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Recombinant synthesis of mouse Zn_3-β and Zn_4-α metallothionein 1 domains and characterization of their cadmium(II) binding capacity

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Abstract. Genetic engineering, coupled with spectroscopic analyses, has enabled the metal binding properties of the α and β subunits of mouse metallothionein 1 (MT) to be characterized. A heterologous expression system in *E.coli* has led to high yields of their pure zinc-complexed forms. The cadmium(II) binding properties of recombinant Zn_4-αMT and Zn_3-βMT have been studied by electronic absorption and circular dichroism. The former binds Cd(II) identically to α fragments obtained from mammalian organs, showing that the recombinant polypeptide behaves like the native protein. Titration of Zn_3-βMT with $CdCl_2$ results in the formation of Cd_3-βMT. The addition of excess Cd(II) leads to Cd_4-βMT which, with the extra loading of Cd(II), unravels to give rise isodichroically to Cd_9-βMT. The effect of cadmium-displaced Zn(II) ions and excess Cd(II) above the full metal occupancy of three has been studied using Chelex-100. The Cd_3-βMT species is stable in the presence of this strong metal-chelating agent.

Key words. Metallothionein domains; recombinant αMT; recombinant βMT; *E. coli* expression; cadmium binding; circular dichroism; UV–VIS difference spectra; Chelex.

Metallothioneins (MT) constitute a well defined group of low molecular weight cysteine-rich proteins, widely distributed in nature and generally considered responsible for heavy metal detoxification in eukaryotes [1, 2]. Although they were discovered in 1957 [3] the first attempts to isolate their corresponding α and β domains were not carried out until 25 years later, when they were obtained from mammalian livers by conventional purification techniques and proteolytic digestion [4, 5]. Classical peptide synthesis in solution by the fragment condensation method [6–9] and automated solid-phase peptide synthesis [10, 11] have also been used to produce MT domains. However, to date the study of the coordinating abilities of the MT α and β domains has been hindered by difficulties associated with their purification and recovery by these methods. The four spectroscopic analyses dealing with the Cd(II) binding properties of the α-fragment are ^{113}Cd-NMR measurement of the native rat liver Cd_4-αMT [12] and the CD measurements of cadmium titrations of either native rat liver Zn_4-αMT [13, 14] or chemically synthesized human liver apo-αMT 2 [10]. Concerning the β-fragment, optical studies of Cd(II) binding have been reported for native calf liver [13] and for chemically synthesized β-domain of human liver MT 2 [8, 10].

The synthesis of individual MT domains could be achieved by recombinant DNA methods, which are

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already used to obtain full-length MT. However, the only expression system reported was unsuccessful for β-domain synthesis and three α-related peptides were obtained as the result of differential bacterial processing at the N-terminus [15]. Thus, in order to obtain sufficient pure recombinant Cd-free MT domains we designed an expression system in *E. coli* using a chimeric glutathione-S-transferase (GST) construct in the pGEX-4T-1 vector. A previous report on a plant (pea) MT expressed from a different vector of the pGEX family [16] showed that this fusion protein bound metals. In our case, recombinant MT fragments were expressed as GST fusion proteins under the *tac* promoter in Zn(II)-supplemented media, yielding high levels of the protein after thrombin cleavage. The Zn_4-αMT and Zn_3-βMT portions were recovered and fully characterized. Results from the Cd(II) titration of Zn_4-αMT 1 showed that recombinant αMT behaves like the native form, thus validating the expression system employed. The pattern of spectra obtained from Zn_3-βMT 1, preliminarily reported in short communications [17, 18], is fully analysed to explore the Cd(II) binding capacity of this fragment.

Materials and methods

Genetic constructs of α and β coding regions. DNA fragments coding for the mouse αMT 1 and βMT 1 domains were constructed by one-step mutagenic PCR using the full-length MT cDNA [19], which was cloned in the plasmid pBX kindly provided by Dr D. Palmiter (University of Washington, USA). The α-domain extends from Ser32 to the C-terminus. As the presence of Lys at the hinge joining the two domains is crucial for proper MT expression [20], we amplified a DNA fragment corresponding to the Lys31 to Ala61 peptide to obtain an αMT coding region. The upstream primer (5′ TCCCGGATCCATGAAGAGCTGCTG 3′) was designed to generate an ATG initiation codon prior to Lys31 and an adjacent *BamH*I restriction site. The downstream primer was 5′ CCCGTCGACATCAGGCACAGCAC GT 3′, which reproduced the stop codon of the MT gene followed by a *Sal*I restriction site. The β-domain extends from Met1 to Cys29, but for the sake of protein stability we amplified the sequence from Met1 to Lys30. The upstream and the downstream primers were 5′ CCCGGATCCATGGACCCCAACTGCTCC 3′ and 5′ CGGGGTCGACCTAC TTGCAGGAGGT 3′, respectively. The upstream primer was used to create a *BamH*I restriction site just before the start of the coding region. The downstream primer added a stop codon after Lys30, and generated an adjacent *Sal*I restriction site.

After PCR amplifications, both αMT 1 and βMT 1 coding regions were obtained as *BamH*I-*Sal*I fragments, suitable to be ligated into the corresponding restriction sites in the expression plasmid pGEX-4T-1 (Pharmacia). The DNA sequence of all the recombinant constructs was confirmed by the dideoxy-termination method of Sanger [21] using the T7 Sequencing Kit from Pharmacia and [^{35}S]dATP[αS] (Amersham). Restriction enzymes were from Promega.

Expression and purification of the recombinant MT domains. To overexpress αMT 1 and βMT 1 the recombinant constructs were introduced into the protease-deficient strain *E.coli* BL21 [22] and transformed cells were cultured in a 70 l total capacity Biostat U (Braun Biotech) fermentor coupled to a Westfalia separator (Braun Biotech), equipped with a CSA-1-06-475 centrifuge, operating at 9800 rpm (6000 × g) and controlled by a TVE-OP 76/0 programer. A total of 2 litres overnight cultures of the transformed host strains in LB supplemented with 100 mg/l ampicillin at 37 °C were poured into the fermentor containing 20 litres of freshly prepared medium. After growth at 37 °C to an A_{600} value of 1.0, the bacteria were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, final concentration). Cells were grown for 30 min and then the culture was supplemented with $ZnCl_2$ to a final concentration of 0.3 mM. After 2.5 h, cells were harvested, washed in phosphate-buffered saline 1.4 M NaCl, 27 mM KCl, 101 mM Na_2HPO_4, 18 mM KH_2PO_4 (PBS) and pelleted by centrifugation at 7700 × *g* for 10 min. For protein purification, cells were resuspended in 5% of the original volume of ice-cold PBS-0.5% v/v β-mercaptoethanol. To prevent oxidation of the metal-free cysteines, pure grade 5.6 argon was bubbled through all the steps of the purification procedure. Suspended cells were sonicated at 4 °C with 20 s pulses for 5 min, and centrifuged at 12000 × *g* for 15 min. The supernatant was recovered and used to purify the GST-αMT and GST-βMT fusion proteins by batch affinity chromatography with Gluthatione-Sepharose-4B (Pharmacia) at a volume ratio 1:10 matrix:sample. The mixture was incubated with gentle agitation for 30 min at room temperature. After three washes in PBS, the matrix was packed in a column (15 cm × 3 cm) for subsequent purification steps. The polylinker of the pGEX vector used included a thrombin recognition site, which allowed cleavage of the fusion protein. To that end, a thrombin-PBS solution (10 units of thrombin (Pharmacia)/mg of fusion protein) was poured into the matrix bed, and digestion was carried out overnight at 23–25 °C. The GST portion of the expressed fusion protein remained bound to the gel matrix, whereas the αMT or βMT portion was eluted together with thrombin. The eluate was then five-fold concentrated using Centriprep Concentrators (Amicon) with a cutoff of 3 kD, and subsequently fractionated using FPLC. A Superdex-75 (Pharmacia) exclusion column was equilibrated with 50 mM Tris-HCl, pH 7.0 and run at 1 ml/min. 1 ml

fractions were collected and analysed for protein content by their absorbance at 254 nm.

Aliquots of the protein-containing Superdex-75 fractions were analysed by SDS-PAGE on 15% gels and stained with Coomassie Blue (fig. 1). Positive samples were pooled, and aliquots were stored at −70 °C for further use.

Analysis of the recombinant αMT 1 and βMT 1. The molecular mass of the MT fragments was determined by electrospray ionization mass spectrometry (ESMS) on a Fisons Platform II Instrument (VG Biotech) calibrated using horse heart myoglobin (0.1 mg/ml). Assay conditions were: source temperature, 120 °C; capillary-counterelectrode voltage, 4500 V; lens-counterelectrode voltage, 1000 V; cone potential, 35 V; m/z range, 1000 to 1800; scanning rate, 5 s/scan; interscan delay, 0.5 s. The carrier was a 10:90 acetonitrile:ammonia water solution at pH 10. The purity of the samples and their zinc content were also determined by inductively coupled plasma (ICP) analysis as explained below. Protein stock solution concentrations were determined from measurements of -SH groups using the reagent DTNB (5,5′-dithiobis(nitrobenzoic acid)) in 3 M guanidine hydrochloride [23], taking into account the fact that the DTNB decomposes with time [24]. At 420 nm, the maximum absorbance for the α and the β fragments was reached at 20 and 10 min, respectively. Oxygen-free conditions are a strict requirement throughout the preparation of the samples. For this reason solutions were prepared in an inert gas box purged with argon, using degassed water obtained after several freeze-vacuum-thaw cycles. Total sulphur content and zinc content were measured by ICP using a Thermo Jarrell Ash, Polyscan 61E at 182.040 nm (S) or at 213.856 nm (Zn) without any previous treatment of the samples [25]. Very good agreement between total sulphur determination by ICP measurements and SH content by Ellman's method was obtained by assuming that the α or β fragments have 11 or 9 HS-Cys groups but 12 or 10 sulphur atoms, respectively, due to the presence of one Met residue in each fragment. Thus, a Zn^{2+}/11 cysteines (=1 mol of protein) ratio of 4.10 was obtained for the α fragment while a Zn^{2+}/9 cysteines (=1 mol of protein) ratio of 3.14 was obtained for the β-fragment. The protein concentrations were 9.72×10^{-5} M for the α and 2.62×10^{-4} M for the β-fragment.

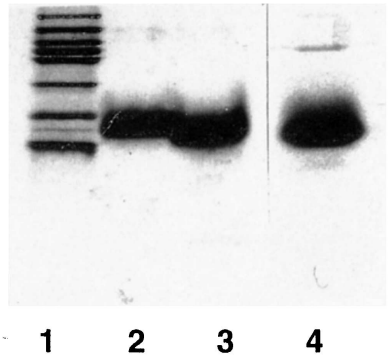

Figure 1. SDS-PAGE analysis of the purified recombinant mouse MT 1 and domains. Coomassie Blue stained 15% acrylamide gels. Lane 1: molecular weight markers, 97.4, 66.2, 55, 42.7, 40, 31, 21.5 and 14.4 kDa. Lane 2: 10 µl of purified recombinant mouse MT 1 solution. Lane 3: 10 µl of purified recombinant α-domain of mouse MT 1. Lane 4: 10 µl of purified recombinant β-domain of mouse MT 1.

Metal ion binding studies. All solutions used in metal ion binding were prepared with Milli-Q purified water and were either argon saturated or vacuum degassed prior to use. Glassware was cleaned with 10% (v/v) nitric acid and repeatedly rinsed with ultrapure water. Molar ratio aliquots of Cd^{2+} (using $CdCl_2$ in water from a Merck AAS Cd^{2+} standard of 1000 ppm) were added sequentially to a single solution of either Zn_4-αMT or Zn_3-βMT. The CD spectra were recorded every 10 min until saturation of the spectral traces. Absorption studies were conducted in parallel with CD studies.

The electronic absorption measurements were performed in 1 cm quartz cuvettes on an HP-8452A Diode array or a Shimadzu UV-2101PC UV-visible spectrophotometer. A Jasco spectropolarimeter (Model J-720) interfaced to an IBM PS/2 computer was used for CD measurements. Both types of spectrum were corrected for the dilution effects and processed with the program GRAMS/386.

The pH for all experiments remained at 7.0 ± 0.1 from beginning to end, although no buffer was added to avoid possible coordination with the metal ions. Prior to use, the Chelex-100 resin was washed with 1 M HCl, rinsed with ultrapure water, followed by 1 M NaOH and then rinsed with distilled water. The final pH was between 10 and 11 [26]. In order to adjust the pH to working conditions the Chelex-100 resin was treated with 50 mM Tris-HCl at pH 7 overnight and rinsed five times with ultrapure water. The final pH was between 6 and 7. Throughout this paper we refer to the mol equivalents of cadmium added in terms of "5 Cd^{2+}" to stand for "5 mol equiv of Cd^{2+}" or "a molar ratio Cd^{2+}:protein of 5".

α-domain. The absorption and CD spectra corresponding to the titration of a 2.66×10^{-5} M solution of mouse Zn_4-αMT 1 with Cd^{2+} at pH 7 were recorded (data not shown). In order to evaluate the effect of Zn(II) in Cd_4-αMT species, 16 mg of Chelex-100 were added after addition of 4 Cd^{2+} to 1.200 ml of a 1.80×10^{-5} M solution of Zn_4-αMT. The mixture was shaken vigorously for 5 min and the spectra

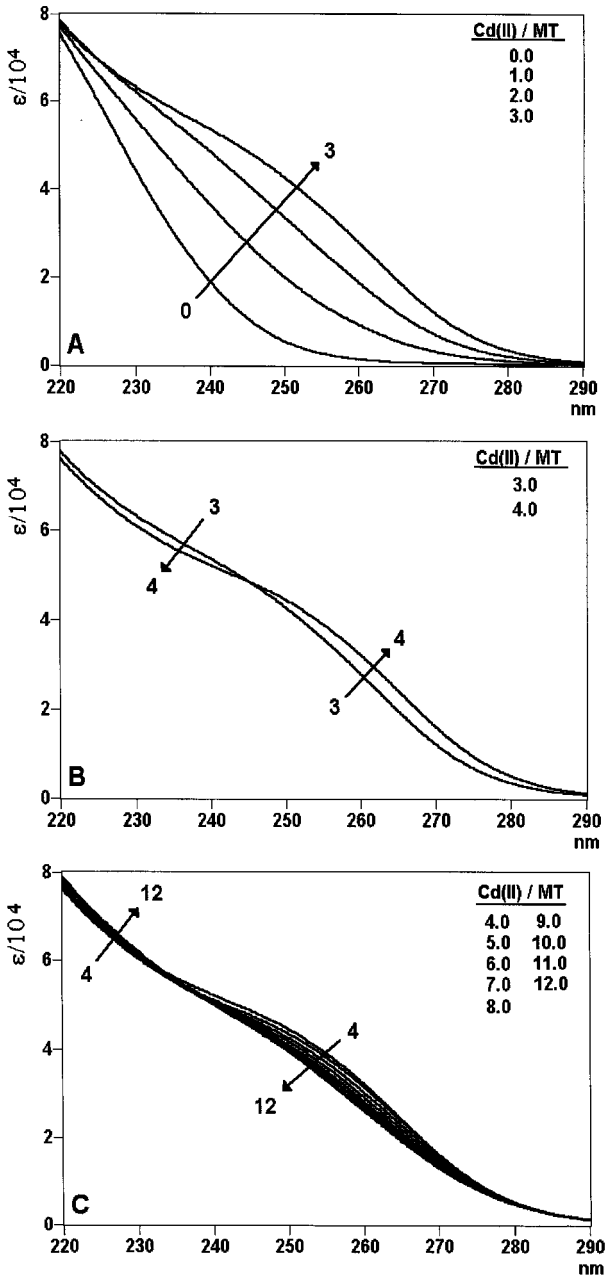

Figure 2. Effects of Cd(II) binding on the absorption spectrum of recombinant mouse Zn_3-βMT 1. Absorption spectra recorded during the titration of a 2.22×10^{-5} M solution of Zn_3-βMT 1 with $CdCl_2$ at pH 7 and room temperature. (*A*) Addition up to 3 mol eq of Cd^{2+}; (*B*) from 3 to 4 Cd(II) mol eq showing the apearance of an isosbestic point at 245 nm; (*C*) from 4 to 12 Cd(II) mol eq. The Cd(II) to MT 1 ratios are indicated within each frame.

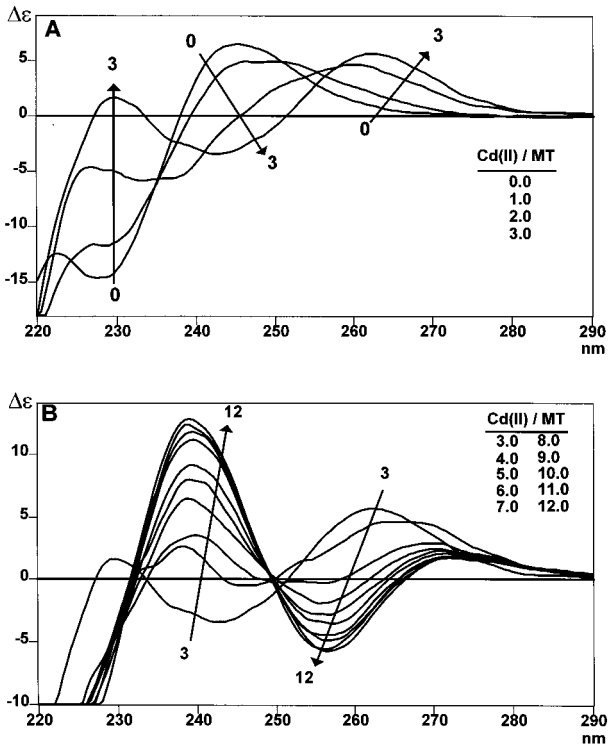

Figure 3. Effects of Cd(II) binding on the circular dichroism spectrum of recombinant mouse Zn_3-βMT 1. CD spectra recorded during the titration of a 2.22×10^{-5} M solution of Zn_3-βMT 1 with $CdCl_2$ at pH 7 and room temperature. (*A*) Addition up to 3 mol eq of Cd^{2+}; (*B*) from 3 up to 12 mol eq of Cd^{2+} showing an isodichroic point at 249.5 nm.

were recorded. The titration was then continued by adding successive volumes of Cd^{2+} to an aliquot of the supernatant. In both cases the CD and UV absorption traces remained unchanged.

β-domain. Figures 2 and 3 show the absorption and CD spectra recorded during the titration of a 2.22×10^{-5} M solution of mouse Zn_3-βMT 1 protein with Cd^{2+} at pH 7. Increment difference absorption spectra are displayed in figure 4. Chelex-100 was added at two stages of the titration. The first portion (30 mg) was added to 2.662 ml of a 2.17×10^{-5} M solution of Cd_3-βMT. Cd^{2+} was then added to an aliquot of the supernatant. After excess Cd(II) had been added, 20 mg of Chelex-100 were poured into the cell. The mixture was shaken and the spectra recorded as a function of time until no further changes were observed. Between recordings, the Chelex-containing solution was shaken vigorously to improve the efficiency of the metal chelating resin.

In all cases, the cadmium, zinc and sulphur content of the supernatant and that of the solution obtained after treatment of the resin with 2 M HNO_3 [27] were analysed by ICP (at 228.802 nm for Cd; for Zn and S see above).

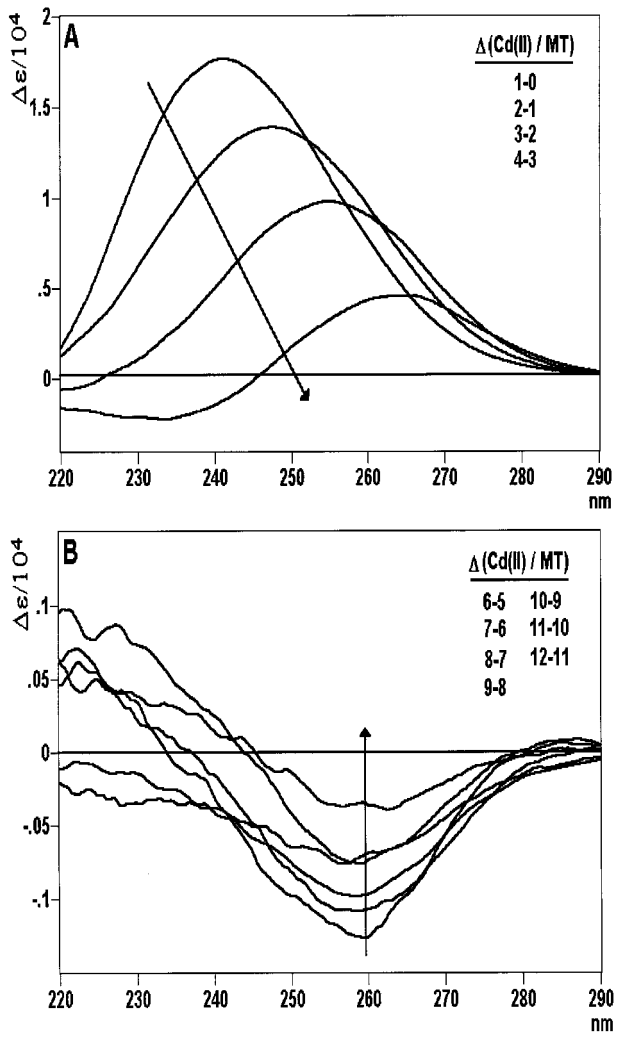

Figure 4. Difference absorption spectra corresponding to the titration of Zn_3-βMT with $CdCl_2$. (*A*) Difference absorption spectra obtained by subtracting successive spectra of figure 2A and 2B. (*B*) Difference absorption spectra obtained by subtracting successive spectra of figure 2C. The first trace in (*A*) (spectrum 1-spectrum 0; 1-0) and subsequent increment difference spectra are labelled within each frame. 3 + Ch refers to a solution of Zn-free Cd_3-βMT species, which has been used to avoid the effect of the Cd-displaced Zn(II) ions in Cd_3-βMT species.

Results and discussion

Synthesis, purification and characterization of Zn_4-αMT and Zn_3-βMT. The final recombinant plasmids, constructed by ligation of the *BamH*I-*Sal*I αMT 1 and βMT 1 coding sequences into pGEX-4T-1, were sequenced and the results confirmed the accuracy of the PCR reaction and the fusion constructs. BL21 *E.coli* cell homogenates were then used as raw material to obtain pure preparations of GST-αMT 1 and GST-βMT 1, at a rate of 2.5 mg/l of culture. The fusion proteins were further digested with thrombin to recover the Zn(II)-complexed αMT 1 and βMT 1 peptides. Figure 1 shows the pure αMT 1 and βMT 1 peptide preparations after FPLC chromatography. Quantification by ICP of total sulphur and zinc in protein samples confirmed the purity of the peptides and the stoichiometry of the metal complexes. For αMT 1 and βMT 1, the metal to protein ratio found was 4.10 and 3.14 respectively, which is consistent with the chelating capacity of these domains. The molecular masses of purified Zn_4-αMT 1 and Zn_3-βMT 1 were found to be 3550.8 and 3348.7, which compare well with the expected molecular weight for a species of 3549.5 (apo-αMT + 4Zn - 8H) and 3348.7 (apo-βMT + 3Zn - 6H), respectively. The expected molecular weights were calculated according to the standard procedure reported in [28] and [29]. The close correlation of the observed and predicted masses supports the purity and stoichiometries of the recombinant peptides.

Electronic absorption and circular dichroism studies of Zn_4-αMT and Zn_3-βMT. Results obtained from the electronic absorption and CD spectra corresponding to the titrations of recombinant Zn_4-αMT and Zn_3-βMT are presented according to the following pattern. For both Zn_4-αMT and Zn_3-βMT the spectral changes observed during the addition of Cd(II) ions up to n Cd^{2+}, where n denotes the number of Zn(II) ions initially coordinated to each MT fragment, are discussed first. The spectral changes corresponding to the addition of excess Cd(II) to solutions containing Cd_3-βMT and Cd_4-αMT species are analysed to evaluate the interaction of each cluster with excess Cd(II) and elucidate the stoichiometry of the resulting species. Finally, to determine whether the displaced Zn(II) ions and excess Cd(II) play a significant role in the structure of Cd_4-αMT and Cd_3-βMT species, the titrations carried out after treatment with Chelex-100 are examined.

α-domain. The recombinant mouse Zn_4-αMT 1 exhibits a characteristic CD trace, which allows us to describe the CD signal as an exciton coupling with a crossover point at 238.5 nm or an inflection point at 235.5 nm. The main features of the UV absorption and CD spectral changes during its Cd(II) titration closely resemble those obtained with native rat liver Zn_4-αMT [13]. Thus, very similar CD profiles are obtained for Cd_4-αMT species regardless of whether the titration departs from recombinant or native Zn_4-αMT [13] or chemically synthesized apo-αMT [10]. Both absorption and CD spectroscopic features reached saturation for 4 Cd(II). Moreover, the addition of excess Cd(II) to recombinant Cd_4-αMT species induced no substantial spectral changes, which indicates a different behaviour of this fragment with respect to Cd_3-βMT. After addition of Chelex-100 resin to Cd_4-αMT 1 no spectral changes were observed (data not shown), indicating that the Zn(II) ions displaced by Cd(II) affect neither the 3D

cluster structure of recombinant Cd_4-αMT 1 species nor the number or nature of Cd-S bonds. ICP data confirmed the presence of four Zn(II) in the resin and 4 Cd(II) in the solution.

β-domain. The electronic absorption and CD spectra clearly illustrate the complexity of the structural changes that occur as Cd(II) is added to Zn_3-βMT 1. Both sets of spectra provide evidence for the formation of the Cd_3-βMT species, which, following the extra loading of Cd(II), first unravels and then rearranges to a new 3D structure.

Electronic absorption (fig. 2A) and CD spectra (fig. 3A) corresponding to the addition of Cd(II) up to 3 eq to recombinant mouse Zn_3-βMT were reported in a preliminary communication [18]. Difference spectra (fig. 4A) show that, up to 3 Cd(II), the ligand to metal charge transfer band red shifts to 255 nm, which agrees with the replacement of Zn(II) by Cd(II). Moreover, the molecular weight obtained by ESMS of 3489.8, corresponding to the final species at the end of this stage, is in agreement with the expected molecular weight for a Cd_3-βMT species of 3489.7 (apoβMT + 3Cd - 6H).

The addition of increments of Cd(II) to Cd_3-ßMT up to 1 Cd(II) in excess is concomitant with the appearance of an isosbestic point at 245 nm in the absorption spectrum (fig. 2B) and thus a decrease in the absorption at lower wavelengths and an increase at higher values is observed. The difference absorption spectrum for this range of Cd(II) added (fig. 4A) shows a red shift from 255 nm to 265 nm, indicating that the extra Cd(II) is bound to the Cd_3-βMT cluster via an increase in the degree of bridging of sulphur atoms [30, 31]. Moreover, the changes observed in the CD spectra during this phase reveal an extensive rearrangement of the Cd_3-βMT clusters with the incorporation of the fourth Cd(II) (fig. 3B).

Over 4 Cd^{2+} added the absorption at 255 nm decreases almost linearly up to 9 Cd(II) through an isosbestically (234 nm) related sequence of spectra, as if the signal was approaching saturation at about 9 Cd^{2+} (fig. 2C). The corresponding difference spectra (fig. 4B) show that each cadmium addition above 4 Cd^{2+} causes the same effect: a decrease in the absorbance centered at 260 nm, which would agree with a decrease in the number of cadmium-sulphur chromophores. Moreover, the blue shift from 265 to 260 nm could well indicate that addition of excess cadmium involves the transformation of bridging, S_b, to terminal, S_t, thiolates. Both effects, the decrease and the blue shift of the absorbance together with the isosbestic (234 nm) and isodichroic (249.5 nm) points appearing in the absorption and CD spectra, suggest that cadmium binding to Cd_4-βMT proceeds in a cooperative way giving a Cd_9-βMT species with fewer Cd-S bonds than the former. One explanation for these results involves the assumption that formation of Cd_9-βMT from Cd_4-βMT leads to a decrease in the number of Cd-S_b bonds. To illustrate this hypothesis we could consider the addition of one further Cd^{2+} ion to the sequence S_t-Cd-S_b-Cd-S_b-Cd-S_t (2 Cd-S_t and 4 Cd-S_b bonds) to give species containing 4 Cd-S_t bonds. Then, the final balance for cadmium binding would be the loss of 2 Cd-S_b bonds and the conversion of 2 Cd-S_b to 2 Cd-S_t. In agreement with the previous assumption the small increase in the absorbance observed below 234 nm may arise from the presence of non-sulphur ligands (e.g. oxygen or chloride) in the coordination sphere of the cadmium ions bound to the protein. The charge transfer bands of such chromophores are expected to occur at higher energies and thus lie outside of the range studied.

The significant rearrangement from Cd_3-βMT to Cd_4-βMT is followed by the unravelling of the latter species and subsequent self-association into a 3D structure. The Cd_9-βMT 1 species forms isodichroically and shows a unique CD spectral fingerprint with strong CD intensities at 239.5(+), 257.5(−) and 274.5(+) nm (fig. 3B) that might arise from the formation of a supercoiled structure. Similar explanations have been proposed for Ag_{18}-MT [32, 33] and Hg_{18}-MT [34]. Studies to confirm the essential requirement for chloride presence in the formation of Cd_9-βMT 1 are now being performed. On the basis of the changes observed in the CD spectra accompanying the dimerization of rabbit liver Cd_7-MT upon exposure to free Cd(II) [31], a dimerization process in recombinant Cd_3-βMT under our experimental conditions may be ruled out.

In a parallel titration of Zn_3-βMT, Chelex-100 was added after incorporation of the first three Cd(II). Surprisingly, unlike the behaviour observed for recombinant mouse Cd_4-αMT, the UV absorption and CD spectra after addition of the resin changed: the absorbance and the characteristic CD signals found for Cd_3-βMT in presence of Zn(II) (229.5(+), 241.5(−) and 263(+) nm) were enhanced. This increase could be attributed to the removal of Zn(II) ions bound to the fragment (ICP analyses confirmed the presence of 3 Zn(II) in the resin and 3 Cd(II) in the supernatant). To determine the number of Zn(II) ions that remain coordinated to the protein after the addition of 3 Cd(II) to Zn_3-βMT another titration in a cell containing Chelex from the starting point was carried out. UV absorption and CD spectra recorded during this titration revealed that only one of the three Cd-displaced Zn(II) ions was coordinated to the protein. This conclusion arises from the observation that there are no changes in either the CD or the UV absorption spectra up to 2 Cd(II) added if compared with the titration carried out in absence of the resin. However, those corresponding to the addition of the third

Cd(II) are different. Spectra obtained for the third Cd(II) added in the presence of the resin coincided with those observed after removal of the three Zn(II) ions displaced in the titration of Zn_3-βMT with Cd(II).

Titration of the zinc-free Cd_3-βMT supernatant with $CdCl_2$ gave rise to the same CD and UV absorption changes as those observed in presence of Zn(II). This coincidence suggests that the fourth Cd(II) added binds to the same binding site of Cd_3-βMT regardless of the presence of Zn^{2+}. Together with the previous assumption that only one Zn(II) ion is coordinated to Cd_3-βMT if displaced-Zn(II) ions are not removed from the solution, this leads us to propose that the fourth Cd(II) displaces the initially coordinated Zn(II) ion. Following this reasoning, it is tempting to suggest that this Zn(II) ion, when bound to Cd_3-βMT, probably occupies the same binding site as the fourth Cd(II) ion added to Cd_3-βMT 1. Moreover, molecular weight measurements by ESMS in Cd_3-βMT containing solutions afforded the same value, 3489.8, irrespective of the presence of displaced Zn(II). These data agree with the assumption that the Zn(II) ion is weakly bound to the Cd_3-βMT cluster.

Previous data indicate that the formation of Cd_9-βMT from Cd_4-βMT proceeds cooperatively, irrespective of the presence of zinc(II) ions. The Cd_9-βMT species could be proposed if it is assumed that the development of a spectral signal that reaches a maximum intensity at a certain stoichiometry of Cd:protein is related to the formation of a single species. The variations of the CD and UV intensities as a function of the molar ratio of Cd^{2+} added to βMT confirm the existence of such species.

After addition of excess Cd(II) to the zinc-free Cd_3-βMT solution, Chelex-100 was added again and the spectral variations were recorded as a function of time. After 48 h the profiles of the CD and UV absorption spectra are very close to those corresponding to zinc-free Cd_3-βMT. ICP results yielded an average S/Cd ratio in the supernatant of 3.56, giving further support to the recovery of the Cd_3-βMT species (10S/3Cd) detected by CD and UV absorption. Moreover, CD data indicate that the transformation of Cd_9-βMT back to Cd_3-βMT follows the reverse pathway to that corresponding to its formation. However, the sequence of electronic spectra shows different pathways for formation of Cd_9-βMT and retrieval of Cd_3-βMT. This could be attributable to charge effects [35] so that the full loaded cadmium species would be retained by the resin and thus induce significant changes in concentration that affect the UV absorption spectra.

Conclusions

A recombinant DNA approach has been used successfully to synthesize in high yield α and β MT domains suitable for spectroscopic studies of their binding abilities.

Recombinant Zn_4-αMT 1 behaves towards Cd(II) analogously to α-fragments of MT obtained from mammalian organs affording Cd_4-αMT. Interestingly, this species is completely resistant to the effects of additional Cd^{2+}.

The spectral envelopes of recombinant Zn_3-βMT 1 and Cd_3-βMT 1 show analogous features to those observed in recombinant M_4-αMT, M = Zn, Cd. In all cases the displacement of Zn(II) by Cd(II) entails a red shift of the electronic absorption and CD spectral traces. Zinc replacement in Zn_4-αMT is also accompanied by a significant increase in the CD spectral intensities, less noticeable for Zn_3-βMT.

Unlike Cd_4-αMT, Cd_3-βMT incorporates more Cd(II). Accordingly, Cd_3-βMT binds via sulphur one extra Cd(II) ion, or displaced Zn^{2+} if not removed from solution, to afford a new Cd_3M-βMT species, M = Cd or Zn. However, spectral data indicate that only for M = Cd(II) the three-metal cluster structure is strongly perturbed, thus becoming very vulnerable. Now, further addition of Cd(II) promotes the cooperative destruction of the metal cluster and formation of the Cd_9-βMT species. On the other hand, treatment with Chelex-100 of the species formed when excess Cd(II) is added to Cd_3-βMT retrieves this species, substantiating its stability even in the presence of the chelating resin.

The Cd(II)-binding properties of the two recombinant MT domains yielded results which allowed a comparison with those obtained from the recombinant full-length MT [36]. Coordinating features of the β-domain, which is assumed to be the hypothetical MT ancestor unit, are particularly interesting as they can contribute to the knowledge of the structure/function relationship in MT and clarify the intrinsic biological role and evolutionary history of these proteins.

Note: All figures related to the titration of recombinant mouse Zn_4-αMT 1 with Cd(II) and to the experiments performed in the presence of Chelex are available upon request from the corresponding author.

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