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Noncooperative cadmium(II) binding to human metallothionein 1a

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ABSTRACT

The two-domain (βα) mammalian metallothionein binds seven divalent metals, however, the binding mechanism is not well characterized and recent reports require the presence of the partially metallated protein. In this paper, step-wise metallation of the metal-free, two-domain βα-rhMT and the isolated β-rhMT using Cd(II) is shown to proceed in a noncooperative manner by analysis of electrospray ionization mass spectrometric data. Under limiting amounts of Cd(II), all intermediate metallation states up to the fully metallated Cd_3-β-rhMT and Cd_7-βα-rhMT were observed. Addition of excess Cd(II), resulted in formation of the supermetallated (metallation in excess of normal levels) Cd_4-β- and Cd_8-βα-metallothionein species. These data establish that noncooperative cadmium metallation is a property of each isolated domain and the complete two-domain protein. Our data now also establish that supermetallation is a property that may provide information about the mechanism of metal transfer to other proteins.

Metallothionein (MT) is a ubiquitous metalloprotein found in bacteria, mammals and invertebrates [1–3]. Characteristic features of mammalian MT include high cysteine content, lack of aromatic amino acids, and a short sequence of amino acids. Although the exact function of MT is unknown, it has been suggested MT is involved in zinc and copper homeostasis, toxic metal detoxification, and, in its partially metallated form, protection against oxidative stress [4–7]. Structural analysis of mammalian MT by NMR and X-ray crystallography have shown it to contain two independent metal binding domains: the C-terminal α-domain with 11 cysteine residues binds four divalent metals and the N-terminal β-domain with 9 cysteine residues binds three divalent metals [8–11]. The overall structure with the two independent domains, α and β, each encapsulating a metal thiolate core is shown in Fig. 1.

MT has long been associated with zinc homeostasis and recent reports suggest it is involved in the longevity of certain human groups [12]. Dynamic equilibria between apo-MT, oxidized MT, and holo-MT have been suggested to affect the stress response in organisms [13]. Mutations of human MT have been associated with hyperglycemia, ischemic cardiomyopathy in some atherosclerotic patients, as well as both diabetes type 2 and related cardiovascular complications [14–16]. MT impacts pathogenesis through both Zn buffering and antioxidant dysfunction, which in turn rely on the metallation status *in vivo*. It is clear that knowledge of the mechanism of metallation is needed to establish the metal status *in vivo*, which will aid in determining the exact involvement of MT in these disorders.

While the stoichiometric ratios of 3 (β) and 4 (α) for divalent metals are well known, there has been considerable debate regarding the mechanism of MT metallation. It has been frequently proposed that the mechanism occurs in a cooperative fashion, that is, the binding of one metal facilitates the binding of the next metal [17,18]. In this mechanism, one would expect only the metal free (apo-MT) and fully metallated (holo-MT) forms to be relevant. However, a noncooperative mechanism is possible in which metallation events occur independently of each other [19–21], in this alternate hypothesis it would be expected that apo-MT, partially metallated MT and completely metallated MT would coexist. Thus the mechanism of MT metallation determines the number of possible physiologically relevant structures that can be present in the cell. Noncooperative metallation of the α-domain [22] has been cited to explain the presence of three distinct zinc affinities in Zn_7-MT [23]. Demetallation of brain-specific MT-3 induced by S-nitrosylation has also demonstrated the persistence of two α-domain-Cd(II) atoms, even in the presence of excess nitric oxide [24]. Enhanced resistance to S-nitrosylation has been reported in MT-1a mutants (Asn27Thr) [14,15]. These results may be reasonably interpreted if a noncooperative mechanism is in effect for the α-domain, however, this has not been proven experimentally to date.

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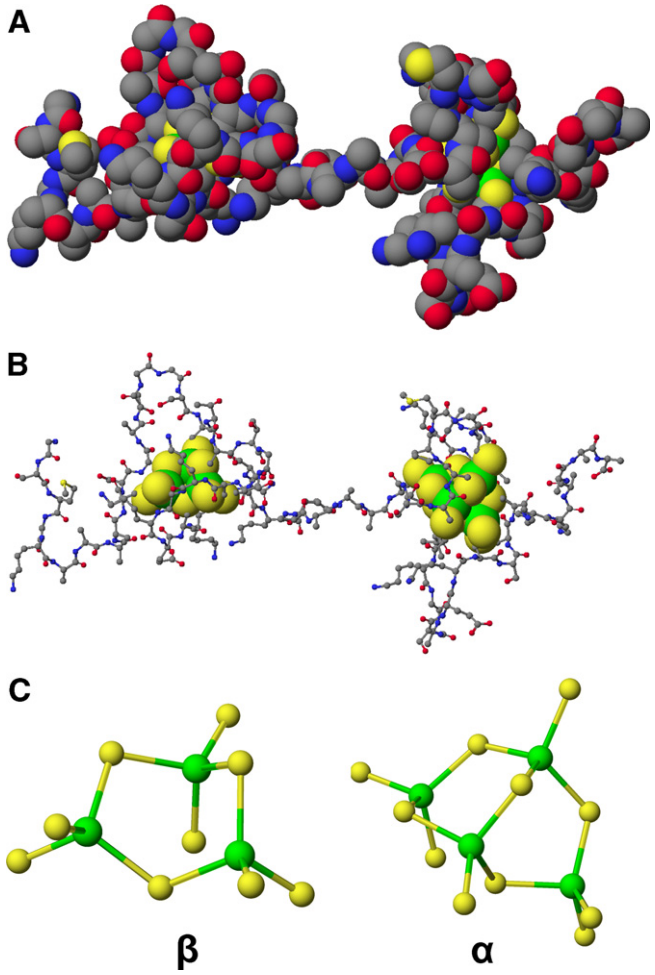

Fig. 1. Molecular mode structure of Cd_7-βα-rhMT with the N-terminal β-domain on the left side and the C-terminal α-domain on the right side: (A) space filling; (B) ball-and-stick with the domains in space filling form; (C) ball and stick models of Cd_3-β- (left) and Cd_4-α- (right) rhMT cadmium-cysteinyl-thiolate clusters. Data of Chan et al. [26].

A recent report has provided convincing evidence for noncooperative binding of cadmium to the isolated α-domain of human MT-1a [22] but not the complete protein or the isolated β-domain. Determination of the mechanism of the β-domain and the complete protein is important, because in a recent paper by Palumaa et al. [25] the authors comment that while the brain-specific MT-3 exhibits noncooperative binding, β-MT domains from other species bind cooperatively. For this reason, we have studied the stepwise metallation of the isolated β-domain and the two-domain βα human protein so that the metallation properties of this human protein can be firmly established.

This study provides the necessary data establishing unambiguously the noncooperative nature of cadmium binding to both isolated domains and the combined βα-domains (βα-rhMT) of human MT-1a. The dominant metallation state (1–3 for β- and 1–7 for βα-) was directly dependent on the stoichiometric ratio of Cd(II) added. Titration of the apo-MT (β or βα) with excess $CdSO_4$ also yielded supermetallated (metallated in excess of the normal maximum levels) species that have been previously proposed from optical studies.

Materials and methods

Chemicals. $CdSO_4$ (J.T. Baker Chemical Company). All chemicals used in this study were of the highest-grade purity from commercial sources. All solutions were made with >14 MΩ cm^{-1} deionized water (Barnstead Nanopure Infinity). Protein purification steps were performed on Hi Trap SP ion exchange columns, fine G-25 Sephadex (Amersham Biosciences) and a stirred ultrafiltration cell (Amicon Bioseparations/Millipore) with a YM-3 membrane (3000 MWCO).

Protein preparation. The expression and purification methods have been previously reported [26]. The β-rhMT and βα-rhMT proteins used in this study were based on the 38-residue and 72-residue sequences, respectively: β-rhMT MGKAAAACSC ATGGSCTCTG SCKCKECKCN SCKKAAAA, βα-rhMT MGKAAAACSC ATGGSCTCTG SCKCKECKCN SCKKAAAACC SCCPMSCAKC AQGCVCKGAS EKCSCCK KAA AA. There are 9 and 20 cysteine residues present in β-rhMT and βα-rhMT, respectively, and no disulfide bonds. The expression system included, for stability purposes, an N-terminal S-tag (MKETAAAKFE RQHMDSPDLG TLVPRGS). Recombinant β-rhMT was expressed in BL21(DE3) *Escherichia coli* (*E. coli*), while βα-rhMT was expressed in ER2566 *E. coli*. Both cell lines were transformed using the pET29a plasmid. Removal of S-tag was performed using a Thrombin CleanCleave™ Kit (Sigma). To impede oxidation of the cysteine residues to disulfide bonds, all protein samples were argon saturated and rigorously evacuated. Concentrated formic acid was used to demetallate protein samples, followed by desalting on a G-25 (Sephadex) column.

ESI-MS procedures. All data were collected on an ESI-TOF mass spectrometer (Waters Micromass Inc.) in the positive ion mode. NaI was used as the calibrant. The scan conditions for the spectrometer were: capillary, +3000.0 V; sample cone, 15 V; extraction cone, 10 V; RF lens, 450 V; desolvation temperature, 20.0 °C; source temperature, 80.0 °C; cone gas flow, 52 L h^{-1}; and desolvation gas flow, 514 L h^{-1}. The *m/z* range was 500.0–2000.0; the scan mode was continuum, with a scan time of 2.4 s and an interscan delay of 0.10 s. Spectra were constructed and deconvoluted using the MASSLYNX v.4.0 software package.

Results

The mass spectra recorded during the titration of apo-β-rhMT and apo-βα-rhMT with Cd(II) are shown in Fig. 2. A graphical summary of the metal status of the β- and βα-proteins is shown in Fig. 3 in which the Cd(II) added to the solutions is compared with the number of Cd(II) ions bound. The data clearly show that stepwise addition of Cd(II) to the initially metal-free protein species results in the formation of partially saturated (β- <3 and βα- <7 Cd(II)) metallated species until saturation occurs with the fully metallated species, which form when stoichiometric amounts of Cd(II) are added. In Fig. 2, the observed charge states (A1–G1 for β- and A2–G2 for βα-) and corresponding deconvoluted (H1–N1 for β- and H2–N2 for βα-)) spectra are shown as a function of increasing concentrations of Cd(II). Cleavage of the S-tag leaves the residues GS attached to the N-terminal of the protein. Theoretical mass values for β-rhMT are as follows: apo-β-rhMT 3753.46 Da, Cd_1 3863.85 Da, Cd_2 3974.24 Da, Cd_3 4084.63 Da and Cd_4 4195.02 Da. However, the mass spectra show that several amino acids of βα-rhMT were truncated either during the cleavage, or in the mass spectrometer itself producing two different species: one in which the N-terminal G residue and C-terminal AA residues were truncated, and a second in which the N-terminal GSMGK as well as A from either the N-terminal or C-terminal were truncated. None of the highly conserved cysteine residues were truncated. By assuming both species of βα-rhMT are equivalently ionized, the first species is more prevalent. Theoretical mass values for the predominant βα-rhMT species are as follows: apo-βα-rhMT 6874.37 Da, Cd_1 6984.76 Da, Cd_2 7095.15 Da, Cd_3 7205.54 Da, Cd_4 7315.93 Da, Cd_5 7426.32 Da, Cd_6 7536.71 Da, Cd_7 7647.10 Da and Cd_8 7757.49 Da.

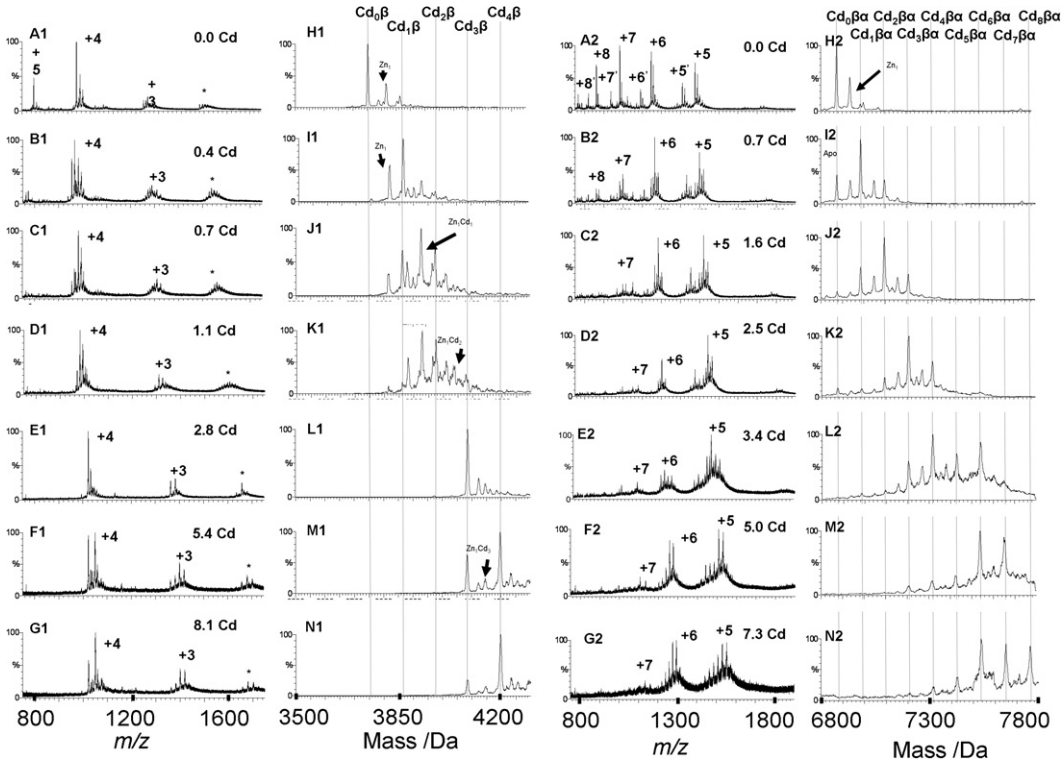

Fig. 2. ESI mass spectra recorded during the titration of apo-β-rhMT (Left), 18.0 μM at pH 9.4, and apo-βα-rhMT (Right), 18.4 μM at pH 8.4, with $CdSO_4$. Spectral changes were recorded as aliquots of Cd(II) (7.1 mM) were titrated into each solution at 22 °C. Spectra of β-rhMT (A1–G1) and the respective deconvoluted spectra (H1–N1) were recorded at Cd(II) molar equivalents of 0.0, 0.4, 0.7, 1.1, 2.8, 5.4, and 8.1. Spectra of βα-rhMT (A2–G2) and the respective deconvoluted spectra (H2–N2) were recorded at Cd(II) molar equivalents of 0.0, 0.7, 1.6, 2.5, 3.4, 5.0, and 7.3. The * refers to a +5 *m/z* charge state from a small fraction of a species corresponding to slight dimer formation of β-rhMT. Duplication in the sets of *m/z* for the βα protein result from the presence of two βα-rhMT species, see text.

In more detail, Fig. 2 shows the bound-metal speciation for β-rhMT as a function of the number of Cd ions added at pH 9.4. One Zn(II) is bound to about 25% of the total protein. The binding persists until the Zn(II) is displaced at the Cd_3 point, (L1) and (M1), when Cd_4-β-rhMT forms. Metal free apo-β-rhMT exhibits significant intensity in charge states corresponding to +5, +4, and +3 at the 0 Cd(II) added point (A1). The +5 charge state intensity is no longer apparent when Cd_1-β-rhMT forms. The loss of the +5 charge state clearly indicates some degree of folding has occurred as a result of the conformational changes required following binding of just the first Cd(II) or the Zn(II) to the apo-β-rhMT to form Cd_1-β-rhMT or Zn_1-β-rhMT (B1). After the disappearance of the +5 charge state, there is very little change in the charge state distribution with further additions of Cd(II).

Cd-loading of the two-domain protein is shown in Fig. 2 with the metal-speciation in βα-rhMT measured as a function of added Cd(II). Cd-species from 1 to 8 at pH 8.4 are observed with stoichiometric addition of Cd(II). A single Zn(II) persists in 25% of the protein as the Zn_1-βα-rhMT species in (H2) and (I2). The Zn(II) is only displaced when Cd_7-species forms. The important features in Fig. 2 are the step-wise increases in Cd(II)-loading as a function of added Cd(II) and the appearance of Cd_8-βα-rhMT with excess added Cd(II). Apo-βα-rhMT (A2) exhibits charge states corresponding to +8, +7, +6, and +5 (+5′ to +8′ correspond to the less prevalent truncated βα-rhMT species). Addition of 1 Cd(II), data in (B2) and (I2), causes a significant reduction in the intensity of the +8 and +7 charge states, again, indicating that a conformational change takes place reducing the exposed basic amino acids. Complete loss of the +8 charge state occurs with formation of Cd_2-βα-rhMT, which corresponds to the complete loss of apo-βα-rhMT species. As the titration proceeds, up to 7 equivalents of Cd(II) are added, the intensity of +6 relative to +5 increases. This reversal in trend in the charge state intensity indicates an increase in volume of the structure. The deconvoluted spectra in (M2) and (N2) show that this corresponds to the emergence of Cd_8-βα-rhMT. The appearance of the lower mass signals at the end of the titration, Cd_3/Cd_4/Cd_5-βα-rhMT, are due to the presence of metallated, truncated βα-rhMT.

We have assembled the relative abundances of each metallated protein species shown in Fig. 2 into Fig. 3. Because there is a small fraction of Zn(II) bound, we have assumed that Zn(II) and Cd(II) bind isomorphously and have counted the Zn(II) as one of the bound metals [27]. Of course, Cd(II) binds with a greater K_F and so displaces the Zn(II) when no other binding sites are available in the noncooperative model, as observed here. Fig. 3 clearly demonstrates that partial metallation occurs; hence the extent of metallation is directly dependent on the amount of metal in solution. At low equivalents of metal only partially metallated species are observed and addition of excess Cd(II) to β-rhMT and βα-rhMT formed Cd_4-β-rhMT and Cd_8-βα-rhMT, respectively.

Discussion

Mammalian metallothioneins bind metals in metal-cysteinyl-thiolate clusters with stoichiometries dependent on the associated metal: Cd_7-MT, Zn_7-MT, Ag_{12}-MT, Cu_{12}-MT, Hg_{18}-MT, and As_6-MT [9,11,28–30]. The details of the metal binding mechanisms are important because of implications in the possible metallation status of the protein *in vivo*, especially, whether partially metallated species exist under normal conditions. There are two overall mechanisms (i) a cooperative mechanism in which only the metal-free (apo-MT) and the metal-saturated (holo-MT) are significantly populated and (ii) a noncooperative mechanism, which would allow for the presence of partially metallated species that could potentially serve a biological function, such as

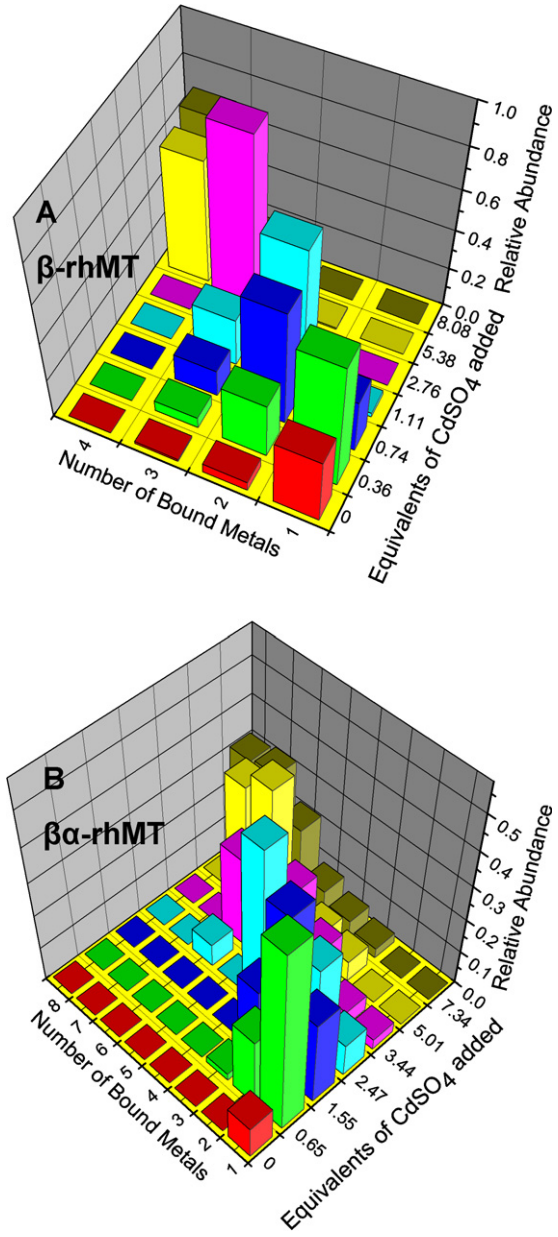

Fig. 3. A plot of metallation state as a function of Cd(II) added. This plot assumes that the binding of zinc and cadmium are equivalent and treats them interchangeably. (A) Refers to the metallation of β-rhMT, whereas (B) refers to the metallation of βα-rhMT. The second βα-rhMT truncation, Fig. 2, accounts for 13% of total signal, which must be spread over several species, no adjustment was made to metal speciation.

controlling the bioavailability of zinc [4,23]. Previous attempts to determine the mechanism of metallation have relied on techniques that do not produce direct evidence for the absence/presence of intermediate species [17,19–21] or may be more sensitive to specific species, for example, the CD spectrum of Cd_4-α-domain dominates the spectrum compared with the partially metallated $Cd_{1\text{-}3}$ [22]. ESI-MS data reported over several years, and the specific recent results for the metallation of the α-domain of rhMT 1a [22,31] show that ESI-MS data exhibit the discriminating power required to detect and quantify the presence of partially metallated metallothioneins.

The present study demonstrates that both isolated domains and the two-domain protein bind Cd(II) in a noncooperative manner. Following step-wise addition of 1 to 4 (β) or 1 to 7 (βα) equivalents of Cd(II), all metallated species are observed ranging from apo-MT to holo-MT depending on the amount of Cd(II) added, Figs. 2 and 3.

In addition to the metallation status, information from the charge state populations provides conformational knowledge. Higher charge states correspond to a more open, solvent exposed, conformation, while lower charge states correspond to more closed, solvent unexposed, conformation. The addition of the first metal ion to both β-rhMT and βα-rhMT result in loss of intensity in the highest charge states, which indicates a metal-induced folding event has occurred. The lack of change in the distribution of the intensity in the charge states with further Cd(II) addition up to 3 (β) or 7 (βα) indicates the first metal locks the structure in place and that, unexpectedly, further metallation does not require significant rearrangement of the polypeptide backbone to occur.

Supermetallated forms of metallothionein were also observed for both the β- and the βα-proteins studied, where we define supermetallation as the metallation into the binding site rather than an adduct at a ratio greater than normally measured. Addition of excess Cd(II), formed Cd_4-β-rhMT as well as Cd_8-βα-rhMT. Supermetallation (for β) has been previously postulated following analysis of CD and UV-visible absorption spectral data [32]. Capdevila et al. have postulated from their spectral data, with excess Cd(II), that Cd_4-β-rhMT subsequently unwound to form Cd_9-β-rhMT in a cooperative manner. It was proposed the unwinding required addition of 9 excess equivalents of Cd(II). The ESI-MS data described here demonstrates that the mechanism is noncooperative for metallation of apo-β-rhMT to Cd_3-β-rhMT, Fig. 3, and subsequently excess Cd(II) forms only Cd_4-β-rhMT. No evidence for a Cd_9-β-rhMT structure was observed, even with very large excess of Cd(II) added.

In summary, addition of Cd(II) to β-rhMT and βα-rhMT results in noncooperative binding of Cd(II) to both. Addition of excess Cd(II) produces Cd_4-β-rhMT and Cd_8-βα-rhMT. The model of metal binding in metallothionein is now clearly more complex than previously thought, with the existence of partially- as well as supermetallated species. With the elucidation of noncooperative metallation of mammalian metallothionein, determination of both the presence and function of intermediate species are now possible.

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