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Interaction of Trivalent Arsenicals with Metallothionein†

Guifeng Jiang,‡ Zhilong Gong,‡ Xing-Fang Li,‡ William R. Cullen,§ and X. Chris Le*,‡

Department of Public Health Sciences, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Alberta, T6G 2G3 Canada, and Department of Chemistry, University of British Columbia, Vancouver, British Columbia, V6T 1Z1 Canada

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Arsenic is a human carcinogen, causing skin, bladder, and lung cancers. Although arsenic in drinking water affects millions of people worldwide, the mechanism(s) of action by which arsenic causes cancers is not known. Arsenic probably exerts some toxic effects by binding with proteins. However, few experimental data are available on arsenic-containing proteins in biological systems. This study reports on arsenic interaction with metallothionein and established binding stoichiometries between metallothionein and the recently discovered trivalent metabolites of arsenic metabolism. Size exclusion chromatography with inductively coupled plasma mass spectrometry analysis of reaction mixtures between trivalent arsenicals and metallothionein clearly demonstrated the formation of complexes of arsenic with metallothionein. Analysis of the complexes using electrospray quadrupole time-of-flight tandem mass spectrometry revealed the detailed binding stoichiometry between arsenic and the 20 Cys residues in the metallothionein molecule. Inorganic arsenite (As^{III}) and its two trivalent methylation metabolites, monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}), readily bind with metallothionein. Each metallothionein molecule could bind with up to six As^{III}, 10 MMA^{III}, and 20 DMA^{III} molecules, consistent with the coordination chemistry of these arsenicals. The findings on arsenic interaction with proteins are useful for a better understanding of arsenic health effects.

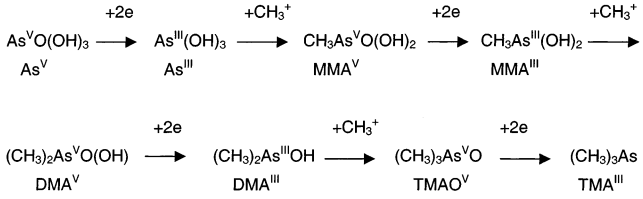
Introduction

Arsenic is classified as a human carcinogen. Epidemiological studies of populations from Taiwan, Argentina, and Chile exposed to high levels of arsenic via ingestion of contaminated drinking water have shown an association between the arsenic ingestion and the prevalence of skin, lung, and bladder cancers (*1*−*4*). Numerous papers from other areas of the world, such as India, Bangladesh, Inner Mongolia (China), and Mexico, also attributed the cancer etiology to the high levels of arsenic in their drinking water (*1*−*7*).

In addition, there is considerable debate about several noncancerous effects, such as hypertension and diabetes, that may result from exposure to low levels of arsenic (*1*, *2*, *8*, *9*). There is a lack of an adequate animal model for arsenic carcinogenesis possibly because of metabolic differences between rodents and humans. Consequently, the mechanism(s) of arsenic toxicity with respect to cancer is not clearly established (*1*, *2*, *8*−*14*). An improved understanding of arsenic toxicity and the dose−response relationship for relatively low levels of arsenic is essential in order to improve the risk assessment process.

The toxicity of arsenic differs dramatically depending on the chemical form of the arsenic. A major process in arsenic metabolism is the biomethylation of arsenic (*14*−*17*). The stepwise methylation process involves a sequence of a two electron reduction of pentavalent to trivalent arsenicals followed by oxidative addition of a methyl group (*15*−*20*), as outlined in Scheme 1. Methyltransferases are responsible for methyl transfer with S-adenosyl-methionine as the methyl donor (*16*, *21*−*23*). Traditionally, the biomethylation of arsenic was considered as a detoxification process (*14*, *24*). The premise was that "organic arsenic species" as represented by the usually detected metabolites, monomethylarsonic acid (MMA^{V}) and dimethylarsinic acid (DMA^{V}), are less toxic than the inorganic arsenic species. However, this view did not take into account the formation of the intermediate metabolites, monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}), which are more reactive and have recently been confirmed to be present in biological systems (*25*−*30*). Toxicological evaluations of these trivalent arsenic methylation metabolites show that MMA^{III} and DMA^{III} are as toxic as, or more toxic than, inorganic arsenite (As^{III}) (*31*−*35*). However, mechanisms of action responsible for the toxicity of these arsenicals

Scheme 1. Biomethylation of Arsenic Involving Alternate Reduction of Pentavalent Arsenic to Trivalent Arsenic Followed by Oxidative Addition of a Methyl Group



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* To whom correspondence should be addressed. Tel: (780)492-6416. Fax: (780)492-7800. E-mail: xc.le@ualberta.ca.

‡ University of Alberta.

§ University of British Columbia.

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are not clear. Binding of arsenic to proteins (enzymes) has been presumed to be a cause of toxicity. Few studies have shown the interactions between arsenicals and proteins in in vitro systems (*36*, *37*). However, no specific or unique arsenic binding protein has been characterized.

Arsenic has been shown to induce metallothionein (MT), a Cys-rich protein (6000−7000 Da) expressed in many cells (*38*−*43*). This small protein can be induced by several heavy metals and stress factors, as a response to toxicity (*38*, *44*). However, the mechanism of MT induction by arsenic is unknown. Furthermore, there is no study on arsenic binding to MT. The objective of this study is to examine binding of MT with trivalent arsenicals including the recently discovered metabolites, MMA^{III} and DMA^{III}.

Experimental Section

Reagents. Rabbit MT II was purchased from Sigma (St. Louis, MO). The protein stock solution (5 mg/mL) was prepared by dissolving 5 mg of MT in 1 mL of water. Working solutions were prepared by serial dilutions of the stock solution with water (or methanol as required). Arsenite stock solution (16.5 mM) was prepared by dissolving arsenic trioxide (Ultrapure, 99.999%, Aldrich) in water.

The source of MMA^{III} was the solid oxide (CH_3AsO), and the source of DMA^{III} was the iodide [$(CH_3)_2AsI$]. The precursors were prepared following literature procedures (*45*, *46*) and were kept at 4 or −20 °C. Dilute solutions of the precursors were prepared fresh in deionized water to form $CH_3As(OH)_2$ (MMA^{III}) and $(CH_3)_2AsOH$ (DMA^{III}), respectively. Analyses of freshly prepared MMA^{III} and DMA^{III} solutions (100 μg/L) showed that their purities were 98 and 97%, respectively. There was a small amount (~2%) of As^{III} and MMA^{V} in the MMA^{III} solution. Approximately 3% DMA^{V} was present in the DMA^{III} reagent. TMAO was obtained from Tri Chemical Laboratory (Yamanashi, Japan). Solutions of other standard arsenic compounds, As^{III}, As^{V}, DMA^{V} (Aldrich, Milwaukee, WI), and MMA^{V} (Chem Service, West Chester, PA), were prepared by appropriate dilutions with deionized water from 1000 mg/L stock solutions, as described previously (*26*, *47*, *48*). Formic acid, water, and methanol (all HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ).

Instrumentation. 1. HPLC−ICPMS. A Perkin-Elmer 200 series HPLC system (PE Instruments, Shelton, CT) was used. A BioSep-SEC 2000 size exclusion column (300 mm × 4.6 mm, Phenomenex, Torrance, CA) was used for separation of MT bound from the unbound arsenicals. The chromatographic separation was carried out using isocratic elution with a mobile phase of 5 mM sodium phosphate (pH 7.3) and a flow rate of 0.8 mL/min. The injection volume was 20 μL. The effluent coming from HPLC was directly detected using an Elan 6100 DRC^{plus} ICPMS (PE Sciex, Concord, ON, Canada), with a Turbochrom Workstation v.6.1.2 software (PE Instruments) for data processing. The operating parameters of ICPMS were optimized to be as follows: RF power, 1150 W; plasma gas flow, 15 L/min; auxiliary gas flow, 1.2 L/min; and nebulizer gas flow, 0.9 L/min. Arsenic and cadmium were detected using the peak-hopping mode of the ICPMS at *m*/*z* of 75 and 114, respectively.

2. Hybrid Quadrupole Time-of-Flight (TOF) MS. A QSTAR Pulsar i mass spectrometer (Applied Biosystem/MDS Sciex) was equipped with a Turbo Ionspray ionization source. Analyte solutions were diluted with 50% methanol and were introduced to the ionspray source by a 1 mL gastight syringe (Hamilton, Reno, NV) and an integrated syringe pump. The flow rate was 10 μL/min. The mass spectrometer was operated in the positive ion mode. The instrument was calibrated daily with a commercial calibration standard. Analyst QS software (Applied Biosystems, Foster City, CA) was used for the spectrum acquisition and data analysis. IgorPro software (WaveMetrics, Lake Oswego, OR) was used to plot the spectra.

In single MS scan mode, the first two quadropoles (Q1 and Q2) were operated in the RF only mode and TOF provided the mass measurements (900−2000 amu). Mass spectra were acquired with an electrospray voltage of 5500 V, a first declustering potential of 65 V, a second declustering potential of 15 V, and a focusing potential of 215 V. The resolution was 10 000 (fwhm) at *m*/*z* 850 with a 10 ppm mass accuracy using internal standard.

In the tandem MS/MS mode, the parent ion was selected by the first quadrupole (Q1) with a mass window of 1 Da at low mass resolution and fragmented in the second quadrupole (Q2) by collision-induced dissociation (CID) with a collision energy of 90 eV and a collision gas setting of 12. The resulting product ions were analyzed by a TOF analyzer. A four anode detector was used with a time-to-digital converter capable of detecting a single ion. In assigning these product ions, the theoretical fragmentation pattern of MT was generated for comparison using the MS product tool in Protein Prospector (http://prospector.ucsf.edu).

Methods. As^{III}, MMA^{III}, and DMA^{III} (1 μM) in deionized water were each incubated with various concentrations of MT (1−15 μM) in PBS for 1−2 h. These solutions were subjected to HPLC−ICPMS analysis of MT−As complexes. The MT-bound and free arsenic species were separated on a size exclusion column with phosphate buffer (pH 7.3) as a mobile phase and were detected using the ICPMS. Both arsenic (*m*/*z* 75) and cadmium (*m*/*z* 114) were detected simultaneously to confirm the binding of arsenic with MT because cadmium was present in the native MT.

To study the binding stoichiometry between MT and As^{III}, MMA^{III}, and DMA^{III} species, a constant concentration of MT (7 μM) was incubated with varying concentrations of the trivalent arsenic species to obtain arsenic to MT molar ratios of 1:20, 1:5, 1:1, 5:1, 20:1, and 100:1. The mixture solutions were incubated at room temperature for 2 h. They were diluted with 50% methanol immediately prior to electrospray MS analysis. The concentration of MT in the dilute solutions was 7 μM, and the concentrations of the arsenic species ranged from 0.35 to 700 μM.

In the preliminary experiments, the MT/arsenic mixture solutions were acidified with 1% formic acid to remove Zn and Cd from the native MT and to simplify the mass spectra of MT−As complexes. Subsequent experiments were carried out without the acidification. The incubation of MT with arsenic species under neutral pH conditions, followed by electrospray MS and HPLC−ICPMS analysis, confirmed the presence of the complexes between the MT and the trivalent arsenic species.

Results

Binding of Arsenic to MT. To demonstrate that trivalent arsenicals bind to MT, we first developed a technique that is based on size exclusion HPLC separation of the MT-bound arsenic from the unbound arsenic followed by specific detection of arsenic using ICPMS. Figure 1 shows typical chromatograms from the HPLC−ICPMS analysis of solutions containing MT and As^{III} and MMA^{III}. The MT-bound As^{III} and MT-bound MMA^{III} are clearly separated from the unbound As^{III} and MMA^{III}. Simultaneous detection of arsenic and cadmium provided a reference of the MT-bound fraction because cadmium is present in the native MT. These results show that trivalent arsenic can be readily bound to MT. Additional experiments carried out using As^{III}, MMA^{III}, and DMA^{III} (1 μM) and MT of various concentrations (1−50 μM) consistently showed the binding between the MT and the trivalent arsenicals.

To further understand the binding of arsenicals to MT and their binding stoichiometry, we used electrospray ionization MS (ESI/MS) to examine various species

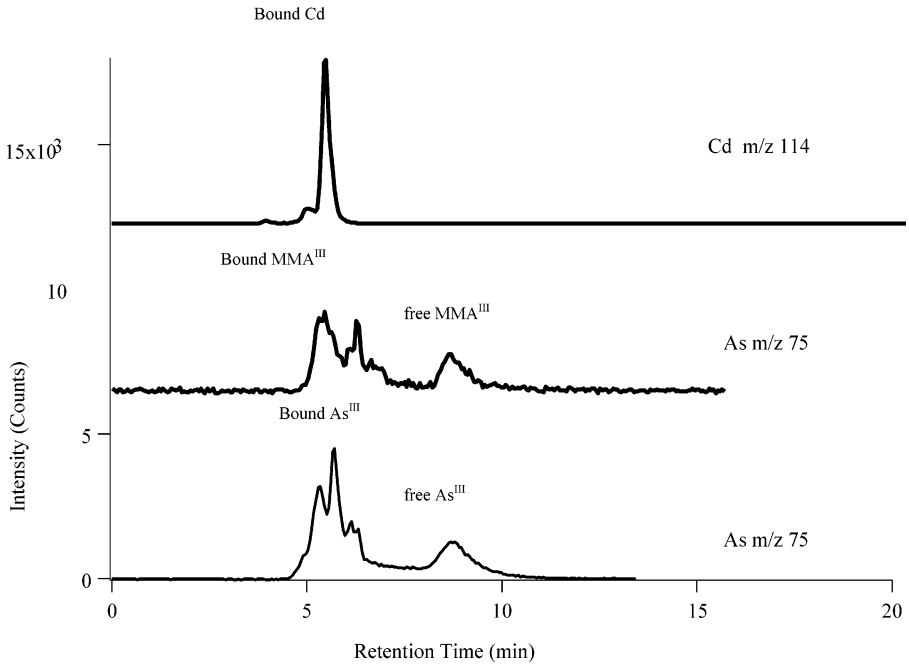
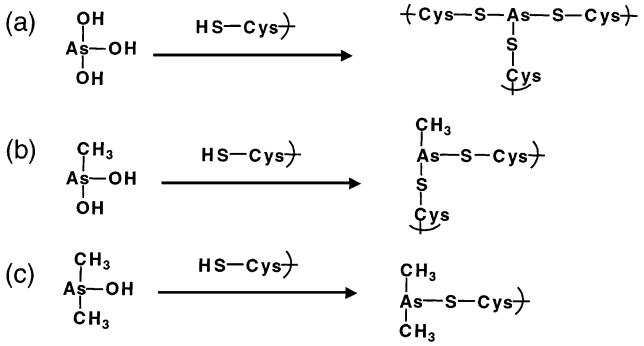


Figure 1. HPLC/ICPMS analysis of reaction mixtures containing MT and As^{III} or MMA^{III} showing the presence of MT−As^{III} and MT−MMA^{III} complexes.

Scheme 2. Schematic Representation of the Binding Stoichiometry between MT and As^{III} (a), MMA^{III} (b), and DMA^{III} (c)[a]



[a] HS−Cys Represents a Cys Residue in MT.

resulting from the binding. The native rabbit MT contained both Zn and Cd. Analyses of MT solutions by ESI/MS revealed that the binding of Zn and Cd with MT was pH-dependent. At pH 6, both Zn and Cd remained intact on the MT. When the pH of the MT solution was decreased to 4.5, zinc was released, leaving only Cd on the MT. When the pH of the solution was further lowered to 2.0, Cd was also released from the protein, resulting in apo−MT. Characteristic ions (*m*/*z*) for the apo−MT detected at pH 2.0 included 1022.0 (6^+ charge state), 1226.2 (5^+ charge state), and 1532.5 (4^+ charge state). These correspond to a molecular mass of 6126 for the apo−MT, consistent with the expected values (6126.3). In addition, sodium adducts of MT were observed at *m*/*z* of 1025.6 (6^+), 1230.6 (5^+), and 1538.0 (4^+) as expected.

To simplify the interpretation of mass spectra, initial studies of MT binding with arsenicals were carried out using apo−MT that was obtained by acidifying the native MT solution to pH 2.0 with 0.5% formic acid. Figure 2 shows typical mass spectra obtained from solutions containing 7 μM MT and increasing concentrations of As^{III} (0.35−140 μM). The molar ratios of As^{III} to MT ranged from 1:5 to 20:1. Multiply charged ions (5^+ and 4^+) were predominant. With the increase of As^{III} concentration at a constant concentration of MT, the number of As^{III} atoms bound to the MT increased.

The numbers marked on the peaks represent the number of arsenic atoms bound to a single MT. At a low concentration of As^{III} relative to MT (Figure 2a), the apo−MT ions, *m*/*z* 1226.2 carrying 5^+ charges and 1532.5 carrying 4^+ charges, were observed as the dominant species. Complex peaks corresponding to the apo−MT bound with one arsenic (peak 1) (*m*/*z* 1240.7 carrying 5^+ and 1550.8 carrying 4^+) were also observed. When the As^{III} concentration was equal to the concentration of MT, the MT−As (peak 1), MT−As_2 (peak 2), MT−As_3 (peak 3), and MT−As_4 (peak 4) complexes along with their sodium adducts were observed (Figure 2b). The MT−As_2 complex (peak 2) was the most abundant species. In the presence of 5-fold excess of As^{III} over MT, MT−As_5 and MT−As_6 complexes were formed (Figure 2c). Increasing As^{III} to an As:MT ratio of 20:1 resulted in the formation of the MT−As_6 complex as the dominant species (Figure 2d). It appears that the maximum number of arsenic atoms bound to the MT was six. A further increase of As^{III} to an As:MT ratio of 100:1 did not show the formation of MT complex with more than six As^{III} moieties.

The binding of each MT molecule with six As^{III} moieties in the presence of excess As^{III} is understandable as illustrated in Scheme 2. MT contains 20 Cys residues. Because each As^{III} is able to bind with three cysteines, the maximum number of As^{III} that can be bound on a MT molecule is six. Results in Figure 2 are consistent with arsenic coordination chemistry. Further studies using MMA^{III} and DMA^{III} binding with MT confirmed the arsenic binding stoichiometry, as will be discussed later.

The assignments of the mass spectral peaks were supported with the accurate mass measurements of the species. Table 1 summarizes the theoretical and the measured masses of the MT and the MT−As species. The measured molecular mass of each species was an average obtained from triplicate measurements of the 5^+ and 4^+

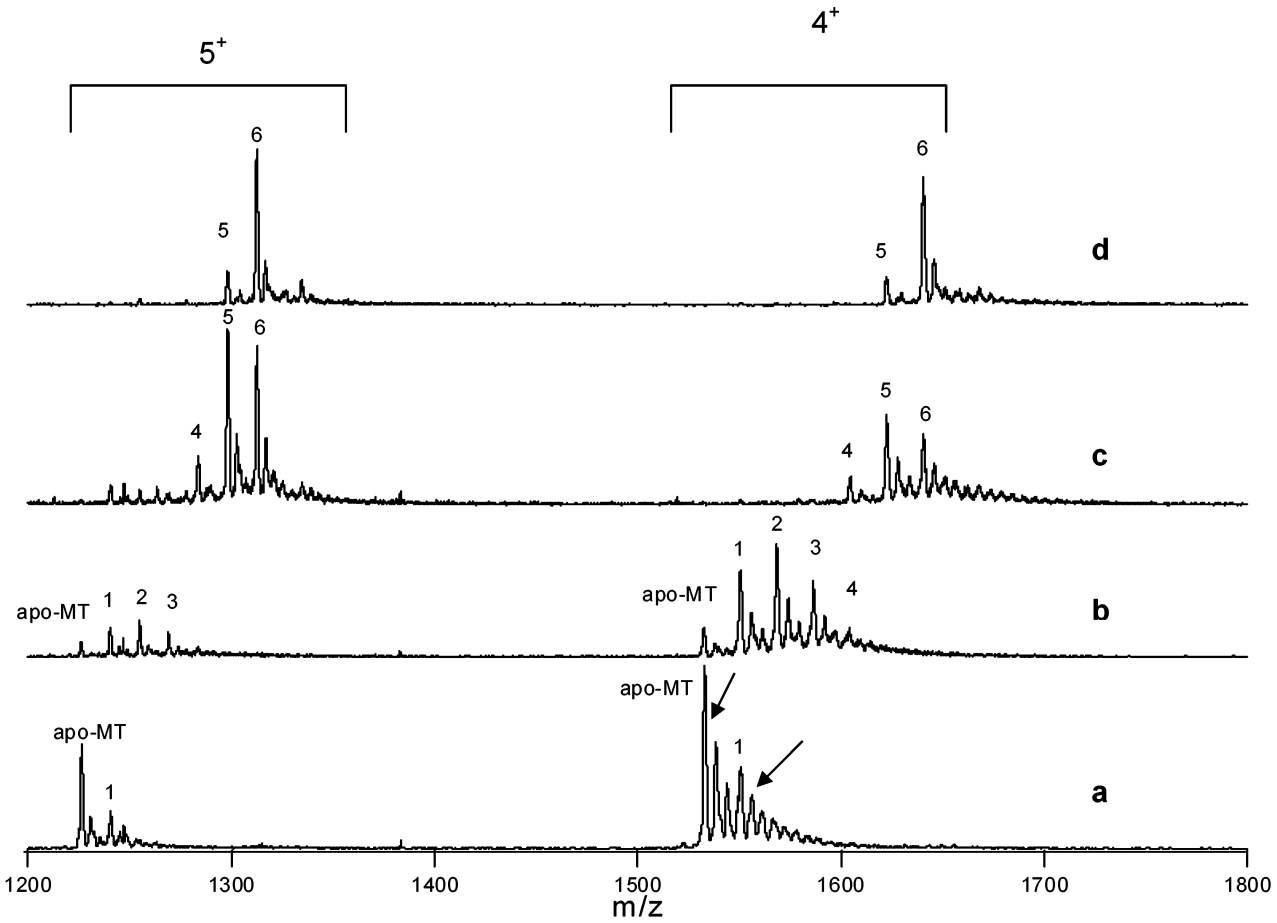


Figure 2. ESI mass spectra from the analysis of solutions containing 7 μM MT and increasing concentrations of As^{III} (0.35, 7, 35, and 140 μM). MT (7 μM) and As^{III} (0.35−140 μM) in deionized water were incubated at room temperature for 2 h. The solution was diluted with 50% methanol and acidified with formic acid to pH 2.0 immediately prior to ESI/MS analysis. The peaks labeled with numbers are complexes of MT and As^{III}, where the numbers on the peaks represent the number of As^{III} bound to the MT molecule. For example, peak 6 represents MT-As_6. The peaks labeled with arrows are sodium adducts of the apo−MT and MT−As complexes. Not all of the sodium adducts are labeled. The ratios of As^{III} to MT are (a) 1:5, (b) 1:1, (c) 5:1, and (d) 20:1.

Table 1. Theoretical and Measured Molecular Masses of the MT and MT−As^{III} Species

	theoretical values (Da)	experimental values				
species	mol mass	5^+ charge state (m/z)	4^+ charge state (m/z)	mol mass (Da)	Δm (Da)	mass accuracy (ppm)
MT	6126.3	1226.20	1532.51	6126.01	−0.29	−46
MT−As	6198.20	1240.66	1550.76	6198.67	0.47	76
MT−As_2	6270.10	1254.74	1568.38	6269.10	1.0	−159
MT−As_3	6341.99	1269.15	1586.74	6341.85	−0.14	−22
MT−As_4	6413.89	1283.97	1604.73	6414.88	0.99	154
MT−As_5	6485.79	1298.18	1622.48	6485.91	0.12	18
MT−As_6	6557.69	1312.65	1640.74	6558.61	0.92	139

ions. They were obtained without using an internal standard for mass calibration. The good match between the measured and the expected values support the assignment of the peaks.

The presence of MT−As complexes was further confirmed with analyses of fragment ions of MT−As species by using tandem MS. CID of the MT−As species resulted in arsenic-containing fragments. Table 2 shows arsenic-containing fragment ions detected from the dissociation of MT−As_6 species (5^+ ion at m/z 1640.7, peak 6 in Figure 2d) with a collision cell energy of 90 eV. The presence of arsenic−Cys fragments suggests that As^{III} is bound to the Cys residues in MT. In addition to these arsenic-containing fragments, other fragment ions were consistent with the theoretical fragmentation pattern of MT protein that was generated by using a MS product tool (http://prospector.ucsf.edu). With a collision cell energy below 40 eV, little fragmentation was observed and the parent ion (MT−As_6) was the most abundant species, suggesting the strong binding of As^{III} with MT.

Table 2. Expected and Experimental Masses of Fragment Ions Containing Arsenic[a]

	expected values (Da)	experimental values (Da)	Δm (Da)	mass accuracy Δm/m (ppm)
As−SCH_2CHN^+	147.920	147.922	0.002	14
As−$SCH_2CHNH_2^+$	149.936	149.935	−0.001	7
As−CysGly	234.954	234.960	0.006	26
As−CysArg	248.964	248.968	0.004	16
As−CysSer	264.964	264.977	0.013	47
As−CysTyr	278.974	278.964	−0.010	36
As−CysGlyArg	305.994	305.992	−0.002	7
As−CysSerArg	336.004	335.981	−0.023	68

[a] The experimental values were observed using tandem MS.

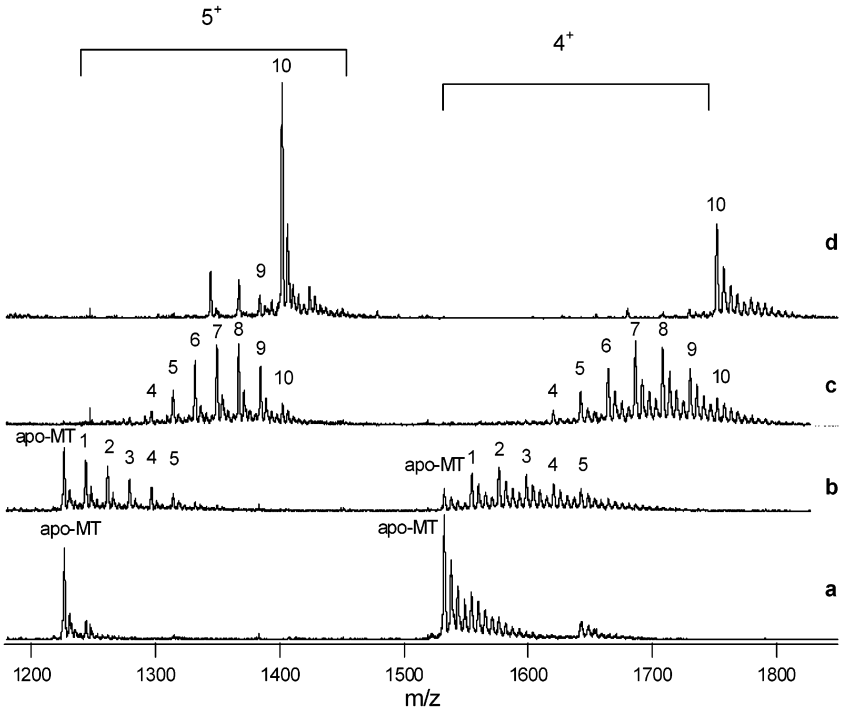


Figure 3. ESI mass spectra from the analysis of solutions containing 7 μM MT and increasing concentrations of MMA^{III}. MT (7 μM) and MMA^{III} (0.35−350 μM) in deionized water were incubated at room temperature for 2 h. The solution was diluted with 50% methanol and acidified with formic acid to pH 2.0 immediately prior to ESI/MS analysis. The peaks labeled with numbers are complexes of MT and MMA^{III}, where the numbers represent the number of MMA^{III} bound to the MT molecule. For example, peak 6 represents MT−$(AsCH_3)_6$. The ratios of MMA^{III} to MT are (a) 1:5, (b) 1:1, (c) 5:1, and (d) 50:1.

We carried out further experiments to confirm that the MT−As complexes were formed in solution and not an artifact of the electrospray process. We examined the formation of MT−As complexes over different incubation periods in solution, and the results indicated that the complex formation was dependent on the incubation conditions in solution. When As^{III} and MT (4:1 molar ratio) were mixed and immediately analyzed using ESI/MS, only MT (1226.2 at 5^+ and 1532.5 at 4^+ charges) and MT−As_1 (1240.7 at 5^+ and 1550.8 at 4^+ charges) species were observed. Repeat ESI/MS analysis of the same mixture after an hour of incubation demonstrated the presence of MT−As_5 and MT−As_6 species (data not shown). These results suggest that the formation of MT−As complexes took place in the liquid phase and depended on the reaction time in solution.

Binding of MMA^{III} and DMA^{III} to MT. Having established the binding of MT with As^{III}, we further examined the binding of MT with the trivalent arsenic metabolites, MMA^{III} and DMA^{III}, to gain further details on the binding stoichiometry. Inorganic As^{III} is able to bind to three thiol groups, forming $As(-Cys)_3$. Therefore, up to six As^{III} could be bound to a single MT as there are 20 Cys residues available in the MT. This is evident from Figure 2 and Scheme 2. MMA^{III} [$CH_3As(OH)_2$] would be able to bind with two thiols. DMA^{III} [$(CH_3)_2AsOH$] would be able to bind to only one thiol group (Scheme 2). Therefore, up to 10 MMA^{III} and 20 DMA^{III} could be bound to each MT when MMA^{III} and DMA^{III} are in excess. Figures 3 and 4 clearly demonstrate this binding stoichiometry.

At a MMA^{III} to MT ratio of 1:5, the apo−MT is the dominant species (Figure 3a). No MT−MMA^{III} complex was evident. Increasing the MMA^{III} concentration to equal the molar concentration of MT resulted in the formation of several MT−MMA^{III} complex species. MT−$AsCH_3$, MT−$(AsCH_3)_2$, MT−$(AsCH_3)_3$, MT−$(AsCH_3)_4$, and MT−$(AsCH_3)_5$ are clearly observed (Figure 3b). Further increasing the MMA^{III} concentration to a 5-fold excess over MT leads to the binding of a maximum of 10 MMA^{III} moieties on the MT, MT−$(AsCH_3)_{10}$ (Figure 3c). With a 50-fold excess of MMA^{III} over MT, the only dominant complex species detected is MT−$(AsCH_3)_{10}$ (Figure 3d).

We further measured the masses of MT−$(AsCH_3)_n$ complexes by using ESI/MS (data not shown). The measured masses for the MT−$(AsCH_3)_n$ complex and the apo−MT differ by 87.9*n*, where *n*, which varies from one to 10, is the number of MMA^{III} moieties bound to MT. This mass difference is consistent with the addition of the expected mass of 87.9294 for $AsCH_3$ and the loss of two protons from thiol groups.

Figure 4 shows spectra from the analysis of DMA^{III} binding to MT. Four spectra were acquired from reaction mixtures containing four different ratios of DMA^{III} to MT. The complex peaks corresponding to MT−$[As(CH_3)_2]_n$ are observed in these spectra, in which *n* varies from one to 20. The observed maximum number of $As(CH_3)_2$ binding with MT is 20 even with a DMA^{III} to MT ratio of 50:1 in solution. The measured mass differences between the MT−$[As(CH_3)_2]_n$ complexes and the apo−MT are 103*n*, where *n* is the number of DMA^{III} moieties bound to MT and varies from one to 20 (data not shown). This is consistent with the addition of the expected mass of 103.9607 for $As(CH_3)_2$ and the loss of a proton from a thiol group.

Tandem mass spectral analysis of the fragment ions derived from *m*/*z* 1686.4 (4^+) (Figure 3) and 1714.0 (4^+) (Figure 4) further confirmed the identity of the MT−$(AsCH_3)_7$ and MT−$[As(CH_3)_2]_7$ species, respectively. For example, $As(CH_3)_2S^+$ (expected, 136.9406; measured, *m*/*z* 136.9428) and $As(CH_3)_2SC_2H_5N^+$ (expected, 179.9828; measured, *m*/*z* 179.9885) were observed, which are

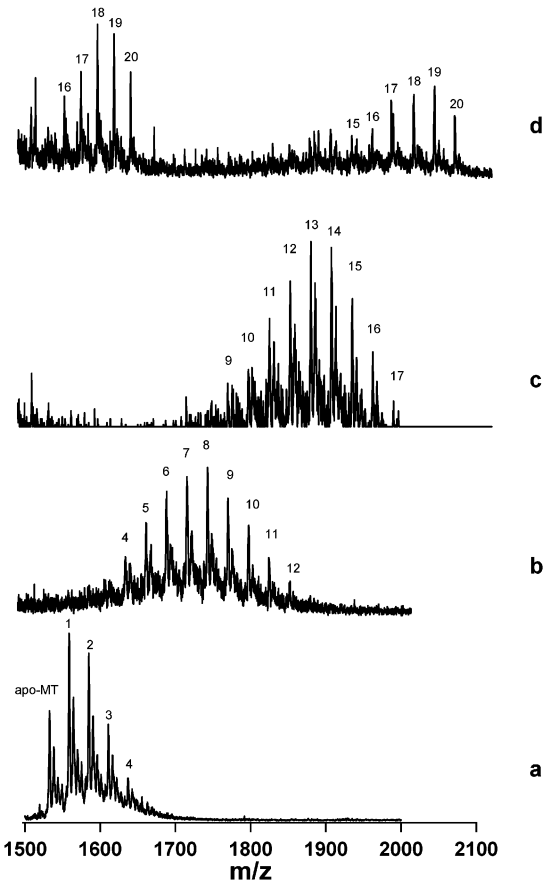


Figure 4. ESI mass spectra from the analysis of solutions containing 7 μM MT and increasing concentrations of DMA^{III}. MT (7 μM) and DMA^{III} (0.35−350 μM) in deionized water were incubated at room temperature for 2 h. The solution was diluted with 50% methanol and acidified with formic acid to pH 2.0 immediately prior to ESI/MS analysis. The peaks labeled with numbers are complexes of MT and DMA^{III}, where the numbers represent the number of DMA^{III} bound to the MT molecule. For example, peak 6 represents MT−$[As(CH_3)_2]_6$. The ratios of DMA^{III} to MT are (a) 1:5, (b) 1:1, (c) 5:1, and (d) 50:1.

evidence of the $As(CH_3)_2$−Cys binding. Observation of these arsenic-containing fragments further supports the binding of trivalent arsenic with MT as shown in Scheme 2.

Reaction between MT and Pentavalent Arsenicals. Experiments were also carried out to explore whether the pentavalent arsenic species, $TMAO^V$, DMA^V, MMA^V, and As^V, would bind with MT. $TMAO^V$, $(CH_3)_3AsO$, could be reduced by MT to $(CH_3)_3As$. However, $(CH_3)_3As$ has no available binding site for thiol groups. We only observed a shift of MT peaks to a lower mass by 20 *m*/*z* units. This corresponds to an oxidation of −SH to disulfide due to reduction of $TMAO^V$ to TMA^{III} (trimethylarsine). The reduced product, trimethylarsine, has no binding site for reacting with Cys. Consistent with arsenic coordination chemistry, our results showed no binding between $TMAO^V$ and MT.

DMA^V could be reduced by the cysteines in MT, and a disulfide bond between two cysteines would be formed in the oxidation. The DMA^{III} that would be produced as the result of the reduction reaction would be expected to form MT−DMA^{III} complexes. There was a reaction as judged by the many new peaks in the mass spectra (data not shown). However, the formation of many species from the redox reaction resulted in complicated mass spectra. We were not able to confirm the presence of any MT−DMA complex from reactions between MT and DMA^V. Similarly, we did not confirm the presence of MT−MMA and MT−As complexes from reactions between the MT and the pentavalent MMA^V and As^V species.

Discussion

The main metabolic process that results from the uptake or ingestion of inorganic arsenic is biomethylation. The intermediate trivalent metabolites, MMA^{III} and DMA^{III}, are at least as toxic as inorganic arsenite (As^{III}). The mechanism(s) of action responsible for the toxicity of the trivalent arsenic species has not been completely elucidated. Our study clearly demonstrates that As^{III}, MMA^{III}, and DMA^{III} readily react with sulfhydryl groups in MT. The novel use of As^{III}, MMA^{III}, and DMA^{III} for this study allowed us to clearly illustrate the binding stoichiometry. Toyama et al. (*50*) have examined the interactions of As^{III} with human MT-II, using UV absorption, ICP atomic emission spectrometry, and matrix-assisted laser desorption ionization MS. Our results on As^{III} binding with MT are consistent with their paper; they also found that the maximum molar ratio of As^{III} to MT was 6:1. They did not examine the binding of MT with other arsenic species. Our study provides further detailed information on the binding stoichiometry of three trivalent arsenic species with MT.

Studies of biochemical interactions between arsenic and proteins are crucial to a better understanding of arsenic health effects. Arsenic has been shown to inhibit several enzymes, such as GSH reductase (*31*, *49*, *51*), thioredoxin reductase (*32*, *52*), lipoamide dehydrogenase (*53*), and pyruvate dehydrogenase (*34*). It may also affect proteins involved in signaling pathways (*54*). Binding of arsenic with proteins via sulfhydryl groups is believed to be responsible for the observed effects of arsenic on these proteins. The present study provides direct evidence of arsenic−protein binding, using MT binding with As^{III}, MMA^{III}, and DMA^{III} as a model system. The concentrations of MT (7 μM, or ∼45 μg/mL) and arsenicals (as low as 0.35 μM) used in this study are relevant to those present in biological systems. The normal concentration of MT in liver and kidney is on the order of tens of μg/g (*55*). The concentrations of trivalent arsenicals in urine samples from humans exposed to elevated arsenic from drinking water are on the order of single digit micromolars (*25*−*29*). The actual concentrations of trivalent arsenicals in the bladder could be higher because the trivalent arsenicals are readily oxidized in the urine (*56*).

MT can be induced by arsenic (*38*−*43*). Since MT was discovered in the 1950s (*57*), it has been the subject of numerous studies (*44*, *55*, *57*, *58*) due to its important role in transport, free radical scavenging, and detoxification of heavy metals, especially cadmium, zinc, copper, and mercury, all of which induce its production. However, the mechanism of MT induction by these cations and the arsenicals is unknown.

Arsenic reacts with protein sulfhydryl groups; thus, MT could be a potentially protective protein. It has a high affinity for many metals and is known to effectively protect cells from cadmium toxicity. Liu et al. (*59*) found that MT-null mice are more sensitive than wild-type mice to the hepatotoxic and nephrotoxic effects of chronic exposure to arsenic. Park et al. (*60*) showed that the LD_{50}

of arsenic for wild-type mice was 1.4-fold higher than for MT-null mice. The present studies on the interaction of MT with arsenic suggest that the protective effect might be a result of binding of the arsenic species by MT.

Matani et al. (*61*) found that As^{III}, but not As^{V}, MMA^{V}, or DMA^{V}, was a modest inducer of MT in the liver of mice. As^{III} did not appear to be bound to MT in appreciable amounts. Kreppel et al. (*62*) reported that pretreatment of mice with zinc, an inducer of MT, protected against the acute toxicity of As^{III}. However, the protective effect of zinc pretreatment did not show a correlation with the extent of induction of MT. There was very little arsenic bound to MT in the cytosol of the Zn-pretreated mice, as determined by gel filtration fractionation analysis. It is still unclear the exact role played by MT in the cellular response to arsenic. There may be some secondary process related to the induction of MT that alters the metabolism or cellular response of arsenic that affects its toxicity.

The interaction of arsenic with MT may serve as a model for the interaction of arsenic with other cellular proteins, including DNA repair proteins. The effect of arsenic on DNA repair is a potential mechanism of action underlying carcinogenesis. The technique described here has the potential to be used in studies of the interaction of arsenic with these other proteins. Study on the stoichiometry and affinity of the arsenicals bound to MT is an important step toward a better understanding of the interaction between arsenic and proteins.

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