



Arsenic transfer between metallothionein proteins at physiological pH

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ABSTRACT

As³⁺ 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 As³⁺ 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 As₆- β - α -hMT occurs. As³⁺ transfer under conditions (pH 7) where the free As³⁺ ion is not stable, provides evidence that Cd²⁺ and Zn²⁺ transfer may also take place through protein–protein interactions and that partially metallated Cd–MT and Zn–MT would be stable.

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1. Introduction

Metallothioneins bind Zn²⁺ 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 Cd²⁺ 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 metallothionein) structure exists for some isoforms of human metallothioneins. In the case of Cd²⁺ binding to the hMT-1a protein, spectroscopic and mass spectrometric characterization demonstrated that the extra metal above the normal complement of four Cd²⁺ in the α domain, three Cd²⁺ in the β domain, and seven Cd²⁺ in the two-domain β - α -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 Cd²⁺ compared with the weaker bound Zn²⁺. 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 As³⁺ in As₆- β - α -hMT-1a transfers readily to the metal-free apo- α -hMT-1a or to the apo- β -hMT-1a allowing the As³⁺ 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 As³⁺ will not bind to the apo-MTs. The neutral pH used here is unlike that used previously; all previous reports of As³⁺ binding were at low pH (approximately pH 3) to stabilize the As³⁺ 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 Zn²⁺ or Cd²⁺ 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 β - α -hMT-1a proteins used in this study were based on the 43-residue,

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

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40-residue and 74-residue sequences, respectively. The sequence for α -hMT is GSMGKAAAC CSCCPMSCAK CAQGCVCCKGA SEKCS CCKKA AAA; for β -hMT is GSMGKAAAC SCATGGSCCTC TGSCCKCK ECK CNSCKKAAAA, and for $\beta\alpha$ -hMT is GSMGKAAAC SCATGGSCCTC TGSCCKCKECK CNSCKKAAAA CCSCCPMSCA KCAQGCVCCKG ASEKC SCCCK AAAA. There are 11, 9, and 20 cysteine residues present in α -hMT, β -hMT and $\beta\alpha$ -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 $_7$ - $\beta\alpha$ -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 μ M for apo- $\beta\alpha$ MT, 11.0 and 8.8 μ M for apo- α -MT and 15.0 μ M 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 As³⁺ were prepared by dissolving As₂O₃ (AnalaR) in concentrated HCl (Caledon) followed by dilution with ultra pure deionized water (Barnstead) and pH adjusted with NH₄OH to pH 2.5. The As³⁺ solutions were evacuated and argon saturated.

As₃- α -hMT was produced by metalation of apo- α -hMT at pH 2.7 with 15 equivalents of aqueous As³⁺ incubated for 1 h at room temperature. As₃- β -hMT and As₆- $\beta\alpha$ -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 As³⁺, 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 $\beta\alpha$ -hMT was incubated at room temperature for 15 min, respectively, with the As³⁺ solutions.

The pH adjustment of each MT sample was monitored by the addition of 10 μ L 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 $\beta\alpha$ -hMT. The sequences of the single metal binding α and β domains, and the two-domain $\beta\alpha$ -hMT are given in the Experimental Section. While in all mammalian MTs reported to date, Zn²⁺ and Cd²⁺ bind with tetrahedral

coordination forming M₃S₉- β -MT, M₄S₁₁- α -MT, and M₇- $\beta\alpha$ -MT species [19–21] (excepting the recently reported supermetallated species), As³⁺ 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 $\beta\alpha$ protein, equilibrium studies have previously reported formation of As₃- β -hMT, As₃- α -hMT, and As₆- $\beta\alpha$ -hMT [17,22]. However, unlike the stabilities of the Zn- and Cd-containing MTs, which are stable near pH 7, all previous reports of As³⁺ 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 As³⁺ 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 Cd₇- $\beta\alpha$ -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 As₃- β -hMT, As₃- α -hMT and As₆- $\beta\alpha$ -hMT, with masses of 3968, 4295, and 7308 Da, respectively (all masses with an error $\pm$ 2 Da).

The As³⁺ protein transfer experiments were carried out by mixing the fully metallated As₆- $\beta\alpha$ -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 As₆- $\beta\alpha$ -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 $\beta\alpha$ 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- $\beta\alpha$ -hMT. The +4 and +3 of the β -fragment (apo- to As₃-) span 900–1000 *m/z* and 1300–1350 *m/z*, respectively, while the $\beta\alpha$ -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 1 $\beta\alpha$:3 α mole equivalent solution (top) we see that the apo- β -hMT coexists with As₁- β -hMT, and a very small amount of As₂- β -hMT, however, no As₃- β -hMT can be discerned in the deconvoluted spectrum or in the charge states. A concomitant reduction in As₆- $\beta\alpha$ -hMT concentration is seen as the As₅- $\beta\alpha$ -hMT and As₄- $\beta\alpha$ -hMT species appear in the mass spectrum. With the 1 $\beta\alpha$:1 α mole equivalent ratio

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