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Cadmium binding mechanisms of isolated domains of human MT isoform 1a: Non-cooperative terminal sites and cooperative cluster sites

Gordon W. Irvine, Martin J. Stillman *

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

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ABSTRACT

A number of biological functions have been ascribed to mammalian metallothioneins (MTs) including zinc and copper homeostatic regulation, redox activity and detoxification of heavy metals like cadmium and mercury. It is unclear how these small, fluxional, cysteine rich proteins manage to play each of these vital roles. Using a combination of cadmium and pH titrations of the isolated domains of human MT isoform 1a monitored by electrospray ionization mass spectrometry and circular dichroism spectroscopy, we report the pH dependencies that control metal binding mechanisms of these domains. We report that the α-domain mechanism is driven by the cooperative formation of the Cd_4MT cluster at slightly acidic pH (≤6.9) switching binding mechanisms over a physiologically relevant pH range, whereas the β-domain metalation mechanism is dominated by terminal coordination of cadmium in a non-cooperative manner above pH 5.5. These results suggest that, in some acidic sub-cellular compartments, cadmium could be sequestered in the α-domain, leaving zinc or copper bound in the β-domain and available for donation to other metalloproteins. We propose that these results can be explained by the intrinsic nature of the two domains, the four-metal α-cluster being more resistant to proton attack due to its lower charge-to-metal ratio, compared with the three-metal β-domain.

1. Introduction

Mammalian metallothioneins (MTs) are a family of cysteine-rich proteins associated with essential metal homeostasis and heavy metal detoxification [1–5]. When fully metalated, the 20 cysteine protein forms two metal binding domains, a 9-cysteine N-terminal domain (β) and an 11-cysteine C-terminal domain (α) [6]. When fully metalated with seven divalent metals the β-domain forms an M_3Cys_9 cluster and the α-domain forms an M_4Cys_{11} cluster [7]. While structures of the fully metalated species are well-known [8], the partially-metalated and apo-structures are fluxional and hard to characterize [9]. Thus, the metalation mechanisms and the factors influencing the metalation pathways of apo-MT1a remain unclear [10]. These mechanisms are essential to our understanding of in vivo metalation processes that control the homeostatic role [1], toxic metal responses [11] and the resistance to metal-based chemotherapeutics associated with cellular metallothioneins [12–15].

The well-known description of the clustered domain structure for MT applies only to the metalated structures after cluster formation but is not accurate in describing the structure of apo- and partially-metalated MT species [9]. To investigate the properties of the individual domains of MT, the isolated domain fragments can be used and metalation studies carried out to determine the behaviour of the individual domains [16]. It should be noted that the isolated domains may have different properties than when joined by the linker sequence due to the possibility of entropic effects in cluster formation and protein folding and also due to inter-domain exchange of metals [17–19]. However, the underlying metal binding structures are the same for the full protein and the two isolated domain fragments, namely terminally bound metals at low metal concentrations leading to clustered domains at saturation. For this reason, examining the isolated domains of MT allows separation of the domain specific spectral properties which are blurred in the complete protein.

MT simultaneously functions as a zinc and copper chaperone, as a heavy metal chelator and as a redox active agent [20–22]. There have been suggestions that different isoforms bind specific metals and have their own unique functions, despite high sequence similarity [23]. Domain specificity of metal binding also remains a controversial topic in the field [24–30]. The MT1 and MT2 isoforms are the most widely expressed in human tissues [31–32] and likely most important isoforms for overall metal homeostasis. They have also been implicated in arsenic induced oxidative stress and cancers [33–34] and tumor resistance to chemotherapeutics [35]. Many chemotherapeutics are metal-based so determining the binding mechanisms operating at all levels of metalation of MT is important for an overall description of its many functions in vivo [12–13,15,36].

Electrospray ionization mass spectrometry (ESI-MS) has emerged as one of the most useful tools for answering questions about MT structure, dynamics and metalation mechanisms [37–40]. This is due to the ability

* Corresponding author.
E-mail address: martin.stillman@uwo.ca (M.J. Stillman).

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of ESI-MS to distinguish all species in solution with different masses (i.e. different numbers of metals bound) and to give semi-quantitative information on their relative abundances [24]. The ionization efficiency of different metal loadings of the same isoform is essentially the same for MTs and thus the relative abundances can be relied upon to accurately reflect solution conditions [41–42].

In this paper we have studied metalation of the isolated α- and β-domains of human MT1a to determine parameters that change the cadmium binding mechanism resulting in either terminally-coordinated or clustered metal binding sites. It is known that the mechanism of the metalation of the full protein is pH-dependent [24,43] but little is known about the individual domain responses to changes in pH. Biologically, MT1a is upregulated in response to cadmium intoxication, due to displacement of Zn from ZnMT and this newly synthesized apoMT can be metalated by cadmium [44–45]. We also reconcile conflicting reports about MT metalation mechanisms and show significant differences in binding preferences for the individual domains of human MT1a.

2. Methods

2.1. Protein preparation

Individual domain fragments of recombinant human metallothionein 1a (β: MGKAAAACSC ATGGSCTCTG SCKCKECKCN SCKKAAAA, α: MGKAAAAC CSCCPMSCAK CAQGCVCKGA SEKCSCCKKA AAA) were expressed separately with an S-tag in BL21 *Escherichia coli* cells which has been described in detail elsewhere [46]. In brief, cells containing the plasmid were grown on kanamycin containing media from a stock culture and grown for 16 h at 37 °C. The colonies were then inoculated into 4 × 1 L broth cultures enriched with 50 μL of 1 M cadmium and incubated in a shaker for approximately 4 h until an absorbance of 0.8 at 600 nm was reached. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to induce expression of MT and 30 min later 150 μL of 1 M cadmium sulfate solution was added to the broth. The cells were collected 3.5 h after induction, centrifuged and stored at −80 °C.

The recombinant cells were lysed using a cell disruptor (Constant Systems, UK) at 20,000 psi. Then, the cell lysate was centrifuged for 1 h to remove cellular debris. The supernatant was filtered and loaded on to a GE healthcare SP ion exchange column with a total volume of 10 mL. The columns were washed with 10 mM Tris (tris-hydroxymethyl-aminomethane) buffer at pH 7.4 for approximately 2 h to remove loosely bound proteins and other organic compounds. The MT fragments were eluted using an increasing gradient of 1 M NaCl + 10 mM Tris buffer at pH 7.4. The eluted MT was concentrated down to <20 mL and the S-tag cleaved using a Thrombin Clean-Cleave kit as per the manufacturers' instructions (Sigma-Aldrich). The mixture was then diluted, desalted and placed on another SP ion exchange column. The S-tag was eluted at low salt concentrations with MT eluting at higher concentrations. The isolated MT fragments were concentrated to approximately 100 μM and stored at −20 °C. After the thrombin cut, the residues at the attachment of the S-tag to the fragments (glycine (gly; G) and serine (ser; S)) were retained by the fragments, so both fragment sequences used for these experiments begin at the N-terminus with GS, then continue as above, MGKAAAA... etc.

To prepare MT for the pH titration experiments, aliquots were first demetalated and desalted using centrifugal filter tubes with a 3 kDa membrane (Millipore) and a 10 mM pH 2.8 ammonium formate buffer. The low pH solutions contained 1 mM dithiothreitol (DTT) to prevent oxidation of the free thiols in MT. The pH was raised by buffer exchange with argon saturated, pH 7.0 10 mM ammonium formate solutions that did not contain DTT. The concentrations of the protein solutions were checked by remetalation of a small aliquot with cadmium using the metal-to-ligand charge transfer band at 250 nm ($\alpha\varepsilon_{250}$ = 45,000 M^{-1} cm^{-1} $\beta\varepsilon_{250}$ = 36,000 M^{-1} cm^{-1}). The solutions were also monitored for oxidation using UV–visible absorption spectroscopy at 280 nm. MT concentrations used were 40–90 μM to ensure good signal-to-noise ratios. In addition to demetalating MT in the presence of DTT, the solutions were vacuum degassed and bubbled with argon to displace any dissolved oxygen. This was done for the 10 mM Cd^{2+}, 0.5% NH_3 and 0.5% formic acid solutions as well to ensure no oxygen was introduced into the system during the titration.

2.2. ESI-MS pH titrations

Mass spectra were collected on a micrOTOF II electrospray-ionization time-of-flight mass spectrometer (Bruker Daltonics) in the positive ion mode. NaI was used as the mass calibrant. The scan conditions for the spectrometer were: end plate offset, −500 V; capillary, +4200 V; nebulizer, 2.0 bar; dry gas flow, 8.0 L min^{-1}; dry temperature, 30 °C; capillary exit, 180 V; skimmer 1, 22.0 V; hexapole 1, 22.5 V; hexapole RF, 600 Vpp; skimmer 2, 22 V; lens 1 transfer, 88 μs; lens 1 pre-pulse storage, 23 μs. The mass range was 500.0–3000.0 *m/z*. Spectra were assembled and deconvoluted using the Bruker Compass data analysis software package.

For the metalation experiment, Cd(II) acetate was added sequentially to the MT solution and a spectrum recorded for each step. The metalation caused a drop in pH due to the displacement of H^+ from the thiol groups so the pH was monitored and adjusted as needed throughout the titration. The pH was confirmed using a micro-pH probe (Accumet).

In addition to metal titrations, a pH titration was carried out at the mid-point of the metal titration to monitor the change in metal distribution as a function of pH. Solutions of oxygen-free 0.5% NH_4OH and formic acid were used to adjust the pH. ESI-MS spectra were recorded for each change in pH. In total, 4 replicate pH titrations were taken for each fragment. No change in the overall the M^{2+}/MT ratio was observed meaning no precipitation of $Cd(OH)_2$ occurred. The error associated with these ESI-MS measurements is estimated to be about ±10% and the error associated with the pH probe is ±0.1 pH unit.

2.3. Circular dichroism pH titrations

For the circular dichroism (CD) spectra 1.5 molar equivalents of cadmium acetate were added to apo-αMT and apo-βMT in a pH 7.0 10 mM ammonium formate solution. The CD spectra (Jasco J810, New Jersey) were measured over the range of 200–310 nm at various pH points. At low pH (<4.0) demetalation occurs and a lowering of signal intensity can be seen in the β-MT CD spectra. Below 220 nm, the CD spectra are skewed due to the absorbance of the ammonium formate buffer and are not shown. The significant cadmium-dependent CD spectral bands lie in the 240–280 nm region [10,47–48]. The overall CD envelope was monitored for a signal characteristic of a CdS MT cluster, with a cross-over point near 250 nm.

3. Results

3.1. The pH dependence of Cd(II) metalation of the αMT fragment

The metalation reaction was carried out over a wide pH range and the αMT speciation recorded via ESI-MS and CD spectroscopy. Of particular importance was detecting the presence or absence of the Cd_4αMT cluster in both sets of spectral data. Unlike, the situation for the full protein βαMT where there are 20 Cys, the formation of the Cd_3- and Cd_4-species in the α-domain fragment must involve bridging cysteinyl thiols due to stoichiometric limitations. The nature of the Cd_xMT species can be identified from the CD spectral envelope characteristics. The Cd_4MT cluster species exhibits a characteristic sigmoidal CD band envelope that arises from exciton splitting of pairs of Cd(II) ions in the cluster [48].

Fig. 1 shows representative deconvoluted ESI mass spectra of the cadmium metalation of the αMT fragment. The spectra are separated into three distinct binding modes, a cluster-dominated,