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Arsenic transfer between metallothionein proteins at physiological pH

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ABSTRACT

As^{3+} bound to the two-domain, recombinant human metallothionein (isoform 1a) is stable at pH 7 and translocates via protein–protein interactions to other metallothionein proteins. The data show As^{3+} transfer from the two-domain β-α-hMT to binding sites in the isolated apo-β-hMT and apo-α-hMT. Under conditions of equilibrium, apo- and partially-metallated species coexist indicating that noncooperative demetallation of the As_6-βα-hMT occurs. As^{3+} transfer under conditions (pH 7) where the free As^{3+} ion is not stable, provides evidence that Cd^{2+} and Zn^{2+} transfer may also take place through protein–protein interactions and that partially metallated Cd–MT and Zn–MT would be stable.

1. Introduction

Metallothioneins bind Zn^{2+} and Cu^{+} *in vivo*, and a wide range of other metals *in vitro* [1–4]. The detailed and wide-ranging equilibrium metal status and physicochemical properties of the metallated protein have been reported for metallothioneins but few reports describe metal-exchange reactions. While reports on the titrations of Cd^{2+} into Zn–MTs, metal-exchange experiments following the mixing of Cd–MT with Zn–MT, and the use of isotopically labelled metals (Zn or Cd) have all indicated that some form of protein–protein interaction may be involved, no direct evidence to support this mechanism has been reported to date [5–9].

Recently, Sutherland, et al. [10], Rigby-Duncan, et al. [11], and Meloni, et al. [12], have reported that a supermetallated (that is metallation in excess of the traditional level for metallothioenin) structure exists for some isoforms of human metallothioneins. In the case of Cd^{2+} binding to the hMT-1a protein, spectroscopic and mass spectrometric characterization demonstrated that the extra metal above the normal complement of four Cd^{2+} in the α domain, three Cd^{2+} in the β domain, and seven Cd^{2+} in the two-domain βα-protein, may be bound in a binding site located in the crevice region of these MTs. These supermetallated species are proposed to be a metal-exchange intermediate during protein–protein metal-exchange reaction that takes place in the cytoplasm as well as *in vitro*. The metal in these structures could be considered to be frozen in place because of the relative binding affinities of the Cd^{2+} compared with the weaker bound Zn^{2+}. Supermetallation suggests that metal-exchange between proteins (either between two MTs or between MT and an apo-enzyme) would proceed via protein–protein interactions.

We now report that the As^{3+} in As_6-βα-hMT-1a transfers readily to the metal-free apo-α-hMT-1a or to the apo-β-hMT-1a allowing the As^{3+} to redistribute across all species in the solutions at physiological pH. The key to the analysis of these metal transfer experiments is that, under these reaction conditions, free As^{3+} will not bind to the apo-MTs. The neutral pH used here is unlike that used previously; all previous reports of As^{3+} binding were at low pH (approximately pH 3) to stabilize the As^{3+} in solution [13–16]. The data described here from detailed mass spectrometric studies are the first to show that metal redistribution between MT proteins must be taking place by protein–protein exchange. These results provide the first direct experimental evidence that protein–protein interactions may operate in the transfer of Zn^{2+} or Cd^{2+} between MT proteins *in vivo*.

2. Materials and methods

Detailed experimental procedures have previously been published (Ngu et al. [17]). In brief, recombinant human metallothionein-1a was expressed in BL21(DE3) *Escherichia coli* cells that were transformed using a pET29a plasmid, which contains an N-terminal S-tag (MKETAAAKFE RQHMDSPDLG TLVPRGS) as previously described [18,19]. The S-tag was removed using a Thrombin CleanCleave™ Kit (Sigma). The α-hMT-1a, β-hMT-1a and βα-hMT-1a proteins used in this study were based on the 43-residue,

Abbreviations: Mt, metallothionein; βα-hMT, βα human metallothionein-1a; β-hMT, β human metallothionein-1a; α-hMT, α human metallothionein-1a; ESI, electrospay ionization; MS, mass spectrometry.

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40-residue and 74-residue sequences, respectively. The sequence for α-hMT is GSMGKAAAAC CSCCPMSCAK CAQGCVCKGA SEKCS CCKKA AAA; for β-hMT is GSMGKAAAAC SCATGGSCTC TGSCKCK ECK CNSCKKAAAA, and for βα-hMT is GSMGKAAAAC SCATGGSCTC TGSCKCKECK CNSCKKAAAA CCSCCPMSCA KCAQGCVCKG ASEKC SCCKK AAAA. There are 11, 9, and 20 cysteine residues present in α-hMT, β-hMT and βα-hMT, respectively, and no disulfide bonds. The Cd_4-α-hMT protein concentrations were determined from the extinction coefficients of 56,000 L mol^{-1} cm^{-1} at 250 nm, the Cd_3-β−hMT protein concentrations were determined from the extinction coefficient of 36,000 L mol^{-1} cm^{-1} at 250 nm, and the Cd_7-βα-hMT protein concentration was determined from the extinction coefficient of 115,000 L mol^{-1} cm^{-1} at 250 nm. The protein concentrations for the ESI–MS experiments were between 8.1 μM for apo-βαMT, 11.0 and 8.8 μM for apo-α-MT and 15.0 μM for apo-β-MT. Oxidation is a significant problem with solutions of MT in these experiments and the apo-proteins were maintained in their reduced state by use of rigorous evacuation and argon saturation within a sealed environment.

Solutions of 5–10 mM As^{3+} were prepared by dissolving As_2O_3 (AnalaR) in concentrated HCl (Caledon) followed by dilution with ultra pure deionized water (Barnstead) and pH adjusted with NH_4OH to pH 2.5. The As^{3+} solutions were evacuated and argon saturated.

As_3-α-hMT was produced by metalation of apo-α-hMT at pH 2.7 with 15 equivalents of aqueous As^{3+} incubated for 1 h at room temperature. As_3-β-hMT and As6-βα-hMT were prepared by adjustment of the Cd-bound proteins to pH 2 with concentrated acetic acid followed by incubation with 9 and 20 equivalents, respectively, of As^{3+}, before purification through a Sephadex G25 column with 5 mM ammonium acetate eluent adjusted to pH 2.7 (Mallinckrodt Baker). The β-hMT was incubated at 40 °C for 1 h and the βα-hMT was incubated at room temperature for 15 min, respectively, with the As^{3+} solutions.

The pH adjustment of each MT sample was monitored by the addition of 10 μL of a 10 mM pH indicator dissolved in methanol as an internal pH indicator since the samples were completely sealed to remain free of oxygen. Previous test experiments show no interference with either the ESI–MS experiment or the mass spectral data recorded for the metallothioneins. Methyl orange was added to samples below pH 4, bromocresol green was added to samples between pH 4 and 5.5, and bromocresol purple was added to samples above pH 5.5. After the ESI–MS data was collected, each sample's pH was confirmed using a pH electrode instrument.

2.1. ESI–MS procedures

All data were collected using a Waters Micromass LCT ESI-TOF mass spectrometer in the positive ion mode. The mass spectrometer was operated using the parameters: 3000 V capillary, 60 V sample cone, 5.0 V extraction cone, 425 V RF Lens, acquisition scan time of 4 s, and interscan delay time of 0.1 s. The ESI–MS data were processed and deconvoluted using the MASSLYNX v4.0 software. The instrument was calibrated using a mixed NaI and CsI solution.

3. Results and discussion

Metallothioneins are composed of approximately 30% cysteine in their sequences with no cross-linked S–S bonds [1–4]. There are 9 Cys residues in the β-hMT fragment, 11 Cys in the α-hMT fragment and 20 Cys in the two-domain βα-hMT. The sequences of the single metal binding α and β domains, and the two-domain βα-hMT are given in the Experimental Section. While in all mammalian MTs reported to date, Zn^{2+} and Cd^{2+} bind with tetrahedral coordination forming M_3S_9-β-MT, M_4S_{11}-α-MT, and M_7-βα-MT species [19–21] (excepting the recently reported supermetallated species), As^{3+} is proposed to bind with pyramidal coordination to three cysteines [15,17,22] in hMT-1a. For the isolated β, and α fragments, and the complete, two-domain βα protein, equilibrium studies have previously reported formation of As_3-β-hMT, As_3-α-hMT, and As_6-β α-hMT [17,22]. However, unlike the stabilities of the Zn- and Cd-containing MTs, which are stable near pH 7, all previous reports of As^{3+} binding to metallothioneins have been at low pH and no data for As–MTs have been reported near pH 7. This means that there are currently no comparisons available of the relative binding affinities of the Zn- and Cd-MTs and the As–MTs.

We now report that As^{3+} bound to the two fragments and to the two-domain protein is stable from pH 1 to 9 (data not shown) including, significantly, the physiological region near pH 7 (Fig. 1). The charge states (m/z) for the three protein species measured near pH 7 are shown on the left-hand side of Fig. 1 (A, B, C) and the corresponding deconvoluted masses (Da) are shown on the right-hand side. Typically for MTs, only two or three charge states exhibit any significant intensity as is seen in these data with two dominant charge states (+4 and +3; in order from high m/z to low m/z). Further, the observed charge states for the two-domain native protein typically are not the sum of the fragment charge states, and this is seen in these data with the charge states of +6 and +5 being dominant, which suggests that in solution the As-containing protein may be more compact than the sum of the two isolated fragments. Rigby-Duncan et al. [23] have reported compact structures for Cd_7-βα-hMT in which the two metallated domains appear to coalesce, which would result in a smaller volume than for two isolated, linked domains that do not interact. Deconvolution of the charge state data provides the parent ions for the three species. The data in Fig. 1 recorded near pH 7 show complete formation of As_3-β-hMT, As_3-α-hMT and As_6-βα-hMT, with masses of 3968, 4295, and 7308 Da, respectively (all masses with an error ± 2 Da).

The As^{3+} protein transfer experiments were carried out by mixing the fully metallated As_6-βα-hMT at pH 7 with a stoichiometric amount (either 1:1 or 1:3) of the metal-free apo-β or apo-α fragment. After a minimum of 30 min mixing time at room temperature to allow equilibrium to be reached, the ESI–MS data were recorded in the same manner as the data shown in Fig. 1. Figs. 2 and 3 show the data obtained when the As_6-βα-hMT was equilibrated with the apo-β-hMT and apo-α-hMT, respectively, at pH 7.

Fig. 2 shows the data recorded for the metal-exchange reaction with the apo-β-hMT at two different mole equivalent ratios, 1:3 (top) and 1:1 (bottom). The charge state data on the left-hand side are more complicated than in Fig. 1 because several sets of charge states are superimposed. The difference in masses between the β-fragment and the βα protein results in the charge states being located in different mass ranges. We should note that the charges on the species recorded are different:+4 and +3 for the apo-and As-containing β-hMT and +6 and +5 for As-containing-βα-hMT. The +4 and +3 of the β-fragment (apo- to As_3-) span 900–1000 m/z and 1300–1350 m/z, respectively, while the βα-hMT +6 charge states are near 1200 m/z and the +5 are near 1450 m/z. These charge state regions can be seen clearly in Fig. 1 for the fully metallated species. The metal-free or apo-species will exhibit similar charge states at lower m/z values. Deconvolution clearly identifies the species present, Fig. 2 right-hand side. Starting with the 1βα:3α mole equivalent solution (top) we see that the apo-β-hMT coexists with As_1-β-hMT, and a very small amount of As_2-β-hMT, however, no As_3-β-hMT can be discerned in the deconvoluted spectrum or in the charge states. A concomitant reduction in As_6-βα-hMT concentration is seen as the As_5-βα-hMT and As_4-βα-hMT species appear in the mass spectrum. With the 1βα:1α mole equivalent ratio

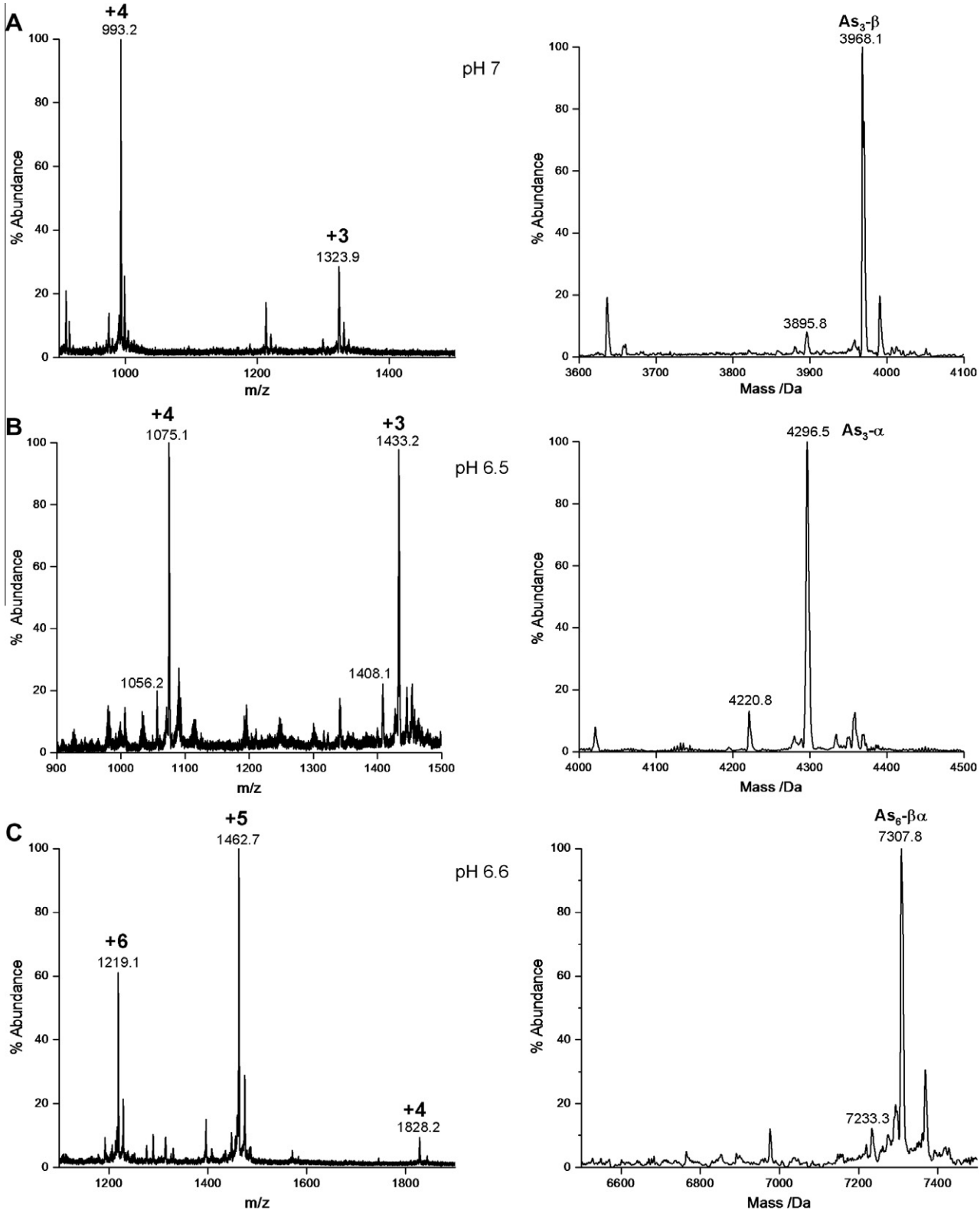

Fig. 1. Stability of As^{3+}-bound to human metallothionein at pH 6.5. ESI mass spectra of pH 6.5 solutions of (A) As_3-β-hMT (15 μM), (B) As_3-α-hMT (11 μM), and (C) As_6-βα-hMT (8.1 μM). Mass to charge ratios (m/z) are shown on the left and deconvoluted masses (Da) are shown on the right.

solution (bottom), we find relatively less As_5-βα-hMT indicating that less As^{3+} has transferred from the As_6-βα-hMT. The 20% abundance of the β-hMT is accounted for by the 33% less apo-β-hMT in this solution in proportion to the βα-hMT. Overall, only partial transfer takes place leaving many empty binding sites on the apo-β-hMT. Indeed, these data convincingly demonstrate that both metallation (of the β-fragment) and demetallation (of the As_6-βα-hMT) take place in a noncooperative manner at pH 7. We will return to discussion of possible reasons for the equilibrium results that were measured by the ESI-mass spectrometer later.

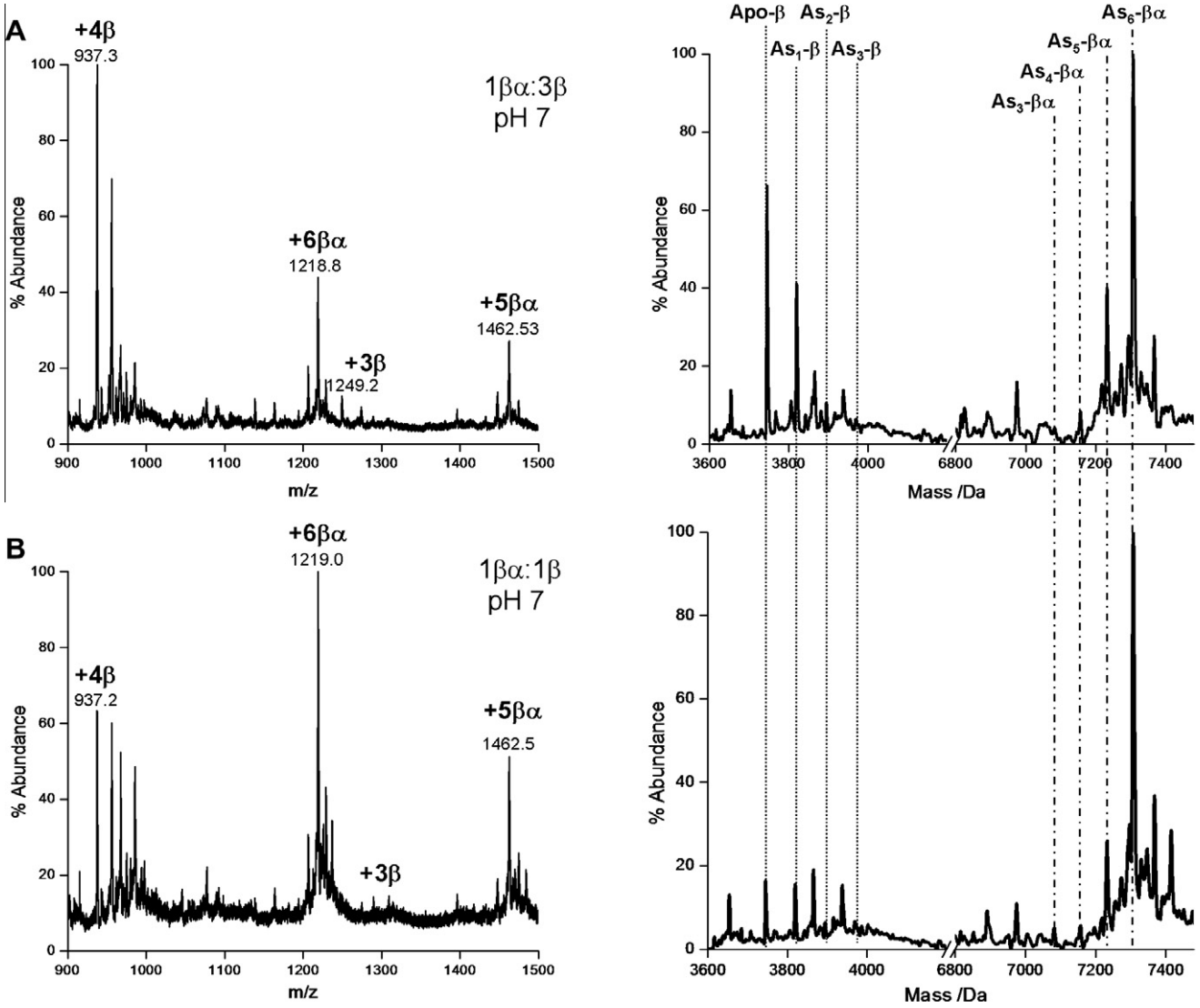

Fig. 2. As^{3+} transfer from As_6-βα-hMT to apo-β-hMT at pH 7. ESI mass spectra were measured 30 min after mixing As_6-βα-hMT (8.1 μM) with (A) 3 equivalents, and (B) 1 equivalent of apo-β-hMT (7.1 μM) at 20 °C and at pH 7. Mass to charge ratios (m/z) are shown on the left and deconvoluted masses (Da) are shown on the right.

Fig. 3 shows the data obtained when apo-α-hMT was added to the fully metallated As_6-βα-hMT at pH 7 with stoichiometric ratios of 1βα:3α (top) or 1βα:1α (bottom). Although the concentrations of the proteins in the solutions are similar to those in Fig. 2, the greater ionization efficiency of the α-fragment in the ESI–MS results in a stronger signal in proportion to the βα-hMT signal. The charge state data again span regions associated with the apo- and partially metallated α fragment and the fully and partially metallated βα-hMT. For the α-fragment the +5 charge state is between 1000 and 1100 m/z, and the +4 state between 1350 and 1400 m/z. The +6 charge state for the βα-hMT spans 1160–1250, while the +5 state lies between 1420 and 1500. Again, the charge states for the fully metallated forms can be found in Fig. 1. Deconvolution is essential in providing information about the As^{3+} transfer shown in the data presented in Fig. 3. Starting with the 1βα:3α solution, we find that the partially-metallated α-fragments (As_1, As_2, and As_3) dominate the mass spectrum recorded following equilibrium indicating that a significant number of As^{3+} ions have been transferred from the As_6-βα-hMT to the α-hMT. The mass spectrum for the βα-hMT species shows that the As_5- species is dominant but As_3, As_4 and As_6 all coexist. Finally, we consider the data for the 1βα:1α solution (Fig. 3, bottom right-hand side). The data systematically follow the predicted effect of reducing the acceptor protein molar ratio by 2/3 in that less As^{3+} is transferred. Although the metallation ratios for the α-fragment are almost the same, it is possible to discern that proportionally less apo-α-hMT remains compared with the As-containing species. This is the effect of the proportional increase in As^{3+} from the 1βα:1α molar ratio. The lack of available empty binding sites from the apo-α-hMT (3 in the 1βα:1α solution compared with 9 in the 1βα:3α solution) for the 6 As^{3+} in the As_6-βα-hMT means that at least twice the fraction of As_6-hMT is expected to remain compared with the amount of As_6-hMT predicted to remain following equilibrium of the 1βα:3α solution.

The data in Figs. 2 and 3 show that As^{3+} transfers in a proportional and predictable manner to the empty sites of the apo-fragments. These are the first data to be reported we believe that demonstrate metal-exchange under experimental conditions for which only protein to protein metal transfer is possible because the As^{3+} ions have been shown to be unable to metallate the apo-α-hMT, apo-β-hMT or the apo-βα-hMT above pH 5.

Before discussion of the significance of this as a property of the metallothioneins, we should briefly consider the known properties of As^{3+} reactions with metallothioneins to put the new results into

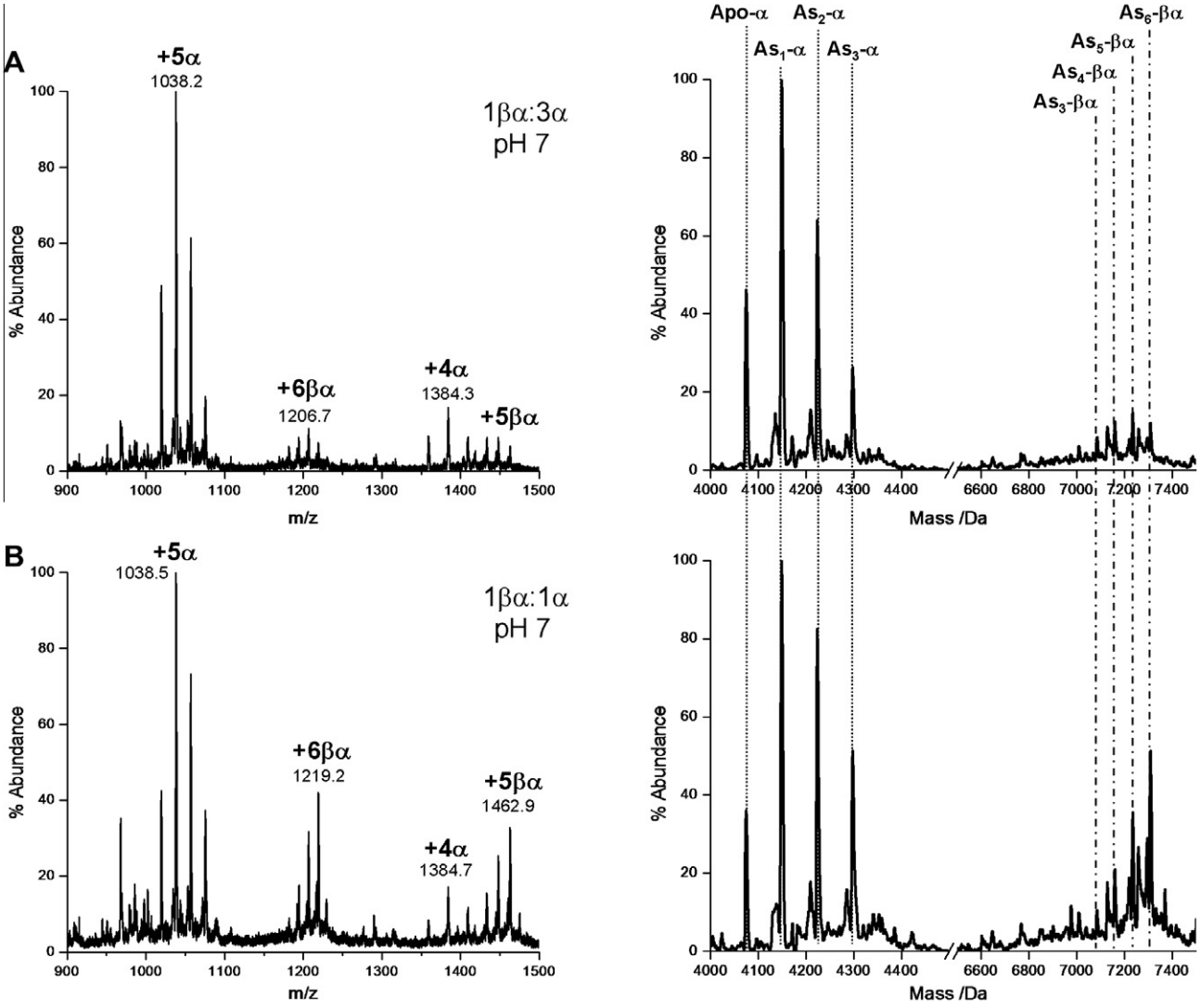

Fig. 3. As^{3+} transfer from As_6-βα-hMT to apo-α-hMT at pH 7. ESI mass spectra were measured 60 min after mixing As_6-ßα-hMT (8.1 μM) with (A) 3 equivalents, and (B) 1 equivalent of apo-α-hMT (7.3 μM) at 20 °C and at pH 7. Mass to charge ratios (m/z) are shown on the left and deconvoluted masses (Da) are shown on the right.

context. Previous reports showed that As^{3+} formed complexes with both mammalian and algal metallothioneins by binding to three thiolates to form a proposed pyramidal geometry [15,17,22,24,25]. Kinetic data were also reported because the reactions were slow enough for all component concentrations to be determined as a function of time and temperature by mass spectrometry [15,17,22,25]. Each of these experiments was carried out at pH values below 4 because of the instability of the As^{3+} salts used.

The data in Figs. 1–3 show that we have been able to stabilize the As–MT species at physiologically more reasonable pH values. This confirms that it is possible for As–MT to exist under physiological conditions in the event of exposure to As^{3+}. The important question to ask is what would happen following the normal turn-over of metallothionein, reported to be every 24 h? If the As^{3+} bound to MT was dissociated from the degraded MT then it is likely that it would not rebind. The data reported here show that in fact the As^{3+} can be translocated to other MT molecules, thus staying bound to the protein. The translocation of the As^{3+} suggests that the metal rearrangements reported in the past for both single metal or mixed metal metallothioneins may take place by protein–protein interactions.

We believe that there is a further significance in the demonstration of metal translocation between two isolated metallothioneins as reported here for As^{3+}. This arises from the question of the existence of partially metallated metallothioneins *in vivo* that Maret and coworkers have discussed over several years [26–28]. There are two aspects of this hypothesis we can relate these results to: Firstly, if partially metallated metallothionein can exist, then there is no need for a cooperative mechanism for metallation and demetallation. Domain specificity is not affected by the lack of requirement for cooperative metallation; however, domain specificity can be accounted for easily if protein–protein metal translocation is possible as is demonstrated in this study. Secondly, Maret and coworkers [26–28] have discussed the role of metallothioneins in the redox activity of the cell. A key requirement is the presence of partial metallation of Zn–MT, so that there are free cysteinyl thiols available for the redox chemistry. The kinetic data for As^{3+} binding to the two isolated fragments and the native human MT provided evidence to support the proposal that there may be metal binding sites with much lower binding affinities, because the previous data showed that the specific rate constants for binding As^{3+} to the metallothioneins decreased significantly as a function of the number of available empty sites. This allows the last metal to bind and dissociate readily, accommodating the proposal by Maret and coworkers that the unprotected cysteinyl thiols could be redox active. The data presented here show also that demetallation of the

As–hMT is not cooperative, meaning that one metal can transferred to a second MT protein molecule leaving an empty binding site, and free cysteinyl thiols as required in the proposal. Cooperative dissociation has been suggested to account for the lack of spectroscopic signature of partially metallated protein. The data presented here clearly demonstrate that the partially metallated MT is stable and viable at pH 7 when bound to As^{3+}.

4. Conclusions

In summary, As–MT species are stable at pH 7, and metal translocation takes place from the As_6-βα-hMT-1a directly to the isolated apo-β and apo-α fragments resulting in stable, partially-metallated species. The formation of the partially metallated fragments requires protein–protein metal transfer because the As^{3+} cannot metallate MT at pH 7.

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