Inorganic Chemistry
Article
pubs.acs.org/IC

The Reaction of Arsenite with Proteins Relies on Solution Conditions

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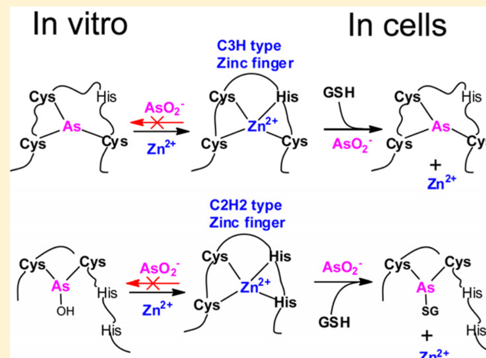
S Supporting Information

ABSTRACT: Arsenic is a biologically interesting element with both antitumor and carcinogenic effects. Zinc finger proteins (ZFPs) have been confirmed to be the cellular targets of arsenite; however, arsenite inhibits ZFPs much less efficiently in vitro than in vivo. The molecular mechanism of this difference is unknown. In this work, we found that the reaction of arsenite with ZFPs relies on the presence of small biomolecules such as glutathione (GSH), histidine, and cysteine (Cys). The weak acidity also enhances the reaction. Further study shows that the coordination of zinc is much more susceptible than that of arsenic to these solution conditions, which enhance the competition of arsenic. Notably, different from C_3H-type ZFPs, the C_2H_2-type ZFPs are more significantly influenced by the presence of thiol-containing molecules in the reaction. GSH and Cys can facilitate the reaction by participation of the coordination to As(III) together with C_2H_2-type ZFPs. Consequently, the reactions are promoted both thermodynamically and kinetically via the formation of ternary complexes GSH-As-ZFP or Cys-As-ZFP. These results indicate that the reactions between arsenite and proteins are considerably modulated by environments such as the small biomolecules and the acidity of the solution. This finding clarifies the discrepancy observed in the reactions of arsenite in vitro versus in cells, and provides an insight into the molecular mechanism of arsenite.



1. INTRODUCTION

Arsenic is a well-known carcinogenic element; however, it has also been successfully used for the treatment of cancer.[1,2] Arsenic contamination in groundwater is a worldwide problem, with more than 100 million people being affected by arsenic toxicosis.[3] Chronic exposure to arsenic is associated with diverse human diseases, including the cancers of skin, liver, lung, kidney, and urinary bladder.[4] On the other hand, arsenic trioxide (As_2O_3) has been therapeutically used in traditional Chinese medicine (TCM) for more than 2400 years.[5] In recent years, this ancient drug has aroused great interest worldwide because it shows remarkable efficacy in the treatment of acute promyelocytic leukemia (APL).[6,7] This success has also led to the exploration of its application in other cancers.[8,9] Although the different biological roles of arsenic have been recognized, the molecular mechanisms of arsenic are complicated and still far from fully understood

The different biological functions of arsenic imply multiple binding targets of this drug in cells. It has been reported that arsenic could inactivate up to 200 enzymes,[10] indicating the nonspecific interaction of arsenic. Thus, the selectivity of arsenic is crucial in the determination of its biological functions. Among various arsenic binding proteins, zinc finger proteins (ZFP) have been suggested to play the most important roles in the dual biological function of arsenite.[11,12] The carcinogenic properties of arsenic are most likely associated with the interaction of arsenic with DNA repair proteins.[11] Arsenite can directly react with the zinc finger domain of these proteins, such as PARP-1, XPA, and FPG, and inhibit DNA repair,[13−15] resulting in increased DNA damage and the risk of cancer development.[16,17] While the binding of arsenic to DNA repair proteins is associated with its carcinogenic property, the interactions of arsenic with some other ZFPs could correlate to its antitumor activity. It has been demonstrated recently that arsenic reacts with the zinc finger domain of PML-RARα, which results in the enhanced SUMOylation and degradation of the PML-RARα fusion protein.[12] Thus, the biological functions of arsenite depend on the reactivity of ZFPs, which determines the target selectivity of arsenite in cells.

Zinc finger proteins contain a conserved zinc binding domain, and zinc coordination plays a crucial role in the stabilization of the structure of ZFPs.[18,19] On the other hand, the zinc coordination residues, cysteines, are also the potential binding site of As(III) based on the hard and soft acid and base (HSAB) principle.[20] Previous studies suggested that the reactivity of ZFPs to arsenite is highly dependent on the number of cysteine residues in the protein.[21] This preference can be explained by the coordination chemistry that As(III) tends to bind to three thiol ligands of proteins. Arsenic binding could displace the zinc ion from ZFPs,[22] resulting in structure perturbation and protein dysfunction.[12] Therefore, arsenite could inhibit the activity of ZFPs both in vivo and in vitro.[12,13,21,23]

Received: November 21, 2013
Published: March 6, 2014

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Although As^{III} interferes with the function of ZFPs in the cell, in vitro studies showed that the binding affinity of ZFPs to Zn^{II} is much higher than that of As^{III}.[22,24] These data are contrary to the result that arsenic can inhibit ZFPs in vivo. The in vitro reactions were usually performed on the apo-ZFPs. On the other hand, experiments also showed that the addition of Zn^{II} could restore the PARP-1 reactivity abolished by As^{III} in cellular systems.[16,25] These observations raise a question of the competition between arsenic and zinc to ZFPs. So far, most cellular results indicate that arsenic is active to ZFPs, whereas in vitro data show that zinc has higher affinity to the protein. Thus, we speculate that the cellular condition could be crucial in making these differences.

In addition to ZFPs, arsenic can also bind to several other proteins in the cell. Tubulin is a proposed target of arsenic in inducing apoptosis of myeloid leukemia cells.[26] By targeting thioredoxin reductase, arsenic could elevate the intracellular level of reactive oxygen species, leading to the apoptosis of cancer cells.[27] Both tubulin and thioredoxin reductase interact with arsenic through two cysteine residues. Although it is suggested that two vicinal cysteines are sufficient for the arsenic binding,[11,26,28,29] in vitro studies demonstrated that arsenite selectively binds to proteins containing three or more cysteine residues.[21,24] In vitro results indicate that arsenic binds to the human thioredoxin (containing five Cys) but not to the *Escherichia coli* thioredoxin (containing two Cys).[30] However, as shown in ZFPs, the in vitro reactivity may not reflect the reaction of proteins in the complicated cell system.

To answer these fundamental questions in the reaction of arsenic with proteins, we have investigated the reactions of arsenite with ZFPs in the presence of different small molecules that are abundant in the cell. The C_2H_2 and C_3H types of ZFPs were used in the reaction since these two types of proteins possess different reactivities to arsenite. The binding and competition of As(III) and Zn(II) with ZFPs were studied under different conditions. Results demonstrated that the acidity and the presence of glutathione (GSH), Cys, or histidine significantly influenced the reactivity of arsenite to ZFPs. In comparison with the C_3H-type ZFPs, the C_2H_2-type ZFPs are more significantly influenced by the presence of GSH and cysteine, as these small thiol molecules participate the coordination to As(III) together with the protein. These findings allow the reconsideration of the reactivity of dithiol proteins with arsenite in cellular systems.

2. EXPERIMENTAL SECTION

Protein Expression and Purification. All proteins used in this work were obtained from the overexpression in *E. coli* as reported previously.[24] The gene coding target protein was amplified by polymerase chain reaction and then inserted into an expression vector to obtain a recombinant plasmid. The recombinant plasmid was transformed into BL21 (DE3) competent cells for the overexpression of fusion protein. The ^{15}N isotopic-labeled protein was obtained by the growth of *E. coli* in the medium containing $^{15}NH_4Cl$ as the sole nitrogen source. The protein was first purified using Ni^{2+} affinity chromatography. The tag was removed by tobacco etch virus protease or by small ubiquitin-like modifier (SUMO) protease. The protein was further purified through gel filtration followed by high-performance liquid chromatography. The protein concentration was determined through UV absorption.

Electrospray Ionization Mass Spectrometry. Electrospray ionization mass spectrometry (ESI-MS) measurements were carried out on a LTQ Orbitrap XL mass spectrometer (Thermo Scientific). Samples were prepared with 100 μM protein in 10 mM ammonium acetate buffer. For arsenite reactions, samples were allowed 30 min of incubation time. All samples were diluted to a final solution with 20 μM protein, 50% methanol. The positive ion mode was used in the ESI-MS experiments.

Fluorescence Measurements. The fluorescence measurements were performed on a RF-5301PC spectrofluorometer (Shimadzu) using a quartz cuvette with the path length of 5 mm. The excitation wavelength was set at 280 nm, and the emission fluorescence spectra were recorded from 300 to 500 nm (observed maximum at 360 nm). The relative intensity of the fluorescence was calculated with the formula $(F - F_S)/(F_0 - F_S)$ (for arsenic) or $(F - F_0)/(F_S - F_0)$ (for zinc), where F is the fluorescence at the given concentration of metal ions, F_0 is the initial fluorescence, and F_S is the final fluorescence of titration. The data were fitted by the equation $Y = \{(P_0 + X + K_d) - ((P_0 + X + K_d)^2 - 4P_0X)^{1/2}\}/2P_0$ using a nonlinear least-squares fitting, where P_0 is the concentration of protein. The apparent dissociation constant K_d was obtained from the data fitting.

For the kinetic study, the fluorescence spectra were recorded on the time scan mode at 22 °C, with excitation at 280 nm and emission at 360 nm. For the binding kinetic measurements, 50 μM Sp1−2 was prepared in 0.5 mM tris(2-carboxyethyl)phosphine (TCEP), 0.25 mM ethylenediaminetetraacetic acid (EDTA), and 20 mM phosphate buffer, pH 7.0. $NaAsO_2$ was prepared in the same buffer in the concentration of 250 μM. $NaAsO_2$ (200 μL) was added to the 50 μL Sp1−2 in the cuvette, and fluorescence intensity was recorded immediately. The control experiment was recorded by adding 200 μL of buffer to the protein solution.

Circular Dichroism. CD measurements were performed on a Jasco J-810 CD spectrometer. The CD spectra were recorded from 280 to 190 nm. The protein samples were prepared to the final concentrations of about 0.1 mg/mL (25 μM for NCp7−2 and 10 μM for PARP1−2) in 20 mM phosphate buffer, pH 7.0 or pH 5.8. The blank spectrum was also recorded on the buffer for baseline corrections. All experiments were repeated three times, and the average values were used in analyses.

NMR Spectroscopy. Heteronuclear single quantum coherence (HSQC) spectra were collected on a Bruker 600 MHz NMR spectrometer at 25 °C. NMR samples were prepared in 100 mM NaCl, 2 mM TCEP, 10% D_2O (v/v), and 50 mM phosphate buffer (pH 7.0 or 6.0). 0.5 mL of 1 mM ^{15}N-labeled NCp7 was used. Zinc chloride (2 mM) was added to obtain the spectrum of Zn-NCp7, while both 2 mM zinc chloride and 4 mM arsenite were added for the competition experiments. The data were processed with NMR pipe and TopSpin software.

Isothermal Titration Calorimetry. Isothermal titration calorimetry (ITC) measurements were carried out on a MicroCal VP-ITC System (GE Healthcare) at 25 ± 0.2 °C. Arsenite (1 mM) in the syringe was injected into 1.443 mL of protein (0.1 mM) in the calorimetry cell. Both the arsenite and proteins were prepared in the same buffer. Titrant (8 μL) was delivered each time with an interval of 2 or 3 min between injections to allow complete equilibration. 34 injections were performed for each titration with the stirring speed of 307 rpm. All experimental solutions were completely degassed before titration. Data were processed with a one-site binding model by the Origin 7.0 software package supplied by MicroCal.

3. RESULTS

Four ZFPs were used in the reaction with arsenite: the NCp7 protein (NCp7, residues 12−55), the second zinc finger domain of NCp7 protein (NCp7−2, residues 34−52), the second zinc finger domain of Sp1 protein (Sp1−2, residues 565−595), and the second zinc finger domain of PARP1 protein (PARP1−2, residues 103−215) (Supporting Information, Scheme S1). Sp1−2 contains a C_2H_2 type of zinc finger domain, while NCp7−2 and PARP1−2 contain a C_3H type of zinc finger domain. Both Sp1−2 and NCp7−2 are characterized by a single tryptophan (Trp) residue that constitutes an intrinsic fluorescence probe for the quantitative analyses of metal binding to the proteins. It has been previously reported

that NCp7−2 is much more reactive than Sp1−2 in their apo forms. PARP1 is a proposed cellular target of arsenite, and PARP1−2 was used in the Study to compare with the reaction of NCp7−2. ITC measurements indicate that the affinity of PARP1−2 is comparable to that of NCp7 and NCp7−2 (Supporting Information, Figure S3).

Competition between As(III) and Zn(II) in the Reactions of ZFPs. The competition between As(III) and Zn(II) was studied on a C_3H zinc finger protein, NCp7−2, which is highly reactive to arsenite.[24,31] As the intrinsic fluorescence of the protein is dependent on the protein folding, the metal binding can be detected by the fluorescence change. The titration results in Figure 1a clearly show the binding of Zn(II) to apo-NCp7−2 led to ∼3-fold increase of the fluorescence, whereas the As(III) binding decreased fluorescence of apo-NCp7−2 to half the intensity. This result suggests that the different structure alterations occurred on the NCp7−2 protein with Zn(II) or As(III) coordination. The titration of Zn to As-NCp7−2 also recovered the fluorescence to the same level of Zn-NCp7−2. This result indicates that Zn(II) can replace the As(III) coordination in NCp7−2, even though arsenic is 10 times in excess over zinc. Therefore, the affinity of Zn(II) for NCp7−2 is much higher than that of As(III), and arsenic cannot substitute for zinc with this protein.

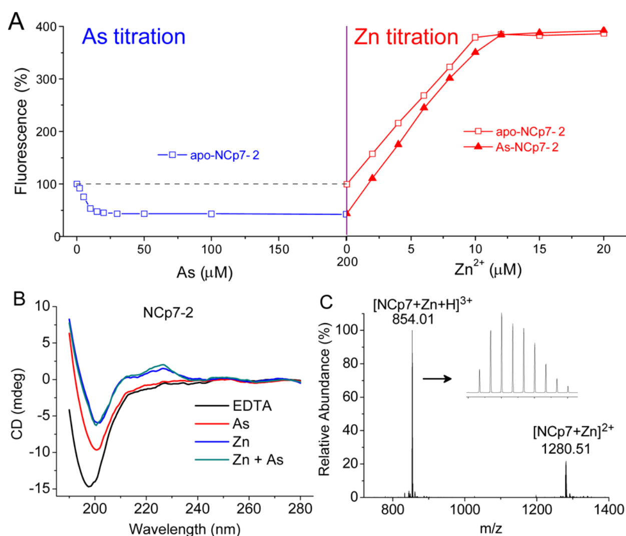

Figure 1. Competition of As(III) and Zn(II) in the binding of NCp7−2 protein. (a) Fluorescence of NCp7−2 titrated with $NaAsO_2$ or $ZnCl_2$. (b) CD spectra of NCp7−2 at pH 7.0. The colors denote the spectrum recorded in the presence of EDTA (black), $ZnCl_2$ (blue), $NaAsO_2$ (red), or both $ZnCl_2$ and $NaAsO_2$ (green). (c) ESI-MS spectrum of NCp7−2 in the reaction with both arsenite and zinc chloride. The spectrum was recorded on 10 μM protein in the presence of 20 μM $ZnCl_2$ and 200 μM $NaAsO_2$ in 10 mM ammonium acetate buffer.

The fluorescence measurements indicate that the NCp7−2 protein could fold differently upon the coordination of zinc or arsenic; therefore, the CD spectra were recorded on the protein in various formats. The spectra of apo- and zinc-bound protein are in accordance with the literature result (Figure 1b).[31] Arsenic coordination resulted in a CD spectrum that is different from either apo- or Zn-NCp7−2, which confirmed the different protein folding of As-NCp7−2. Notably, when apo-NCp7−2 was mixed with both arsenite and zinc chloride, the CD spectrum was the same as it was for Zn-NCp7−2. This result confirms that Zn(II) is much more competitive than As(III) in the reaction with NCp7−2. Similar results were observed on another C_3H zinc finger protein PARP1−2, which is a proposed arsenic target in cells (Supporting Information, Figure S1).

To further confirm the substitution of arsenic with zinc in NCp7−2, the product identity was analyzed with ESI-MS. Our previous study showed that arsenic forms a 1:1 complex with apo-NCp7−2.[24] The ESI-MS result in this work indicates that the addition of zinc to the As-NCp7−2 complex led to the release of arsenic and generated the Zn-NCp7−2 complex (Figure 1c). The observed molecular weight is in agreement with the calculated data of Zn-NCp7−2 (obsd. 2561.03; cald. 2561.02) (Supporting Information, Table S1). Therefore, it can be concluded that Zn(II) can substitute for As(III) in the coordination of NCp7−2 and PARP1−2 even with excess As(III). Since both NCp7−2 and PARP1−2 are C_3H-type ZFPs and are proposed to be highly reactive to arsenite, these results highly suggest that Zn(II) is much more competitive than As(III) in the coordination to ZFPs, and arsenite cannot interfere with the zinc-bound ZFPs in vitro.

Effect of Acidity on the Competition of As(III) and Zn(II). Since arsenite cannot react with Zn-bound ZFPs in vitro, we next investigated what conditions could influence this reaction. To compare with some literature results,[12] and also for considering the more acidic environment in tumors and in some subcellular compartments,[32,33] we tested whether the acidity could affect the reaction. The reaction was verified using two-dimensional (2D) $^1H-^{15}N$ heteronuclear single-quantum coherence (HSQC) NMR spectra on the ^{15}N isotopic-labeled Zn-NCp7. In the absence of arsenite, the spectrum of Zn-NCp7 is well-dispersed, showing the well-folded structure of the protein. Adding arsenite to Zn-NCp7 at pH 7.0 did not influence the NMR spectrum (Figure 2a), which confirms the result that As(III) cannot replace Zn(II) in the protein at neutral conditions. On the contrary, when the reaction was carried out at pH 6.0, the spectrum of Zn-NCp7 is clearly disturbed by arsenite (Figure 2b). This result indicates that

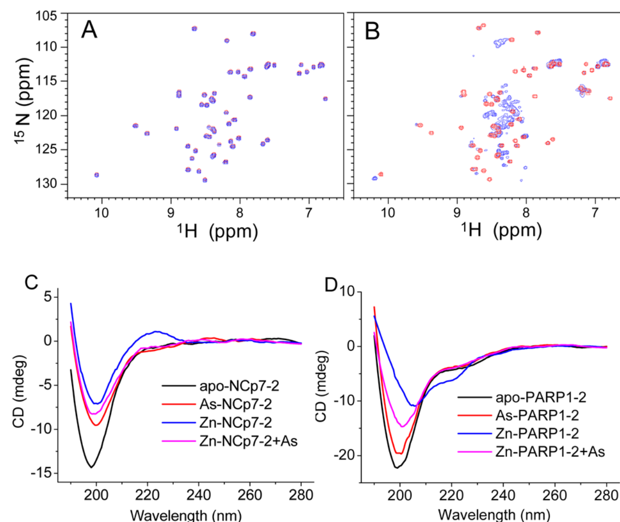

Figure 2. Effect of acidity on the reaction of arsenite with Zn-bound ZFPs. (a, b) Overlay of 2D $^1H-^{15}N$ HSQC NMR spectra of Zn-NCp7 in the absence (red) and in the presence (blue) of arsenite at pH 7.0 (a) or pH 6.0 (b). (c, d) CD spectra of NCp7−2 (c) and PARP1−2 (d). Spectra were recorded at pH 5.8 in the presence of EDTA, $ZnCl_2$, $NaAsO_2$, or both $ZnCl_2$ and $NaAsO_2$.

dx.doi.org/10.1021/ic402891t | Inorg. Chem. 2014, 53, 3054−3061

arsenite becomes more reactive to Zn-NCp7 in acidic conditions. In addition, the spectrum appeared less dispersed after the reaction of arsenite, indicating that the As(III) binding disrupted the protein folding of NCp7.

CD spectra were also recorded for the reaction at two pH values. Data show that the spectrum of NCp7−2 in the presence of both Zn(II) and As(III) is similar to that of As-NCp7−2 at pH 5.8 (Figure 2c). In comparison to the reaction in the neutral condition (Figure 1b), this result indicates that arsenite becomes more competitive than zinc in the reaction of NCp7−2 at pH 5.8. The same result is also observed in the reaction of PARP1−2 (Figure 2d).

Effect of Biomolecules on the Reaction of Arsenite with Zn-ZFPs. A number of small biomolecules in the cell, such as glutathione and histidine, possess high capabilities of metal coordination. These molecules could also influence the interaction of arsenite with ZFPs. For instance, glutathione is present in high concentrations (0.5−10 mM) in cells and plays an important role in arsenic metabolism.[34] To investigate whether these molecules affect the reaction of arsenite, we performed the fluorescence titration of Zn-NCp7−2 in the presence of 5 mM GSH, Cys, or histidine. The control experiment shows that, in the absence of other biomolecules, the titration with arsenite decreased the fluorescence of Zn-NCp7−2 at pH 5.8, but not at the neutral condition (Figure 3).

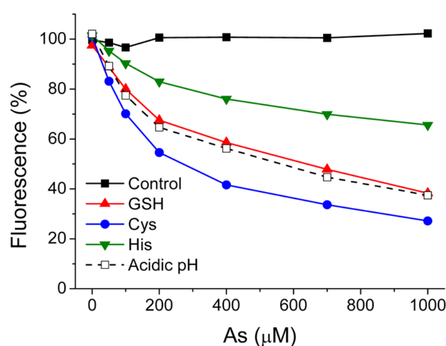

Figure 3. Effect of small biomolecules on the reaction of arsenite with Zn-bound ZFPs. The fluorescence titrations were performed on 10 μM Zn-NCp7−2 in 20 mM phosphate buffer at pH 7.0. Symbols denote the control in the absence of additional molecules (■: pH 7.0, □: pH 5.8), or in the presence of 5 mM GSH (▲), Cys (●), or His (▼) at pH 7.0. The dashed line indicates the reaction at pH 5.8.

This is consistent with previous results that arsenite can react with Zn-NCp7−2 only in acidic conditions. However, in the presence of 5 mM GSH, arsenite clearly decreased the fluorescence of Zn-NCp7−2 at pH 7.0. This observation indicates that GSH significantly enhances the reactivity of arsenite with the protein. Similar results were observed on the reactions in the presence of cysteine or histidine, although the degree of the fluorescence change was different (Figure 3). Therefore, it can be concluded that cellular molecules are capable of modulating the reaction of arsenite with ZFPs. The combined effect of small molecules and pH value could be more significant.

Effect of Solution Conditions on the Coordination of As(III) and Zn(II) to ZFP. To understand how these solution conditions modulate the interaction of arsenite with the protein, we studied the binding affinity of As(III) and Zn(II) to NCp7−2 under different conditions. The binding constants were determined using the fluorescence titration of arsenite or zinc ions with apo-NCp7−2 (Figure 4). The apparent dissociation constants (K_d(app)) were obtained by fitting the titration curves (Table 1). Results show that the binding affinity of Zn(II) is 3 orders of magnitude larger than that of As(III) to NCp7−2, with K_d(app) in 1.24 nM and 1.14 μM, respectively. The K_d(app) of Zn(II) with NCp7−2 is in agreement with the literature.[22] These data are consistent with the preceding results from binding competition experiments.

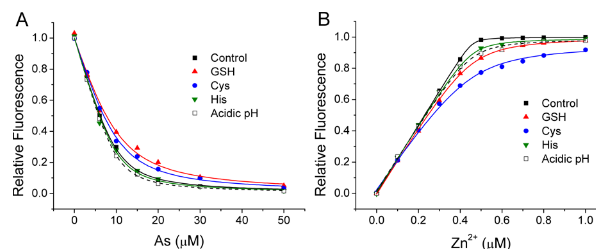

Figure 4. Fluorescence titration of apo-NCp7−2 in different conditions. (a) 10 μM apo-NCp7−2 was titrated with $NaAsO_2$; (b) 0.5 μM apo-NCp7−2 was titrated with $ZnCl_2$. All reactions were performed in 0.1 mM TCEP, 20 mM phosphate buffer, pH 7.0. Symbols denote the control in the absence of additional molecules (■: pH 7.0, □: pH 5.8), or in the presence of 5 mM GSH (▲), Cys (●), or His (▼) at pH 7.0. Curves were from the nonlinear least-squares fitting of experimental data. The dashed line indicates the reaction at pH 5.8.

The titration results also show that the binding affinities of As(III) and Zn(II) were influenced differently by the solution conditions. While the K_d(app) of As(III) are only slightly influenced, the binding affinity of Zn(II) decreases up to 30 times in the presence of small molecules (Table 1). This discrepancy could result from the different contributions of small biomolecules to the coordination of As(III) and Zn(II). In addition, the weak acidity (pH = 5.8) had little influence on the coordination of As(III); however, it clearly decreased binding affinity of Zn(II). The different pH effects can be expected from the coordination sites of these two metal ions. The histidine residue usually has a pK_a of about 6.8; therefore, lowering the pH to 5.8 weakens the coordination of histidine to Zn(II). However, As(III) preferentially coordinates to the cysteine residue, which is not influenced by such a pH change. Taken together, these data indicate that the reaction conditions, such as small biomolecules and weak acidity, could decrease the binding affinity of Zn(II) much more than that of As(III). These different influences could enhance the competition of As(III) in the cellular environment.

Thiol Ligands Enhance the Binding of Arsenic to Dithiol-Proteins. In addition to ZFPs, some other proteins with two vicinal Cys residues, such as tubulin and thioredoxin reductase, have also been proposed to be the cellular targets of arsenic. However, in vitro results suggest that arsenite selectively binds to proteins with three or more cysteine residues.[21,24] For instance, Sp1−2, a C_2H_2-type ZFP protein, exhibits 2 orders of magnitude lower binding affinity than the C_3H or C_4 types of ZFPs.[24] Since Sp1−2 provides only two cysteine residues for As(III) coordination, a hydroxyl coordination is present in the product.[24] This observation suggests that the ligands in solution also contribute the binding of As(III) in the dithiol-proteins. Therefore, we speculated that GSH could provide an additional thiol for arsenic binding to

Table 1. Apparent Dissociation Constant (K_d) of apo-NCp7-2 to As(III) and Zn(II)

	control	GSH	Cys	His	acidic pH[a]
As (μM)	1.14 ± 0.12	2.63 ± 0.77	2.02 ± 0.34	0.98 ± 0.26	0.79 ± 0.06
Zn (nM)	1.24 ± 0.31	18.8 ± 2.2	36.1 ± 10.9	6.51 ± 1.90	9.53 ± 3.63

[a]pH = 5.8.

the dithiol protein and then influence the coordination of As(III) to the protein.

To verify this hypothesis, we studied the reaction of arsenite with Sp1−2 in the presence of GSH or Cys. The fluorescence titration demonstrate that both GSH and Cys significantly promoted the reaction of arsenite with Sp1−2, and more significant effects were observed with higher concentrations of GSH and Cys (Figure 5a). The K_d(app) decreased from 184 μM to 10.9 μM and 16.0 μM in the presence of 5 mM GSH and 5 mM Cys, respectively (Table 2). These data confirm the hypothesis that thiol ligands promote the binding affinity of arsenite to the C_2H_2-type ZFPs. On the contrary, thiol ligands slightly decrease the binding affinity of arsenite to C_3H-type ZFP (Figure 4a). The ESI-MS spectra also confirmed that small thiol ligands contribute differently between C_2H_2- and C_3H-type ZFPs in the As(III) coordination (see below). These data reveal that GSH and Cys play different roles in the reactions of C_2H_2-type ZFPs in comparison to C_3H-type ZFPs.

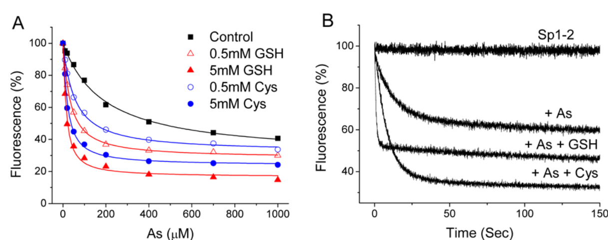

Figure 5. Thiol ligands promote the reaction of arsenite with dithiol protein. (a) Fluorescence spectra of Sp1−2 titrated with arsenite. Symbols denote the reaction performed in control (■) or in the presence of GSH (○ 0.5 mM; ● 5 mM) or Cys (△ 0.5 mM; ▲ 5 mM). (b) Time-dependent fluorescence spectra of Sp1−2 in the reaction with arsenite. Reactions were performed on 10 μM Sp1−2 and 200 μM arsenite in 20 mM phosphate buffer at pH 7.0. The control experiment was recorded on a 10 μM Sp1−2 solution without addition arsenite. Three reactions were performed on arsenite (+As), arsenite with 5 mM GSH (+As + GSH), and arsenite with 5 mM Cys (+As + Cys).

In addition to the binding affinity, the binding rate of arsenite to Sp1−2 was also analyzed with fluorescence measurements (Figure 5b). Results show that the presence of GSH or Cys also promoted the reaction rate of Sp1−2. As arsenite is in much more excess than Sp1−2 (20 mol equiv), the reaction can be considered as a pseudo-first-order kinetic process. By fitting the time-dependent fluorescence quenching, the rate constants were obtained. The presence of GSH and Cys increased rate constant from 0.07 s^{-1} to 0.10 s^{-1} and 0.71 s^{-1}, respectively. These data indicate that thiol ligands can promote the reaction kinetically as well as thermodynamically. Moreover, GSH enhances the binding affinity more efficiently, while Cys promotes the reaction rate more significantly.

To confirm the hypothesis that small thiol molecules act as the third ligands in the coordination of arsenite to Sp1−2, the product composition was analyzed using ESI-MS. Results show that, in the absence of GSH and Cys, the direct reaction between arsenite and Sp1−2 led to the deprotonation of two Cys residues and the formation of HO-As-[Sp1−2] adduct (Figure 6). This result is in accordance with our previous data

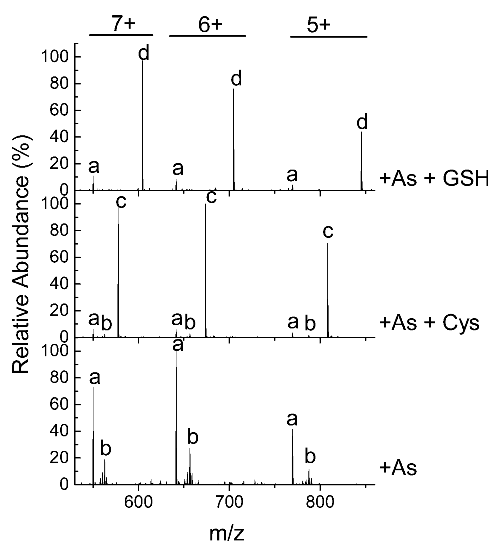

Figure 6. The binding of small thiol ligands to the As(III)-coordinated Sp1−2. ESI-MS mass spectra of Sp1−2 reacted with arsenite. Three reactions were performed on 0.1 mM Sp1−2 with 1 mM $NaAsO_2$ in the absence of additional thiol ligand (+As) or in the presence of 5 mM Cys (+As + Cys) and 5 mM glutathione (+As + GSH), respectively. Four major products from these reactions were detected: a, Sp1−2; b, HO-As-[Sp1−2]; c, Cys-As-[Sp1−2]; and d, GSH-As-[Sp1−2].

that the C_2H_2-type ZFPs can only provide two coordination sites for As(III) and that a hydroxyl group is required to fit the tricoordinated As(III). In the meantime, the product signals are rather weak due to the low binding affinity of Sp1−2. On the contrary, the As-bound products are dominant when the reactions were performed in the presence of GSH or Cys. The m/z peaks indicate the covalent binding of GSH or Cys to the products, forming ternary complexes GSH-As-[Sp1−2] and Cys-As-[Sp1−2], respectively (see Table 3 for the peak assignment). Although the peak intensity in ESI-MS spectra is not directly corresponding to the concentration of each

Table 2. Apparent Dissociation Constant (K_d) of As(III) Binding to Sp1−2 in Various Concentrations of Thiol Ligands

		GSH		Cys	
	control	0.5 mM	5 mM	0.5 mM	5 mM
K_d (μM)	184 ± 17	28.7 ± 1.0	10.9 ± 1.5	49.2 ± 3.4	16.0 ± 2.1

Table 3. Mass Spectra Analyses of Sp1−2 after the Reaction with Arsenite in the Presence of GSH or Cysteine (Data from Figure 6)

peak	composition	molecular formula	molecular weight obsd.[a]	molecular weight cald.	m/z (charge)
a	Sp1−2	$C_{165}H_{257}N_{55}O_{46}S_3$	3842.9	3842.9	549.99 (+7)
					641.48 (+6)
					769.58 (+5)
b	HO-As-Sp1−2	$C_{165}H_{256}N_{55}O_{47}S_3As$	3932.8	3932.8	562.83 (+7)
					656.47 (+6)
					787.56 (+5)
c	GSH-As-Sp1−2	$C_{175}H_{271}N_{58}O_{52}S_4As$	4221.9	4221.8	604.13 (+7)
					704.65 (+6)
					845.38 (+5)
d	Cys-As-Sp1−2	$C_{168}H_{261}N_{56}O_{48}S_4As$	4035.8	4035.8	577.55 (+7)
					673.64 (+6)
					808.16 (+5)

[a]The observed molecular weights are calculated from the average of the m/z peaks.

species, the relatively higher abundance of product signals is consistent with fluorescence titration results that indicate Sp1−2 is more reactive in the presence of GSH or Cys. These results indicate that small thiol molecules promote the reaction of arsenic to C_2H_2-type ZFPs by coordination to the third binding site of arsenic. In contrast to the reaction of Sp1−2, NCp7−2 showed only minor signals of ternary complexes Cys-As-NCp7−2 and GSH-As-NCp7−2 (Supporting Information, Figure S4), indicating different roles of small thiol ligands in the reactions of C_2H_2- and C_3H-type ZFPs.

4. DISCUSSION

ZFPs are the most abundant DNA binding proteins in human transcription factors and participate in various cellular processes, including transcription and translation, DNA replication and repair, cell proliferation, and apoptosis.[35] A large variety of genes, including housekeeping genes and tumor-developing genes, are regulated by ZFPs.[36,37] Therefore, the disruption of ZFPs could lead to different biological responses, depending on the selectivity of inhibitors. It has been proposed that both anticancer and carcinogenic activities of arsenite are correlated to the reaction of ZFPs, suggesting multiple cellular targets for the arsenic. Thus, the reactivity of different ZFPs is crucial for the function of arsenite.

The zinc coordination plays a central role in the stabilization of the structure of ZFPs.[38] On the other hand, the zinc coordination sites, Cys and His residues, could also be the potential target of heavy metals such as platinum and arsenic anticancer drugs.[12,39] Arsenic could inhibit the activity of the ZFPs by coordinating to Cys and releasing the zinc ion from ZFPs, which disrupts the structure and function of the protein. Thus, the reactivity of ZFPs to arsenic determines the targetability of the protein. Previous studies showed that arsenite preferentially binds to ZFPs containing three or more Cys residues.[24] However, even for ZFPs having more than three Cys, their affinity to arsenite is about 3 orders of magnitude lower than that to Zn(II). In this work, we confirmed that As(III) cannot substitute for Zn(II) in ZFPs, while Zn(II) can replace As(III) in the protein. These results provide a basic understanding of the competition between arsenic and zinc for ZFPs in vitro.

A number of in vitro studies, including our previous report, showed that arsenite can react with apo-ZFPs efficiently.[21,24] In addition, some other studies also investigated the reactions between arsenite and Zn-bound ZFPs.[12,16] The ESI-MS results showed that arsenic can interfere with Zn-bound ZFPs; however, the MS analyses were carried out in the presence formic acid.[16,31] At acidic condition (pH = 5.6), the NMR study showed arsenic binding disturbed the structure of promyelocytic leukaemia protein (PML).[12] As we demonstrated in this work, acidity enhances the competition of As(III) with Zn(II). Although the zinc release assays using the zinc dye 4-(2-pyridylazo)resorcinol (PAR) indicated that the reaction of arsenite led to the zinc ejection from ZFPs in neutral,[40] PAR can significantly lower the binding affinity of Zn(II) to ZFPs due the coordination to Zn(II) (log K = 12.3).[41] Taken together, these data indicate that the reaction of arsenite with ZFPs requires additional solution conditions to enhance the reactivity.

An in vivo study demonstrated that low concentrations ($\leq$2 μM) of arsenite effectively inhibited ZFPs activity.[17] However, in vitro studies have shown that the arsenite inhibition of ZFPs requires high concentrations (mM) of arsenite.[40] These observations suggest that the cellular conditions play important roles for arsenite to inhibit ZFPs. Results in this work reveal that GSH, one of the most abundant thiol-containing biomolecules, significantly enhances the competition of As(III) with Zn(II) in the reaction of ZFPs. Other small molecules, Cys, and histidine showed a similar influence on the reaction. This result indicates that metal-binding molecules in the cell regulate the reaction of arsenite with ZFPs. Because of the high concentration of zinc-binding molecules such as metallothionein and GSH in the cell, the cellular conditions should generally reduce the competition of Zn(II) relative to As(III). A recent study reveals that the concentration of free zinc ion is approximately 5−10 pM in cytoplasm and nucleus.[42] Such conditions enable the inhibition of ZFPs by arsenic in the μM range in vivo.

Although ZFPs are the important cellular targets for arsenic, many other proteins, such as GSH reductase, GSH S-transferase, thioredoxin reductase, DNA ligases, pyruvate dehydrogenase, tubulin, actin, and estrogen receptor α, have been shown to be inhibited by arsenic.[10] Some of these proteins, for example, thioredoxin reductase, pyruvate dehydrogenase, and tubulin, do not contain three vicinal thiols to fit the coordination of As(III).[26,27,43] It has been suggested that arsenite is able to react with proteins containing two vicinal thiols.[9,26] These reactions could perturb the protein secondary

structures, depending on the location of the two thiols.[44,45] On the other hand, the positions of two Cys residues also influence the binding affinity of arsenic.[46] However, in vitro results suggest that dithiol proteins possess much lower binding affinity to arsenite as these proteins lack enough coordination sites for As(III). Results in this work demonstrate that small thiol-containing biomolecules such as GSH and Cys can promote the reaction of arsenite with dithiol-proteins by acting as the third ligand. The reaction generates ternary complexes GSH-As-ZFP or Cys-As-ZFP. Quantitative analyses revealed that the participation of GSH or Cys significantly promotes the reaction both thermodynamically and kinetically. The Cys modification prevents the coordination to As(III), which further confirms the binding site of thiol groups (Supporting Information, Figures S5 and S6). The physiological concentration of GSH enhances the affinity of arsenic binding to Sp1–2 by 18 times. These data explain the observations that arsenite is more inhibitory to dithiol-containing proteins in cells than in vitro.[10]

5. CONCLUSIONS

In summary, this Study investigated the influence of solution conditions on the reaction between arsenite and proteins. Results demonstrate that small biomolecules, including glutathione, Cys, and histidine, significantly regulate the reactions of As(III) and Zn(II) with ZFPs. The binding affinity measurements indicate that Zn(II) has 3 orders of magnitude higher coordination constant than As(III), resulting in greater selectivity for zinc than for arsenic in the protein binding. Therefore, arsenite appears unreactive to the zinc-bound ZFPs in vitro in the absence of other agents. However, the presence of physiological concentrations of GSH significantly enhances the competition of As(III) with Zn(II) and leads to the substitution of Zn(II) with As(III) in the protein. Weak acidity can further promote the reaction. The binding of arsenic causes zinc release from the protein and disrupts the secondary structure of ZFPs. In addition, the reactions of C_2H_2- and C_3H-type ZFPs are influenced differently by thiol-containing molecules. GSH and Cys can further enhance the binding affinity to C_2H_2-type ZFPs by participation in the reaction as the third ligand of As(III). These small thiol molecules promote the reactions both thermodynamically and kinetically. The reactions with GSH and Cys generate ternary complexes GSH-As-ZFP and Cys-As-ZFP, respectively. These data clarify the discrepancy from the in vitro reactions relative to the in vivo biological response of arsenite. These findings reveal that the cellular conditions are crucial for the function of arsenite.

■ ASSOCIATED CONTENT

Supporting Information

CD, ESI-MS, and ITC data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the National Science Foundation of China (U1332210, 21171156), the National Basic Research Program of China (973 Program, 2012CB932502), the Ministry of Education of China (SRFDP 20133402110041), USTC-NSRL Association Funding (KY2060190005), and the Fundamental Research Funds for the Central Universities.

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