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RESEARCH ARTICLE

The Effect of Salts in Promoting Specific and Competitive Interactions between Zinc Finger Proteins and Metals

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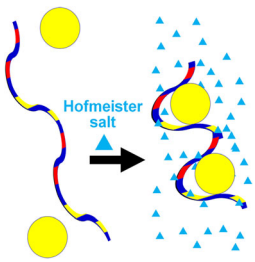

Abstract. Specific protein–metal interactions (PMIs) fulfill essential functions in cells and organic bodies, and activation of these functions in vivo are mostly modulated by the complex environmental factors, including pH value, small biomolecules, and salts. Specifically, the role of salts in promoting specific PMIs and their competition among various metals has remained untapped mainly due to the difficulty to distinguish nonspecific PMIs from specific PMIs by classic spectroscopic techniques. Herein, we report Hofmeister salts differentially promote the specific PMIs by combining nanoelectrospray ionization mass spectrometry and spectroscopic techniques (fluorescence measurement and circular dichroism). Furthermore, to explore the influence of salts in competitive binding between metalloproteins and various metals, we designed a series of competitive experiments and applied to a well-defined model system, the competitive binding of zinc (II) and arsenic (III) to holo-promyelocytic leukemia protein (PML). These experiments not only provided new insights at the molecular scale as complementary to previous NMR and spectroscopic results, but also deduced the relative binding ability between zinc finger proteins and metals at the molecular scale, which avoids the mass spectrometric titration-based determination of binding constants that is frequently affected and often degraded by variable solution conditions including salt contents.

Keywords: Protein–metal interactions, Hofmeister salt, Zinc finger protein, Promyelocytic leukemia protein, Mass spectrometry

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Introduction

Protein–metal interactions (PMIs) have long been acknowledged to be inextricably critical for many biological regulations and disease developments [1–3]. For example, almost half of all enzymes were found to be associated with at least one particular metal to function properly [2]. To understand why each metal–protein partnership arose and how it is maintained has become increasingly important [4].

Hofmeister salts, including ammonium acetate (NH_4OAc), have been increasingly investigated regarding the influence of ions on the physical behavior of a wide variety of aqueous processes, such as the "salting in" and "salting out" of proteins [5]. Actually, the stabilizing effect of Hofmeister salts for multiprotein complex has been extensively investigated [5–10]. However, the role of Hofmeister salts in promoting specific PMIs has not been systematically investigated so far, although the solution conditions were reported with significant influences on the PMIs [11].

Typical tools for PMI studies are spectroscopic measurements, including fluorescence (FL), circular dichroism (CD) [11, 12], and NMR [13–15] to reveal solution PMI structures. Though great achievements have been made by these pioneering techniques, it is still challenging to reveal specific

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PMIs in certain aspects such as stoichiometry and discrimination between specific and nonspecific PMIs. In another word, those spectroscopic techniques generally report the overall binding events but are often unable to reveal the diversity between specific and nonspecific PMIs. Interestingly, as one of the powerful and complementary techniques for analyzing protein interactions, nanoelectrospray ionization mass spectrometry (nESI-MS) has been developed and combined with other biophysical tools to provide structural and molecular insights into the stoichiometry and concomitantly enables the direct readout of specific and nonspecific PMIs [16–19]. Notably, pioneering work in competitive PMIs, specifically zinc finger proteins (ZFPs) binding with various metals, have been reported by using nESI-MS [20–25].

Herein, the role of a series of Hofmeister salts in promoting the specific PMIs was systematically explored and first illustrated with two typical ZFPs, Sp1-2 and NCp7. Sp1-2, the second zinc finger domain of specificity protein 1 (Sp1), contains a C2H2-type zinc finger domain, whereas NCp7 contains two C3H-type zinc finger domains [26, 27]. Recently, we found that reactions of ZFPs with metals are considerably modulated by solution environments such as the small biomolecules and the acidity [11]. In this study, we further investigated how solution conditions, specifically the salt content of solution, affect these specific PMIs by using nESI-MS and spectroscopic techniques (FL and CD). For the first time, we also found that only with the presence of concentrated salts could one observe the phenomenon that As^{3+} displaces Zn^{2+} from the zinc-binding domain of PML protein.

Experimental

Chemicals

Two zinc finger proteins (ZFPs) were used: the NCp7 protein (NCp7, residues 12–55) and the second zinc finger domain of Sp1 protein (Sp1-2, residues 565–595). β-Mercaptoethanol, Tris, kanamycin, and IPTG (isopropyl β-D-1-thiogalactopyranoside) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Other chemicals (such as NH_4OAc, NaCl, and $ZnSO_4{\cdot}7H_2O$) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Acetonitrile (ACN) was purchased from Honeywell Burdick & Jackson Inc., (Muskegon, MI, USA). No further purifications were performed for all reagents. All solvents used in this study were of HPLC grade. Purified water (conductivity of 18.2 MΩ.cm) was obtained from Milli-Q Reference System (Millipore Corp., Bedford, MA, USA). Hofmeister salts used in this study include NH_4OAc, $Mg(OAc)_2$, NaOAc, $Ca(OAc)_2$, KOAc, $(NH_4)_2HPO_4$, NH_4ClO_4, NH_4Cl, and $(NH_4)_2SO_4$.

ZFP Expression and Purification

A pST-GB1 expression vector that contains DNA fragment of ZFP was transformed into *E. coli* BL21 (DE3) cells. The cells were grown at 37 °C until reaching an OD_{600} to 0.8, and protein expression was induced by adding 0.4 mM IPTG at 25 °C for 10 h.. Then the cells were harvested by centrifugation and the pellets were resuspended in the binding buffer (20 mM Tris, 200 mM NaCl, 5 mM citrate, 10 μM $ZnCl_2$, 5 mM β-Me at pH 7.4) and disrupted by sonication for 10 min on ice. The insoluble debris was removed by centrifugation at 16,000 rpm for 30 min at 4 °C. The supernatant was loaded on the Ni-NTA column, which was pre-equilibrated with binding buffer. The fusion protein was eluted by the elution buffer (20 mM Tris, 200 mM NaCl, 5 mM citrate, 10 μM $ZnCl_2$, 250 mM imidazole, 5 mM β-mercaptoethanol at pH 7.4). The His_6-GB1 tag was removed by incubating with TEV protease overnight. Protein was further purified via gel filtration on a Superdex 75 16/60 column pre-equilibrated with binding buffer. Zn^{2+} was removed from protein by AKTA system equipped with a RPC 3 mL column, and the fractions were lyophilized to obtain the final protein.

Nanospray Mass Spectrometry

In all spray experiments, the spray emitters were pulled from borosilicate glass capillaries (1.2 mm o.d., 0.9 mm i.d.) by using a P-2000 laser-based micropipette puller (Sutter Instruments, Novato, CA, USA) with the size of ~10 μm. Experiments for identifying protein isotopic distributions were conducted with an Exactive Plus (Thermo Fisher Scientific, CA, USA) mass spectrometer, maximum ion injection time 10 ms with 1 micro-scan, MS inlet temperature 275 °C. Other experiments were carried out with a LTQ-Velos Pro (Thermo Fisher Scientific, CA, USA) mass spectrometer. MS operation conditions are as follows (unless stated otherwise): nanospray voltage dc 2 kV, maximum ion injection time 30 ms, and three micro-scans for each individual scan. Isolation width was set as 1.0. MS inlet temperature of 275 °C.

Fluorescence Measurement

The fluorescence measurements were performed on a RF-5301PC spectrofluorometer (Shimadzu) using a quartz cuvette with the path length of 10 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).

Circular Dichroism (CD) Characterization

CD measurements were performed on a Jasco J-810 CD spectrometer. The CD spectra were recorded from 280 to 190 nm. 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.

Results and Discussion

Effect of Salts on ZFP Binding States

Hofmeister salts have been widely used for protein-MS analysis [5–10]. As one of Hofmeister salts, NH_4OAc is a commonly used buffer during ESI-MS analysis of proteins because of its volatility and compatibility with MS detection [28–31]. It is also observed that NH_4OAc affects protein charge state distributions (CSDs). Surprisingly, in addition to CSD changes, from Figure 1, we also observed totally different binding ratios of NCp7 and Zn^{2+} with or without the presence of NH_4OAc. Without NH_4OAc, around 60% of NCp7 are in ZFP-Zn^{2+} (1:1) form, and meanwhile, only less than 20% are in its holo-form (ZFP-2Zn^{2+}), although the concentration of Zn^{2+} and ZFP solution is 2:1. This observation indicated that only ~20 % of the Zn^{2+} binds with ZFP, which is obviously inconsistent with the known structure of NCp7. Interestingly, with NH_4OAc (bottom in Figure 1), more than 90% of NCp7 are in holo-form (ZFP-2Zn^{2+}), which means ~90 % of Zn^{2+} binds with ZFP in a reasonable stoichiometry. That result is in good accordance with previous study showing NCp7 tends to bind with two Zn^{2+} [27]. It should also be noted that the CSD of NCp7 between that with or without NH_4OAc has significantly changed, that is, the most abundant charge state dramatically shifts from 9+ to 5+ and the range of charge window becomes sharply narrow. In addition to NCp7-Zn^{2+} binding system, NH_4OAc also works for other systems like Sp1-2 and Cu^{+} binding system (Supplementary Figure S1). In a word, the ZFPs with relatively high content salts showed more biologically relative binding states than that in pure water.

To understand how the concentration of NH_4OAc affects specific PMIs, a series of nESI-MS and FL experiments were performed using ZFP-Zn^{2+} binding system (Figure 2). Taking NCp7 as a model, we tested various concentrations of NH_4OAc from 0, 0.01, 0.05 to 10 mM. All forms of the NCp7 showed regular changing trends in the salt-titration experiments (Figure 2a). Both the apo-ZFP and ZFP-Zn^{2+} (1:1) forms

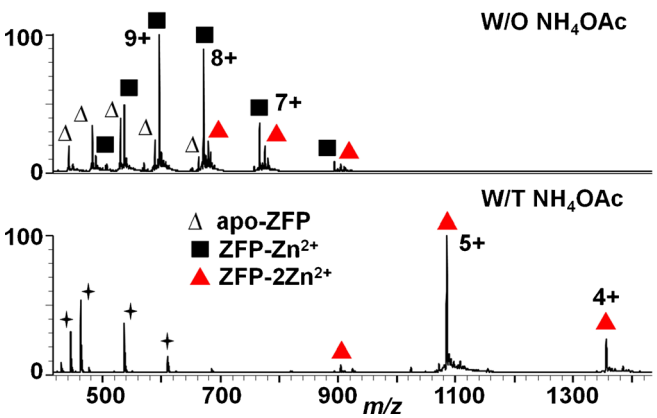

Figure 1. Effect of NH_4OAc on the binding ratios of a zinc finger protein (ZFP, with two Zn^{2+}-binding domains, NCp7) revealed by native nESI-MS. ZFP, 7.0 μM. Zn^{2+}, 14.0 μM. Background peaks were marked with plus signs. NH_4OAc, 10 mM. W/O, without. W/T, with

decreased similarly with a slightly different response factor upon increasing NH_4OAc concentrations. Notably, from the holo-form (ZFP-2Zn^{2+}) curve, the specific binding ratio between ZFP and Zn^{2+} was tuned from less than 20% to more than 90%. In order to further understand the underlying principles, a series of corresponding FL experiments were performed. Similar titration phenomenon (Figure 2b) was observed under various NH_4OAc concentrations. It should be noted that while the overall trend in MS signal increase for the holo-form of the protein-Zn^{2+} complex is similar to that observed from fluorescence measurements, there is some notable difference in the rate of increase in signal with the concentration of NH_4OAc. In particular, the trend of signal increase in Figure 2b is much sharper than that in Figure 2a. This observation suggests that some of the protein–Zn^{2+} complex did not survive under the ESI-MS condition at low NH_4OAc concentrations. Additionally, the promoting effect of NH_4OAc for specific PMIs was also observed in other PMI systems (Supplementary Figure S3).

As part of the mechanistic investigation, we have made a series of measurements, including critical NH_4OAc concentration, acidity, change in secondary structure, and other salts. First, we tentatively measured the critical concentration of NH_4OAc for promoting specific PMIs of different proteins with different binding domain numbers. We compared the specific binding curves of NCp7 and Sp1-2 along with a control of non-Zn^{2+}-specific binding peptide (KCTCCA). To obtain the salt-dependent protein–metal coordination, ZFPs as well as Zn^{2+} with certain concentrations were exposed to various concentrations of NH_4OAc. Under the same protein concentration, three model proteins showed different behaviors (Supplementary Figure S4a). Nonspecific binding between KCTCCA and Zn^{2+} was kept at low binding level throughout concentration range of NH_4OAc. Yet, the specific binding of Sp1-2 and NCp7 showed regular and controllable increment with the gradual elevation of NH_4OAc concentration. We also found that the critical NH_4OAc concentration for promoting the specific binding to the maximum level (e.g., 90% binding ratios) for Sp1-2 and NCp7 is approximately 1 mM and 5 mM, respectively. In spite of the difficulty for the quantification of NH_4OAc needed for an extended PMI system, the promoting effect of NH_4OAc on PMIs is fundamentally demonstrated and might be applicable for other binding systems. A recent report [31] on the effect of the concentration of NH_4OAc on protein–ligand interactions partly supported our finding.

Second, we have checked the pH values throughout the NH_4OAc concentration range studied here, and also performed corresponding circular dichroism (CD) experiments to reveal the change in secondary structure associated with the variation of NH_4OAc concentration. Recently, we have reported that the acidity is vital for specific PMIs [11]. However, in this case, Supplementary Figure S4b showed that pH values remained constant for various NH_4OAc concentrations. Meanwhile, CD results (Figure 3a) showed that NCp7-Zn adopted more secondary structures in NH_4OAc-bufferd solutions than that without NH_4OAc, all

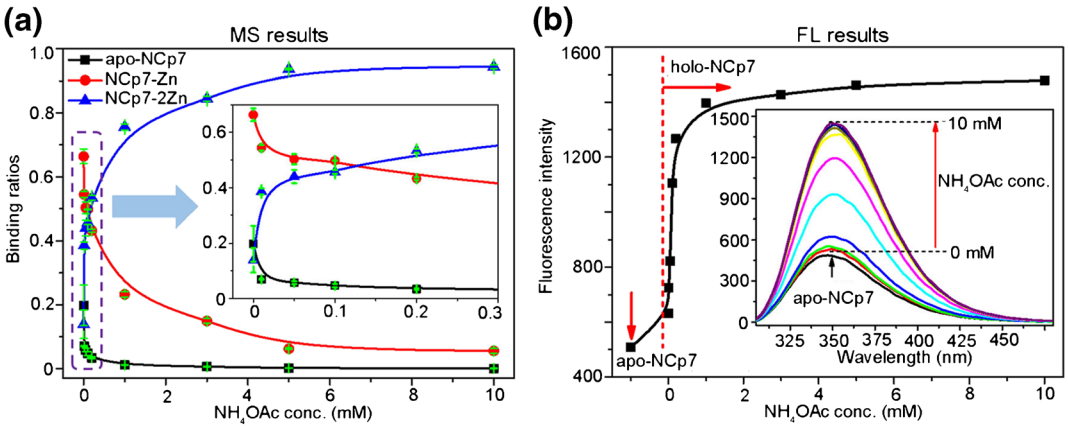

Figure 2. NH_4OAc-mediated PMIs probed by direct nESI-MS characterization **(a)**, and corresponding representative FL measurements **(b)**. NCp7, 7.0 μM. Zn^{2+}, 14.0 μM. Mass spectrum can be found in Supplementary Figure S2. For the direct comparison, the FL value of apo-NCp7 in pure water (without NH_4OAc) was placed in the negative X axis **(b)**

of which have more secondary structure than apo-NCp7. The drastic change of NCp7 structure upon the addition of Zn^{2+} indicates that Zn^{2+} plays an important role in maintaining the structure of NCp7, and NH_4OAc promotes the folding of metalloproteins. These conformational changes in NH_4OAc-mediated specific PMIs could even be more significant in Sp1-2-Zn system (Figure 3b).

Third, in addition to NH_4OAc, a series of Hofmeister salts were observed to differentially affect the binding states of PMIs. Results showed the promoting effect of Hofmeister salts for ZFP in our system was mostly in accordance with the initial order listed by Hofmeister but with some exceptions (Figure 4). Meanwhile, the average charge state values (ACS, in Figure 4) decreased concomitantly with the elevation of specific PMI binding efficiency, which further indicated that this salt-mediated PMIs might be related with the conformational change of proteins [32–36]. The promoting effects could also be evidenced with different binding efficiencies (Figure 4) for varied salt species at the same concentration. This result indicated that ionic strength is not the key factor for salt-mediated PMIs, which was also supported by corresponding FL measurements (Supplementary Figure S5). The binding efficiency of ZFP-Zn^{2+} mediated by Hofmeister salts seems to be more susceptible to anions (Figure 4b) than cations (Figure 4a). This observation could be further validated by recombination of anion and cation (Supplementary Figure S6). From the trend curve of ACS change for various cations, differential promoting effect was observed between divalent cations and monovalent cations. While we have observed less ZFP-Zn binding ratios (resulting from less compact structures) with the presence of Ca^{2+} than that of Na^+, the ACS value of protein with Ca^{2+} is relatively lower (reflective of more compact structures) than Na^+. This is a somewhat typical exception, even though we have a general trend that is similar with previous report. That is, multiply charged cations (e.g., Mg^{2+} and Ca^{2+} here) showed higher ability to preserve protein structures than singly charged cations (e.g., K^+ here) [8]. Combining the findings of secondary structural change in FL/CD measurements (Figure 2b and Figure 3) and CSD variations in MS results (Figure 4, Supplementary Figures S1–3 and Supplementary Figure S6), we can conclude that the effect of salts in promoting specific PMIs, especially ZFPs and metals, is more likely a matter of salt-mediated conformational change.

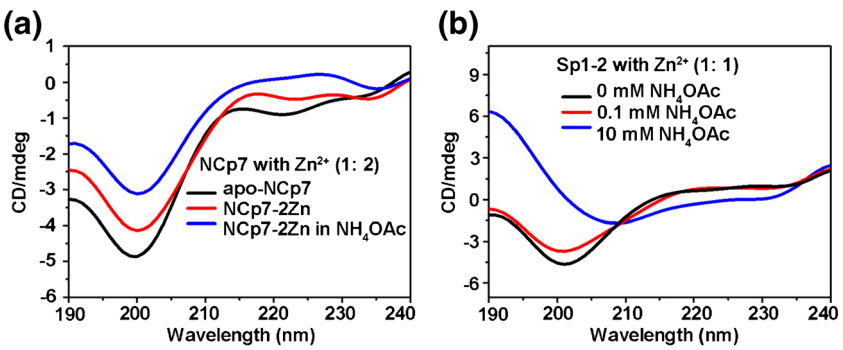

Figure 3. CD spectrum of NCp7 **(a)**, and Sp1-2 **(b)** recorded from protein solution same with MS measurements. NH_4OAc in **(a)**, 0.1 mM

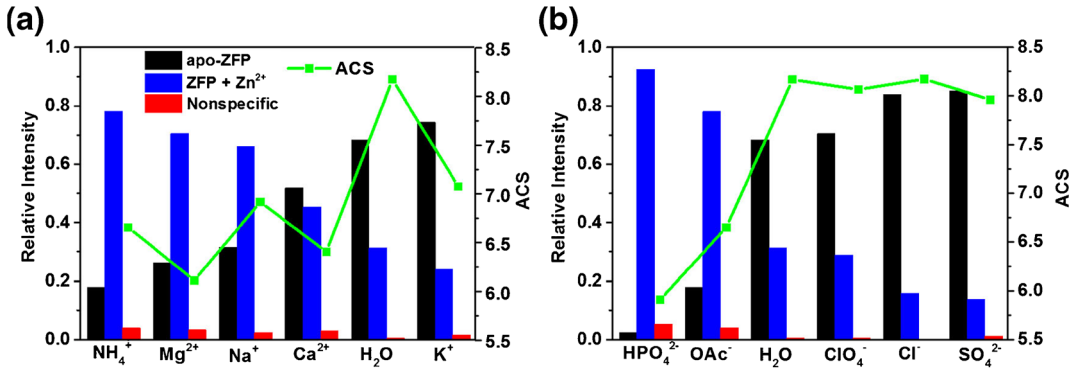

Figure 4. Comparison of the promoting effects of different Hofmeister salts on PMIs. Both **(a)** cations and **(b)** anions were tested, and the starting salt of all these experiments is NH_4OAc. Then NH_4^+ and OAc^- were successively replaced by those cations and anions listed above. For cations, OAc^- was used as the pair ion, and for anions, NH_4^+ was used as the pair ion. The average charge state (ACS) values were also indicated. ZFP, Sp1-2, 5.0 μM. Zn^{2+}, 5.0 μM. Individual salt concentration, 0.055 mM. Corresponding FL results can be found in Supplementary Figure S4

Effect of Salts on Competitive Binding of Zinc (II) and Arsenic (III) to Holo-Promyelocytic Leukemia Protein Lastly, to test the applicability of the promoting effect of salts, we then studied the comparative binding of promyelocytic leukemia protein (PML), a typical ZFP relating to human health, with zinc (Zn) and arsenic (As) under various salt conditions. As is known, As_2O_3 has attracted worldwide interests because it shows distinctive anticancer activity in patients with acute promyelocytic leukemia [13, 37]. Previous NMR and spectroscopic studies revealed that As_2O_3 directly binds to PML and then promotes the degradation of the oncogenic protein [13]. In this study, we found that the concentration of salt is fatal for the binding between PML and metals. According to a previous study, As_2O_3 functions in vivo regularly at pH ~6.0 [13]. Under this specific condition (pH 6.0), the specific PML binding with Zn (+2Zn, Figure 5a) could only be observed with the presence of NH_4OAc (e.g., 100 mM). This is further validated by the high-resolution MS identification of specific PML-Zn interactions. In the case of PML-As binding, similar protective effect of NH_4OAc is also observed (Figure 5b). More than 90% of

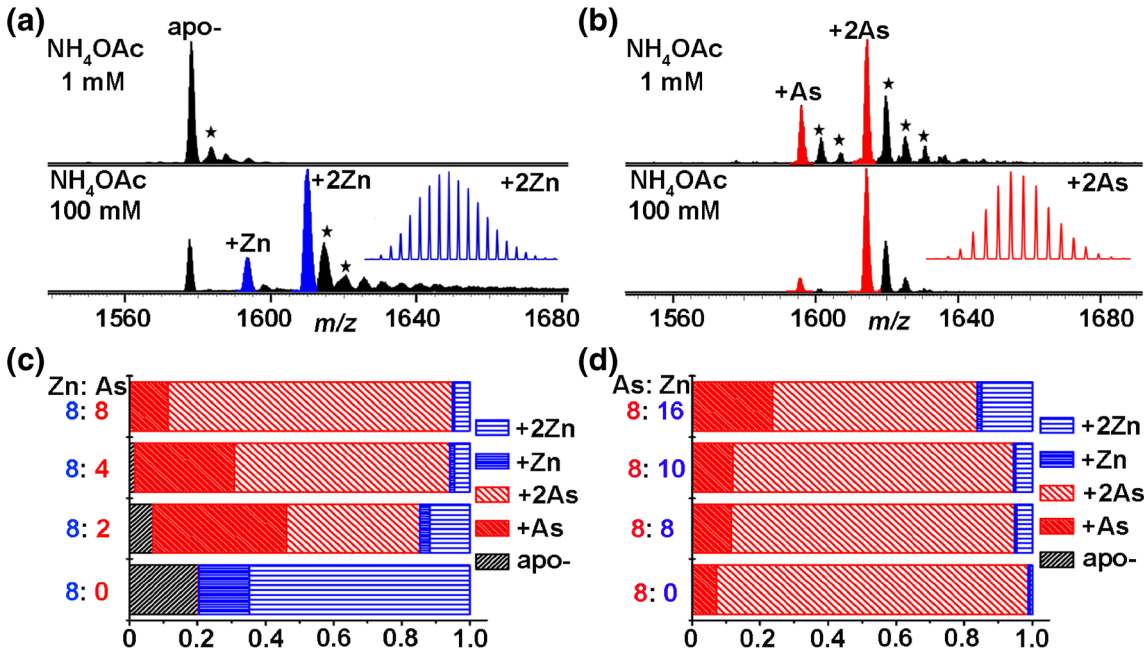

Figure 5. Competitive binding of Zn^{2+} and As^{3+} to PML with the presence of NH_4OAc (100 mM, pH 6.0). The salt effects on the binding between PML and Zn/As were presented in **(a)** and **(b)** individually. Insets, isotopic distributions for 4+ charge of holo-PML (+2Zn or +2As). Sodium adducts are marked with stars and are included in respective species when calculating binding ratios. PML, 6.0 μM. Initial concentrations of Zn^{2+} in **(a)** and **(c)**, 8.0 μM. Initial concentrations of As^{3+} in **(b)** and **(d)**, 8.0 μM. Shown in **(c)** and **(d)** are the competitive binding between PML and Zn or As. Note that "+As" and "+2As" denote the number of arsenic in the protein

PML are in holo- form binding with two As(III) with 100 mM NH_4OAc, but for 1 mM NH_4OAc, almost 40% of PML bind with only one As(III).

Therefore, in order to obtain the exact molecular mechanism involved in the therapy of promyelocytic leukemia with As_2O_3, we designed a series of competitive binding experiments (Figure 5c and d) between PML complex and Zn or As, using NH_4OAc (100 mM) solution at pH 6.0. From our nESI-MS results, we found that upon the addition of $NaAsO_2$ to the PML-Zn binding system (e.g., As, 2 μM, Figure 5c), the ratio of PML-Zn complex sharply decreased, and the ratio of the PML-As rose quickly to dominate all forms of PML with the presence of As (e.g., As, 2 μM, Figure 5c). We also noted that the PML-As complex is gradually changed from partial holo-form (+As, Figure 5c) to complete holo-form (+2As, Figure 5c). This unique changing trend indicated that As(III) competes with and gradually replaces the pre-coordinated Zn^{2+} in PML complex. Interestingly, this is in good accordance with previous NMR observations under similar conditions in which arsenic might replace the zinc in PML-Zn [13]. Conversely, the PML-As binding was not disrupted by Zn^{2+} even with the addition of a sufficient amount (from 8 μM to 16 μM, Figure 5d) of Zn^{2+}. These results indicated that the PML-As binding affinity surpasses that of PML-Zn under this condition, which might provide molecular insights into the reactions between As_2O_3 and protein PML as a complementary evidence to previous NMR data.

Conclusions

In summary, using nESI-MS and other spectroscopic techniques including FL and CD, we presented new insights into how solution conditions, specifically salt content, might affect specific PMIs. Our results may support the relationship between diverse protein functions and their binding states. That is, specific PMIs associated with the remarkable conformational change facilitate the activation of protein functions involved in certain cellular processes. This study not only provided new molecular insights into the underlying reaction mechanisms of As_2O_3 as complementary to previous NMR and spectroscopic results, but also deduced the relative binding ability between zinc finger proteins and metals at the molecular scale, which avoids the mass spectrometric titration-based determination of binding constants that is frequently affected and often degraded by variable solution conditions. The present finding of Hofmeister salt-mediated PMIs, typically the PML-Zn(II)-As(III) competition system, also indicated that the conventional functional investigation in vitro might not be simply applied for in vivo studies. However, because cellular environment cannot be duplicated precisely, more cautions might be raised about the functional correlation between in vivo and in vitro results.

Acknowledgements

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Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests.

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