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Differential Binding of Monomethylarsonous Acid Compared to Arsenite and Arsenic Trioxide with Zinc Finger Peptides and Proteins

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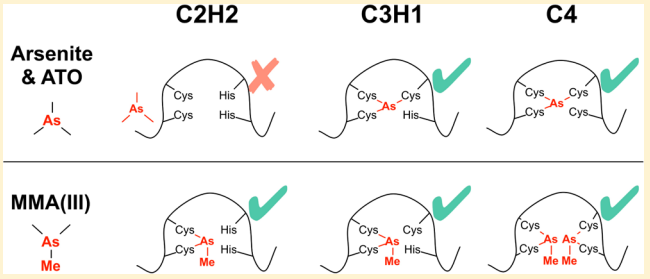
ABSTRACT: Arsenic is an environmental toxin that enhances the carcinogenic effect of DNA-damaging agents, such as ultraviolet radiation and benzo[*a*]pyrene. Interaction with zinc finger proteins has been shown to be an important molecular mechanism for arsenic toxicity and cocarcinogenesis. Arsenicals such as arsenite, arsenic trioxide (ATO), and monomethylarsonous acid (MMA(III)) have been reported to interact with cysteine residues of zinc finger domains, but little is known about potential differences in their selectivity of interaction. Herein we analyzed the interaction of arsenite, MMA(III), and ATO with C2H2, C3H1, and C4 configurations of zinc fingers using UV−vis, cobalt, fluorescence, and mass spectrometry. We observed that arsenite and ATO both selectively bound to C3H1 and C4 zinc fingers, while MMA(III) interacted with all three configurations of zinc finger peptides. Structurally and functionally, arsenite and ATO caused conformational changes and zinc loss on C3H1 and C4 zinc finger peptide and protein, respectively, whereas MMA(III) changed conformation and displaced zinc on all three types of zinc fingers. The differential selectivity was also demonstrated in zinc finger proteins isolated from cells treated with these arsenicals. Our results show that trivalent inorganic arsenic compounds, arsenite and ATO, have the same selectivity and behavior when interacting with zinc finger proteins, while methylation removes the selectivity. These findings provide insights on the molecular mechanisms underlying the differential effects of inorganic versus methylated arsenicals, as well as the role of *in vivo* arsenic methylation in arsenic toxicity and carcinogenesis.



INTRODUCTION

Arsenic is a significant public health concern due to its toxicity and carcinogenesis. Chronic arsenic exposure is related to many adverse health effects,[1] such as increased risk of cancers of the skin, lung, and urinary tract.[1−5] More than 140 million people worldwide are believed to be exposed to arsenic levels above the World Health Organization maximum contaminant level of 10 ppb.[6] Arsenic exists in inorganic and organic forms. Environmental routes of exposure to arsenic include ingestion and inhalation of inorganic arsenic. Arsenite is an inorganic trivalent arsenic compound widely present in water, soil, and food.[1] In contrast, arsenic trioxide (ATO, As_2O_3), another trivalent arsenic compound, is the most common inorganic arsenical in airborne dust.[1] Inhalation of ATO has been shown to alter immune function.[7] Organic arsenicals mainly consist of mono- and dimethylated arsenic metabolites, derived from biomethylation of inorganic arsenicals in cellular environment.[8−11] A trivalent monomethylated arsenic metabolite, monomethylarsonous acid (MMA(III)), has been shown to display greater toxicity and/or carcinogenic potential than inorganic arsenite.[12−14]

Interaction with zinc finger proteins is considered to be an important mechanism of arsenic toxicity and carcinogenesis. Substitution of zinc with another metal, such as arsenic, is believed to disrupt the coordination sphere in the finger environment and consequently the zinc finger function.[1,15] Furthermore, both inorganic and organic trivalent arsenic compounds interact with zinc finger proteins. Zinc finger proteins, poly (ADP-ribose) polymerase 1 (PARP-1), and xeroderma pigmentosum group A (XPA) are both involved in DNA repair and have been validated as direct molecular targets for arsenite and MMA(III).[1−5,16−18] ATO is also known to interact with cysteine-rich zinc finger proteins.[6,19] We have investigated extensively the interaction of arsenite with zinc finger proteins in recent years. Our findings demonstrate that arsenite can replace zinc in the zinc finger moiety, leading to changes of structure and loss of protein function.[20] In addition, we found that arsenite selectively interacts with zinc finger motifs with C3H1 or C4 configurations by coordinating with three cysteine residues.[1,21] This suggests that subsets of zinc finger proteins are more sensitive molecular targets of arsenite than others. Since a methyl group already occupies one of the three covalent bonds in MMA(III), it is likely that MMA(III)

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will not be able to bind with three cysteine residues as arsenite does. Therefore, we hypothesize that binding selectivity of MMA(III) will be different from that of arsenite.

In this study, we tested the differential binding selectivity hypothesis by investigating interactions of arsenite, ATO, and MMA(III) with three different configurations of zinc finger peptides and proteins: C2H2 (aprataxin, APTX), C3H1 (PARP-1), and C4 (XPA). A variety of analytical approaches were utilized to determine whether the three arsenicals display differential binding selectivity toward these zinc finger configurations, and potential consequences of these interactions in terms of structural or functional changes. Our results demonstrate that the binding selectivity indeed differs among the methylated versus nonmethylated arsenicals, which provides insightful understanding for the molecular mechanisms underlying the differential effects of inorganic versus organic arsenicals in arsenic toxicity and carcinogenesis.

EXPERIMENTAL PROCEDURES

Chemicals. Peptides derived from the finger motifs of APTX, XPA, and the first zinc finger motif of PARP-1 (sequences in Table 1) were commercially synthesized by Genemed Synthesis Inc. (San Antonio, TX). Purity confirmed by HPLC was greater than 95%. Diiodomethylarsine (MMAIII iodide, CH_3AsI_2, >98% pure) was prepared by the Synthetic Chemistry Facility Core (Southwest Environmental Health Sciences Center, Tucson, AZ) and kindly provided by Dr. A. Jay Gandolfi, University of Arizona. As_2O_3 (ATO, >99.95%) was obtained from Mallinckrodt Chemical Works (St. Louis, MO). Cobalt chloride, zinc chloride, and sodium arsenite were obtained from Fluka Chemie. All other chemicals were obtained from Sigma-Aldrich.

Table 1. Sequences of Zinc Finger Peptides

Name	Sequence
APTX (C2H2)	PLRCHECQQLLPSIPQLKEHLRKHWTQ
PARP-1 ZF 1 (C3H1)	GRASCKKCSESIPKDKVPHWYHFSCFWKV
XPA (C4)	DYVICEECGKEFMDSYLMNHFDLPTCDNCRDADDKHK

Cobalt Spectrometry Analysis of Free Metal Binding Sites on Zinc Finger Peptides. Lyophilized zinc finger peptides were suspended at 1 mM in 20 mM Tris (pH 7.8) containing 0.1 mM Tris(2-carboxyethyl)phosphine (TCEP) to protect the cysteine residues from oxidation prior to incubations. Solutions of arsenic compounds were prepared freshly in 20 mM Tris (pH 7.8) before incubation with zinc finger peptides. Zinc finger peptides diluted to 100 μM were incubated with 50, 100, or 200 μM arsenic compounds at room temperature for 30 min, then cobalt chloride was added to a final concentration of 200 μM. The absorption spectra from 260 to 800 nm were collected at 25 °C on a SpectraMax M2 spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA). Absorbance at 660 nm indicates the formation of a cobalt and zinc finger peptide complex,[1,22,23] and therefore, the cobalt spectrum A_{660} value represents the amount of sites on zinc fingers that are still available for metal ions to bind after treatments of arsenic compounds.

UV−Vis Spectrometry Analysis of As−S Bond Formation on Zinc Finger Peptides. Aliquots of 100 μM zinc finger peptides in 20 mM Tris (pH 7.8) were incubated with various concentrations (0−200 μM) of arsenic compounds for 30 min at 25 °C, then the UV−vis absorption spectra of the mixtures from 260 to 500 nm were collected at 25 °C on a SpectraMax M2 spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA). A_{270} is used as the indication of As−S bond formation due to arsenic interaction with cysteine residues on zinc finger peptides.[8−11,24]

Mass Spectrometry Analysis. Lyophilized peptides were suspended at a concentration of 1 mM in 20 mM Tris (pH 7.8) containing 0.1 mM TCEP to protect the cysteine residues from oxidation. Stock solutions of arsenic compounds were freshly prepared at a concentration of 1 M in 20 mM Tris (pH 7.8). Aliquots of 100 μM zinc finger peptides were incubated with 100 μM arsenic compounds for 30 min at 25 °C. The samples were then diluted 50 times in 5 mg/mL α-cyano-4- hydroxycinnamic acid (Sigma-Aldrich) in a 1:1 (v/v) water/acetonitrile solution, and 1 μL of each sample was deposited in duplicate on the MALDI plate, allowed to dry at 37 °C, and MALDI-TOF-MS analyses performed on an Applied Biosystems 4700 Proteomics Analyzer (TOF/TOF) operating in MS reflector-positive ion mode. The total acceleration voltage was 20 kV. Desorption was performed using a neodymium/yttrium−aluminum-garnet laser (355 nm, 3 ns pulse width, and 200 Hz repetition rate). Mass spectra were acquired with laser pulses over a mass range of m/z from 1000 to 5000 Da using focus mass of 3500. Final mass spectra were the summation of 10 subspectra, each acquired with 200 laser pulses.

Intrinsic Fluorescent Analysis of Arsenical Binding to Zinc Fingers. Aliquots of 100 μM zinc finger peptides were incubated with different concentrations of arsenic compounds or 100 μM zinc chloride for 30 min at 25 °C. After that, the emission fluorescent spectra from 300 to 400 nm were collected at 25 °C on a SpectraMax M2 fluorescent spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA). The excitation wavelength was 280 nm. The intensity of fluorescence is related to the chemical environments of phenylalanine, tyrosine, and tryptophan. The intrinsic fluorescence intensity of zinc finger peptides undergoes a dramatic change on folding/unfolding. This allows for the tertiary structure change of zinc finger peptides to be monitored by fluorescence spectroscopy. Fluorescent intensity at 350 nm was used to represent the status of the tertiary structure of zinc finger peptides with different treatments.

Cell Culture and Zinc Finger Protein Isolation by Immunoprecipitation. The human keratinocyte cell line (HaCaT) was a kind gift from Dr. Mitch Denning (Loyola University Medical Center, Maywood, IL). Cells were maintained as described previously.[12−14,16] After exposure to 2 μM arsenic compounds for 24 h, cells were harvested in RIPA cell lysis buffer (25 mM Tris-HCl at pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS), sonicated, and centrifuged at 14,000 rpm for 15 min at 4 °C to remove cellular debris. Protein (500 μg in 500 μL) was incubated with 5 μL of rabbit polyclonal antibody (APTX, Abcam #31841; PARP-1, Cell Signaling #9542 or XPA, Abcam ab85914) for at least 2 h at 4 °C. Protein A beads (Invitrogen) were added in a 1:1 slurry, and samples were incubated for an additional 2 h at 4 °C. The beads were recovered by centrifugation at 10,000 rpm for 5 min at 4 °C and washed five times with 1 mL of lysis buffer. To elute protein, the pellets were incubated with 100 μL of 100 mM citric acid (pH 3.0) for 30 min, followed by centrifugation at 14,000 rpm for 5 min at 4 °C. The supernatant was adjusted to pH 7 with 10 M NaOH.

Measurement of Zinc Content in Protein. Proteins obtained from cells by immunoprecipitation were incubated with 10 mM H_2O_2 for at least 2 h at 4 °C to release zinc from proteins. Zinc content was measured by adding 10 μL of 1 mM 4-(2-pyridylazo)resorcinol to 100 μL of protein sample followed by scanning the UV−vis spectra at 350 to 550 nm on a SpectraMax M2 spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA). The absorbance of resorcinol shifts from 411 to 493 nm in the presence of zinc, and the 493 nm peak is recorded and compared with a standard curve for calculation of zinc content in protein samples.[21,23]

RESULTS

Cobalt Spectrometry Analysis of the Differential Selectivity of Occupying Metal Binding Sites on Zinc Fingers. Zinc binding within zinc finger motifs is critical for the maintenance of the tertiary structure and activity of zinc finger proteins. Occupation of metal binding sites by arsenic is an indicator of arsenic interaction with zinc fingers. To determine whether an arsenic compound is capable of occupying the metal binding sites and to investigate differences in metal binding site occupation by MMA(III), arsenite or ATO, we used cobalt as a probe to detect available metal

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binding sites on zinc finger motifs after incubation with arsenic compounds. Cobalt binding with zinc finger motifs generates absorbance at 660 nm,[22,23] which is used to quantitatively determine the remaining free metal binding sites after arsenic occupation. The zinc finger peptides derived from the zinc finger domains of C2H2 (APTX), C3H1 (PARP-1), and C4 (XPA) were incubated with arsenicals, and then the cobalt spectrum was analyzed. In PARP-1 and XPA peptides, arsenite incubation decreased subsequent cobalt binding in a concentration-dependent manner but not in APTX peptides (Figure 1A). This indicates that arsenite selectively occupies the metal binding site on C3H1 and C4 but not the C2H2 zinc fingers. This result is consistent with our published findings.[21] In contrast, incubation of peptide with MMA(III) led to a concentration-dependent decrease in cobalt binding to all three peptides indicating that MMA(III) occupied the metal binding sites on all three configurations of zinc fingers (Figure 1B). ATO showed the same selectivity as arsenite (Figure 1C). These results demonstrate that arsenite and ATO selectively occupy metal binding sites on C3H1 and C4 zinc fingers, while MMA(III) occupies metal binding sites on each of the C2H2, C3H1, and C4 zinc finger peptides.

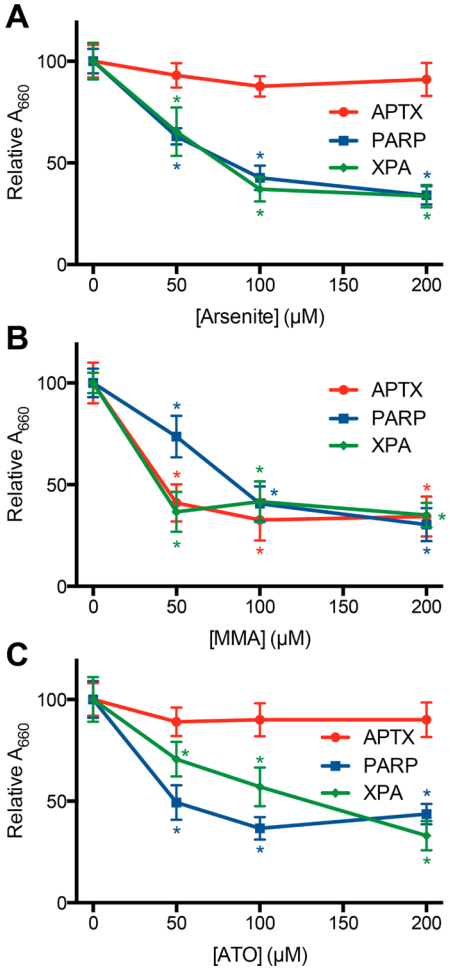

Figure 1. Cobalt spectrometry analysis for arsenicals binding with zinc fingers. APTX, PARP-1, and XPA zinc finger peptides were preincubated with arsenic compounds for 30 min. After that, 200 μM cobalt was added into the system. Absorbance at 660 nm increased when cobalt bound to zinc finger motifs. (A) Cobalt binding signal decreased in PARP-1 and XPA zinc fingers after preincubating with increasing concentrations of arsenite. For the APTX zinc finger, the free metal binding site was always available. (B) Cobalt binding signal decreased in all three configurations of zinc fingers in a MMA(III) concentration-dependent manner. (C) ATO performed the same in cobalt spectrum detection as arsenite. Data were presented as the mean ± SD, *p < 0.05 vs corresponding [As] = 0 group, n = 3.

UV−Vis Spectral Analysis of Differential Selectivity of As−S Bond Formation with Zinc Fingers. When arsenicals occupy metal binding sites on zinc fingers, they coordinate with cysteine residues, forming an As−S bond. Therefore, the As−S bond is a key structure of arsenic interacting with the thiol group on cysteine residues of zinc fingers. In order to determine whether arsenic interaction with thiol groups is consistent with binding site occupation on the zinc finger motif, we analyzed As−S bond formation between arsenic and cysteine residues on the zinc finger peptide. In the UV−vis spectrum, an As−S bond can generate absorbance from 260 to 340 nm.[24] We used A_{270} in UV−vis spectra as the indicator of As−S bond formation. Varying concentrations of arsenic compounds were incubated with 100 μM of different configurations of zinc finger peptides for 30 min at room temperature, and the UV−vis spectra of the mixtures were recorded. In arsenite treated samples, A_{270} values increased in a concentration-dependent manner for PARP-1 and XPA zinc finger peptides, but not for APTX (Figure 2A), showing that arsenite selectively forms an As−S bond with C3H1 and C4 zinc fingers, as expected from our previous report.[21] In contrast, in MMA(III) treated samples, A_{270} values increased for all three types of zinc fingers in a MMA(III) concentration-dependent manner (Figure 2B), indicating that MMA(III) could form an As−S bond with each zinc finger. ATO formed As−S bonds with PARP-1 and XPA zinc fingers but not APTX (Figure 2C), showing the same binding selectivity for C3H1 and C4 configurations as arsenite in terms of forming As−S bonds. These results indicate that interaction with Cys residues by forming As−S bonds is the molecular mechanism for zinc binding site occupation by arsenic. Among the three arsenicals, arsenite and ATO showed the same selectivity in forming As−S bonds with C3H1 and C4 zinc fingers, but MMA(III) could form As−S bonds with all three configurations of zinc finger peptides.

Mass Spectrometry Analysis of Arsenicals Interacting with C2, C3, and C4 Zinc Fingers. To further understand the differences in binding selectivity, MALDI-TOF mass spectrometry was utilized to analyze the precise molecular weights of the arsenic−zinc finger complex. Zinc finger peptides with different configurations (100 μM) were treated with 100 μM arsenic compounds. The mass spectra of apo-zinc finger peptide for APTX, PARP-1, and XPA are shown in Figures 3A, B, and C, respectively. Arsenite showed no binding to the APTX zinc finger (Figure 3D) but bound with PARP-1 and XPA zinc finger peptides, both giving +72 m/z shift against the apo-peptide signals (Figures 3E and F), indicating that arsenite coordinates with zinc fingers with the arsenic atom alone (m/z = 75), releasing three hydrogen atoms (m/z = −3) at the same time. This result is consistent with our previous published data.[21] MMA(III) induced a +88 m/z shift to the APTX zinc finger peptide (Figure 3G). The interpretation of a +88 m/z shift is that MMA(III) bound to the APTX zinc finger peptide with As-CH_3 (m/z: 75, As+12, C+3, 3H = 90), losing 2H (m/z: −2) from Cys residues on zinc finger peptides. MMA(III) also bound to the PARP-1 zinc finger (Figure 3H) with a +88 m/z shift, showing that MMA(III) used 2 cysteine

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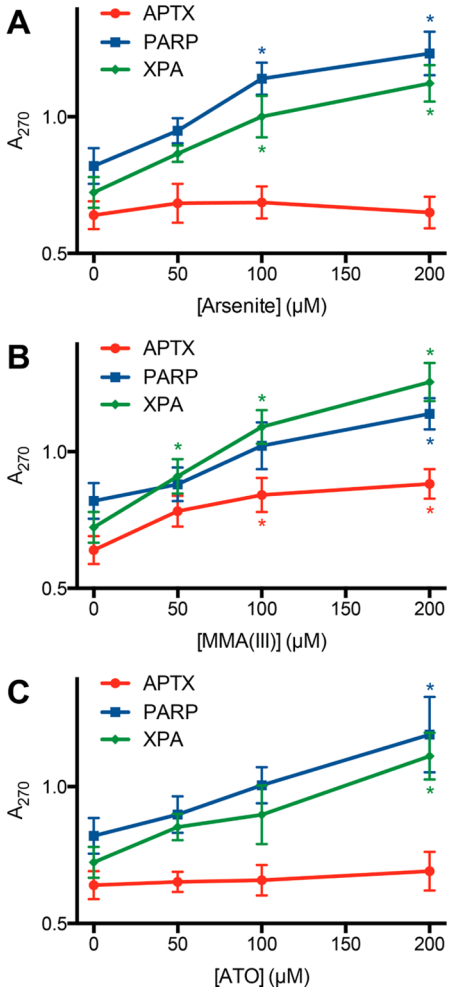

Figure 2. As−S bound formation analysis using UV−vis spectrometry. Arsenic compounds (A, arsenite; B, MMA(III); C, ATO) were incubated with 100 μM of the indicated zinc finger peptides at room temperature for 30 min. Then UV−vis spectrometry analysis was performed as described in the Experimental Procedures section. Absorbance at 270 nm represents the As−S bound formation after arsenic binds to zinc fingers. (A) Arsenite bound to C3 and C4 zinc fingers selectively. (B) MMA(III) bound to all three configurations of zinc fingers. (C) ATO showed the same zinc finger binding selectivity as arsenite. Data were presented as the mean ± SD, * $p < 0.05$ vs corresponding [As] = 0 group, $n = 3$.

residues for binding. For XPA, 1 molecule of MMA(III) bound to the XPA zinc finger, giving a +88 m/z shift to the apopeptide. At the same time, we detected the signal of 2 molecules of MMA(III) bound to the same XPA zinc finger peptide (Figure 3I), giving a +88 m/z shift for each. Since the XPA zinc finger has 4 cysteine residues, when 1 molecule of MMA(III) bound to the zinc finger peptide, occupying 2 cysteine residues, there were still 2 free cysteine residues available for another molecule of MMA(III) coordination. This result further confirms that, unlike arsenite, MMA(III) only occupies 2 Cys during binding with zinc fingers. The mass spectra for ATO was the same as arsenite; it did not bind to the APTX zinc finger (Figure 3J), but bound with PARP-1 or XPA zinc fingers, giving a +72 m/z shift (Figure 3K and L). Therefore, the selectivity of ATO binding with zinc fingers was the same as arsenite. Furthermore, the +72 m/z shift indicates that ATO bound with zinc fingers in the same manner as arsenite, i.e., coordinating with 3 Cys residues. Together, the mass spectrometry results show that MMA(III) coordinated with 2 Cys but that arsenite and ATO both occupy 3 Cys when binding with zinc fingers.

Intrinsic Fluorescence Analysis of the Alternation of Tertiary Structure of Zinc Fingers. Next, we investigated whether arsenic binding could lead to structural changes of the zinc finger peptides. Zinc finger motifs of DNA repair proteins are frequently responsible for DNA recognition and DNA binding.[25,26] Maintaining a correct tertiary structure is critically important for DNA binding and DNA repair capability. In order to investigate conformational changes due to arsenic binding to the zinc fingers, intrinsic fluorescence was used to analyze tertiary structure alteration on zinc finger peptides after treatments with arsenic compounds, as compared to zinc incubation. Intrinsic fluorescence is primarily generated from tryptophan and tyrosine residues (phenylalanine also contributes a small portion), representing the chemical environment of these amino acids. The intensity of fluorescence usually increases while peptides fold and side chains of Trp and Tyr are located in a relatively hydrophobic environment.[27] We treated different configurations of zinc finger peptides (100 μM) for 30 min at room temperature with varying concentrations of arsenic compounds. After that, we collected the fluorescent spectra of each sample under the excitation wavelength of 280 nm and emission from 300 to 400 nm. Treatment with 100 μM zinc chloride was used as a control to show the natural folded conformation of the zinc finger peptides. Finally, fluorescent intensity at 350 nm was used to represent the tertiary structure change of zinc fingers. As shown in Figure 4A, the fluorescent signal of the APTX zinc finger could be decreased in a concentration-dependent manner only by MMA(III), but not arsenic or ATO, while zinc treatment generated the highest fluorescent signal (shown as a single data point in the top left corner of Figure 1A, B, and C). This result indicates that the APTX zinc finger forms a defined structure with zinc ions but that MMA(III) treatment could unfold the structure in a concentration-dependent manner. Arsenite or ATO showed no effect, which is consistent with the lack of binding based on the selectivity data (Figures 1, 2, and 3). For the PARP-1 zinc finger peptide, all 3 arsenic compounds decreased the fluorescent intensity in a concentration-dependent manner (Figure 4B). Results on the XPA zinc finger exhibited a trend similar (Figure 4C) to that of PARP-1 (Figure 4B). Together, these results indicate that MMA(III) alters the tertiary structure of all 3 conformations of zinc fingers, while arsenic and ATO selectively disrupted the tertiary structure of C3H1 and C4 zinc fingers. Furthermore, the findings demonstrate that the alteration in the tertiary structure of the zinc finger is a direct consequence of arsenic binding and that the selectivity of structural changes induced by arsenicals is consistent with the binding selectivity.

Selective Loss of Zinc from Zinc Finger Proteins in Cells Exposed to Arsenicals. Finally, in order to test whether the selectivity and behavior of MMA(III), arsenite, and ATO binding with zinc finger proteins are applicable in cells, zinc content in DNA repair proteins from cells treated with arsenicals was analyzed. We have reported that zinc loss from zinc finger proteins is a direct consequence of arsenic binding and a key event for protein function loss and arsenic toxicity in cells.[20] Human keratinocyte (HaCat) cells were treated with 2 μM arsenite, ATO, or MMA(III) for 24 h. APTX, PARP-1, and XPA protein were immunoprecipitated from cell extracts using

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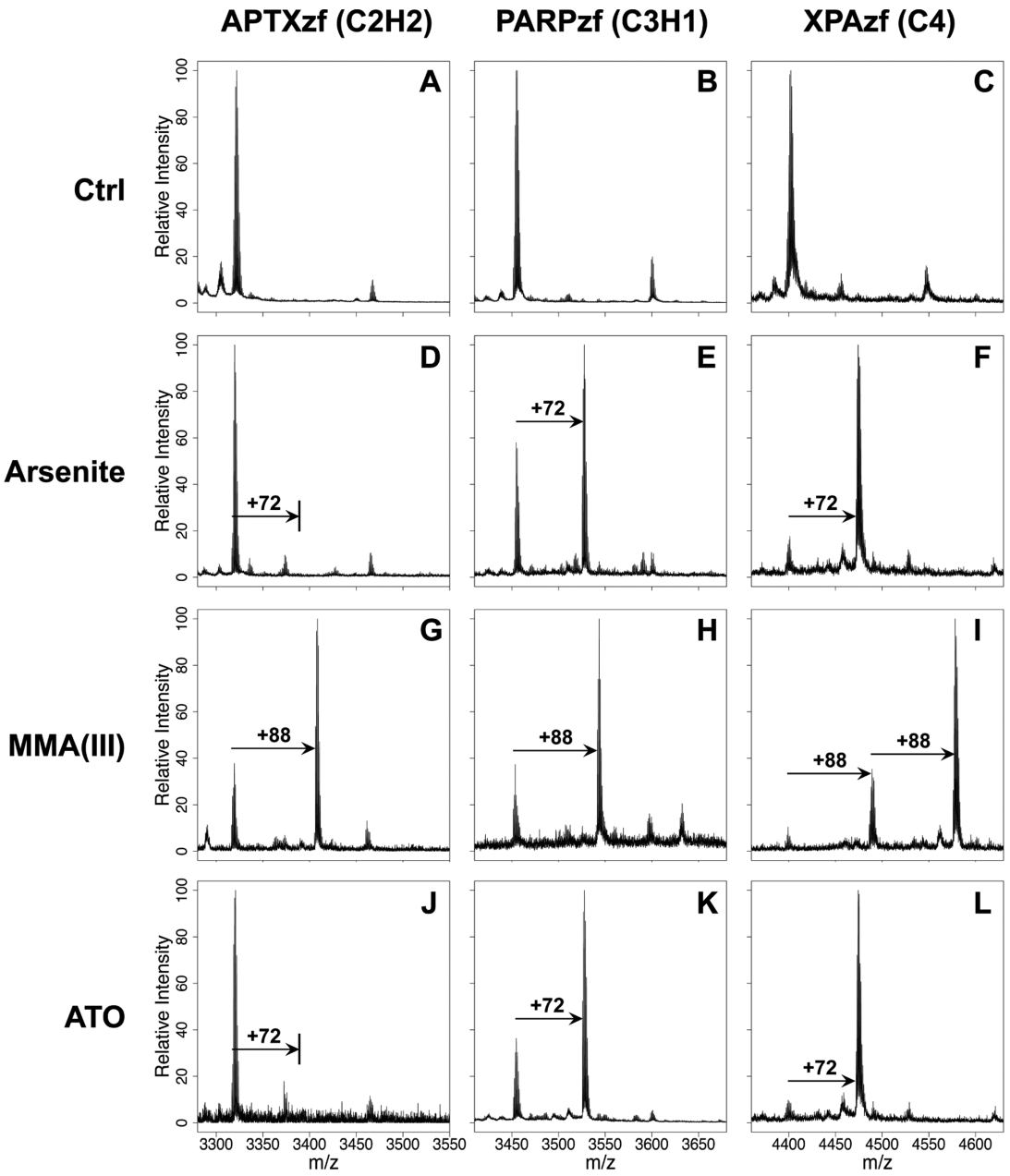

Figure 3. Zinc finger binding behaviors analyzed by MALDI-TOF mass spectrometry. One hundred micromolar arsenic compounds were incubated with 100 μM of the indicated zinc finger peptides at room temperature for 30 min, then MALDI-TOF mass spectrometry analysis was performed as described in Experimental Procedures section. (A, B, and C) Apo-APTX, PARP-1, and XPA zinc fingers had m/z at 3319, 3454, and 4400 in mass spectra. (D) Arsenite did not bind to the C2H2 zinc finger (APTX). (E and F) Arsenite bound to C3H1 (PARP-1) and C4 (XPA) zinc fingers, giving a +72 m/z shift. (G, H, and I) MMA(III) could bind to all three configurations of zinc fingers, giving +88 m/z shift to zinc fingers. In I, 1 or 2 molecules of MMA(III) bound to C4 zinc finger (XPA). Each molecule of MMA(III) gave +88 m/z shift. (J, K, and L) ATO showed the same zinc finger binding selectivity as arsenite. ATO and arsenite gave the same +72 m/z shift to C3H1 and C4 zinc fingers.

corresponding antibodies, and the zinc content in each protein sample was determined. As shown in Figure 5, MMA(III) caused zinc loss from all three configurations of zinc finger proteins, while arsenite and ATO selectively displaced zinc from PARP-1 and XPA proteins isolated from cells. These results demonstrate that arsenite and ATO selectively interacted with C3H1 and C4 zinc fingers in the context of native protein but that MMA(III) interacted with all 3 configurations of zinc finger proteins in HaCat cells.

DISCUSSION

Targeted interaction with zinc finger domains is considered an important mechanism for arsenic toxicity and cocarcinogenesis. In the present study, by using cobalt spectrometry, we demonstrated that both inorganic and organic arsenicals interacted with zinc fingers by direct occupation of metal binding sites. UV−vis spectra demonstrated that all three arsenicals formed As−S bonds with Cys residues on zinc fingers, illustrating the importance of Cys residues for arsenic binding. Mass spectrometry analysis further confirmed the formation of As−S covalent bond. In addition, loss of hydrogen

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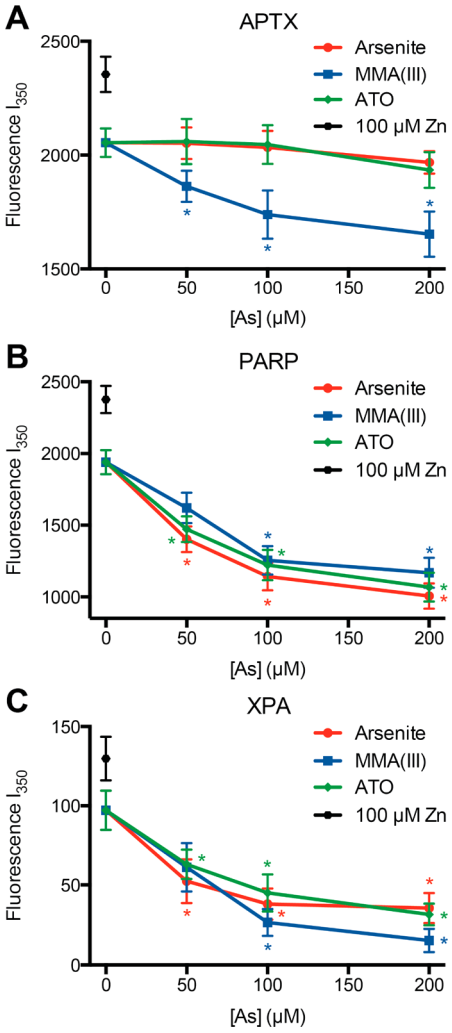

Figure 4. Conformational changes of zinc fingers induced by arsenic binding. Intrinsic fluorescence analysis was performed as described in the Experimental Procedures section. Intensities of fluorescence at 350 nm were used to represent the conformation/folding status of zinc finger peptides. The fluorescent intensities of 100 μM zinc treatment on zinc finger peptides are shown (top left corner) as controls. (A) Natural conformation of APTX could be altered by MMA(III) in a concentration-dependent manner, while arsenic and ATO showed no effect. (B) All three arsenic compounds could cause conformational change on the PARP-1 zinc finger in a concentration-dependent manner. (C) All three arsenic compounds could cause conformational change on the XPA zinc finger in a concentration-dependent manner. Data were presented as the mean ± SD, * $p < 0.05$ vs corresponding [As] = 0 group, $n = 3$.

atoms on the complexes confirmed that arsenicals interacted with Cys residues but not His residues on zinc fingers, which is different from the mechanism of zinc binding with zinc fingers. This may also be one of the reasons that arsenic binding changes conformation of zinc fingers, which we demonstrated by intrinsic fluorescent analysis. Collectively, from the data offered by these diverse techniques, we show that arsenite, MMA(III), and ATO occupy metal binding sites on zinc fingers by directly coordinating with Cys residues to form As−S bonds, leading to conformational change as well as zinc loss from the zinc fingers.

For the two trivalent inorganic arsenicals, arsenite and ATO, their patterns of interaction with zinc fingers are the same. This

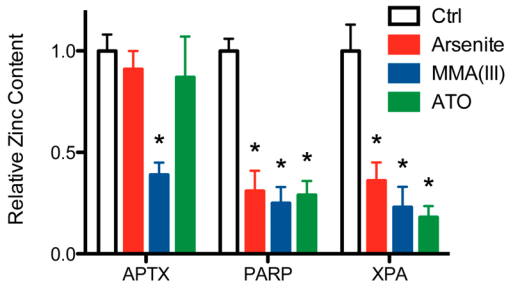

Figure 5. Zinc loss from zinc finger proteins isolated from cells treated with arsenicals. HaCat cells were treated with 2 μM of different arsenic compounds for 24 h. Zinc finger proteins were immunoprecipitated from the cell extract, then the zinc content in each specific protein was analyzed with the colorimetric assay as described in the Experimental Procedures section. Zinc content in APTX protein was sensitive only to MMA(III) treatment, but zinc in PARP-1 and XPA was decreased by all three arsenic compounds. That is, MMA(III) could remove zinc from all three configurations of zinc finger proteins, while arsenic and ATO selectively remove zinc from C3H1 and C4 zinc finger proteins in cells. Bar plot shows the mean ± SD, * $p < 0.05$ vs Ctrl group (no treatment), $n = 3$.

conclusion is drawn from the cobalt spectra showing the occupation of metal binding sites, the UV−vis spectra showing the formation of As−S bonds, and the mass spectra showing the coordination with 3 Cys residues on zinc fingers. As shown by mass spectrometry, arsenite and ATO gave exactly the same +72 m/z shift to C3H1 or C4 zinc fingers, indicating that both arsenite and ATO bind to zinc fingers using the arsenic atom only and coordinate with 3 Cys on zinc finger motif, with the release of three hydrogens. This behavior may explain the selectivity in binding with C3H1 and C4 zinc fingers (as illustrated in Figure 6, top row). Kitchin and Wallace reported that arsenite bound C3H1 and C4 complexes are over 2 orders of magnitude more stable than a C2H2 complex (155 versus 1.29 min) in kinetic studies.[28] Therefore, it is reasonable to suggest that trivalent arsenite or ATO may form two As−S bonds with C2H2 zinc fingers while leaving the third bond unoccupied but that the resulting product is too unstable to accumulate to high enough a concentration to be detected by our analytical approaches due to the presence of an unoccupied bond. This binding selectivity toward zinc fingers with 3 or more Cys could have potential biological significance. In terms of structural/functional consequences, arsenite and ATO led to selective conformational changes of C3H1 and C4 zinc fingers and induced zinc loss selectively in PARP-1 and XPA proteins in cells. There was no significant difference in the efficiency of arsenite and ATO in changing the structure of zinc fingers as well as zinc release from proteins. Circular dichroism (CD) and nuclear magnetic resonance (NMR) spectra indicate that arsenic binding to a C3H1 or C4 zinc finger motif result in an unfolded structure.[29,30] These results, together with the findings here, provide evidence to the alteration of zinc finger structure by arsenic binding and further support that arsenic interaction with zinc finger proteins will likely disrupt protein function. Since C3H1 and C4 zinc finger proteins are a minority in the whole zinc finger protein family (less than 20%), inorganic arsenicals could target some C3H1 and C4 zinc finger proteins in cells more effectively even at low concentrations.

It has been reported that that MMA(III) binds to PARP-1 and XPA zinc finger peptides.[31,32] Here, we further demonstrated that MMA(III) could occupy metal binding

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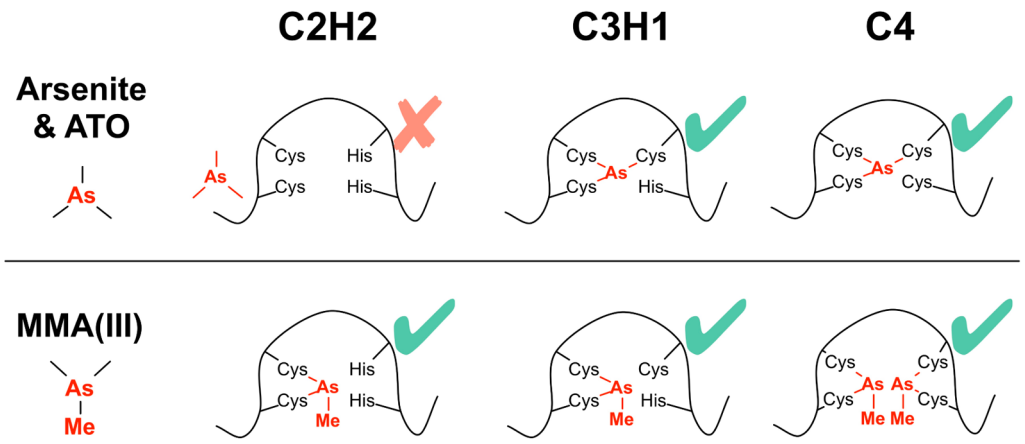

Figure 6. Schematic illustration of arsenite, ATO, and MMA(III) binding to zinc fingers. Arsenite and ATO bind to zinc fingers by coordinating with 3 Cys, which leads to selective binding with C3H1 and C4 zinc fingers. MMA(III) binds to zinc fingers together with the methyl group (-Me), using 2 Cys instead of 3, which causes nonselective binding with all 3 configurations of zinc fingers.

sites (Figure 1A) and form As−S bonds (Figure 2A) on all 3 configurations of zinc fingers, nonselectively. This nonselective interaction is also confirmed structurally and functionally using conformation and zinc content analysis (Figures 4 and 5). The molecular mechanism behind the nonselectivity is that MMA(III) covalently binds to two Cys residues, releasing two hydrogen, as shown in mass spectrometry (Figures 3G, H, and I). In contrast to arsenite and ATO binding with three Cys, MMA(III) only binds with two Cys on zinc fingers. This is demonstrated by the +88 m/z shift, as well as the binding of two MMA(III) molecules to the C4 zinc finger (Figure 3I). The +88 m/z shift and loss of 2H is consistent with previous findings by Wnek et al.[31] In addition, a complex of two MMA(III) molecules with the C4 XPA zinc finger peptide has been detected by Piatek et al.[32] In this work, we confirmed these findings using MALDI-MS and put these together to explain the mechanism of differential binding of MMA(III) to zinc finger peptides at the molecular level. Although arsenic in MMA(III) is still trivalent, one bond is already occupied by the methyl group, leaving the remaining two bonds for binding with Cys. The +88 m/z shift in mass spectrometry studies with C2H2, C3H1, and C4 zinc fingers confirm that when MMA(III) binds to zinc fingers, the methyl group is still bound to arsenic. In other words, the presence of the methyl group on MMA(III) changed the binding behavior and selectivity of this trivalent arsenical (as illustrated in Figure 6, bottom row), enabling it to bind with two Cys on zinc fingers. Importantly, unlike the situation with inorganic arsenite, the product derived from MMA(III) binding with two Cys is stable since there is no longer an unoccupied bond existing on the molecule. This result indicates that the methylation of arsenic could dramatically change the preference and profile of arsenic interaction with zinc finger proteins. It is possible that the greater breadth of zinc finger protein binding may lead to differences in toxicity and carcinogenic potential. Some recent studies showed that MMA(III) is more toxic than inorganic arsenic in terms of certain parameters of carcinogenesis, such as cell transformation.[33,34] As for the possible molecular mechanism, Piatek et al. demonstrated that MMA(III) acts more effectively than arsenite in destroying the structure of C4 zinc fingers.[32] Meanwhile, in studies of PARP activity, up to 90% inhibition is readily evident at submicromolar concentration of arsenite in human keratinocytes,[16,21] but exposure to 1 μM MMA(III) caused about 30% PARP activity inhibition in urothelial cells.[31] These findings might suggest that arsenite causes a greater magnitude of PARP inhibition than MMA(III) or that the findings may simply reflect cell type differences. Apparently, further research is needed to investigate the relationship between binding selectivity and toxicity/carcinogenesis, i.e., whether the change of selectivity enhances the effect of arsenic *in vivo* or simply dilutes arsenic interaction across the large family of zinc finger proteins.

In conclusion, this work demonstrates that arsenite and ATO have the same selective effect in binding with C3H1 and C4 zinc finger proteins, whereas MMA(III) interacts with all three configurations of zinc finger proteins. Methylation of trivalent inorganic arsenicals is responsible for the change in binding selectivity. These findings provide insightful understanding of the molecular mechanisms underlying the differential effects of inorganic versus methylated arsenicals, as well as the role of *in vivo* arsenic methylation in arsenic toxicity and carcinogenesis.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

MMA(III), monomethylarsonous acid; ATO, arsenic trioxide; PARP-1, poly(ADP-ribose) polymerase-1; APTX, aprataxin; XPA, Xeroderma pigmentosum group A; UV−vis spectroscopy, ultraviolet−visible spectroscopy; MALDI-TOF-MS, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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