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Arsenic-metalation of triple-domain human metallothioneins: Support for the evolutionary advantage and interdomain metalation of multiple-metal-binding domains

Thanh T. Ngu [1], Janice A. Lee [1], Tyler B.J. Pinter, Martin J. Stillman *

Department of Chemistry, University of Western Ontario, London, ON, Canada N6A 5B7

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ABSTRACT

Metallothionein (MT) is a prominent metal-binding protein and in mammalian systems contains a two-domain βα motif, while in lower life forms MT often consists of only a single-domain structure. There are also unusual MTs from American oysters that consist of multiple domains (from one to four α domains). This report details the study of the As^{3+}-metalation to two different concatenated triple β and α domain MTs using time-resolved electrospray ionization mass spectrometry (ESI MS). Analysis of kinetic ESI MS data show that ααα human MT and βββ human MT bind As^{3+} in a noncooperative manner and involves up to 11 sequential bimolecular reactions. We report the complete progress of the reactions for the As^{3+}-metalation of both triple-domain MTs from zero and up to 9 (βββ) or 10 As^{3+} ions (ααα). The rate constants for the As^{3+}-metalation are reported for both the βββ and ααα human MT. At room temperature (298 K) and pH 3.5, the sequential individual rate constants, k_n (n = 1–9) for the As^{3+}-metalation of βββhMT starting at $k_{1βββ}$ are 40, 36, 37, 26, 27, 17, 12, 6, and 1 M^{-1} s^{-1}; while at room temperature (298 K) and pH 3.5, the sequential individual rate constants, k_n (n = 1–10) for the As^{3+}-metalation of αααhMT starting at $k_{1ααα}$ are 52, 45, 46, 42, 38, 36, 29, 25, 14, and 6 M^{-1} s^{-1}. The trend in the rate constant values reported for these two triple-domain MT proteins supports our previous proposal that the rate constant values are proportionally related to the total number of equivalent binding sites. The rate of binding for the 1st As^{3+} is the fastest we have determined for any MT to date. Additionally, we propose that the data show for the first time for any MT species, that interdomain metalation occurs in the binding of the 10th and 11th As^{3+} to αααhMT.

1. Introduction

The metal-binding properties of metallothionein were first characterized in 1957 by Margoshes and Vallee [1] when they studied cadmium in horse kidney. Since then, the ubiquitous nature of metallothionein (MT) has suggested an important role for this cysteine-rich metalloprotein in cellular life. Mammalian MT consists of two metal-binding domains: a M_4Cys_{11} α domain at the C-terminal and a M_3Cys_9 β domain at the N-terminal (where M represents a divalent Group 12 metal) [2–5]. On the other hand, many lower life forms consist of a single metal-binding domain MT [6–10]. While the typical multiple metal-binding domain MT contains a βα motif, there are examples of MTs that have more than two-domains. In the American oyster *Crassostrea virginica*, there are MT species that range from a single α domain up to four α domains [11]. Further, a previous report has suggested the use of multi-domain MTs (between 3 and 9 domains) to remediate metals [12]. Notably, there is a large conserved sequence in many single-domain and two-domain MTs, which points to a common evolutionary ancestor [5,13]. Moreover, the evolution of a two-domain MT in vastly diverse species such as sea urchins and mammals [13,14] over a single-domain suggests an important function for this structural motif [14,15].

Arsenic is a toxic metalloid found ubiquitously in the environment. For this reason, almost all organisms, from *Escherichia coli* to humans, have some form of a detoxification mechanism [16]. Arsenic has been reported to induce MT production [17] and As^{3+} binds to MT [18–23]. Previous reports show that As^{3+} binds three cysteines in cysteine-rich metalloproteins/peptides [24–28], this is consistent with our previous As^{3+} binding studies to MT that show that the number of As^{3+} ions bound is directly related to the number of groups of three cysteines available [18–20].

The two-domain metal-binding motif of MT was first reported by Armitage and coworkers, as well as by Wüthrich and coworkers in 1980 [15] and 1988 [29], respectively, using NMR techniques

* Corresponding author. Tel.: +1 519 661 3821; fax: +1 519 661 3022.
E-mail address: Martin.Stillman@uwo.ca (M.J. Stillman).
URL: http://www.stillmangroup.ca (M.J. Stillman).
[1] Both the authors contributed equally to the experimental data.

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and was later confirmed by Stout and coworkers in 1991 from an X-ray crystal structure [30]. The functional importance of the two-domain structure has been speculated on since the structure of MT was first published. Studies by Nielson and Winge showed that the β domain preferentially bound Cu^{+} while the α domain preferentially bound Cd^{2+} or Zn^{2+} [31], and that the metal-dependent folding of the protein to form the metal–thiolate clusters occurred independently of the other domain [32]. On the other hand, Maret and coworkers showed that the cumulative properties of the individual domains were insufficient to describe the two-domain MT structurally or functionally; simply put, the properties of the two-domain were not the sum of the properties of the isolated domains [33]. Attempts to prove interdomain interactions by NMR spectroscopy showed no interdomain NOEs but this may have been the result of flexibility in the linker [34]. We have recently proposed an evolutionary advantage and explanation for the two-domain MT structure observed in a diverse number of higher life forms. Based on the As^{3+}-metalation studies of the two-domain βα human metallothionein and the individual domain fragments [18,20], we have shown that the metal-free (apo) two-domain protein binds the first metals much faster than the isolated individual domains [18]. This increase in rate for the two-domain protein compared with the rate for the one-domain isolated fragments may be explained by the statistical increase in the number of equivalent binding sites available to the incoming metal, thus a two-domain protein will be able to bind metals faster and be a more efficient metal scavenger as long as all the sites are considered equivalent [18].

The increase in rate observed for a two-domain MT may be considered a form of indirect interdomain interaction because it was found in the previous analysis that all the binding sites for the two-domains were cumulatively available to each incoming metal starting with the first incoming metal [18]. Previous studies have suggested that MT transfers metals between the two-domains following initial binding, i.e. the kinetic product rearranges to the thermodynamic product [35–37]. For instance, during the Co^{2+} metalation of βαMT, it has been postulated that any metals that bind to the β domain are transferred to the α domain until the Co_4S_{11}-α domain is formed [36]. Interdomain interactions, such as the interdomain metal transfer, might be expected to occur through a short-lived intermediates where the metal is simultaneously coordinated by both domains.

We now report the complete analysis of the step-wise arsenic metalation of two three-domain proteins: the 27-cysteine recombinant βββ human metallothionein 1a, and the 33-cysteine recombinant ααα human metallothionein 1a, using time-resolved electrospray ionization mass spectrometry (ESI MS). We report that each protein binds the As^{3+} ions in a series of consecutive bimolecular reactions. Further, the ααα protein binds a 10th and 11th As^{3+} ion using the six additional cysteines in its sequence over the βββ protein. The time-resolved ESI MS data show the complete progress of the reaction from 1 As^{3+} bound to 9 As^{3+} bound for βββhMT and from 1 to 10 As^{3+} bound for αααhMT. The data allow for the determination of each of the individual rate constants and the construction of a simulation that shows the progress of the metalation for each of the As^{3+}ions. The trend in rate constants that we now report support a conceptual model in which metallothionein may not be fully metalated and the unoccupied sites may take part in redox chemistry [38,39].

2. Materials and methods

2.1. Chemicals

All chemicals used in this study were of the highest-grade purity from commercial sources. Metal salts used were $CdSO_4$ (Fisher Scientific) and arsenic trioxide (AnalaR). Hi Trap SP ion exchange columns and G-25 Sephadex (Amersham Biosciences) were used for protein purification.

2.2. Protein preparation

Experimental procedures have previously been published; please refer to Ngu et al. [20] for further details. The recombinant βββ human metallothionein (βββhMT) protein was based on the 100-residue sequence: MGKAAAACSC ATGGSCTCTG SCKCKECKCN SCKKAAAACS CATGGSCTCT GSCKCKECKC NSCKKAAAAC SCATGGSCTC TGSCKCKECK CNSCKKAAAA. There are 27 cysteine residues present and no disulfide bonds. The recombinant ααα human metallothionein (αααhMT) protein was based on the 109-residue sequence: MGKAAAACCS CCPMSCAKCA QGCVCKGASE KCSCCKKAAA ACCSCCPMSC AKCAQGCVCK GASEKCSCCK KAAA ACCSCC PMSCAKCAQG CVCKGASEKC SCCKKAAAA. There are 33 cysteine residues present and no disulfide bonds. In addition to the sequences from βββhMT and αααhMT, the expression systems include the amino acid residues of the stabilizing S-peptide tag (MKETAAAKFERQHMDSPDLGTLVPRGS) on the N-terminus of each protein [40,41]. Recombinant βββhMT-1a and αααhMT-1a were expressed and purified as previously reported [20,21]. The Cd_9-βββhMT and Cd_{12}-αααhMT protein concentrations were determined from the extinction coefficients of 130,000 L mol^{-1} cm^{-1} and 160,000 L mol^{-1} cm^{-1}, respectively, at 250 nm. The extinction coefficients were calculated by measuring the Cd concentration of the Cd-bound MT using atomic absorption spectroscopy and relating the MT concentration to the absorbance at 250 nm in the UV absorption spectrum. The protein concentrations for the time-resolved ESI MS experiments were, in typical experiments, 20.3–31.8, and 7.8 μM for apo-βββhMT and apo-αααhMT, respectively. Oxidation can be a significant problem with solutions of MT in these experiments and the apo-proteins were maintained in their

Table 1
Calculated mass (Da) and mass/charge (m/z) for As^{3+}-metalated βββhMT species based on the primary amino acid sequence of βββhMT.

	Mass (Da)	Charge State and predicted m/z for each species						
		+8	+9	+10	+11	+12	+13	+14
Apo-βββhMT	12545.8	1569.2	1395.0	1255.6	1141.5	1046.5	966.1	897.1
As_1-H_{24}-βββhMT	12617.7	1578.2	1403.0	1262.8	1148.1	1052.5	971.6	902.3
As_2-H_{21}-βββhMT	12689.6	1587.2	1411.0	1270.0	1154.6	1058.5	977.1	907.4
As_3-H_{18}-βββhMT	12761.5	1596.2	1418.9	1277.1	1161.1	1064.7	982.6	912.5
As_4-H_{15}-βββhMT	12833.4	1605.2	1426.9	1284.3	1167.7	1070.4	988.2	917.7
As_5-H_{12}-βββhMT	12905.3	1614.2	1434.9	1291.5	1174.2	1076.4	993.7	922.8
As_6-H_9-βββhMT	12977.2	1623.1	1442.9	1298.7	1180.7	1082.4	999.2	927.9
As_7-H_6-βββhMT	13049.1	1632.1	1450.9	1305.9	1187.3	1088.4	1004.8	933.1
As_8-H_3-βββhMT	13121.0	1641.1	1459.9	1313.1	1193.8	1094.4	1010.3	938.2
As_9-βββhMT	13192.9	1650.1	1466.9	1320.3	1200.4	1100.4	1015.8	943.4