Identification of the Major Arsenic−Binding Protein in Rat Plasma As the Ternary Dimethylarsinous−Hemoglobin−Haptoglobin Complex

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Identification of the Major Arsenic-Binding Protein in Rat Plasma As the Ternary Dimethylarsinous−Hemoglobin−Haptoglobin Complex

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Chronic exposure to arsenic causes a wide range of diseases such as hyperkeratosis, cardiovascular diseases, and skin, lung, and bladder cancers, and millions of people are chronically exposed to arsenic worldwide. However, little is known about the mechanisms underlying these toxic actions. The metabolism of arsenic is essential for understanding the toxic actions. Here, we identified the major arsenic-binding protein (As-BP) in the plasma of rats after oral administration of arsenite by the use of two different HPLC columns, gel filtration and anion exchange ones, coupled with an inductively coupled argon plasma mass spectrometer (ICP MS). The molecular mass of the As-BP was estimated to be 90 kDa based on results using the former column, and arsenic bound to this protein only in the form of dimethylarsinous acid (DMA^{III}) in the plasma in vivo. In addition, the purified As-BP was shown to consist of two different proteins, haptoglobin (Hp) of 37 kDa (three bands) and the hemoglobin (Hb) α chain of 14 kDa (single band), using sodium dodecylsulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and matrix-assisted laser desorption ionization−time-of-flight mass spectrometry (MALDI-TOF MS), respectively, suggesting that the As-BP was the ternary DMA^{III}−Hb−Hp complex. To confirm the present observations, an arsenic-binding assay was carried out in vitro. Although DMA^{III} bound directly to fresh rat plasma proteins, they were different from that identified in vivo. However, when a DMA^{III}-exposed rat RBC lysate (DMA^{III} binds to Hb in rat RBCs) was added to control rat plasma, a new arsenic peak increased at the expense of the arsenic-Hb one. Furthermore, this new arsenic peak was consistent with the As-BP identified in the plasma in vivo, suggesting that arsenic bound to Hb further binds to haptoglobin (Hp), forming the ternary As−Hb−Hp complex.

Introduction

Exposure to arsenic is considered to be a significant worldwide public health problem for humans (*1, 2*) because it has a number of adverse effects on them, including vascular diseases, and skin, lung, and bladder cancers (*3, 4*). Contrary to these adverse health effects, arsenic has been used as a therapeutic agent for acute promyelocytic leukemia (APL)[1] and is also being newly applied for lymphoproliferative and autoimmune syndromes (*5, 6*). However, little is known about the biological and toxicological mechanisms underlying arsenic-induced cytotoxicity, carcinogenicity, and apoptosis in organs and body fluids. Namely, it is poorly understood which arsenic metabolites or how specific toxic metabolites have these biological and toxicological effects. As the toxicity of arsenic is to be related to the chemical forms (metabolites) or chemical reactions occurring during its metabolism, arsenic metabolites have to be identified and correlated with their toxicity.

Inorganic arsenic taken up orally is transformed through the first pass in the liver into metabolites through consecutive reductive methylations in a form bound to proteins (*7*), and then the metabolites are distributed to organs/body fluids or excreted into the urine mostly in the form of dimethylarsinic acid (DMA^{V}) in mammals (*8, 9*). Interestingly, arsenicals are more abundantly and promptly excreted into the urine of hamsters and humans than into that of rats (*10*). These observations have been explained by the difference in the metabolism of arsenic between the two groups of animals due to the participation of red blood cells (RBCs). Namely, DMA^{III} excreted from the liver is efficiently taken up by RBCs and binds to hemoglobin (Hb) in rats. On the other hand, DMA^{III} is less efficiently taken up by RBCs, and/or hardly binds to Hb, and oxidized to DMA^{V} in the bloodstream, resulting in much more prompt excretion into the urine in humans and hamsters. This is because the affinity of DMA^{III} for rat Hb is much higher than those of human and hamster Hb (*10–12*). Thus, owing to the difference in the affinity of DMA^{III} for Hb, arsenic is much more abundantly and promptly excreted into the urine in the form of DMA^{V} in the arsenic-sensitive animal group than in the tolerant one (*13*).

Trivalent arsenicals are known to be highly reactive with cysteinyl residues in proteins, and to inhibit the activities of several enzymes such as glutathione peroxidases (*14*) and thioredoxin reductase (*15*). These trivalent arsenicals seem to bind to cysteinyl residues in proteins more selectively when two thiol groups are close to each other (*16*).

Recently, Raab et al. (*17*) identified the glutathione (GSH)-conjugated form of pentavalent dimethylmonothioarsinic acid ($DMMTA^{V}$) in plants on exposure to DMA^{V}. However, we have previously shown that only trivalent arsenic compounds, that is, not pentavalent ones, can bind to the thiol groups of proteins

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[1] Abbreviations: arsenate, iAs^{V}; arsenic-binding protein, As-BP; arsenite, iAs^{III}; arsenotriglutathione, $As^{III}(GS)_3$; dimethylarsinic acid, DMA^{V}; dimethylarsinous acid, DMA^{III}; glutathione, GSH; haptoglobin, Hp; hemoglobin, Hb; inductively coupled argon plasma mass spectrometry, ICP MS; matrix-assisted laser desorption ionization-time-of-flight mass spectrometry, MALDI-MS; sodium dodecyl sulfate−polyacrylamide gel electrophoresis, SDS−PAGE.

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in animals (*7*), and only trivalent arsenic-GSH conjugates, such as arsenotriglutathione [$iAs^{III}(GS)_3$] and monomethylarsenodiglutathione [$MMA^{III}(GS)_2$], can be detected in the bile of rats (*18, 19*). We have explained the discrepancy regarding the binding of pentavalent arsenicals to thiol groups. Namely, $DMMTA^V$ is the form of DMA^V activated by sulfide, and this activated form of pentavalent arsenical can bind to GSH (thiol groups) to form the energetically more stable $DMMTA^V$−GSH complex under acidic conditions depending on the pK_a values of the pentavalent arsenical (*20*). Thus, although activated pentavalent arsenicals may bind to thiol groups, it is considered that arsenicals bind to thiol groups in most cases in a trivalent state.

In our previous studies, we found that $DMMTA^V$ taken up by cells was immediately hydrolyzed to pentavalent DMA^V in epidermoid carcinoma A431 cells and was hard to detect in its intact form (*21*). Interestingly, although arsenic of $DMMTA^V$ origin can bind to rat Hb on incubation of pentavalent $DMMTA^V$ with a rat RBC lysate, the arsenical was identified as trivalent DMA^{III} but not pentavalent $DMMTA^V$, that is, not $DMMTA^V$-Hb but the DMA^{III}-Hb complex was formed (unpublished data), suggesting that only trivalent arsenicals, that is, not pentavalent ones can bind to the thiol groups of proteins under physiological conditions.

Arsenic-binding proteins (As-BPs) have been studied by several groups, mainly through the use of an arsenic affinity resin [phenylarsine oxide-agarose (PAO-agarose)], and have been identified as protein-tyrosine kinase, insulin-dependent glucose transporter proteins GLUT4 and galectin 1, the glutathione *S*-transferase P-form (GST-P), and thioredoxin peroxidase II (TPX-II) in 3T3-L1 cells, and Chinese hamster ovary (CHO) cells (*22, 23*). Although the mechanisms underlying the roles of As-BPs are not well understood, trivalent arsenicals have been shown to exert their toxic effects through interaction with the SH-groups of proteins (*24–26*).

As-BPs are commonly detected in rat and hamster plasma on exposure to arsenite (iAs^{III}), and more abundantly in the plasma of rats after exposure for a long period (*10*). A similar finding was made for rat plasma on exposure to DMA^V for a long period, and the arsenic was mostly detected in the forms of As-BPs in the plasma (*12*). Zhang et al. (*27*) also detected an As-BP in the plasma of uremic patients and identified it as transferrin. However, in our preliminary study involving rats, although trivalent arsenicals were bound to proteins on incubation in vitro with rat plasma, they were different from that detected in vivo, suggesting that in contrast with transferrin in human serum, arsenic may not bind to transferrin in rat plasma. Thus, the As-BPs in rat plasma have not yet been identified, and little is known as to which arsenic species bind efficiently to which plasma proteins.

In the present study, we hypothesized that the major As-BP in rat plasma may be produced in some organs and excreted into the plasma or that a protein of some organ origin may form a complex with a plasma protein (As-BP) through a protein–protein interaction. The most major As-BP in the plasma of rats on exposure to arsenite was purified by HPLC on a gel filtration Protein KW-803 column and an anion exchange ES-502 column coupled with an inductively coupled argon plasma mass spectrometer (ICP MS). The major As-BP purified by HPLC was confirmed by SDS−PAGE and then subjected to analysis with a matrix-assisted laser desorption ionization mass spectrometer (MALDI-MS).

Materials and Methods

Reagents. All reagents were of analytical grade. Milli-Q water (Millipore, MA, USA) was used throughout. Trizma HCl, Trizma Base, and 1,3-diaminopropane were purchased from Sigma (St. Louis, MO, USA). Hydrogen peroxide (30%), hydrogen chloride, ammonium acetate, acetic acid, a 28% ammonia solution, sodium arsenite, and dimethylarsinic acid [$(CH_3)_2AsO(OH)$] (DMA^V) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The glycopeptidase F (N-gycosidase F) was purchased from TaKaRa Bio. INC (Otsu, Japan). The arsenic standard solution (1000 μg/mL) for ICP MS was purchased from SPEX CentiPrep (Metuchen, NJ, USA). Stock solutions of all arsenic compounds (10 mM) were prepared from the respective standard compounds. All stock solutions were stored in the dark at 4 °C. Diluted standard solutions for analysis were prepared daily prior to use.

HPLC-ICP MS Analysis. The HPLC system consisted of a PU-610 liquid chromatograph solvent delivery pump and a DG 660B-2 degasser (GL Sciences Co., Tokyo, Japan). Polymer-based gel filtration columns (Shodex Asahipak GS-220 HQ, 300 mm × 7.6 mm i.d., and Protein KW-803 7G, 500 mm × 7.6 mm i.d., Showa Denko, Tokyo) with exclusion limits of 3 and 170 kDa were used to separate low and high molecular weight constituents, respectively. Twenty or 200 μL aliquots of sample solutions were applied to the columns, and then the columns were eluted with 50 mM ammonium acetate buffer (pH 6.5 at 25 °C) or 50 mM Tris-HNO_3 buffer (pH 7.4 at 25 °C), respectively, at the flow rate of 0.6 mL/min. An anion exchange ES-502N 7C column (Shodex Asahipak 100 mm × 7.6 mm i.d.) was eluted with a linear concentration gradient of 20−200 mM 1,3-diaminopropane buffer (pH 9.0 at 25 °C), at the flow rate of 1.0 mL/min. One-hundred microliter aliquots of sample solutions were applied to this column.

Arsenic in the eluate was monitored with an Agilent HP 7500 ICP MS (Yokogawa Analytical Systems, Hachiouji, Japan) equipped with an octopole reaction system (ORS) with a D_2 gas flow of 1.0 mL/min (*28*) to prevent molecular interference by $^{40}Ar^{35}Cl^+$ (signal at *m*/*z* 77). Online ICP MS data were processed with software developed in-house.

Animal Experiments. All animal experiments were carried out according to the "Principles of Laboratory Animal Care" (NIH version, revised 1996) and the Guidelines of the Animal Investigation Committee, Graduate School of Pharmaceutical Sciences, Chiba University, Japan.

Male SD rats (SLC Japan Co., Tokyo) were purchased at 7 weeks of age and housed in a humidity-controlled room maintained at 22–25 °C with a 12 h light-dark cycle. The rats were given free access to a commercial diet (CE-2; Clea Japan Co.) and tap water. After a 1-week acclimation period, rats weighing 200–250 g were assigned to experimental groups.

Arsenite was dissolved in purified water at the concentration of 3.0 mg As/mL and then administered orally at the dose of 3.0 mg of As/kg of body weight to rats of 8 weeks of age, which were euthanized at 3 and 6 h, and 1, 3, 5, and 7 days after the administration. Controls were administered an equivalent volume of purified water. Heparinized blood (or nonheparinized blood) was obtained from the left ventricle of the heart and then immediately centrifuged at 1500*g* at 4 °C for 10 min to obtain plasma (or serum).

Purification of the Arsenic-Binding Protein by Gel Filtration and Anion Exchange Column Chromatography. Proteins were detected based on the absorbance at 280 nm with a UV detector (Shimadzu, Kyoto, Japan). In the first separation step, the As-BP in the rat plasma was eluted from a gel filtration KW column with 50 mM Tris-HNO_3 buffer (pH 7.4 at 25 °C) at a flow rate of 0.6 mL/min, and the As-BP fraction between the retention times of 13 and 15 min was collected.

In the second step, the fraction obtained with the gel filtration column was concentrated and desalted by ultrafiltration with an Amicon Ultra-15 centrifuge filter device on a 10 kDa cutoff membrane (Millipore) and then separated on an anion exchange ES column by elution with a linear concentration gradient of 1,3-diaminopropane (pH 9.0, adjusted with concentrated hydrochloric

acid). Afterward, the column was eluted with buffer A [20 mM 1,3-diaminopropane (pH 9.0 at 25 °C)] for the initial 5 min and then with a linear concentration gradient of 0% buffer A to 100% buffer B [200 mM 1,3-diaminopropane (pH 9.0 at 25 °C)] over 15 min, followed by 100% buffer B for an additional 15 min (flow rate, 1.0 mL/min). The As-BP fraction (collected between the retention times of 16 and 18 min) was concentrated and desalted by ultrafiltration with an Amicon Ultra-15 centrifuge filter device. Finally, the concentrated fraction was again applied on a KW column.

SDS−PAGE Analysis. Each protein fraction (1 μg of protein/sample) was subjected to separation by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) according to the method of Schägger and von Jagow (1987), using a 4.75% stacking gel and a 10−15% separation gel. The separated protein bands on the gel were detected by silver staining (Wako). After scanning and destaining, the protein bands were subjected to tryptic digestion.

Identification of the Arsenic-binding Protein by Matrix-Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS). The protein bands obtained on SDS−PAGE were cut into slices and then subjected to in-gel tryptic digestion at 37 °C for 20 h. After the digestion, the samples were desalted and then concentrated to obtain peptides using a ZipTip C18 pipet tip (Millipore). Freshly prepared 1,2-dimethoxy-4-hydroxycinnamic acid (Sigma Aldrich) was used as a matrix solution at a concentration of 10 mg/mL in 50% acetonitrile containing 1% trifluoroacetic acid. An approximately 1.5 μL portion of the desalted and concentrated sample/matrix mixture in the ZipTip C18 pipet tip was directly spotted onto the MALDI target and dried in air. The MALDI-MS measurements were performed with an AXIMA CFR-plus time-of-flight mass spectrometer (Shimadzu/Kratos, Kyoto) equipped with a pulsed N_2 laser at λ 337 nm, with a pulse width of 3 ns and a frequency of 10 Hz. MALDI mass spectra in the range of *m*/*z* 1−5000 were obtained in the positive reflection mode by averaging 200 individual laser shots for 450 μm^2 in the raster scan mode. Mass calibration was carried out according to the steps mentioned below. Provisional peak assignment was performed by calibration using the two peaks of bradykinin fragment 1–7 ($[M + H]^+$, *m*/*z* 757.4) and ACTH fragment 18–39 ($[M + H]^+$, *m*/*z* 2465.2). Proteins (according to the peptide fragments of protein) were identified with an online search engine, Mascot Peptide Mass Fingerprint (www.matrixscience.com) with following parameters: (i) NCBInr; (ii) one missed cleavage; (iii) fixed modifications, carbamidomethylation (C); (iv) peptide tolerance up to 100 ppm. A mowse score of higher than 45 was accepted to indicate a reliable protein or homologue.

In Vitro Incubation System. The major As-BP in the purified fraction at each step was incubated with glycopeptidase F (GPF) (at 1 mU GFP per 25 μg of glycoprotein) at 37 °C for 40 h and then confirmed by SDS−PAGE.

Results

Time-Related Changes in the Distribution of Arsenic in Plasma after a Single Oral Administration of Arsenite to Rats. The arsenic distributions in rat plasma (or serum) obtained at 3 and 6 h, and 1, 3, and 5 days after a single oral administration of iAs^{III} were determined on a gel filtration GS 220 column, because this column is known to separate various low molecular weight arsenicals and arsenic metabolites, while separating high molecular weight proteins from these low molecular weight metabolites (*29*). Figure 1 shows that, although the As-BP (retention time, 9.0 min) was detected as a small arsenic peak even in the control rat plasma, it increased significantly with time on day 1 and remained at the elevated level until day 5. In addition, DMA^V was also detected at an early time point together with arsenobetaine (AsB), both as free forms (nonprotein bound forms), in the plasma. On the other hand, we observed in our previous study involving rats that

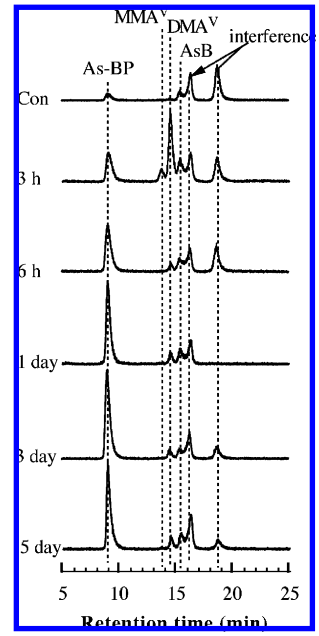

Figure 1. Time-related changes in the distribution of arsenic in rat plasma after a single oral administration of arsenite. The distribution of arsenic in plasma obtained from SD rats was determined on a GS column by HPLC-ICP MS. Plasma was obtained from rats sacrificed at 3 and 6 h, and on 1, 3, and 5 days after a single oral administration of iAs^{III}. Control plasma was prepared from rats orally administered purified water. Under the present conditions, arsenate and arsenite were eluted at the retention times of 13.8 and 23 min, respectively.

arsenic was excreted into the urine mostly within 1 day after a single oral administration of iAs^{III} and had decreased to the control level by day 3 (*10*). On the basis of the present plasma and previous urine observations, we assumed that the major As-BP in the plasma of rats was not excreted into the urine within a short time period after a single administration of iAs^{III}.

Distribution of Arsenic in Rat Plasma (or Serum) Determined on a Protein KW Column by HPLC-ICP MS. Although low molecular weight arsenic metabolites can be well separated on a GS 220 column, arsenic bound to high molecular weight proteins cannot be separated on this column. Therefore, we used a Protein KW column to separate high molecular weight As-BPs according to the method reported elsewhere (*7*). The HPLC-ICP MS profiles in Figure 2 show the distributions of arsenic (^{75}As) and selenium (^{82}Se), as a reference, in rat plasma or serum. Only a single As-BP peak (^{75}As) was detected at the retention time of 14.2 min for the plasma (with heparin), as shown in Figure 2A. On the other hand, two major selenium peaks were detected at the retention times of 11.2 and 14.2 min in the ^{82}Se profile, which corresponded to extracellular glutathione peroxidase (eGPx) and selenoprotein P (Sel P), and the latter was eluted at the same retention time as that of the major As-BP peak, suggesting that arsenic may bind to Sel P in plasma (Figure 2A). Sel P is known to be a heparin-binding protein (high affinity to heparin), and the Sel P−heparin complex is eluted faster than the free Sel P (*30*). Then, the arsenic and selenium in the serum (without heparin) were detected on the same column to determine whether or not arsenic and selenium are coeluted. Only the Sel P peak, that is, not the arsenic one, for the serum shifted, that is, from the retention time of 14.2 min for plasma to that of 16.2 min for serum, as shown in Figure 2B. Thus, it was concluded that arsenic did not bind to Sel P, and the molecular mass of the As-BP was assumed to be approximately 90 kDa based on those of Sel P (or Hb) and eGPx. At the same time, we found that the major As-BP was

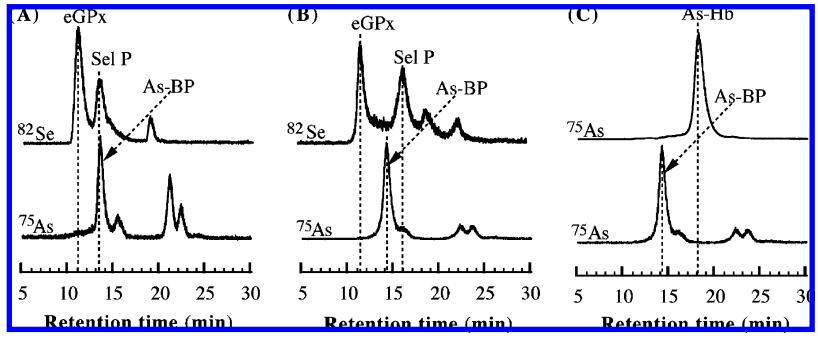

Figure 2. Arsenic-binding proteins and selenoproteins in rat plasma (or serum) determined on a KW column by HPLC-ICP MS. As-BP and selenoproteins (GPx and Sel P) in rat plasma (A) and serum (B) were determined on a KW column, the column being eluted with 50 mM Tris-HNO_3 (pH 7.4) at the flow rate of 0.6 mL/min. The DMA^{III}−Hb complex in the rat RBC lysate obtained on a single oral administration of iAs^{III} to rats (C) was also determined on the same column. The same arsenic profile as that for serum (B) is also shown in (C) for reference. Arsenic and selenium were monitored at *m*/*z* 75 and 82, respectively, by ICP MS.

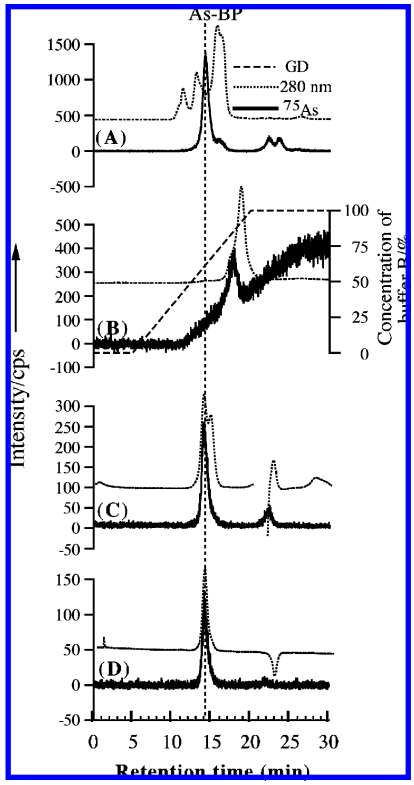

Figure 3. Arsenic-binding protein in rat plasma purified by four steps on gel filtration and anion exchange columns by HPLC-ICP MS. The major As-BP in rat plasma was separated on a KW column (A), and the As-BP fraction was concentrated by ultrafiltration. Then the concentrated solution was applied to an anion exchange ES column (B), and the column was eluted with a linear concentration gradient of 1,3-diaminopropane, from 20 to 200 mM, pH 9.0. The As-BP fraction was combined and concentrated/desalted by ultrafiltration, and then it was purified twice on a KW column, as shown in (C) and (D). Arsenic was monitored at *m*/*z* 75 by ICP MS (solid line), and proteins were detected based on the absorbance at 280 nm with a UV detector (dotted line). The gradient program is depicted as a thick dotted line.

not consistent with the arsenic−Hb complex on this column, as shown in Figure 2C, indicating that the major As-BP in the plasma was not the arsenic−Hb complex.

Purification of the Arsenic-binding Protein in Rat Plasma on Gel Filtration and Anion Exchange Columns by HPLC-UV-ICP MS. To identify the As-BP in plasma, it was purified on two different columns, that is, gel filtration KW and anion exchange ES ones, by four steps, as shown in Figure 3A−D. Rat plasma obtained on day 1 after a single administration of iAs^{III} was selected as the plasma source throughout the present experiment based on the time-related changes shown in Figure 1. The major As-BP in the plasma was separated, retention times 13−15 min, on a KW column (Figure 3A). The UV (280 nm) absorbance indicated that most proteins were removed at this step (dotted line), suggesting that the As-BP is not a major protein. The major As-BP fraction obtained with the KW column was subjected to anion exchange column chromatography with elution with a concentration gradient of 1,3-diaminnopropane, as shown in Figure 3B. The As-BP in this fraction was separated from major contaminating proteins, and the fraction obtained between retention times of 16.0 and 17.8 min with an ES column was concentrated by ultrafiltration for further separation on a KW column, as shown in Figure 3C. Two sharp overlapping protein peaks (dotted lines) were clearly detected in the UV absorbance profile, the major arsenic peak being eluted at the same retention time as that of the faster eluting one. The fraction obtained between retention times of 14.2 and 16.0 min was concentrated by ultrafiltration and then subjected to the same gel filtration chromatography on a KW column, as shown in Figure 3D. A single protein peak was coeluted with the arsenic peak, suggesting that the major As-BP was purified under the present separation and detection conditions.

To identify the arsenic species in the purified As-BP, the purified protein was oxidized with H_2O_2. Only DMA^V, that is, not other arsenic species, was detected in the oxidized reaction solution (data not shown), suggesting that arsenic bound to the major As-BP in the form of a dimethylated arsenical, most probably as trivalent DMA^{III}.

SDS−PAGE Analysis of Protein Fractions Obtained with Gel Filtration and Anion Exchange Columns. To confirm the step of purification of the major As-BP in plasma, the purified fraction at each step was subjected to SDS−PAGE (10% polyacrylamide gel) analysis, as shown in Figure 4. Four protein bands increased with the purification steps (A−D for steps 1–4), three of them corresponding to a molecular mass of 35 kDa (bands 1, 2, and 3), and the other to one of 15 kDa (band 4). However, the present SDS−PAGE results were not consistent with those obtained on the column chromatography shown in Figure 3. Namely, the result of column chromatography on a KW 803 column suggested a molecular mass of 90 kDa, while that of SDS−PAGE indicated one of 35 kDa, suggesting that the As-BP is a complex formed through a protein–protein interaction that can be separated into the individual proteins on treatment with SDS. When the purified As-BP fraction was subjected to SDS−PAGE (15% polyacrylamide gel), the 15 kDa protein band (band d) was separated into two bands corresponding to 15 and 14 kDa (bands 5 and 6) (data not shown).

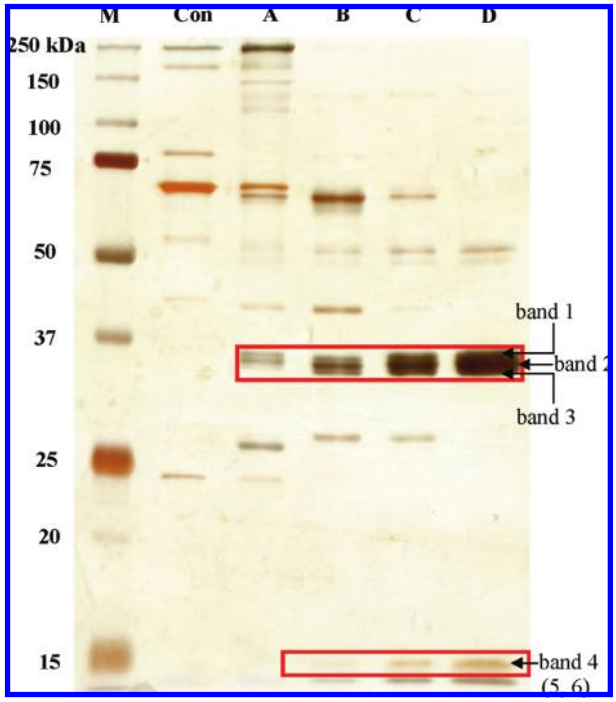

Figure 4. SDS−PAGE profile of each protein fraction obtained on gel filtration and anion exchange columns by HPLC-ICP MS. The major As-BP fraction obtained on the column chromatography shown in Figure 3 was subjected to SDS−PAGE (15% polyacryamide gel), the concentration of proteins in the applied fraction being adjusted to 1 μg of protein/sample. Proteins were detected on SDS−PAGE by silver staining. The marker proteins are shown to the left (lane M). Lane Con is that of nonpurified crude plasma, and lanes A, B, C, and D are those of the As-BP fractions separated in the four purification steps.

MALDI-TOF Mass Spectrometry of the Major Arsenic-Binding Protein after Digestion with Trypsin. Figure 5 shows the mass spectrum of a total tryptic digest of the major As-BP (bands 1, 2, and 3 corresponding to 35 kDa in Figure 4), typical peptide fragments for band 2 out of the three bands being presented as an example. Ten peptide fragment peaks (a−j) were clearly detected in the spectrum, and the amino acid sequences were determined, as shown in Table 1, indicating that the three bands corresponding to 35 kDa were of the same protein, that is, haptoglobin (Hp), as shown in Table 2. The amino acid sequence of Hp is shown in Figure 6. Thus, the molecular weight was determined to be 39 kDa, which was different from that estimated on SDS−PAGE (35 kDa). On the other hand, the 15 kDa protein (band 4) was identified as the Hb α chain (Table 2) because it was further separated into two bands (bands 5 and 6). No peptide that matched the Hb β chain was detected in band 6.

Regarding the three 35 kDa bands for Hp obtained on SDS−PAGE in Figure 4, we assumed that Hp might contain different sugar derivatives because Hp is a glycoprotein. In fact, the three 35 kDa bands were converted into a single band on treatment of the major As-BP with glycosidase, suggesting that Hp is modified differently by different carbohydrates (data not shown).

Protein–Protein Interaction between a Rat RBC Lysate and Plasma Proteins. The formation of a complex between arsenic, Hb and Hp was examined in vitro by incubation of a rat RBC lysate (that contained the arsenic−Hb complex in the form of DMA^{III}-Hb) with plasma (that contained Hp and/or the Hb−Hp complex). The RBC lysate was prepared from rats injected with iAs^{III}, while the plasma was from control rats. Arsenic in the RBC lysate was mostly detected as a single arsenic−Hb peak on a KW column, as shown in Figure 7A. On the other hand, arsenic was not detected in the plasma. When the RBC lysate was incubated with the plasma, the arsenic−Hb decreased, with a shift from the original arsenic−Hb peak to a new peak at the retention time of 14.2 min, which corresponds to the major As−BP peak observed in vivo, as shown in Figure 7B. Thus, the major As−BP in rat plasma was suggested to be the ternary complex DMA^{III}−Hb−Hp.

Discussion

Hp is a heterotetrameric ($\alpha_2\beta_2$) plasma glycoprotein that is secreted from the liver. One of the functions of Hp is to bind free Hb released from RBCs on lysis due to inflammation and trauma (*31*). It forms a stable 1:l complex with Hb in which each Hp β-subunit binds to an $\alpha\beta$ dimer of Hb. The Hb−Hp complex is removed from the bloodstream by parenchymal and reticuloendothelial cells in the liver with subsequent degradation of the protein moiety and conservation of iron (*32*). On the other hand, unbound free Hb oxidizes lipids and proteins and causes tissue (kidney glomerulus) damage by catalyzing the production of iron-derived hydroxyl radicals through Fenton reactions. The formation of a complex between Hb and Hp also prevents the damage to tissues/organs caused by free Hb (*33, 34*). In the present study, we isolated the major As-BP present in rat plasma after a single oral administration of arsenite, and identified it as the ternary DMA^{III}−Hb−Hp complex for the first time.

Inorganic iAs^{III} is known to be metabolized through consecutive reductive methylations in a form bound to proteins in animals (*7*). Among arsenic metabolites, trivalent dimethylated arsenical DMA^{III} is known to exhibit high affinity to Hb in rat RBCs (*11, 12*), and the DMA^{III}−Hb complex is retained for a long time in rat RBCs. However, nothing is known of the mechanism underlying the metabolism of the arsenic accumulating in RBCs, that is, metabolism leading to its excretion into the urine. Recently, a number of reports indicated that erythrocytes are the main target for arsine gas (AsH_3) because AsH_3 induces hemolysis in the living body (*35*). However, no reports have been published on the hemolysis induced by DMA^{III} in vivo or in vitro. In the present study, we hypothesized that As-BPs such as the DMA^{III}−Hb−Hp complex in plasma might be produced through hemolysis under physiological and pathological conditions. This is because the biological half-time of RBCs is about 120 days in rats, and aged or damaged RBCs are mainly removed from the circulation through the phagocytic activity of macrophages in the liver and spleen (*36*). A few aged RBCs are also hemolyzed in the circulation. Recent studies have also indicated that hemolysis can be induced by trace elements such as selenium compounds (ebselen and diethyldithiocarbamate selenotrisulfide) through their production of reactive oxygen species (ROS), which then attack the RBC membrane (*37*).

Even though DMA^{III} may bind to thiol groups on any proteins in control plasma, and more to thiol groups on Hb in rats, very little of the arsenic−Hb−Hp complex was detected in control rat plasma, suggesting that the level of Hb−Hp complex produced on physiological hemolysis is very low in control plasma. In fact, when an arsenic-exposed rat RBC lysate was added to fresh control plasma, the arsenic−Hb−Hp complex was readily produced consistently in an in vivo experiment (Figure 7). We proposed the mechanism underlying the formation of the As−Hb−Hp complex, as shown in Figure 8. Namely, DMA^{III} binds to Hb in RBCs of rats, and then the DMA^{III}−Hb complex is released on hemolysis into the plasma, where it further binds to Hp to form the ternary DMA^{III}−Hb−Hp complex. However, although DMA^{III} binds to plasma proteins

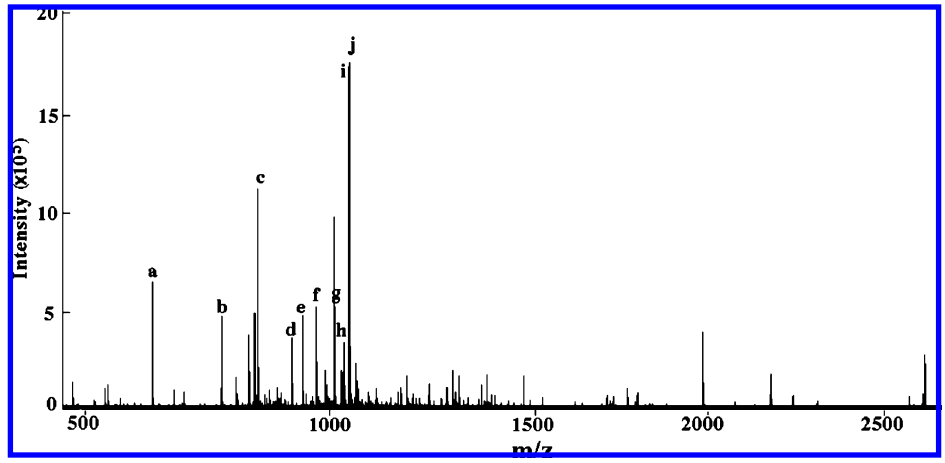

Figure 5. Identification of the major arsenic-binding protein in rat plasma by MALDI-TOF mass spectrometry. The major As-BP (band 2 in Figure 4) was digested with trypsin, and the resulting peptides were subjected to MALDI-TOF MS. Peptides that could be identified from mass data are listed in Table 1, and are labeled as a−j according to their molecular masses. These mass sizes and sequence numbers along with the amino acid sequence of Hp are presented in Table 1.

Table 1. Peptide Sequences Detected on MALDI-TOF MS

peak[a]	residue numbers	peptide sequence	m/z_{obs} [b]	m/z_{calc} [c]	no. of mass values matched	miss sequence
a	228–232	NVNFR	649.27 ± 0.10	648.34	7	0
b	212–218	DYVAPGR	777.27 ± 0.05	776.38	3	0
c	177–183	VVLHPER	849.77 ± 0.53	848.77	10	0
d	112–119	GSFPWQAK	920.83 ± 0.57	919.56	6	0
e	184–192	SVVDIGLI	943.44 ± 0.06	942.57	3	0
f	169–176	NQLVEIEK	973.05 ± 0.52	971.58	6	0
g	219–227	MGYVSGWGR	1012.05 ± 0.12	1011.50	10	0
h	278–286	HTFCAGLTK	1034.34 ± 0.04	1033.50	2	0
i	203–211	VMPICLPSK	1045.26	1044.28	1	0
j	86–94	CEAVCGKPK	1048.03 ± 0.62	1047.48	3	0

[a] Letters indicate peaks obtained by the corresponding letters in Figure 5. [b] m/z_{obs}, the observed mass to charge ratios. [c] m/z_{exp}, the calculated mass to charge ratios.

Table 2. Arsenic-Binding Protein in Rat Plasma Identified on MALDI-TOF MS

band no.	name of protein	MW (Da)	total score	sequence coverage (mass values[b])
band 1	haptoglobin	39	22	22% (26)
band 2	haptoglobin	39	55	23% (51)
band 3	haptoglobin	39	32	10% (19)
band 5	haptoglobin (alpha chain)	15	11	32% (45)
band 6	haptoglobin (beta chain)		ND[a]	

[a] ND, not detected. [b] Matched number of mass values.

of control rats in vitro, any of these DMA^{III}-plasma proteins is not consistent with the DMA^{III}−Hb complex observed in vivo; that is, DMA^{III} did not form the DMA^{III}−Hb−Hp complex but bound to the iron-binding protein that corresponded to transferrin (data not shown). On the basis of these findings, the accumulation of arsenic in the plasma of rats on exposure to iAs^{III} can be explained either that arsenic may accelerate hemolysis by producing ROS, which cannot take place under physiological conditions, or that the biological half-time of the arsenic−Hb−Hp complex is longer than that of the Hb−Hp complex due to the binding of arsenic. However, this assumption requires supporting data.

Arsenic compounds have long been recognized to have adverse health effects on humans (*1–3*). In particular, epidemiological studies have indicated that a peripheral vascular disease, known as "blackfoot disease," or a variety of vascular diseases such as Reynaud's phenomenon, cardio- and cerebrovascular diseases, arteriosclerosis, and hypertension (*4, 38, 39*) are associated with arsenic exposure. However, the mechanisms underlying these arsenic-induced diseases are not precisely understood. Recent studies indicated that oxidative stress is a major cause of cellular injury, and induces and increases plasma lipid peroxides in people in arsenic-affected districts through the production of ROS (*40*).

In the present study, although we obtained the first evidence that the major As-BP in the plasma is the DMA^{III}−Hb−Hp complex, little is known about the mechanism underlying the formation of the As-BP in the bloodstream. In fact, although we observed high scores for preprohaptoglobin (total scores, 45, 70, and 40) for matched peptides in the three bands (bands 1, 2, and 3) according to the Mascot search results, it is still not clear whether Hp in the ternary complex is the mature Hp or the primary transcription product preprohaptoglobin. However, in the biosynthesis and processing of Hp, preprohaptoglobin contains both α- and β-subunit regions, and it is finally secreted into the plasma as mature Hp and not in the form of preprohaptoglobin (*41*). On the other hand, the As-BP appearing in rat plasma in vivo had significantly increased by 24 h after the administration of iAs^{III}, and the level remained unchanged until day 5 (Figure 1). Our previously obtained data for rats indicated that more than 75−80% of the dose was recovered in RBCs in the form of DMA^{III} by 24 h after a single oral administration of iAs^{III}, and the amount of excreted arsenic in the urine was unchanged from day 3 to 7 (low, a negligible control level) (*10*).

CD163 is known to be a cysteine-rich scavenger/receptor expressed in monocyte-macrophage lineage for Hb, mediating endocytosis of the Hb−Hp complex, whereby Hb is degraded into bilirubin and free iron by heme-oxygenase (HO) (*42*). We hypothesize that the DMA^{III}−Hb−Hp complex may not be

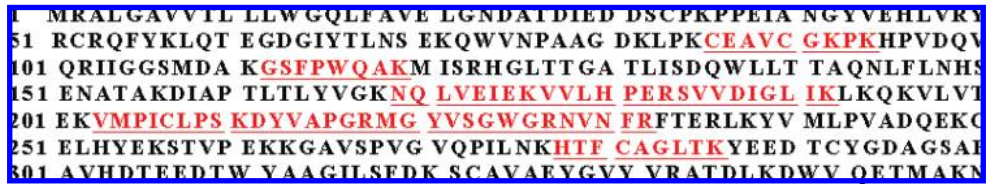

Figure 6. Animo acid sequence of haptoglobin in rat plasma. The red-colored amino acid sequences with underlining are the sequences assigned according to the mass sizes obtained for the tryptic digests of rat Hp on MALDI-TOF MS (see Table 1 and Figure 5).

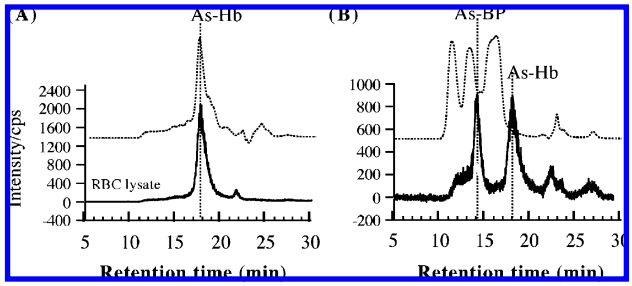

Figure 7. Protein–protein interaction between a rat RBC lysate and plasma determined on a KW column by HPLC-ICP MS. A 20% rat RBC lysate containing the As−Hb complex (A) was obtained from rats sacrificed 1 day after a single oral administration of iAs^{III}, and incubated with control rat plasma in the ratio of 1:20 (B). The distributions of arsenic (solid lines) and proteins (dotted lines) were determined on a KW column by HPLC-ICP MS and with UV, respectively.

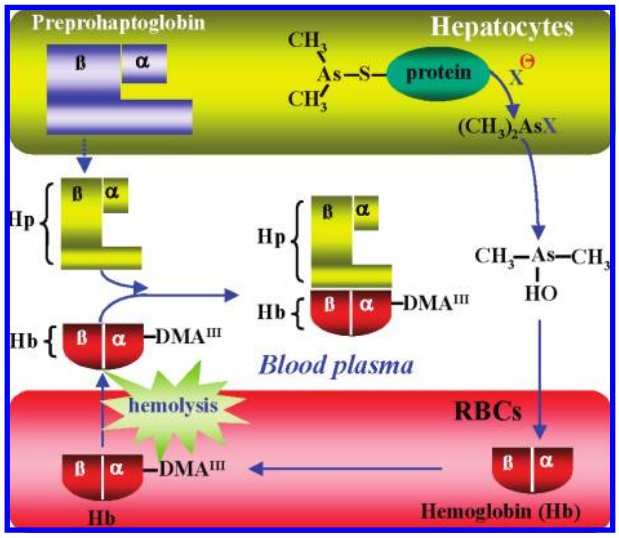

Figure 8. Proposed mechanism underlying the production of DMA^{III}−Hb−Hp complex in the bloodstream on exposure of iAs^{III} to rats. The production of the ternary complex in the bloodstream of rats was proposed: Preprohaptoglobin produced in hepatocytes is excreted into the plasma after the post-translational processing to mature haptoglobin (Hb), whereas, inorganic arsenic is methylated to dimethylarsonous acid (DMA^{III}) in hepatocytes and excreted in the form of DMA^{III}, which is promptly taken up by RBCs in rats to form the DMA^{III}−Hb complex in RBCs. The DMA^{III}−Hb complex remained in RBCs in rats. However, the DMA^{III}−Hb complex is released into the bloodstream by hemolysis either due to lifespan or toxic effect of arsenic, and then the DMA^{III}−Hb complex binds to Hp by the high affinity between Hb and Hp according to the physiological role to sequester Hb.

recognized (or degraded) by the CD163 scavenger/receptor since DMA^{III} binds to Hb.

Although the As-BP identified in human plasma was transferrin (*27*), that is, different from the present As-BP in rat plasma, it is not known whether or not arsenic bound to transferrin and Hb−Hp has different biological and toxicological effects. Further studies are needed to elucidate the mechanisms underlying the effects of arsenic in the bloodstream in animal models.

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