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Evidence for non-isostructural replacement of Zn^{2+} with Cd^{2+} in the β-domain of brain-specific metallothionein-3

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Abstract Metallothionein-3 (MT-3) is a brain-specific MT, which is downregulated in Alzheimer's disease. The N-terminal region of CdMT-3 is highly dynamic and has escaped structural characterization by nuclear magnetic resonance. We have used electrospray ionization mass spectrometry to probe conformational states of cadmium- and zinc-substituted metalloforms of MT-3 and can demonstrate that the N-terminal β-domain of MT-3 filled with Cd^{2+} has a more open conformation than that filled with Zn^{2+}. The results suggest that the larger Cd^{2+} ions cannot isostructurally replace zinc in the β-domain of MT-3 whereas in the case of MT-1 and MT-2 the replacement is isostructural. Specific metal binding properties of the β-domain of MT-3 may be essential for fulfilling the specific role of MT-3 in the brain. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Metallothionein-3; Growth inhibitory factor; Charge state distribution analysis; Electrospray ionization time of flight mass spectrometry; Conformation of clusters

1. Introduction

Metallothioneins (MTs) constitute a class of small cysteine-rich proteins adapted for the binding of essential (Zn^{2+} and Cu^{+}) and toxic (Cd^{2+}, Hg^{2+}) transition metals [1]. Mammalian MTs contain 60–68 amino acid residues with an absolutely conserved pattern of 20 Cys residues. MTs are polymorphous proteins and the known mammalian MT isoforms are grouped into four classes: MT-1, MT-2, MT-3 and MT-4, according to the total charge and length of the polypeptide chain [2]. MT-1 and MT-2 are expressed in almost all tissues [3], whereas expression of MT-3 and MT-4 is cell-specific, and is regulated by different mechanisms [4,5]. MT-3 (also known as growth inhibitory factor), is a brain-specific MT, which in contrast to common MTs inhibits the growth of cultured neurons and is downregulated in Alzheimer's disease [6,7]. Evidently, MT-3 fulfils biological roles distinct from those of MT-1/2, which should rely on a different structure and/or chemical properties (binding of metals, reactivity towards oxidants and other electrophiles, etc.) of MT-3.

The structure of MTs has been extensively studied by multinuclear nuclear magnetic resonance (NMR) [8] and by X-ray crystallography [9]. In these studies ^{113}Cd-NMR has proven to be indispensable and a powerful tool in the elucidation of the correct 3D structure of mammalian MT-1 and MT-2 [8,10]. In all resolved structures mammalian MTs bind seven divalent metals (zinc and/or cadmium) and the protein exposes two domains, both folded into compact metal–thiolate clusters. The N-terminal β-domain (amino acid residues 1–31) contains a cluster of three metals coordinated with nine thiolates and the C-terminal α-domain contains a cluster of four metals coordinated with 11 thiolates [10]. In the case of the common MTs, the overall fold of zinc- and cadmium-substituted metalloforms is practically identical [11] which confirms that cadmium replaces zinc isostructurally in the case of common MTs.

MT-3 consists of 68 amino acid residues and has two inserts – a single amino acid insert (Thr) in the N-terminal region and a hexapeptide glutamate-rich insert in the C-terminal region of the protein [6]. Although MT-3 has approximately 70% identity with other MT sequences, it has escaped full structural characterization so far [12–14]. The structural studies of CdMT-3 by NMR have resolved only the structure of the C-terminal half of MT-3, which is folded into a four-metal cluster similar to that of other MTs [12]. The N-terminal region of CdMT-3 exposes enhanced conformational dynamics and has structurally not been characterized so far. The dynamic phenomena observed in CdMT-3 are commonly explained by the conformational dynamics in the N-terminal Cd_3S_9 cluster of Cd_7MT-3 [12–14]. We have recently demonstrated by electrospray ionization mass spectrometry (ESI MS) that MT-3 binds metals non-cooperatively and exists in solution as a dynamic mixture of multiple metalloforms, which could be an additional reason behind the unusual behavior of CdMT-3 in NMR studies [15]. We have also noticed that CdMT-3 is more heterogeneous than ZnMT-3. Therefore, it is not excluded that the conformational state of CdMT-3 is different from that of ZnMT-3.

In the present work, we have used ESI MS for conformational analysis of Cd- and Zn-substituted metalloforms of MT-3 and can demonstrate that Cd-substituted metalloforms of MT-3 have more open conformations in solution than Zn-substituted metalloforms, which complicates application of

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Abbreviations: MT, metallothionein; NMR, nuclear magnetic resonance; DTT, 1,4-dithiothreitol; ESI TOF MS, electrospray ionization time of flight mass spectrometry

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CdMT-3 for structural studies, but gives additional evidence for specific metal binding properties of MT-3.

2. Materials and methods

2.1. Isolation of MT-1 and MT-3

Rabbit MT-1 was isolated from livers of cadmium-exposed rabbits and the apoMT-1A form was purified to homogeneity by reverse phase HPLC as described [15]. Recombinant human MT-3 was expressed in *Escherichia coli* and the apoMT-3 form was purified by reverse phase HPLC [15]. Lyophilized apoMT-1A and apoMT-3 were used for reconstitution experiments.

2.2. Reconstitution of MT metalloforms

MT-3 was reconstituted by addition of various concentrations of metals (Cd^{2+} acetate or Zn^{2+} acetate) to apoMT-3 (20 μM), in degassed 5 mM ammonium acetate buffer, pH 7.5, containing 25 μM 1,4-dithiothreitol (DTT). Samples were injected into the mass spectrometer using a syringe pump. MT-1A was reconstituted by addition of metal salts (Cd^{2+} acetate or Zn^{2+} acetate) to apoMT-1A (10 μM) in degassed 20 mM formic acid, pH 2.7, containing 25 μM DTT, and pH of the solution was raised to pH 8.5 with ammonia solution. Before MS analysis, the buffer was exchanged to 5 mM ammonium acetate, pH 7.5, using MicroSpin™ G-25 columns and the eluate was injected into the MS instrument using a syringe pump. MS spectra were recorded on an Ettan™ electrospray time-of-flight mass spectrometer (ESI TOF MS, Amersham Biosciences, Uppsala, Sweden).

3. Results and discussion

3.1. Reconstitution of MT-3 with Zn^{2+} and Cd^{2+} ions

MS spectra of MT-3 reconstituted with increasing stoichiometries of Zn^{2+} ions are presented in Fig. 1. At stoichiometries of added zinc up to 7, metalloforms of MT-3 expose mainly the charge state +5 and only at higher stoichiometries of bound metals (8–10), the proportion of charge state +6 increases in the MS spectra.

MS spectra of MT-3 reconstituted with increasing stoichiometries of Cd^{2+} ions are presented in Fig. 2. All CdMT-3 metalloforms display proportionally more ions in the charge state +6 than the corresponding ZnMT-3 metalloforms. How-

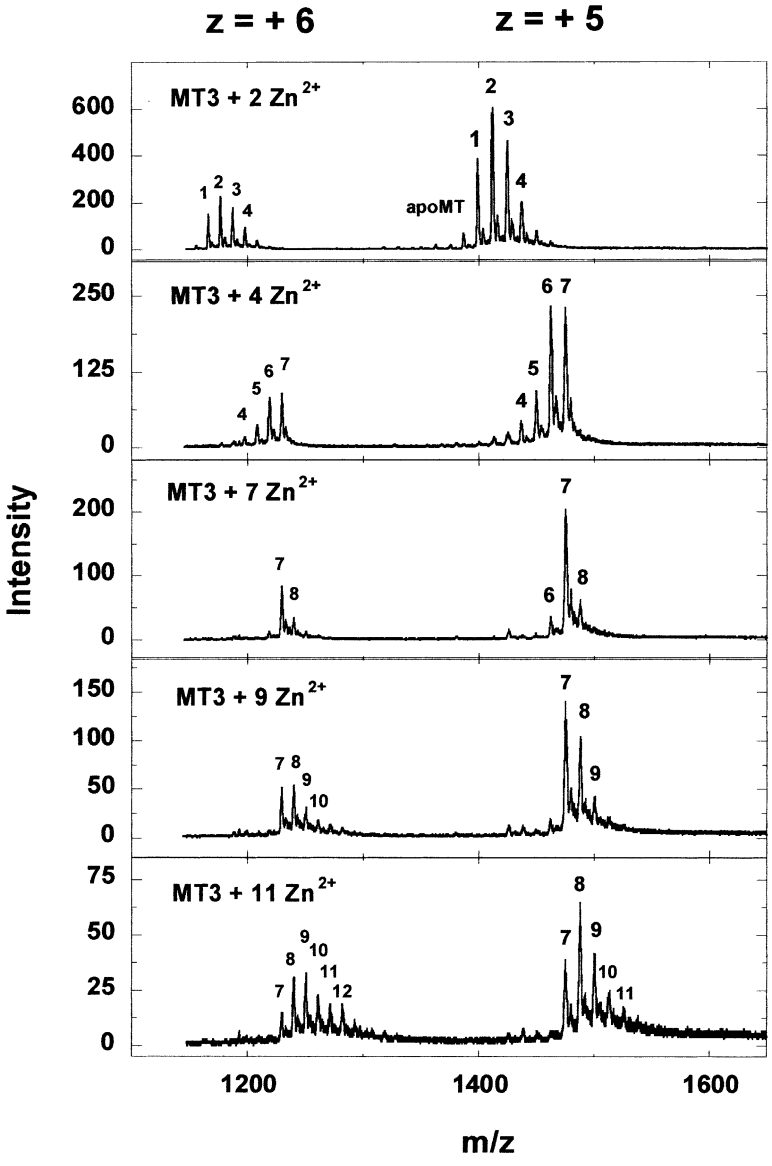

Fig. 1. ESI TOF spectra of MT-3 (10 μM) reconstituted with different concentrations of Zn^{2+} in 5 mM ammonium acetate, pH 7.5, containing 25 μM DTT, 25°C. Numbers on the peaks denote the metal stoichiometry of the complex.

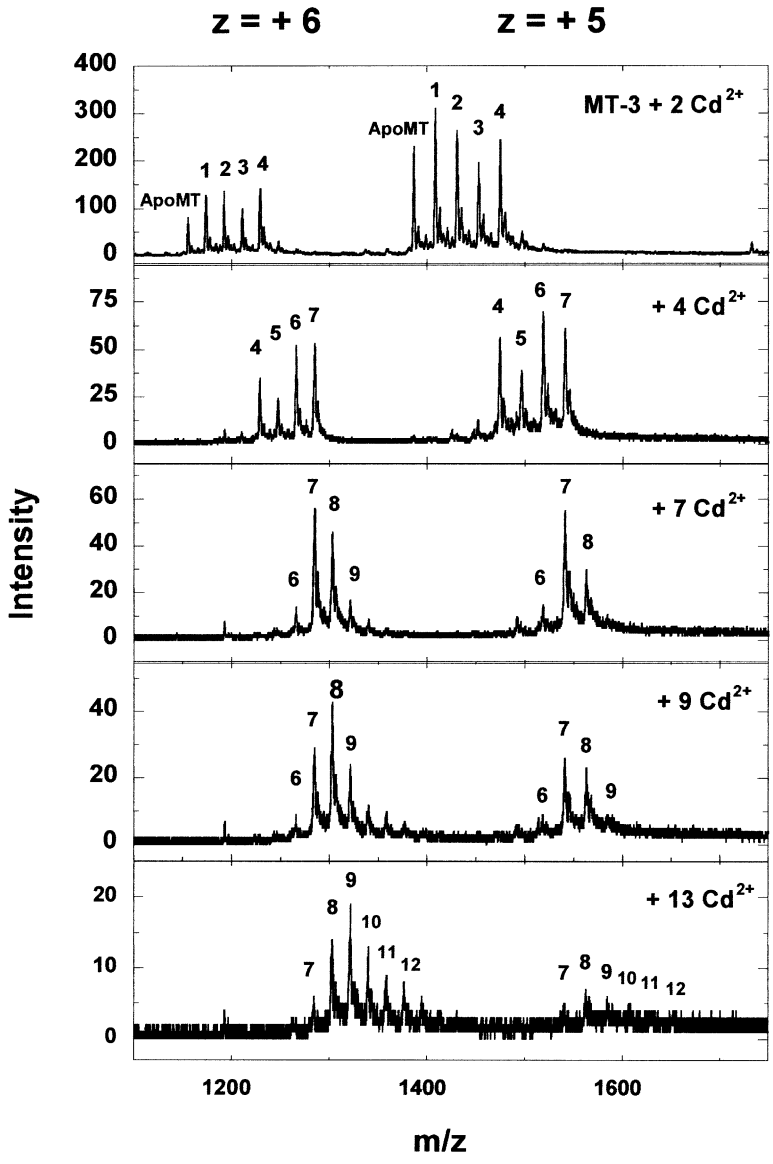

Fig. 2. ESI TOF spectra of MT-3 (10 μM) reconstituted with different concentrations of Cd^{2+} in 5 mM ammonium acetate, pH 7.5, containing 25 μM DTT, 25°C. Numbers on the peaks denote the metal stoichiometry of the complex.

ever, in the case of CdMT-3 there is a continuous increase of the population of +6 ions in the MS spectra in parallel with an increasing metal stoichiometry of metalloforms.

ESI MS spectra of Zn_7MT-1 and Cd_7MT-1 are presented in Fig. 3. Both metalloforms display in a similar manner a main fraction of +5 ions and minor fraction of +6 ions in the spectra.

3.2. *Application of charge state distribution analysis for probing protein conformations*

ESI MS has developed into a powerful technique, which could, besides mass determination, be applied also for probing conformation and dynamics of proteins under a variety of conditions. The latter approach is based on the assumption that in the process of electrospray ionization compact protein conformations produce lower charge state ions than more open conformations, which is most probably caused by enhanced electrostatic repulsion of charges [16]. A number of studies with well-studied model proteins have demonstrated that the assumption above is valid and charge state distribution analysis can be effectively used for analysis of protein conformations [17,18].

In the present work, we have used ESI MS for comparison of conformational states of Cd- and Zn-substituted metalloforms of MT-3. The results demonstrate that Zn- and CdMT-3 forms differ substantially in their charge state distribution in ESI MS spectra (Fig. 4). Generally, all CdMT-3 metalloforms display proportionally more ions in the higher charge state (+6) than in the lower charge state (+5), which is not the case for the corresponding ZnMT-3 forms. In the case of ZnMT-3 the charge state distribution is similar for all metalloforms with metal stoichiometry up to 7, and starts to increase in favor of the higher charge state (+6) at higher metal stoichiometries. This result indicates that the conformation of ZnMT-3 is compact up to metal stoichiometry 7, and opens up at elevated metal stoichiometries. Metalloforms of CdMT-3 with a low metal stoichiometry (1–4) expose a slightly higher proportion of charge state +6 than corresponding ZnMT-3

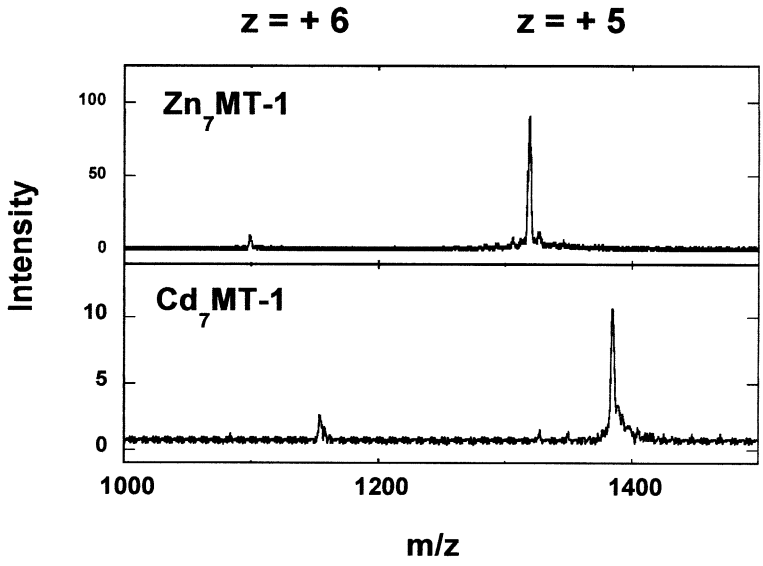

Fig. 3. ESI TOF spectra of MT-1 (20 μM) reconstituted with Zn^{2+} and Cd^{2+} in 5 mM ammonium acetate, pH 7.5, containing 25 μM DTT, 25°C. Numbers on the peaks denote the metal stoichiometry of the complex.

metalloforms, which demonstrates that the four-metal cluster of MT-3, which is filled first [15], is slightly more open than the four-metal cluster of ZnMT-3. However, metalloforms of CdMT-3 with metal stoichiometries 5, 6, and 7 expose a sufficiently higher proportion of ions in charge state +6 than corresponding ZnMT-3 forms, which demonstrates that MT-3 forms with Cd^{2+} in the β-domain have significantly more open conformations than Zn^{2+}-substituted forms. Charge state distribution of MT-3 with Cd^{2+} stoichiometries higher than 7 is even further shifted towards the charge state +6 (Fig. 4). The results demonstrate that the β-cluster of MT-3 cannot accommodate Cd^{2+} into an equally compact structure as is the case for Zn^{2+} ions, and provide evidence for a non-isostructural replacement of Zn^{2+} with Cd^{2+} ions in the β-cluster of MT-3. The situation is specific for MT-3, as Zn_7MT-1 and Cd_7MT-1 display the same distribution of

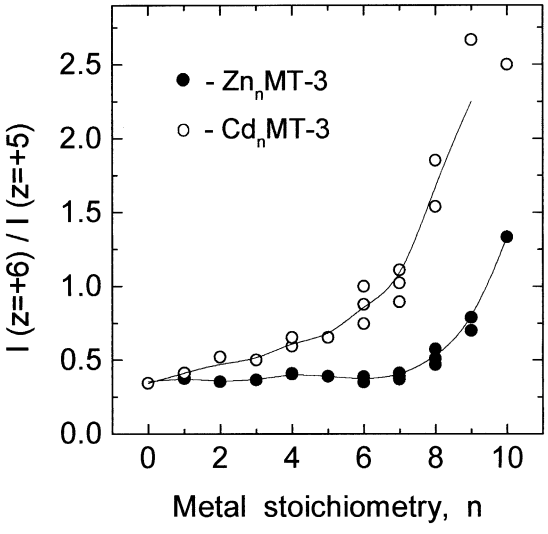

Fig. 4. Ratio of charge state +6 and charge state +5 ion intensities in the ESI TOF MS spectra of Zn- and Cd-substituted metalloforms of MT-3.

charged states in MS spectra, which additionally confirms that Zn_7MT-1 and Cd_7MT-1 have similar conformations in solution [11]. As Cd^{2+} ions have very similar coordination preferences and expose similar or higher chemical affinities towards different ligands as compared to Zn^{2+}, the observed conformational differences between Cd- and Zn-substituted MT-3 could be reasonably explained only with the different ionic radii of Cd^{2+} (0.97 Å) and Zn^{2+} (0.71 Å). It is feasible that the β-cluster of MT-3, in contrast to common MTs, cannot accommodate metals as large as Cd^{2+} into a compact cluster structure. EXAFS studies of ZnMT-3 and its β-cluster demonstrate that the Zn–Zn distance in the β-cluster of MT-3 is equal to 3.2 Å [19], which is unusually short as compared to 3.8 Å for the Zn–Zn distance in the structure of MT-2 [9]. Thus EXAFS data demonstrate that the β-cluster of MT-3 is more compact than the β-cluster of common MTs, which supports our conclusion that Cd^{2+} ions are size-excluded from the β-cluster of MT-3. Our data present the first example for non-isostructural replacement of Zn^{2+} with Cd^{2+} ions in the case of MTs which complicates application of CdMT-3 for structural studies as the more open conformation of Cd-substituted protein may cause higher heterogeneity of the CdMT-3 sample as compared with ZnMT-3 [15] and lead to enhanced dynamics of CdMT-3 observed in NMR studies.

The presented results give additional evidence for specific metal binding properties of MT-3 as compared with common MTs, which may be related to the specific physiological function(s) of this particular MT isoform. MT-3 is specifically expressed in the brain and it is not involved in detoxification of Cd^{2+} ions, which is one of the commonly accepted functions of MT-1/MT-2 [3,20]. Therefore Cd^{2+} is a non-native metal ion for MT-3 and it is not surprising that MT-3 is not well accommodated for its binding. Although MT-3 isolated from native tissues contains some amount of copper [21], the native metal for MT-3 in the brain is most likely zinc. The most evident confirmation for this conclusion has been obtained from transgenic mouse experiments, where MT-3 overexpression resulted in an increase of the zinc content in the brain, but did not influence the copper content [22]. Previ-

ously we have proposed that the specific metal binding properties of MT-3 may be effectively used for buffering of highly fluctuating concentrations of zinc and transfer of zinc into synaptic vesicles in zincergic neurons [15]. Specific metal binding properties of the β-cluster of MT-3, demonstrated in the present paper, may also support the functioning of MT-3 in the brain; however, the mechanism(s) involved are currently unknown. At the same time it is also not excluded that specific metal binding properties of MT-3 are connected with specific antioxidative properties of MT-3 [23,24], which are responsible for growth inhibitory activity of MT-3 towards cultured neurons in vitro [23] but which may also have physiological relevance [24].

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