The Zn- and Cd-Clusters of Recombinant Mammalian MT1 and MT4 Metallothionein Domains Include Sulfide Ligands

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Recombinant (*E. coli*) synthesis of mammalian MT1 and MT4 domains as separate peptides in Zn(II) and Cd(II) enriched growth media has rendered metal complexes containing sulfide anions as additional ligands. The Cd preparations show higher sulfide content than the Zn preparations. Also, the βMT1 and βMT4 fragments exhibit higher sulfide/peptide ratios than the respective α fragments. Titration of Zn_3-βMT1 with Cd(II) followed by addition of several sodium sulfide equivalents shows that the Cd(II)-βMT1 species can incorporate sulfide ligands *in vitro*, with a concomitant evolution of their UV-vis and CD fingerprints to those characteristic of the Cd-S^{2-} chromophores. Current results have also provided full understanding of previous data collected by this group in the characterization of the Cd-βMT1 preparations obtained from large-scale fermentor synthesis by allowing identification of at least $2S^{2-}$ ligands per Cd-βMT1 species. Furthermore, the results here presented have revealed that synthesis of βMT4 in Cd-supplemented cultures yielded Cd,S^{2-}-containing clusters instead of the proposed heterometallic Zn,Cd-βMT4 complexes. Finally, a global evaluation of our results suggests that the higher the Cu-thionein character of a MT peptide, the higher is its tendency to harbor nonproteic ligands (i.e., sulfide anions) when building divalent metal clusters, especially Cd-MT complexes. Exp Biol Med 231:1522–1527, 2006

Key words: α domain; β domain; metallothionein; MT1; MT4; sulfide ligands

Introduction

Metallothioneins (MTs) are a superfamily of atypical small proteins, ubiquitous but probably polyphyletic, which coordinate heavy metal ions through metal-thiolate bonds established by the highly abundant cysteine residues of their sequence (1). Currently, and after half a century of multidisciplinary research, the biological structure of MTs and their contribution to a variety of physiological processes in the most diverse organisms still remain undetermined (2, 3). Two main reasons should be connected with this fact. First, most of the existing data refer to mammalian MTs, while the extreme sequence heterogeneity of this superfamily of proteins precludes any homology-driven structural, functional, or evolutionary inference (cf. Web page: http://www.expasy.ch/cgi-bin/lists?metallo.txt). Second, the difficulties found when trying to obtain homogeneous native metal-MT complexes, together with the impossibility of purifying them of some organisms or in non-metal-induced forms, forced the use of indirect methods for their preparation, always on the assumption that genuine and functional native MT structures were reproduced. The most common methodology applied relied on the *in vitro* reconstitution of metal-MT complexes from native apoforms obtained after heavy acidification, or even from synthetic peptides, which does not necessarily guarantee the physiological significance of the recovered species. A further advance was attained through the recombinant synthesis of MTs in heterologous hosts, which enabled both the study of metal-MT species conformed *in vivo* (i.e., in a physiological environment) and the application of nonaggressive purification strategies.

Nearly ten years ago we developed an *Escherichia coli* expression system that allows the biosynthesis of Zn^{II}-, Cd^{II}- and Cu^{I}-MT intact MT complexes, isolated domains, and mutant variants, with sufficient quantity and purity to allow for analytical, spectrometric, and spectroscopic characterization. Since studies on the mouse Zn-MT1 system (4, 5) fully validated the correspondence between native and recombinant complexes, studies were expanded from mammalian MT1 (6–8) to mammalian MT4 (9), the crustacean MTH (10), the *Drosophila* MTN (11) and MTO (12), and the plant *Quercus suber* QsMT (13). Recently, after the recombinant synthesis of several MTs of different organisms in *E. coli* and the thorough analysis of the

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chemical/structural features of their *in vivo* conformed metal-MT complexes, we identified a third component in the metal-MT clusters: inorganic sulfur anions behaving as sulfide ligands. Hence, using analytical, spectroscopic, and spectrometric techniques, we provided qualitative and quantitative evidence that S^{2-} ligands were present in nearly all the Zn^{II}- and Cd^{II}-MT complexes of the studied MTs, always in a larger quantity in the latter than in the former (14). The general features of these clusters correlated well with those of the plant and yeast Zn- or Cd-γ-glutamyl peptides, thus bridging the gap between both kinds of metal binding biomolecules (15, 16). Also, this finding enlarges the physiological potential of these poorly understood proteins, in view of the emerging evidence of S^{2-} involvement in relevant cell events, such as electron transfer (17), redox equilibrium (18), and neurotransmission and neuromodulation (19).

While the crucial question of whether sulfide ligands are also present in native MT forms is addressed, we have also focused our efforts in analyzing the sulfide ligand presence in the separate β and α domains of the mammalian MT1 and MT4 isoforms. This is of great interest, first, due to the intrinsic importance of the mammalian proteins in biomedical research, and second, because characterization of the recombinant mammalian domains had been carried out before the discovery of the S^{2-} presence in the MT recombinant preparations. Therefore, we present here the results of the analysis of the Zn(II)- and Cd(II)-species of the separate βMT1, βMT4, αMT1, and αMT4 domains obtained by the same rationale used to detect and quantify sulfide in the full length MTs (14). Furthermore, this work includes the comparison of their features with those of the entire MT1 and MT4, and the reconsideration of some data reported in the literature for the Cd(II)-complexes of the MT1 and MT4 separate domains (6, 9).

Material and Methods

Recombinant Syntheses of the Metal-MT Complexes. The construction of the cDNA encoding for the separate MT domains and their cloning in the pGEX expression vector have been previously reported (MT1 [6], MT4 [11]). The synthesis and purification of the corresponding Zn(II)- and Cd(II) complexes in *E. coli* LB (Luria Bertani) culture media supplemented with 300 μ*M* Zn^{II} or Cd^{II} final concentration, respectively, has also been carried out as explained there.

Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) and Amino Acid Analysis. S, Zn, and Cd sample contents were determined by ICP-AES, using a Polyscan 61E (Thermo Jarrell Ash; Thermo Electron Corporation, Waltham, MA) spectropolarimeter and measuring S at 182.040 nm, Zn at 213.856 nm, and Cd at 228.802 nm, and sample preparation was performed according to two alternatives. *Conventional* ICP implied no previous treatment of the sample, while *acid* ICP included an acidification of the sample (incubation in 1 *M* HCl at 65°C for 5 mins) prior to ICP measures (14). In both cases, protein concentration was calculated from the S content of the sample, assuming that S was only contributed by the Cys and Met residues of the MT. In addition, protein concentration was assessed by standard amino acid analysis (hydrolysis in 6 *M* HCl at 110°C for 22 hrs) on an Alpha Plus Amino Acid Autoanalyzer (Pharmacia LKB, Cambridge, England). Ser, Lys and Gly contents were used to extrapolate MT concentrations.

***In Vitro* Cadmium and Sulfide Binding Studies.** Cd(II) binding studies of Zn_3-βMT1 were undertaken following the procedures already described in the literature for Cd(II) titrations (4). After four Cd(II) equivalents added to Zn_3-βMT1, aliquots of a standard 3.14 mM Na_2S solution, prepared as described in (14), were added until a ratio of 5 S^{2-} per βMT1 was achieved.

Mass Spectrometry and CD-UV Spectroscopy. The molecular mass of the metal-MT species was determined by electrospray ionization mass spectrometry (ESI-MS) on a Fisons Platform II Instrument (Fisons Instruments Inc, Beverly, MA), equipped with MassLynx software and calibrated with horse-heart myoglobin (0.1 mg/ml). The assay conditions were as follows: 20 μl of protein solution injected at 40 μl/min; the use of an HPLC Uptisphere (Interchim, Montluçon, France) C_4 33 mm × 2 mm × 5 μm column to separate analytes; capillary counterelectrode voltage, 4.5 kV; lens counterelectrode voltage, 1.0 kV; cone potential, 60 V; source temperature, 120°C; m/z range, 850-1950; scanning rate, 3 secs/scan; interscan delay, 0.3 secs. In all cases, the running buffer was an appropriate mixture of acetonitrile and 5 m*M* ammonium acetate/ammonia, pH 7.5. Electronic absorption measurements were performed on an HP-8453 diode array UV-visible spectrophotometer. A Jasco spectropolarimeter (J-715; Jasco, Easton, MD) interfaced to a computer (GRAMS/32 software) was used for CD determinations. The temperature for all measurements was kept at 25°C by means of a Peltier PTC-351S apparatus (TE Technology Inc., Traverse City, MI).

Gas Chromatography with Flame Photometric Detection (GC-FPD). GC-FPD (14, 20) was used to measure sulfide at low concentrations without the need of a derivatization step. H_2S was generated by strong sample acidification (H_2SO_4, pH 0.0) in order to ensure the metal-MT complex disruption as well as to avoid the precipitation of the insoluble ZnS and CdS which might have been generated. From a nominal 1000 ppm S^{2-} solution (14), dilute standards of 0, 0.25, 0.5, 1, 1.5, 2.5, and 3 ppm sulfide concentration were used to draw the corresponding calibration curve. Sample aliquots, as well as the standard solutions, were transferred to airtight 2-ml vials, acidified to a final volume of 0.5 ml, and immediately sealed. Vials were then incubated at 40°C for 2 hours with agitation (250 rpm) in order to accelerate the evolution of hydrogen sulfide from the aqueous phase and equilibration of gas phase in the

Table 1. Analytical Characterization of the Zn- and Cd-Species Identified in the Recombinant Preparations of the Separate β and α Domains of Mammalian MT1 and MT4, and Comparison with Data of the Corresponding Full-Length Proteins

Metal-MT	[MT][b] ($\times 10^{-4}$ M)	[MT][c] ($\times 10^{-4}$ M)	Metal/MT[d]	Metal/MT[f]	S^{2-}/MT[g] (GC-FPD)
Zn-MT1[a]	1.38	1.13	6.0	7.3	1.5
Zn-βMT1	3.74	3.10	2.8	2.9	0.9
Zn-αMT1	1.02	0.75	3.8	4.1	3.0
Cd-MT1[a]	1.90	1.13	3.7	6.4	3.1
Cd-βMT1	0.17	0.13	2.6	3.0	4.2
Cd-αMT1	1.14	1.03	3.9	4.1	2.6
Zn-MT4[a]	2.18	1.18	6.7	7.0	3.5
Zn-βMT4	1.47	1.35	3.0	3.0	1.6
Zn-αMT4	2.50	1.86	4.1	4.4	0.8
Cd-MT4[a]	0.19	0.11	5.5[e]	6.9[e]	14.0
Cd-βMT4	0.67	0.43	3.6	3.9	5.0
Cd-αMT4	2.13	1.27	3.9	4.6	1.7

[a] The data for the full-length mammalian MT1 and MT4 isoforms are from (14).
[b] Protein concentration calculated from *conventional* ICP-AES results.
[c] Protein concentration calculated from *acid* ICP-AES results. Amino acid analysis led to identical figures ±0.01.
[d] Metal-to-MT ratio deduced from the metal content measured by ICP-AES and (b).
[e] In this case, "metal" accounts for the summation of the zinc and cadmium content.
[f] Metal-to-MT ratio deduced from the metal content measured by ICP-AES and (c).
[g] N/D, nondetectable.

headspace. Five hundred microliters of the headspace gas were subjected to gas chromatography (HP 5890 Series II coupled to a FPD80 CE Instruments [Thermo Finnigan detector; Thermo Electron Corporation]). The gaseous mixture was carried by a 6.6 ml/min flux of He through the GC glass column (SPB 608 30 m × 0.25 mm I.D. with 0.5 μm of particle size). Both the injection and the detection port were kept at 110°C, while the column was operated at a constant temperature of 35°C. The H_2S peak generated from MT samples was readily identified by its retention time. All determinations were done in duplicate to ensure reproducibility.

Results and Discussion

Zn- and Cd-Complexes of Mammalian βMT1 and αMT1 Domains. Analytical data in Table 1 show that recombinant synthesis of the βMT1 and αMT1 domains as separate peptides in metal supplemented media renders S^{2-}-containing Zn- and Cd-species, in correspondence with the results obtained for the entire MT1 (14). In the case of the β clusters, the presence of the sulfide ligands is quantitatively more important for the cadmium than for the zinc preparations, whereas both α cluster preparations exhibit approximately equivalent sulfide content.

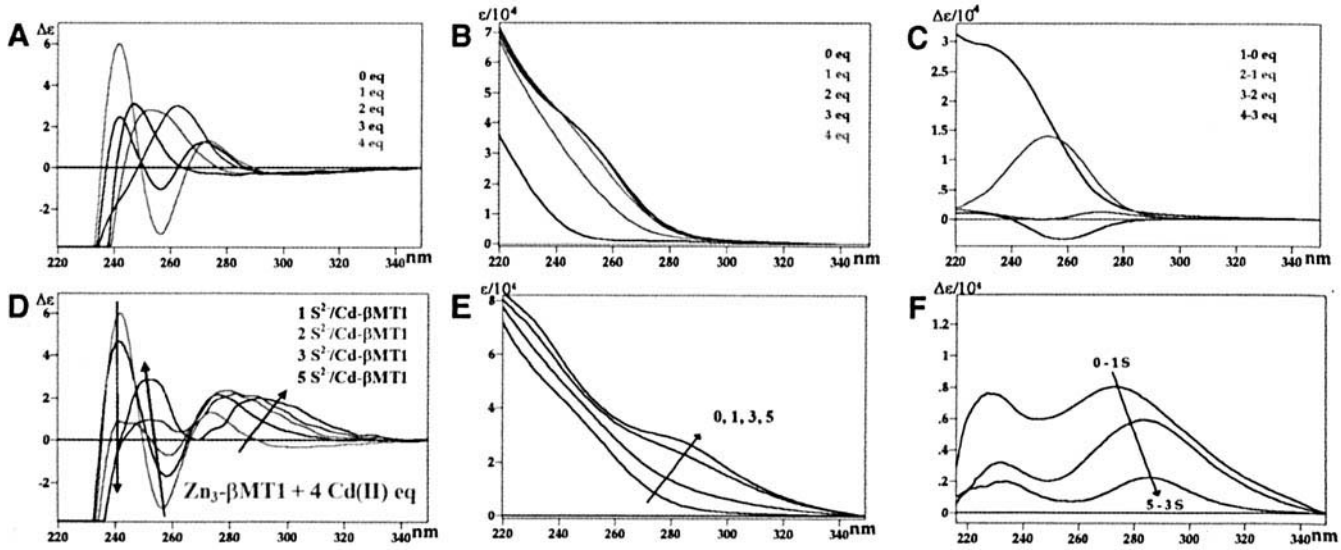

Figure 1. Effects of Cd(II) binding (A–C) and subsequent S^{2-} binding (D–F) on the circular dichroism (A, D), UV-vis (B, E) and UV-vis difference (C, F) spectra of recombinant mouse Zn_3-βMT1. The Cd(II) and S^{2-} to Zn_3-βMT1 ratios are indicated within each frame. The arrows show the evolution of the spectra when the indicated number of sulfide equivalents was added. Color figure available in on-line version of journal.

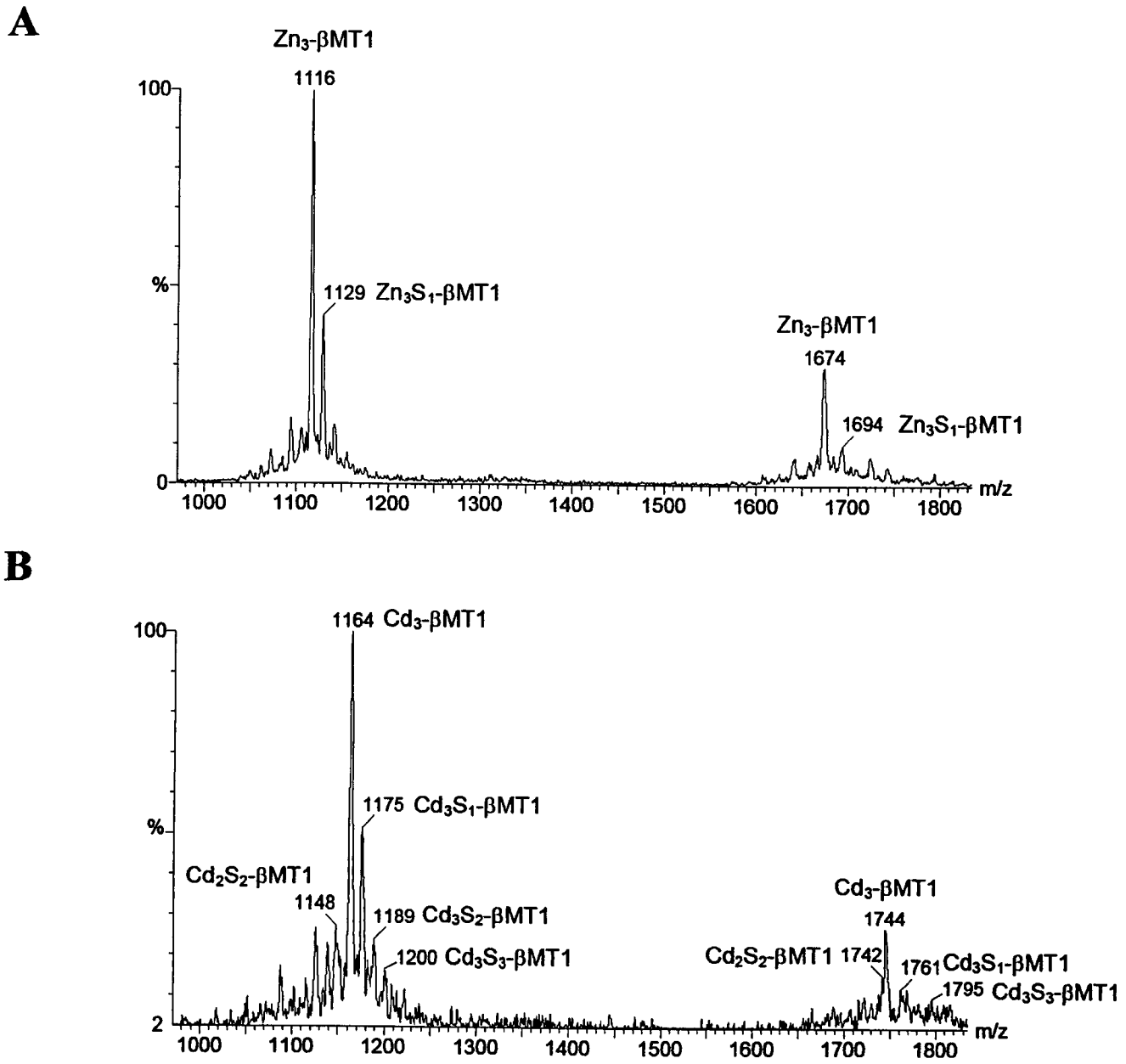

Figure 2. ESI-MS spectrum of the *in vivo* synthesized recombinant mouse Zn_3-βMT1 species (A) and ESI-MS spectrum recorded after the *in vitro* addition of four Cd(II) equivalents and one S^{2-} equivalent to Zn_3-βMT1 (B).

Given the significant content of S^{2-} ligands in the Cd-βMT1 preparation, and taking into account our previous report on divergent compositions of the Cd-βMT1 preparations depending on culture conditions (6), we decided to go further into this particular case. To this end we prepared Zn_3-βMT1, which was then titrated with a standard Cd(II) solution to a Cd(II)/Zn_3-βMT1 ratio of 4, calculated on the basis of the *conventional* ICP-AES quantification of the initial Zn_3-βMT1 sample. This procedure pursued two objectives. First, to reproduce the reported Cd titration of the Zn_3-βMT1 species (4), and second, to ensure an excess of Cd(II) over the canonical expected species, Cd_3-βMT1. Interestingly, the set of CD, UV-vis, and UV-vis difference spectra recorded (Fig. 1A, B, and C, respectively) fully reproduced those reported, although for lower metal-to-protein ratios. Interpretation of these results are rather straightforward, considering that the Zn_3-βMT1 solution used in this work contains a non-negligible amount of S^{2-}, as revealed by the analytical results (*cf.* Table 1) and also confirmed by the ESI-MS spectrum (Fig. 2A). Therefore, quantification of the initial Zn_3-βMT1 sample by *conventional* ICP-AES led to an overestimation of the actual MT concentration, and consequently the number of equivalents added during the titration were also overestimated (for a full explanation of *conventional vs. acid* ICP-AES MT quantification methodology, see 14). After the final stage of the Cd(II)/Zn_3-βMT1 titration, increasing amounts of S^{2-}, from one to five equivalents per Cd_3-βMT1, were added to the sample. Spectroscopic data (Fig. 1D, E, and F) and ESI-MS

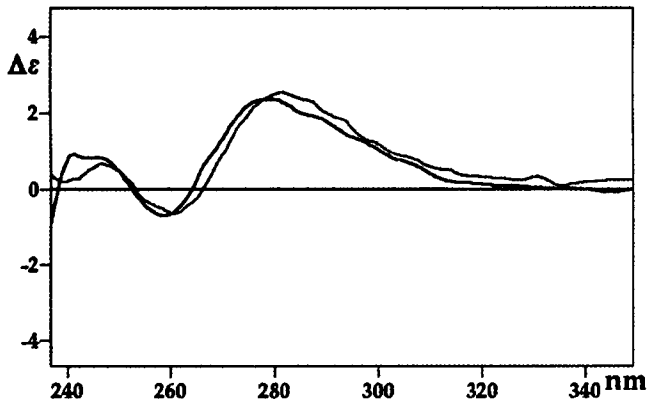

Figure 3. Comparison of the circular dichroism spectra of the *in vivo* synthesized Cd-βMT1 species obtained from a *large-scale* culture (6) (grey line) and that recorded after the *in vitro* addition of four Cd(II) equivalents and two S^{2-} equivalents to Zn_3-βMT1 (black line).

results (Fig. 2B) unequivocally evidenced the incorporation of S^{2-} ligands to the metal clusters. This implies the conversion of the 250-nm centred exciton coupling, attributable to the $Cd(SCys)_4$ chromophores, to a Gaussian band at the same wavelength, together with a redshift of the initially 275-nm centered band to *ca.* 300 nm. In parallel, UV-vis and difference UV-vis spectra also show increase in the absorptions between 260 and 320 nm, with a clear redshift of the maxima, in accordance with literature data correlating higher wavelength absorptions with bigger Cd-S crystallites (21).

The CD envelopes recovered after the *in vitro* additions of S^{2-} to the Cd_3-βMT1 sample closely resembled that previously obtained for the *large scale* recombinant synthesis of Cd-βMT1 (6). To be precise, the CD spectrum reported on that occasion is nicely reproduced by that corresponding to the addition of two S^{2-} equivalents to Cd_3-βMT1 (Fig. 3). In spite of the lack of conclusive results of the ESI-MS analysis on that occasion, our current results strongly suggest the presence of S^{2-} ligands in the Cd-βMT1 species recovered after large-scale synthesis, at an approximate ratio of 2 S^{2-}/βMT1. This significant presence of S^{2-} ligands per βMT1 peptide would also easily explain the low Cd/βMT1 stoichiometry then reported, in accordance with our verification that *conventional* (i.e., *non-acid*) ICP-AES measurements lead to an overestimation of the protein concentration in S^{2-}-containing MT samples, and thus to false low metal-to-protein ratios (14). A plausible explanation of the reasons determining large-scale synthesis yielding metal-MT species with increased S^{2-} content remains to be put forward. These productions are carried out in 50-liter fermentors, with good aeration systems (6). This implies that the *E. coli* culture grows on aerobiosis, and some of the cell responses to adapt to oxidative status may end up favoring metabolic pathways that increase intracellular sulfide availability.

Zn- and Cd-Complexes of Mammalian βMT4 and αMT4 Domains. Regarding the MT4 isoform, Table 1 shows that not only the entire protein, but also its two constituting β and α domains, contain sulfide ligands in their Zn- and Cd-species recovered after recombinant synthesis. As expected, the Cd-preparations show higher S^{2-} content than the Zn samples, and in particular, Cd-βMT4 shows the highest value found in any mammalian MT fragment. Interestingly, this allowed us to revisit some of the results we recently reported for this MT isoform. Hence, when the *in vivo* Cd-βMT4 preparations were characterized (9), we interpreted the ESI-MS data as corresponding to a mixture of Cd_3, Cd_2Zn_1, and Cd_1Zn_2 species. However, the ICP-AES figures somewhat questioned this possibility, since Cd(II) was the unique metal ion detected in the samples. By considering the data obtained for *in vivo* Cd-βMT4 in the current work, we can now suggest that all the assumed heterometallic Zn,Cd-βMT4 species were in fact Cd-βMT4 complexes that included sulfide ligands, on the basis of the mass equivalence between one Zn and two S. Consequently, the ESI-MS spectra of the *in vivo* Cd-βMT4 preparation should be reinterpreted as shown in Figure 4, which shows the coexistence of the Cd_3- and Cd_2S_2-βMT4 species. This reinterpretation of the ESI-MS results corresponding to the *in vivo* synthesis of Cd-βMT4 cannot be directly extended to the full-length MT4 protein, which also gave rise to a mixture of Zn,Cd-species, as in that case the ICP-AES data also confirmed their heterometallic nature. However, in view of the high S^{2-} content measured in the Cd-MT4 preparations (Table 1), it is sensible to postulate now that probably part of the alleged Zn,Cd-MT4 clusters are in fact Cd,S^{2-}-MT4 species.

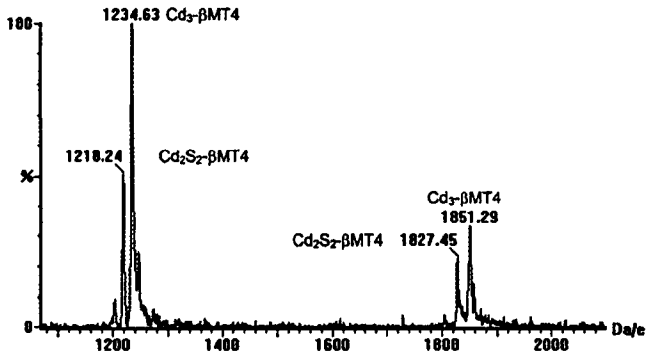

Figure 4. Reassignation of the ESI-MS spectrum of the recombinant mouse Cd-βMT4 preparation.

General Conclusions

This work, properly completing the results reported in (14), shows that recombinant synthesis of mammalian MT1 and MT4 domains in the presence of Zn(II) and Cd(II) gives rise to sulfide-containing clusters. The Cd-complexes have a higher number of sulfide ligands than the Zn-species, and among the former, those of the β domain higher than those of the α domain. Interestingly, βMT1 and βMT4 have been described by this group as Cu-thioneins (9, 10), with βMT4, which harbors the highest sulfide content among all the

peptides analyzed (Table 1), exhibiting a higher Cu-thionein character than βMT1. Consequently, it seems sensible to hypothesize that the higher the Cu-thionein character of a MT peptide, the higher the sulfide content in its divalent-metal, especially cadmium, complexes. This is also patently clear if considering the *Drosophila* MTs, MTN and MTO, both classified as Cu-thioneins (11, 12, 14). Maybe the higher predisposition of a polypeptide to accommodate the *small* Cu(I) ions in linear or trigonal coordination environments is correlated with a relatively higher difficulty in providing Zn and Cd divalent ions, and especially the *bulkier* Cd(II), with a strict tetrahedral coordination environment, and thus the participation of extra (nonproteic) ligands to stabilize their metal complexes would be significantly favored.

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