each piece contained a lane with 2 μg horse spleen ferritin next to a lane containing 30 μg of radiolabeled rabbit liver cytosol protein. The three resulting gel pieces were processed as follows. One gel was stained for nonheme iron proteins as previously described (85). Briefly, the gel was soaked in a solution containing 100 mM potassium ferricyanide and 100 mM NaCl in 50 mM Tris-HCl pH 7.5 under dark conditions at 25°C for 10 min. The gel was then developed in solution containing 10% methanol and 10% trichloroacetic acid. Royal blue bands indicate the presence of nonheme iron proteins. Autoradiography was performed on one gel to detect [73As]-arsenic binding proteins by wrapping the gel in polyethylene and exposing it to Kodak XRP-1 X-ray film for 6 days prior to development, after exposure this gel was also stained for nonheme iron. The remaining gel was analyzed by Western blot using standard procedures. The gel proteins were transferred to a nitrocellulose membrane and probed with rabbit anti-ferritin antibodies. After subsequent incubation with secondary anti-rabbit IgG alkaline phosphatase antibodies, the ferritin signal was visualized colorimetrically with Bio-Rad BCIP/NBT Color Development Solution.
RESULTS

Interaction between arsenic and ferritin during ferritin formation. Arsenic binds ferritin but not apoferritin, and iron appears to be critical for binding. The elution profiles of the ferritin reconstitution reactions injected onto a 1 cm x 44 cm Sephacryl S-300 column can be seen in Figure 3.1. The horse spleen ferritin in the control reaction eluted in fractions 14-20 (Figure 3.1A). The $[^{73}\text{As}]$-sodium arsenate in the control reaction containing no iron or apoferritin eluted in fractions 29-38 (Figure 3.1B). The apoferritin and $[^{73}\text{As}]$-sodium arsenate in the ferritin reconstitution reaction containing no iron eluted in fractions 14-20 and fractions 29-38 respectively (Figure 3.1B). In the complete ferritin reconstitution reaction containing iron, the ferritin and $[^{73}\text{As}]$-arsenic co-eluted in fractions 14-20 (Figure 3.1D). This clearly demonstrates that arsenic binds ferritin, the fact that arsenic does not bind apoferritin suggests that it is the iron in ferritin that arsenic is interacting with.

Relationship between arsenic-ferritin binding and ferritin-ferric oxide hydrate concentration. Arsenic-ferritin binding is directly dependent on the iron content of ferritin. Figure 3.2 shows that ferritin reconstitution reactions containing increasing concentrations of iron from 0.0 μM to 250.0 μM ferrous ammonium sulfate resulted in an increase from 0.0 nmol to 37.1 ± 0.8 nmol bound $[^{73}\text{As}]$-arsenic/mg ferritin respectively. In addition, as the iron concentration of the ferritin reconstitution reactions increased, the iron uptake by ferritin determined by the formation of ferric oxide hydrate increased from 0.0 nM to 943.3 ± 14.5 nM ferric iron (Figure 3.2). This clearly demonstrates that the amount of arsenic that can bind ferritin is dependent on the iron content of ferritin.
**Figure 3.1** Arsenic Binds Ferritin *in vitro.* (A) Elution profile of 1.0 mg horse spleen ferritin, the ferritin elutes in fractions 14-20. (B) Elution profile of 60 μM [\(^{79}\text{As}\)]-sodium arsenate, the radiolabeled arsenic elutes in fractions 29-38. (C) Elution profile of ferritin reconstitution reaction in the absence of iron but in the presence of 1.0 mg apoferritin and 60 μM [\(^{79}\text{As}\)]-sodium arsenate, the apoferritin and [\(^{79}\text{As}\)]-arsenic elute in fractions 14-20 and 29-38 respectively. (D) Elution profile for the complete ferritin reconstitution reaction containing 300 μM ferrous ammonium sulfate, 1.0 mg apoferritin, and 60 μM [\(^{79}\text{As}\)]-sodium arsenate, the ferritin and [\(^{79}\text{As}\)]-arsenic co-eluted in fractions 14-20.
Figure 3.2 Arsenic-Ferritin Binding as a Function of Iron(III) Concentration. Ferritin-bound arsenic increases proportionally with the amount of iron in ferritin as determined by absorption of ferric oxide hydrate at 420 nm ($\varepsilon = 100$).
Oxidative status of ferritin-bound arsenicals. The predominant species of inorganic arsenic bound to ferritin is arsenate – not arsenite. The ferritin-bound $^{73}\text{As}$-arsenic produced by incubating 60.0 $\mu$M HPLC purified $^{73}\text{As}$-sodium arsenite with 1.0 mg horse spleen ferritin under highly reducing conditions, was removed competitively by incubation with sodium phosphate, cold sodium arsenate, and cold sodium arsenite. The resulting non-ferritin bound $^{73}\text{As}$-arsenic was determined to be 97.0 ± 1.2 % arsenate by HPLC analysis. Since the cold arsenate and arsenite protected the oxidation state of any radiolabeled arsenic removed by competition, this data demonstrates that arsenite is oxidized to arsenate by ferritin in a highly reducing environment, and that arsenate is arsenical which binds ferric iron. This is consistent with our model.

Effect of phosphate on arsenic-ferritin binding. Phosphate competes with arsenate for binding sites in ferritin. In Figure 3.3 a decrease in the amount of ferritin-bound arsenic can be seen when 1.0 mg “phosphate free” horse spleen ferritin and 50 $\mu$M $^{73}\text{As}$-sodium arsenate were incubated with increasing amounts of sodium phosphate. The amount of ferritin-bound $^{73}\text{As}$-arsenic decreased from 28.3 ± 0.1 nmol/mg ferritin at 0.0 $\mu$M sodium phosphate to 15.3 ± 0.1 nmol/mg ferritin at 50.0 $\mu$M sodium phosphate – a reduction of arsenic-ferritin binding of approximately 54%. A reduction of roughly 50% in arsenic-ferritin binding in the presence of an equimolar amount of phosphate suggests that both arsenate and phosphate bind ferritin competitively and have similar affinities for ferritin.

Effect of arsenic on iron uptake during ferritin formation. Arsenate inhibits the uptake of iron by ferritin in vitro. In Figure 3.4 it can be seen that increasing sodium ars-
Figure 3.3 Competition Between Phosphate and Arsenic-Ferritin Binding.
Figure 3.4 Effect of Arsenate on Iron Uptake by Ferritin. Increasing oxyanion concentrations of arsenate and phosphate during ferritin reconstitution retard iron uptake by ferritin.
nate concentrations in ferritin reconstitution reactions decreased the amount of iron uptake by ferritin as sodium phosphate has been reported to do (37). Also during ferritin reconstitution, sodium arsenate decreased ferritin iron content from 8.8 ± 0.3 μmol iron/mg ferritin in the absence of sodium arsenate to 4.2 ± 0.3 μmol iron/mg ferritin in reactions containing 100 μM sodium arsenate. The 100 μM sodium arsenate decreased ferritin iron uptake by 52.3% compared to the 75.8% decrease in iron uptake measured at equal concentrations of sodium phosphate (Figure 3.4). This result demonstrates that arsenate is similar to phosphate in its ability to retard iron uptake by ferritin as my model predicts.

**Arsenic-ferritin binding in vivo.** Arsenic binds ferritin in vivo. Figure 3.5A shows a gel loaded with 2.0 μg Horse Spleen ferritin in a lane adjacent to 30 μg rabbit liver cytosol incubated with [\(^{73}\text{As}\)]-sodium arsenite, the gel was stained for nonheme iron. The royal blue bands indicate two proteins containing nonheme iron in the [\(^{73}\text{As}\)]-arsenic labeled rabbit liver cytosol which correspond in size to ferritin and its dimer seen in the horse spleen ferritin standard. Figure 3.5B is the Western blot analysis which shows two bands in the [\(^{73}\text{As}\)]-arsenic labeled rabbit liver cytosol corresponding in size to ferritin and its dimer in the horse spleen ferritin standard. Figure 3.5C is the gel used for autoradiography and subsequently stained for iron (Figure 3.5D) which reveals the co-localized [\(^{73}\text{As}\)]-arsenic and iron signals which correspond to the positions of ferritin and its dimer which were observed in Figures 3.5-A and 3.5-B. The co-localization of iron, ferritin, and arsenic in rabbit liver cytosol prepared from a rabbit challenged with [\(^{73}\text{As}\)]-arsenic with ferritin standards demonstrates arsenic-ferritin binding in vivo.
Figure 3.5 Page Analysis of Ferritin and [\(^{73}\text{As}\)]-Arsenic Labeled Binding Proteins. (A) PAGE gel with 2.0 µg horse spleen ferritin and 30 µg [\(^{73}\text{As}\)]-sodium arsenite labeled rabbit liver cytosol in adjacent lanes stained for nonheme iron with potassium ferricyanide. Royal blue bands indicate nonheme iron-containing proteins. (B) Western Blot of 2.0 µg horse spleen ferritin and 30 µg [\(^{73}\text{As}\)]-sodium arsenite labeled rabbit liver cytosol in adjacent lanes probed with rabbit anti-ferritin antibodies. (C) Autoradiogram of gel loaded with 30 µg [\(^{73}\text{As}\)]-sodium arsenite labeled rabbit liver cytosol. Arsenic binding proteins were detected after 6 days of exposure. (D) The same gel used for the autoradiogram stained for nonheme iron after autoradiography.
DISCUSSION

The *in vitro* experiments describing the chromatographic behavior of ferritin reconstitution reactions clearly demonstrate arsenic-ferritin binding. In the ferritin reconstitution reaction containing radiolabeled arsenate, but no iron, apoferritin and arsenic eluted separately at their characteristic elution volumes, indicating that arsenate does not bind apoferritin – the protein shell without iron (Figure 3.1C). However, chromatography of the same reaction with iron added had a different elution profile, the $[^{73}\text{As}]$-arsenic and ferritin co-elute at ferritin’s elution volume, clearly demonstrating that arsenic-ferritin binding occurs, and that iron plays a major role in that binding (Figure 3.1D). This is strongly supported by the results showing that arsenate-ferritin binding increases proportionally with ferritin iron content (Figure 3.2).

The predominant form of inorganic arsenic bound to ferritin is arsenate – not arsenite. The two experiments that demonstrated arsenate-ferritin binding used potassium iodate (a strong oxidant) in the ferritin reconstitution reactions to facilitate the oxidation and uptake of iron by ferritin. Arsenite was not used because the KIO$_3$ would immediately oxidized it to arsenate. However, although we have demonstrated that arsenate binds iron in ferritin, it is not clear if arsenite also binds ferritin. To investigate this, HPLC purified $[^{73}\text{As}]$-arsenite was incubated with horse spleen ferritin under highly reducing conditions. The ferritin-bound $[^{73}\text{As}]$-arsenic was removed by competition with phosphate in the presence of both cold arsenate and arsenite in order to protect its oxidation state. HPLC analysis determined the previously ferritin-bound arsenic to be $97.0 \pm 1.2 \%$ arsenate. This demonstrates that the ferric iron (Fe$^{III}$) of ferritin oxidized the arsenite (As$^{III}$) to
arsenate (As$^V$) – a well characterized redox reaction (13, 38), and that arsenate is the predominant form of inorganic arsenic bound to ferritin.

Arsenate can compete with phosphate for binding sites in ferritin. Ferritin reconstitution reactions containing increasing amounts of sodium phosphate decreased the amount of ferritin-bound arsenic. At equal concentrations of phosphate and arsenate, arsenic-ferritin binding was reduced approximately 54%, compared to arsenic-ferritin binding which occurred in the absence of phosphate suggesting that arsenate and phosphate have similar affinities for ferric iron binding sites (Figure 3.3).

In addition to competing with phosphate for ferritin binding sites, arsenate inhibits iron uptake by ferritin in a manner similar to phosphate. A number of factors contribute to the iron content of ferritin; however, it is known that high iron content corresponds with low phosphate content and vice versa. It is thought that phosphate interferes with the microcrystalline formation of the iron core, forcing the formation of new microcrystalline cores. Experimental evidence shows that ferritin formed under high phosphate conditions contains multiple microcrystalline iron cores of smaller size versus one large core, thus reducing ferritin’s ability to take up and store iron (37). To determine if arsenate had the same effect, ferritin reconstitution reactions with increasing arsenate concentrations were analyzed for ferritin iron content. A parallel experiment was done with phosphate as a control. The results indicate that arsenate, like phosphate, inhibits iron uptake by ferritin (Figure 3.4). This result could be very significant in terms of arsenic toxicity. It has already been shown that arsenic exposure releases iron from ferritin (42). If arsenic releases iron from ferritin, the resulting increase in the iron pool
will stimulate new ferritin synthesis to protect the cell from ROS produced by iron via the Fenton Reaction. If our *in vitro* result accurately reflects what is going on *in vivo*, arsenic interfering with the re-uptake of newly released ferrous iron (Fe\(^{II}\)) produced by the Fe\(^{III}/As^{III}\) redox reaction may also contribute to a larger free iron pool and further oxidative damage.

The animal experiment clearly demonstrated that arsenic-ferritin interactions occur *in vivo*. Cytosol prepared from a rabbit administered 1.0 mCi \(^{73}\text{As}\)-arsenite was analyzed for \(^{73}\text{As}\)-arsenic, iron, and ferritin protein. The autoradiogram for \(^{73}\text{As}\)-arsenic, the Western blot, and the gel stained for nonheme iron proteins, as well as the gel used for the autoradiogram which was subsequently stained for iron, all contained positive signals which corresponded in size to ferritin and its dimer (Figure 3.5). The co-localization of ferritin, \(^{73}\text{As}\)-arsenic, and nonheme iron during PAGE with horse spleen standards clearly demonstrate arsenic-ferritin binding *in vivo*.

The ability of arsenic to limit iron uptake by ferritin and the *in vivo* results shown here combined with another report demonstrating that arsenic liberates iron from ferritin (42), suggests that arsenic-iron redox reactions in ferritin can increase the free iron pool resulting in higher ROS concentrations. This is strongly supported by data demonstrating DNA damage and mutagenesis from arsenic induced ROS in general (43, 44) and more specifically in the presence of ferritin (45). Recent reports also suggest that key enzyme inactivations by arsenic are not due to direct arsenic-enzyme interactions as previously thought (86, 87), but via ROS generated by arsenic (46, 47). In light of these studies our results suggest that arsenic-iron redox reactions occurring in ferritin may be the major
source of arsenic-mediated ROS. Since each ferritin molecule can store a maximum of 4,500 iron atoms with an iron to phosphate ratio of 10:1, ROS generated by arsenic-iron redox reactions in ferritin could occur at very significant levels.

This study only examines ferritin interactions with inorganic arsenic, however, previous work showing that arsenite releases iron from ferritin, also demonstrated that the trivalent and pentavalent forms of monomethylarsonic acid and dimethylarsinic acid release more iron from ferritin than the inorganic species (42). Other studies have also demonstrated that significant amounts of methylated arsenic species are protein bound (88, 89). Further work is needed to determine if the methylated arsenic species bind and interact with ferritin like their inorganic counterparts, and to what extent.

In conclusion, our results demonstrate that arsenic-ferritin interactions occur both in vitro and in vivo and suggest that the source of arsenic induced ROS production are the redox reactions occurring between arsenite and ferric iron in ferritin. Epidemiological studies suggesting that different human populations show variable susceptibility to arsenic’s carcinogenic effects have examined genetic polymorphism, dietary intake of zinc, antimony, selenium, in order to explain these differences (90). To my knowledge no studies have examined iron intake as a parameter in populations challenged with arsenic. Future studies examining arsenic related cancers in conjunction with iron intake may provide some insight to this public health problem. In addition, more research is needed to determine if iron chelation therapy may be an appropriate treatment in conjunction with arsenic chelation therapy for patients suffering from acute arsenic challenge.
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