J Biol Inorg Chem (2011) 16:991–1009
DOI 10.1007/s00775-011-0827-2

MINIREVIEW

Zn- and Cu-thioneins: a functional classification for metallothioneins?

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Received: 3 May 2011 / Accepted: 25 July 2011 / Published online: 8 August 2011

Abstract This report intends to provide the reader with a deeper insight in the chemical, and extensively biological, characteristics of the metallothionein (MT) system. We have devoted nearly 20 years to the study of MTs and this has allowed us to form what we believe is a more complete picture of this peculiar family of metalloproteins. At the beginning of the 1990s, the landscape of this field was quite different from the overall picture we have now. Many researchers have contributed to the readjustment of this part of scientific knowledge. In our case, we implemented a unified method for obtaining MTs, for characterizing their metal-binding features, and for applying a unified research rationale. All this has helped to enlarge the initial picture that was mainly dominated by mammalian MT1/MT2 and yeast Cup1, by introducing approximately 20 new MTs. It has also allowed some characteristics to be clarified and examined in more detail, such as the cooperativity or the coexistence of multiple species in the metal-substitution reactions, the availability of Ag(I) or Cd(II) for use as respective probes for the Cu(I) and Zn(II) binding sites, the participation of chloride or sulfide ligands in the metal coordination spheres, and the feasibility of using in vitro data as representative of in vivo scenarios. Overall, the results yield enough data to consider new criteria for a proposal of classification of MTs based on MT metal-binding features, which complements the previous classifications, and that can shed light on the still controversial physiological functions of this peculiar superfamily of metalloproteins.

Keywords Metallothionein, Cu-thionein, Zn-thionein, biosynthesis · Metal binding, mass spectrometry, spectroscopic data · Protein engineering · Protein folding · Structure–function relationship

This article is part of a JBIC special issue on metallothioneins.

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State of the art of metallothionein research at the beginning of the 1990s

Metallothioneins (MTs) were discovered at the end of the 1950s when looking for the agent provoking Cd(II) accumulation in certain mammalian tissues [1], and soon received this name owing to their high Cys content and consequent capacity to bind heavy metal ions [2]. The first known MT was purified from horse kidney after the treatment of the organism with Cd(II) salts. Afterwards, another mammalian isoform and several subisoforms were identified and some other similar polypeptides, such as those found in crab [3], sea urchin [4], and fish [5], which shared the characteristics of the two initially isolated MT1 and MT2 proteins, were aggregated to the mammalian isoforms to constitute this superfamily we know today under the name "metallothionein" (MT).

After 30 years of MT research (1960–1990), two international conferences solely devoted to MTs—in 1978 [6] and 1985 [7]—and some good reviews [8–13], it seemed

that the landscape of this research field was quite well defined. There were many different MTs known (among the 42 entries in UniProtKB [14] at the end of 1990s, 26 corresponded to mammalian MTs, but also crab (two), yeast and fungi (five) fruit fly (two), pigeon (two), nematode (two), plaice (one), sea urchin (one), and cyanobacteria (one) MTs were found) and the purification methods from the native organisms were already well developed [15]. Also, an initial classification into three classes had been proposed [7], and a good number of spectroscopic techniques had been applied to the metal–MT systems, rendering valuable information [15]. Therefore, a literature search on MTs at the beginning/middle of the 1990s would have left the reader with the following general ideas, widely accepted at that time by the scientific community but, as shown later, not necessarily accurate:

1. Any polypeptide characterized by a low molecular weight (corresponding to 40–100 amino acids) and a high content of Cys residues (approximately 30%), which confer them with an increased metal-binding capacity, can be considered an MT.
2. They do not contain His or aromatic amino acids, a fact that facilitates their spectroscopic study.
3. All known MTs can be classified into three classes, depending on whether they are homologous to the mammalian MT1/MT2 isoforms (class I) or not (class II) or whether they are enzymatically synthesized peptides (class III or phytochelatins and cadistins) [16].
4. Higher organisms have bidominial MTs that bind Zn(II), whereas lower organisms synthesize monodominial Cu(I)-binding MTs.
5. All the apo-MTs are devoid of secondary structure elements (random coil). Polypeptide folding is achieved by metal binding to render the holo-MT forms.
6. The chemistry of the metal–MT systems is similar to that known for the metal–thiolate complexes, but is more complex because of the nuclearity of the final species.
7. The metal-substitution reactions for Zn-MT or the reconstitution of apo-MT preparations are quite fast and the number of metal equivalents added to a protein solution accounts for the final metal–MT stoichiometry, until the saturation of the protein is reached.
8. Ag(I) and Cd(II) can be used as respective models of Cu(I) and Zn(II) for the study of the metal-binding sites of MTs, particularly in those techniques that require isotopically active nuclei.
9. Some "magic numbers" are recurrent in the literature: especially seven divalent ions [Zn(II), Cd(II), Hg(II), Pb(II), etc.] and 12 monovalent ions [Ag(I), Cu(I)] for mammalian MTs.

Finally, it has to be taken into account that all research studies were, by necessity, undertaken under in vitro conditions, because the natural concentration of MT in tissues was too low to allow any type of analytical, spectroscopic, or chemical characterization of in vivo samples using the techniques developed at that time. Consequently, all results obtained in vitro were assumed to reproduce the putative in vivo performance of the polypeptides studied and were therefore considered representative of their physiological functionality.

This glance at the literature in the first three decades of MT research also reveals the intense work of several research groups, such as those led by (in alphabetic order and the most chemically oriented), Armitage, Kägi, Otvos, Petering, Shaw, Stillman, Vallee, Vasak, Weser, or Winge, as well as the limitations of the techniques and methods available. Purifying a sufficient amount of a given MT for study of it was, at that time, a difficult process, with many intermediate stages and low final yields, which meant scientists had to concentrate their efforts on the major MT isoforms of those organisms that were easier to handle (e.g., rats, rabbits, yeast). This explains why a high percentage of the publications in those decades were devoted to the characterization of the features of the MT1 and MT2 mammalian (rabbit, mouse, rat, or human) isoforms or of the yeast Cup1 protein. Actually, more than the 90% of our current knowledge of MTs (current research and accumulated literature) still refers to mammalian MTs and maybe a further 5% (getting up to 95% of the total number of publications available) comes from the yeast Cup1 MT.

Therefore, at the beginning of the 1990s, the landscape of the MT field was quite different from the overall picture we have now. Many researchers have contributed to the readjustment of this part of scientific knowledge, and all these contributions have been recently compiled in a review [17]. In our case, we implemented a unified method for obtaining MTs, for characterizing their metal-binding features, and for applying a unified research rationale. All this has helped to enlarge the initial picture that was mainly dominated by mammalian MT1/MT2 and yeast Cup1, through the analysis of approximately 20 new recombinant MTs. This has led us to propose an alternative classification for MTs, which complements the previous classifications, and that aims at deepening knowledge of this family from a functional point of view by shedding light on the still controversial physiological functions of this peculiar superfamily of metalloproteins.

Our first steps in the MT field

In the framework described in the previous section, with a solid background in the field of the chemical and structural

characterization of metal thiolates, and in the application of genetic engineering techniques, we started working with MTs in the middle of the 1990s. Taking into account that mammalian MT1 and MT2, together with yeast Cup1, only represented a very small fraction of the living organisms where MT peptides had been identified (all animal phyla, most fungi and plants, and some prokaryotes) (Fig. 1) and the huge heterogeneity observed in their amino acid sequences, especially among those of the denominated class II (Table 1), prompted us to devise and implement a new method that would allow us to study the very distinct, and otherwise difficult to obtain, MTs, and avoid the drawbacks associated with their purification from native organisms.

At that time, most of the spectroscopic and chemical studies on the metal-binding properties of MTs were performed with proteins isolated from native sources. However, it is difficult to obtain homogeneous native preparations, owing to the normal presence of several MT isoforms in most animal and plant species, the proteolysis generated during the purification steps, and the extremely low amounts of MTs synthesized in many organisms even under induction conditions. Synthetically prepared MTs were not a useful alternative either, owing to the inherent difficulties of synthetic preparation of Cys-rich peptides. The use of recombinant-DNA approaches was revealed as an optimum way for obtaining significant amounts of highly pure MTs of the most diverse organisms, and directly in the form of the desired metal complex. Despite this fact, at the beginning of the 1990s, the first attempts to synthesize mouse MT in *Escherichia coli* had limited success [18, 19], probably owing to translational and stability constraints of this singular eukaryotic protein in a prokaryotic environment. On the other hand, some cases of heterologous MT expression were satisfactorily achieved as a fusion protein in *E. coli* [20–22]. However, most of those studies only reported data on the synthesis and metal content of the fusion protein, or provided a rough evaluation of MT production by metal resistance and bioaccumulation in the host cells. Preparation of purified recombinant MT synthesized from improved recombinant expression systems allowed its characterization and use for refined spectrophotometric analyses, such as metal cluster formation in sea urchin MT [23]. Unfortunately, these expression systems were based on Cd(II) addition to the culture medium and therefore the recovered MT was

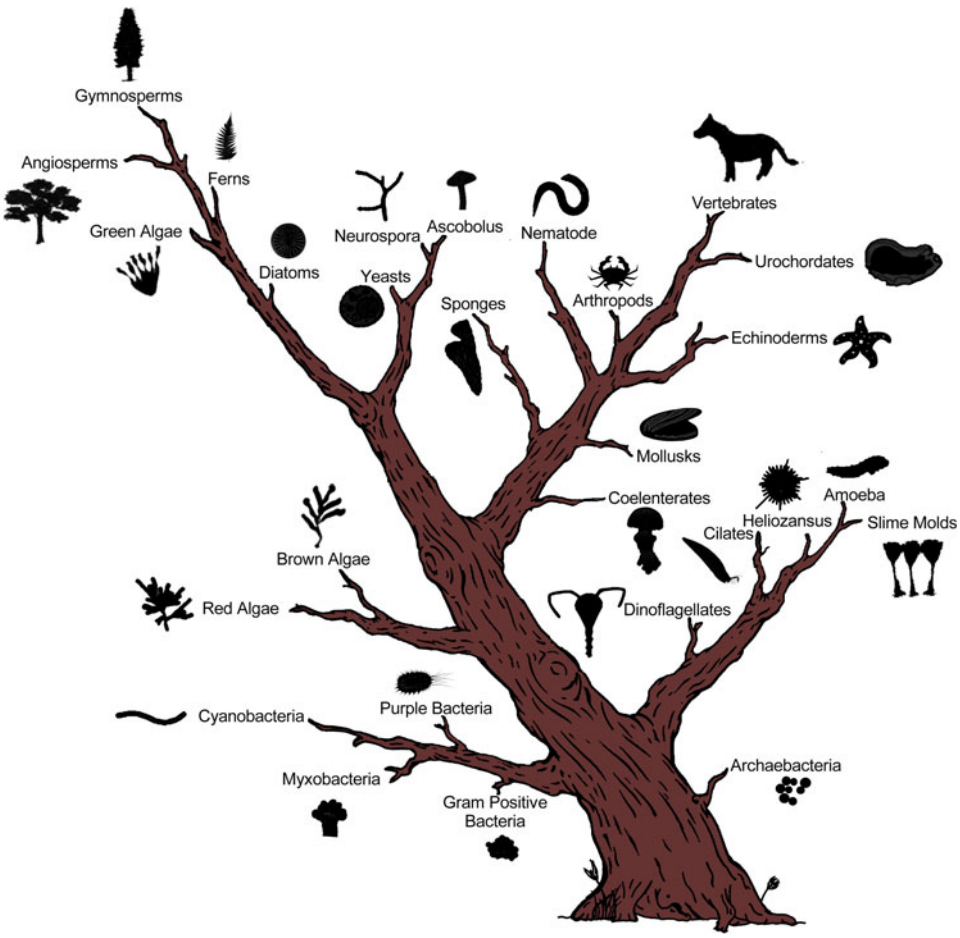


Fig. 1 The tree of life showing the diverse branches in which living organisms can be classified

Table 1 Examples of members of some of the 15 metallothionein (*MT*) families proposed by Binz and Kägi (http://www.biochem.unizh.ch/mtpage/classif.html) showing the high heterogeneity in length and primary structure of the MT sequences

Vertebrate	*M. musculus* MT1	MDPNCSCTTGGSCACAGSCKCKECKCTSCKK-CCSCCPVGCAKCAQGCVCKGSSEKCRCCA	(P02802)
Mollusca	*M. edulis*10MTIV	MPAPCNCIETNVCICDTGCSGEGCRCGDACKCSGADCKCSGCKVVCKCSGSCACEGGCTGPSTCKCAPGCSCK	(P80249)
	H. pomatia CdMT	MSGKGKGEKCTSACRSEPCQCGSKCQCGEGCTCAACKTCNCTSDGCKCGKECTGPDSCKCGSSCSCK	(P33187)
Díptera	*D. melanogaster* MtnA	MPCPCGSGCKCASQATKGSCNCGSDCKCGGDKKSACGCSK	(P04357)
Nematoda	*C. elegans* MT1	MACKCDCKNKQCKCGDKCECSGDKCCEKYCCEEASEKKCCPAGCKGDCKCANCHCAEQKQCGDKTHQHQGTAAAH	(P17511)
Ciliata	*T. thermophila* MTT1	MDKVNNNCCCGENAKPCCTDPNSGCCCVSETNNCCKSDKKECCTGTGEGCKWTGCKCCQPAKSGCCCGDKAKACCTDPNSGCCCSSKTNKCCDSTNKTECKTCECCK	(Q8T6B3)
Fungae and yeasts	*N. crassa* MT	MGDCGCSGASSCNCGSGCSCSNCGSK	(P02807)
	C. glabrata MT1	MANDCKCPNGCSCPNCANGGCQCGDKCECKKQSCHGCGEQCKCGSHGSSCHGSCGCGDKCECK	(P15113)
	S. cerevisiae CUP1	MFSELINFQNEGHECQCQCGSCKNNEQCQKSCSCPTGCNSDDKCPCGNKSEETKKSCCSGK	(P07215)
Prokariota	*Synechococcus* SmtA	MTTVTQMKCACPHCLCIVSLNDAIMVDGKPYCSEVCANGTCKENSGCGHAGCGCGSA	(P30331)
Plants (Type 1)	*A. thaliana MT1*	MADSNCGCGSSCKCGDSCSCEKNYNKECDNCSCGSNCSCGSNCNC	(P43392)
Plants (Type 2)	*A. thaliana MT2*	MSCCGGNCGCGSGCKCGNGCGGCKMYPDLGFSGETTTTETFVLGVAPAMKNQYEASGESNNAENDACKCGSDCKCDPCTCK	(P25860)
Plants (Type 3)	*A. thaliana MT3*	MSSNCGSCDCADKTQCVKKGTSYTFDIVETQESYKEAMIMDVGAEENNANCKCKCGSSCSCVNCTCCPN	(O22433)
Plants (Type 4 or EC)	*A. thaliana MT4*	MADTGKGSSVAGCNDSCGCPSPCPGGNSCRCRMREASAGDQGHMVCPCGEHCGCNPCNCPKTQTQTSAKGCTCGEGCTCASCAT	(P93746)

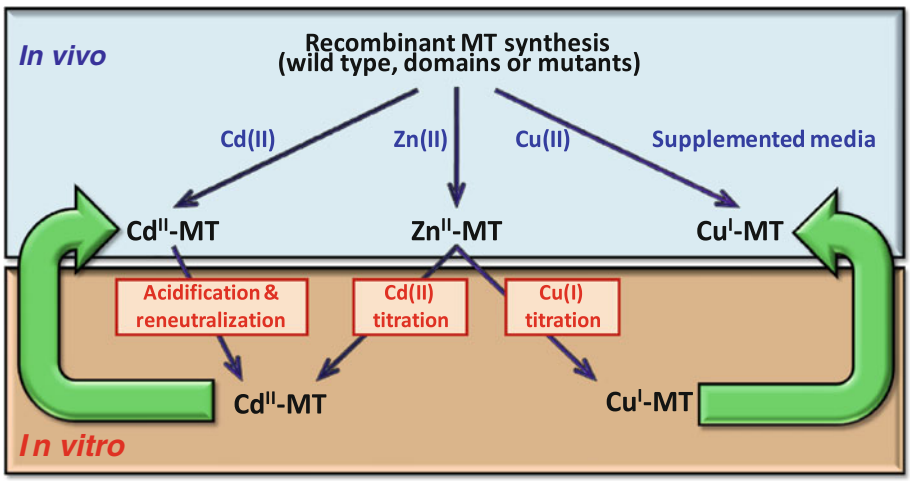
Cys are highlighted in *bold*, Cys triplets in *red*, His in *blue*, other aromatic residues in *pink*, and spacers are *underlined*. UniProtKB (Swiss-Prot) accession codes are included after each sequence

cadmium-complexed, thus requiring further manipulation to obtain the corresponding apo form, and from there the desired metal complex. In this scenario, we were successful in setting up a new expression system, based on the synthesis of glutathione *S*-transferase–MT fusion proteins, which when subsequently processed yielded metal–MT complexes that had been folded into the cell cytoplasm, and that therefore do not require further manipulation, but are representative of in vivo folding processes. Validation assays of this method were conducted with preparations of the entire recombinant mouse Zn_7-MT1, and that of the αMT1 (Zn_4S_{11}) and βMT1 (Zn_3S_9) domains, which showed equivalence with the already characterized corresponding native forms [24, 27]. Afterwards, this equivalence was invariably confirmed in different cases, in which characterization of the corresponding native form had been accomplished previous to our studies with recombinant MTs, significantly with, e.g., *Helix pomatia* (pulmonate snail) [25] or *Saccharomyces cerevisiae* (baker's yeast) [26] MTs.

The use of this method later allowed our group to synthesize high amounts of any MT (Table 2). It has also allowed the easy production of protein fragments or domains (Table 2), of site-directed mutant forms (Table 2), and of putative MT proteins, i.e., proteins which have been predicted in silico as open reading frames. This is the case of the amphioxus MTs, which are currently being characterized by our group.

The characterization of the in vivo conformed and of the in vitro generated metal–MT complexes of all these MTs has been attained by the use of spectroscopic and spectrometric techniques. UV–vis absorption spectroscopy and circular dichroism spectroscopy and its variants have been very useful owing to the information they provide as regards the nature of the metal-binding sites and on the protein folding about the metal ions. Also, the ever-increasing use of electrospray ionization mass spectrometry (ESI–MS) in this field has been crucial for a better understanding of the behavior of MTs when binding different types of metal ions, as it allows the molecular distribution of the species present in solution to be observed and identified, as well as their exact stoichiometry.

For more than 15 years we have been studying the metal-binding properties of a wide selection of MTs of distinct origin, covering most of the different branches of the tree of life, by always following a common approach that encompasses the steps shown in Scheme 1. Initially, the particular MT is recombinantly synthesized in *E. coli* cultures enriched with Zn(II), Cd(II), or Cu(II). The preparations obtained in this way are denominated in vivo Zn-MT, Cd-MT, and Cu-MT preparations, although they can contain either a single species or a mixture of metal–MT complexes, which can simultaneously be of homonuclear or heteronuclear nature. In vivo Zn-MT preparations are then titrated with Cd(II)- or Cu(I)-containing salts to generate what we call in vitro Cd-MT and Cu-MT preparations. These can also contain a single species or a mixture of complexes, both of homonuclear or heteronuclear nature. Also, in some cases, it is advisable to demetalate the in vivo Cd-MT preparations by acidification to produce the apo-MT form. This sample is then reneutralized to yield another in vitro Cd-MT preparation, which may, or may not, match the features of the former sample and that obtained by Cd(II) titration of the Zn-MT form. It is important to note here that during the years devoted to this work, we have realized that the analytical and chemical characterization of all these in vivo and in vitro preparations has to be attained by using all the spectroscopic and



Scheme 1 Experimental approach commonly used in our research groups to recombinantly produce MTs in diverse metal-supplemented media, the so-called Zn-MT, Cd-MT, and Cu-MT in vivo preparations, and the in vitro manipulations which produce the denominated Cd-MT and Cu-MT in vitro, the features of which are compared with those of the corresponding in vivo preparations

spectrometric techniques available in parallel, and by trying to match all the, sometimes discordant, results if a conclusive picture of the system is to be achieved.

Once these characterization steps have been overcome, the comparison of the features of the in vitro preparations with those corresponding to the in vivo synthesis provides information on (1) the "preferences" of the MT studied for the particular metal ions, (2) the feasibility of in vitro generation of samples analogous to those obtained in vivo, (3) the mechanisms of metal displacement operating in the system, and (4) the folding and unfolding pathways of the protein about the metal ions.

The exhaustive, parallel, and comparative analysis of the results achieved with all the distinct MTs studied has shed light on many, until recently unknown, features of the metal–MT system, which are detailed in the following sections. Now, and with all this knowledge in our hands, we are searching for some common links that will give us information about the evolutionary patterns underlying the apparent heterogeneity of the MT family.

Unexpected findings that draw a new landscape for the MT field

As mentioned already, our initial studies intended to ensure that recombinant techniques yielded metal–MT preparations that could reproduce the features and behavior of the samples obtained from native organisms. For this reason our first works were devoted to mouse MT1 and the corresponding independent α and β domains.

The recombinant Zn-MT1, Zn-αMT1, and Zn-βMT1 preparations, obtained from the respective pGEX expression constructs, were very pure, highly concentrated, and showed intense and good spectroscopic fingerprints. Furthermore, the findings of the study of their metal-binding abilities perfectly matched, and also enlarged, the previous knowledge available [24, 28, 45–47, 50, 51], all this validating our methodological approach for the MT synthesis.

Once we started supplementing the *E. coli* cultures with metal ions other than Zn(II), i.e., Cd(II) or Cu(II), to respectively obtain in vivo folded mouse Cd-MTs or Cu-MTs, we started to obtain some unexpected results. This was, for instance, the case for βMT1, which yielded distinct preparations with Cd(II) depending on the volume of the *E. coli* cultures [48], or at least this was our interpretation at that time. Later, we observed that the volume of the culture was in fact only revealing the influence of another factor never considered before, the degree of oxygenation of the cultures and/or the presence of sulfide ligands in the metal–MT complexes (see later).

Also, the study of the binding abilities of the recombinant mouse MT1 peptide with distinct metal ions alerted us that some of the general ideas accepted until then required further attention. This was the case for (1) the number of metal ions required to achieve certain metal-to-MT stoichiometries, (2) the reliability of the spectroscopic data for proposal of cooperative processes in the metal-loading or metal-displacement processes in MTs, (3) the time required to reach a thermodynamically stable species—or a mixture of species—and (4) the need for Zn(II) ions to maintain/favor certain metal–MT species, something that elsewhere we attributed to a structural role of Zn(II) and/or to the denominated zinc-template effect. All these aspects will be treated in detail in the following sections. These sections will also include other findings that have arisen from the study of nonmammalian MTs, and that have substantially changed our perception of the MT world. In any case, it is our purpose to highlight here that the finding of all these unexpected results in the study of MTs has helped us to reconsider how the metal binding studies should be performed, in order to avoid a biased view of the system or misinterpretation of some of the results.

Table 2 Enumeration of all the recombinant peptides from different organisms studied in our group up to now: wild-type proteins, engineered fragments, and mutant forms of metallothioneins

Organisms	Proteins	References
Wild-type proteins		
Mus musculus	MT1	[24, 27, 28]
	MT2	–[a]
	MT3	–[a]
	MT4	[29]
Drosophila melanogaster	MtnA	[30]
	MtnB	[31]
	MtnC	[32]
	MtnD	[32]
	MtnE	[33]
Homarus americanus	MTH	[34]
Quercus suber	QsMT	[35–37]
Gallus gallus	ckMT	[38]
Saccharomyces cerevisiae	Crs5	[39]
	Cup1	[26]
Tetrahymena pyriformis	TpyMT1	[40]
Mytilus edulis	MT-10-IV (MeMT)	[41]
Caenorhabditis elegans	CeMT1	[42]
	CeMT2	[42]
Helix pomatia	HpCdMT	[25]
	HpCuMT	[25]
Cornu aspersum	CaCdMT	[43]
	CaCdCuMT	[43]
	CaCuMT	[43]
Strongylocentrotus purpuratus	SpMTA	–[a]
	SpMTB	
Branchiostoma floridae	BfMT1	–[a]
	BfMT2	
Megathura crenulata	McMT	[44]
Tetrahymena thermophila	MTT1	–[a]
	MTT2	
	MTT3	
	MTT4	
	MTT5	
Cryptococcus	cMT1	–[a]
	cMT2	
Glycine max	GmMT1	–[a]
	GmMT2	
	GmMT3	
	GmMT4	
Engineered fragments		
Mus musculus	αMT1	[45]
	βMT1	[45–48]
	αMT2	–[a]
	βMT2	
	αMT3	–[a]
	βMT3	
	αMT4	[29]
	βMT4	
Caenorhabditis elegans	Nt CeMT1	[42]
	Ct CeMT1	
	Nt CeMT2	[42]
	Ct CeMT2	
Strongylocentrotus purpuratus	αSpMTA	–[a]
	βSpMTA	
	αSpMTB	–[a]
	βSpMTB	
Mutant forms		
Mus musculus	MT1	[49]
Caenorhabditis elegans	CeMT2	[42]
Helix pomatia	HpCuMT	–[a]
Quercus suber	QsMT	[37]
Saccharomyces cerevisiae	Crs5	[39]

[a] Unpublished results

Cooperative processes and metal-to-MT stoichiometry in relation to the number of metal equivalents added to an MT

Before ESI–MS became a routine procedure to provide information on the molecular distribution of the species formed during the studies of the metal-binding abilities of MTs, different spectroscopic techniques (UV–vis absorption spectroscopy, CD spectroscopy, magnetic CD spectroscopy, etc.) were the most used methods [52]. These allowed the metal-displacement reactions undergone by a Zn–MT complex to be followed when increasing amounts of metal ions were added. By this time, NMR spectroscopy was already providing information about nonaverage peptide structures, even in the case of zinc-loaded MTs, but preparations containing a unique or a clearly major species were required. Probably on the basis of existing knowledge of the chemical reactivity between transition metal ions and thiolate ligands, and because spectroscopy only provides average information for the system, the only way to determine the metal-binding capacity of each MT studied was to reach its saturation point by adding increasing amounts of a metal ion solution. This approach led to the assumption that the stoichiometry of the final species formed—which was supposed to be the unique complex present in solution—was directly related to the amount of metal (i.e., the number of metal equivalents) added to the initial protein solution. Nowadays, it is easy to find examples in the literature where the formation of a particularly stable species dominates, in a certain way, the speciation of the distinct steps of a metal-addition process.

A clear example is that provided by the titration of recombinant mouse αMT1 with Ag(I) [28]. Here the ESI–MS data revealed, from the very beginning of the titration, the presence of the Ag_9-αMT1 species, this suggesting a special importance of this complex. A similar behavior was observed during the addition of Cu(I) to Zn_3-βMT4 [29], where the addition of only 4 eq of Cu(I) provoked the formation of a practically unique Cu_6-βMT4 species, which remained as the major complex in solution, even in the presence of excess Cu(I). Finally, it is worth mentioning that the addition of some equivalents of a metal ion with high affinity for Cys does not necessarily imply displacement of the previously bound ion which shows lower affinity for thiolate ligands. This is the case observed on the addition of Cd(II) to Zn_7-MeMT (*Mytilus edulis* MT-10-IV isoform) [41]. Here the addition of seven Cd(II) yielded a heterometallic Zn_1Cd_6-MT species that required the addition of Cd(II), until a total of 15 eq, to displace the remaining Zn(II) bound to the protein (see later).

Furthermore, it was also implicit in most of the literature data of the first decades that cooperative processes [53–55] in the formation of metal–MT species were proposed when isosbestic and/or isodichroic points were observed during the titration of MTs with metal ions. The implementation of the use of ESI–MS in the study of the above-mentioned systems has called into question these intriguing assumptions, as one of its major advantages is that it allows the observation and identification of the species present in solution at every stage of the titration [50], thus facilitating the distinction between the cooperative [56, 57] and the noncooperative [28, 50] processes. Interestingly, two works with the mouse MT4 isoform performed by different researchers have reveled that formation of the Cd_7-MT4 species proceeds cooperatively when Cd(II) is added to apo-MT4 [57], but noncooperatively when the Zn(II) initially bound in Zn_7-MT4 is replaced by Cd(II) [29].

Rapidity of the metal-substitution reactions

The general rapidity of the inorganic reactions, in comparison with the organic ones, and the reaction rates reported in the literature for the formation of most of the metal thiolates, together with the fast substitution times observed in the binding of Cd(II) to mammalian MTs [58], provoked the widely accepted assumption that the binding of metal ions to MTs occurs in a rapid way, let us say between 10 and 30 min. However, when Cd(II) was changed for other metal ions, we soon realized that this was not always necessarily accurate. The first evidence for this assertion was obtained when we analyzed Ag(I) binding to recombinant mouse MT1 and its independent α and β domains [28]. Here, the evolution of the CD spectrum corresponding to a certain number of equivalents added to the protein indicated to us that some stages of the titration required more than 1 h to allow the existing species to reach adequate folding about the metal–MT clusters formed. The need for a long stabilization time was even more evident when the Hg(II) binding to recombinant mouse MT1 was studied [51]. The addition of a certain amount of Hg(II) to a solution of MT1 yielded a CD fingerprint that continuously changed with time. These changes could last for hours or even days (Fig. 2a), and revealed that the folding of the protein was evolving probably owing to the well-known lability of the $Hg–S_{thiolate}$ bonds. Also, a time-dependent evolution of the Pb-MT and ZnPb-MT species formed when Pb(II) binds to recombinant mouse MT1, αMT1, and βMT1 has been observed by means of ESI–MS [59] and recorded spectrophotometrically (Fig. 2b). As stated above, the lability of the $Hg–S_{Cys}$ and $Pb–S_{Cys}$ bonds provides a reasonable explanation for these observed facts; however, it is not so easy to justify the evolution with time observed in the binding of 4 eq of Cu(I) to Zn-Cup1 [26], the paradigmatic Cu-thionein of yeast, which requires a stabilization time of 24 h (Fig. 2c) to produce a mixture of Cu_4-Cup1 and Cu_8-Cup1 species by total displacement of the initially bound Zn(II). Consequently, our observations suggest that, although in most of the cases the reaction rates of metal binding to MTs are probably going to be fast, the time requirements of the reaction must be checked beforehand. This is just to ensure that the formation of the thermodynamically stable species is going to be achieved at each step of the metal addition, and that the metal–MT species formed have reached their definite folding before further amounts of metal ion are added, thus avoiding the operation of distinct reaction pathways.

Importance of the purity of the Zn-MT samples used

One of our main concerns when analyzing the metal-binding abilities of MTs was to obtain very pure in vivo Zn-MT preparations in order to be able to perform in vitro studies. We soon realized that this statement of "very pure" had different meanings for chemists and biologists. For a chemist it means a unique metal–MT species in solution, whereas biologists consider a sample pure if it contains only the desired protein, irrespective of how many metal–MT complexes are present in the sample. Once we moved on from the mouse MT1 isoform, which yields a very pure and unique Zn_7-MT1 species when recombinantly produced, the protein biosynthesis of the Zn-MT preparations of other MTs yielded "biologically" pure protein samples that however contained several Zn–MT complexes. This is the case for the CeMT2 isoform of *Caenorhabditis elegans* [42], which is synthesized in vivo as a mixture of major Zn_6-CeMT2, Zn_5-CeMT2, and

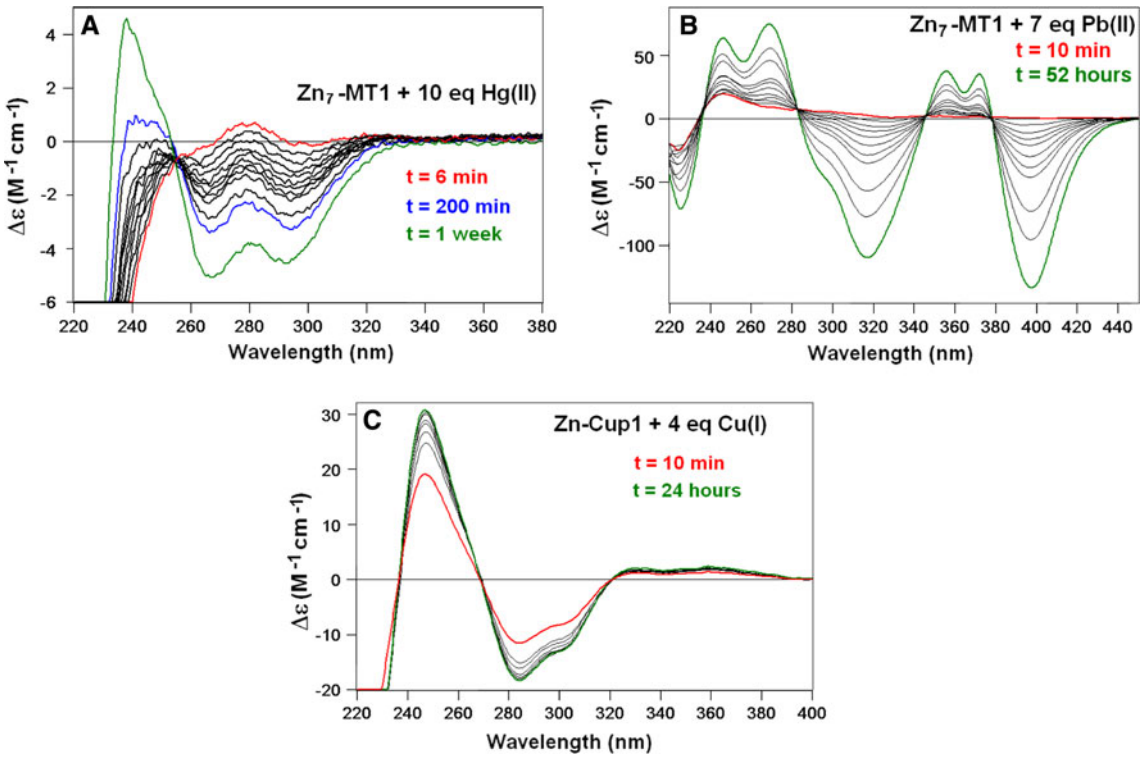


Fig. 2 Evolution with time of the circular dichroism (CD) spectra of samples corresponding to the addition of different amounts of metal ions to the zinc-loaded forms of distinct MTs: **a** 10 eq of Hg(II) and **b** 7 eq of Pb(II) added to mouse Zn_7-MT1, and **c** 4 eq of Cu(I) added to yeast Zn-Cup1

Zn_4-CeMT2 complexes (Fig. 3a). Interestingly, in the case of its recombinant production in cadmium-enriched media, it rendered a unique Cd_6-CeMT2 species (Fig. 3b). Our in vitro studies revealed that, regardless of the speciation of the original zinc sample, addition of 6 eq of Cd(II) yielded a unique and well-folded Cd_6-CeMT2 species (Fig. 3c), thus reproducing the properties of the in vivo preparation (Fig. 3d) and showing the preference of this isoform for cadmium binding rather than for zinc binding in accordance with the formation of a unique and thus stable Cd-CeMT2 species.

The role of zinc as a structural ion

The structural role that Zn(II) plays in several metalloproteins in which it is coordinated to four Cys residues has been well documented in the literature [60]. Nevertheless, and in spite of the typical $Zn(SCys)_4$ coordination environments that this metal ion adopts in most MTs, a structural role has not been widely proposed for Zn(II) in these metalloproteins. In fact, we were, to our knowledge, the first authors to propose that Zn(II) has a key role in the structure of the physiologically relevant mouse Cu-MT1 species [27] and to highlight the requirement of Zn(II) for MT1 to maintain its bidominial structure during the Zn/Ag replacement process [28].

More interestingly, our studies with nonmammalian MTs have revealed the importance of the Zn(II) ions in some MTs, which refuse to have these ions replaced by other incoming metals, even in spite of the higher affinity of the latter for the SCys binding sites. This is in agreement with some studies demonstrating that not all the Zn(II) ions bound to MTs show the same binding strength [61, 62], and could be related either to an important structural role of Zn(II) in these proteins or to deeply buried $Zn(SCys)_4$ sites that are "protected" by the protein folding about the metal clusters. Just to mention a few examples, we recall the Cd(II) binding studies conducted with the Zn_7–MT complexes of the *C. elegans* nematode [42] and *M. edulis* mollusk [41]. CeMT1 and CeMT2, the two MT isoforms identified in *C. elegans*, exhibit a clear preference for divalent metal ions, rather than for Cu(I). Nevertheless, the analysis of their metal-binding abilities has shown a marked preference of CeMT1 for Zn(II), whereas CeMT2 prefers Cd(II). Here, both the biosynthesis of CeMT1 in a cadmium-enriched medium and the Zn/Cd replacement process in Zn_7-CeMT1 after a significant excess of Cd(II) had been added yielded a unique Zn_1Cd_6-CeMT1 species (Fig. 4a). In parallel, the analysis of the addition of Cd(II) to a solution of Zn_7-MeMT, the MT-10-IV isoform of the *M. edulis* mussel, illustrates the reluctance of the protein to liberate at least one of its initially bound Zn(II) ions

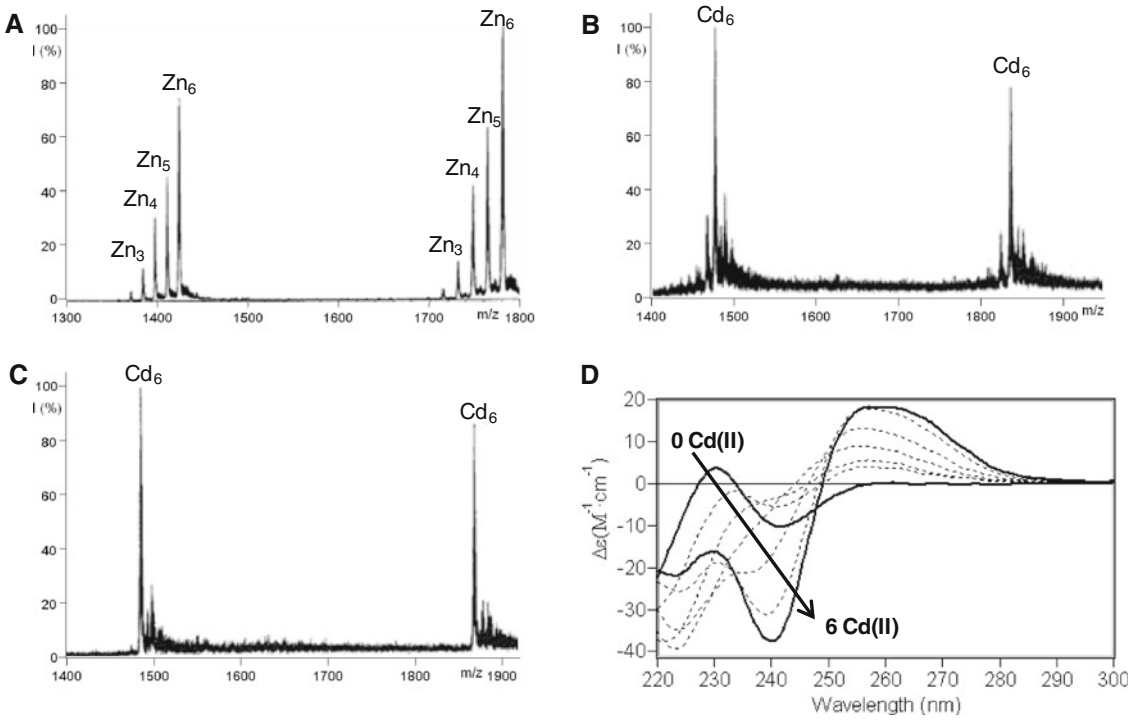


Fig. 3 Spectrometric data corresponding to **a** in vivo Zn-CeMT2, **b** in vivo Cd-CeMT2, and **c** in vitro Cd-CeMT2 preparations. **d** The evolution of the CD spectra of the in vivo Zn-CeMT2 after the subsequent addition of Cd(II) ions until the formation of a Cd_6-CeMT2 species, which shows the same CD fingerprint as the in vivo Cd-CeMT2 preparation

(Fig. 4b). Therefore, after the addition of 9 eq of Cd(II), the major species is Zn_1Cd_6—followed by Cd_7-MeMT. These complexes invert their abundance relationship after the addition of 11 eq of Cd(II), but 15 Cd(II) equivalents are needed to make the Zn_1Cd_6 totally convert into the expected Cd_7-MeMT species, more than double the number of Cd(II) ions needed stoichiometrically.

Thus, summarizing, the reluctance of an MT to replace Zn(II) by another incoming metal ion of higher affinity for its binding site is a feature not shared by all MTs, and points out some particularities and potential functionalities of these proteins. Consequently, this is one of the bases we have considered for the proposal of a new classification of the members of this large family of metalloproteins (see later).

Ag(I) and Cd(II) as respective models of Cu(I) and Zn(II)?

The experience accumulated in the field of metal thiolates [63], their membership of the same groups of the periodic table, which confers on them similar binding features when binding to sulfur-donor ligands, and the existence of NMR-active isotopes for silver and cadmium has resulted in these two metals being commonly used when trying to solve the difficulties associated with the NMR structural determination of metalloproteins containing Cu(I) or Zn(II). In the field of MTs, Cd(II) has traditionally been used as a probe for Zn(II)-binding MTs, whereas Ag(I) has been employed as a model for Cu(I) [64].

As regards the use of Ag(I) as a model of Cu(I) coordination in MTs, some time ago we demonstrated [28], by metal titration of the recombinant mouse MT1 isoform, that Ag(I) behaves similarly to Cu(I), i.e., they both give rise to species of identical stoichiometry and close folding about the metal ions, only when at least one Zn(II) ion still remains bound to the protein. Once the last bound Zn(II) has been displaced by the incoming metal ions, or when these are being added to the apo-MT1, Ag(I) clearly behaves differently from Cu(I). However, we were not the first researchers to realize the drawbacks of using Ag(I) as a model for Cu(I) as stronger arguments became apparent during the NMR determination of the 3D structure of the first monovalent-metal-ion-binding MT: the *Saccharomyces cerevisiae* Cup1 isoform [65, 66]. The structures of Cu_7-Cup1 and Ag_7-Cup1 were determined and compared. Finally, the resolution of the crystal structure of Cu(I)-Cup1 [67] revealed a Cu_8-Cup1 instead of a Cu_7-Cup1 species. Obviously, it was also shown that the Cu-SCys connectivities were not the same as those depicted from the Ag_7-Cup1 structure.

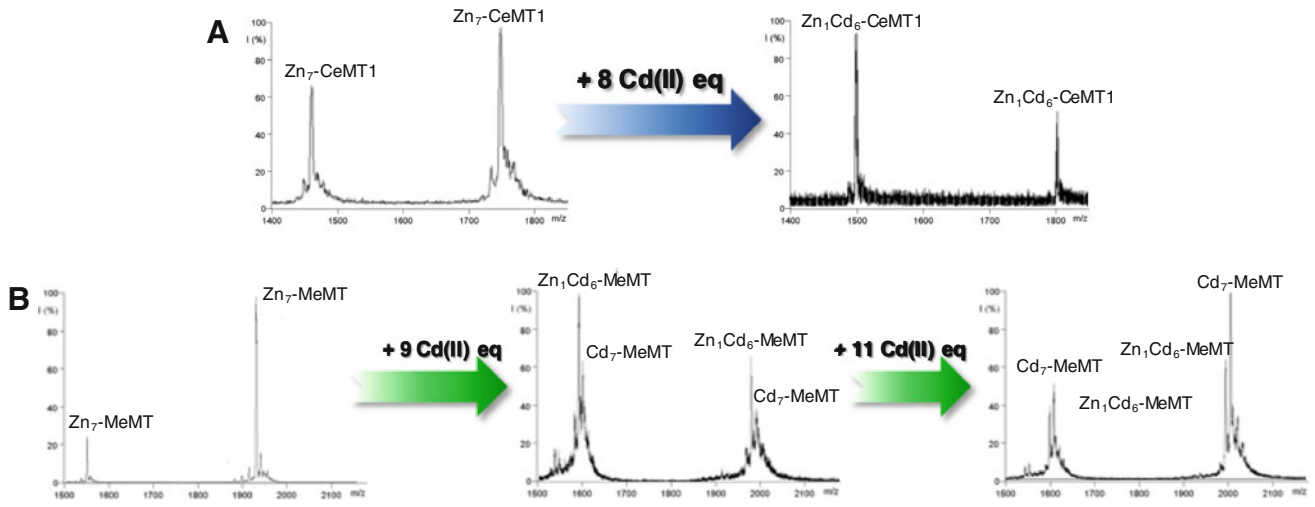


Fig. 4 **a** Effect of the addition of Cd(II) to the *C. elegans* Zn_7-CeMT1 species, where the remaining Zn(II) ion can be observed by electrospray ionization mass spectrometry. **b** For *M. edulis* MT, the substitution of Zn(II) by Cd(II) from the initial Zn_7-MeMT preparation renders a Zn_1Cd_6-MeMT species, which coexists with Cd_7-MeMT even in a clear Cd(II) excess

As regards the use of Cd(II) as a model for Zn(II) in their binding to MTs—and particularly in terms of NMR structural determinations [68–70]—it should be noted that it has been, and still is, a widespread and frequent strategy. This approach is valid for the most studied MTs, namely, mammalian MT1 and MT2 isoforms and related subisoforms, as well as for other MTs which can been directly related to Zn(II) binding (Zn-thioneins, see later). But there are many cases in the recent literature that do not support this assumption. Most of the examples showing differences between Zn(II) and Cd(II) in MTs are related to the presence of S^{2-} ligands, a fact that will be further discussed later.

Probably, the most interesting case supporting this idea is that found after the bioproduction of Cup1, a well-known Cu-thionein, in the presence of Cd(II) [26]. Here, we observed that the in vivo recombinant synthesis of Cup1 in a zinc-supplemented medium (Zn_4-Cup1 as the major species) was completely different from the native and recombinant synthesis in cadmium-supplemented media (mainly Cd_5-Cup1), especially in terms of the number and stoichiometry of the species formed, as well as in their spectroscopic features (Fig. 5). Here, the presence of additional S^{2-} ligands in the Cd(II) productions was the main differential characteristic. Interestingly, the in vitro replacement of Zn(II) by Cd(II) in the initial Zn_4-Cup1 preparation rendered samples with the same features as those obtained in vivo, thus confirming that the observed differences between the Zn(II) and Cd(II) productions are due to the binding preferences of the protein and are not an artifact associated with the synthesis or purification procedures.

Other examples can be found when analyzing the binding features of other Cu-thioneins such as mouse MT4 [29], *Drosophila melanogaster* MT isoforms [30–32], *H. pomatia* HpCuMT isoform [25], and *Quercus suber* QsMT [35, 37]. Even considering the high diversity of sequences among them, and their different metal-binding properties, they exhibit some interesting common features that must be taken into consideration: in all cases, they display a higher preference for Cu(I) than for divalent metal ions; their recombinant production in zinc(II)-supplemented media yields mixtures of species of different compositions; and also, the presence of S^{2-} ligands is observed in the samples obtained with Cd(II).

All these examples make it obvious that Cd(II) is not always a good model of Zn(II) in MTs, especially in the case of Cu-thioneins, and this is associated with the fact that regularly MTs do not show isostructural zinc and cadmium forms (see the next section).

Nonisostructural Zn-MT and Cd-MT forms

As already detailed, the recombinant production of any desired MT enables it to be synthesized under in vivo conditions and loaded with any desired metal ion. This allows a comparison between the physiologically relevant Zn-MT and Cd-MT forms of all MTs studied. Interestingly, and regardless of obvious facts, such as those cases in which the number and/or stoichiometry of the species formed clearly differ, we are in a position to affirm that among the MTs we have studied, only recombinant mouse MT1 and sea urchin SpMTA give rise to preparations that on the basis of their spectroscopic fingerprints can be expected to

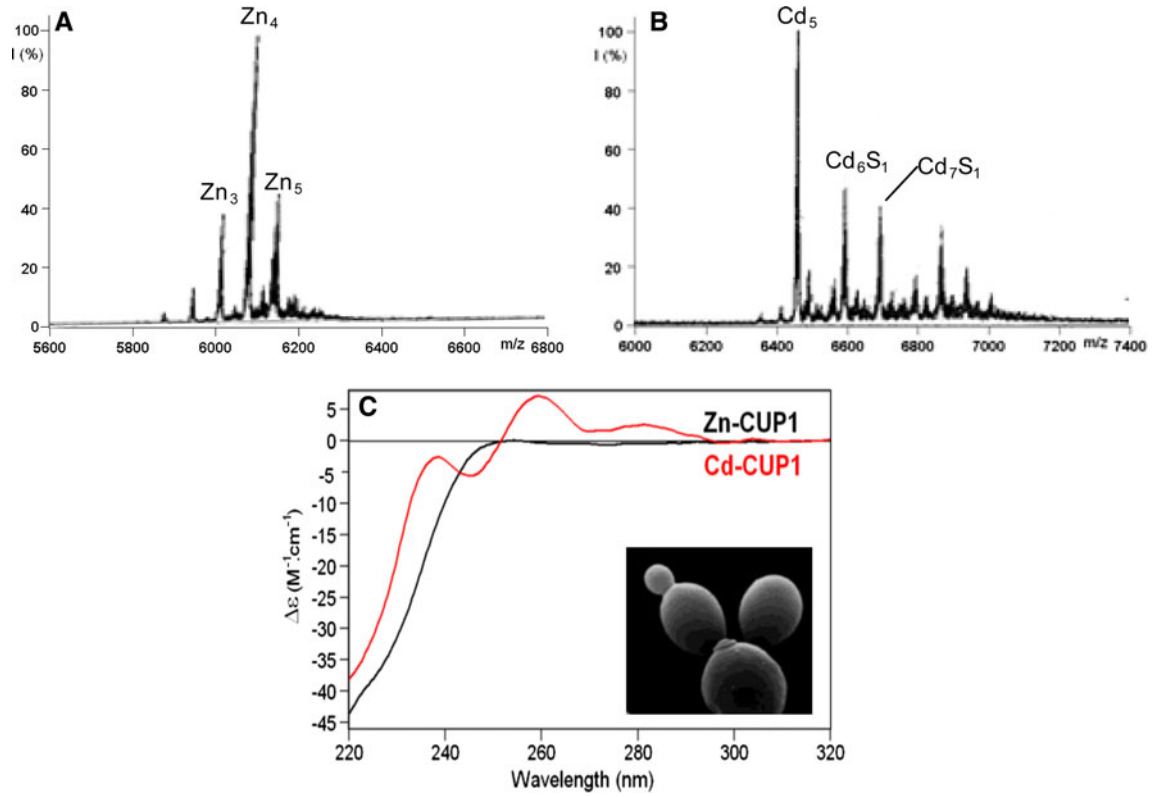


Fig. 5 Deconvoluted mass spectra of recombinantly synthesized yeast Cup1 isoform in **a** zinc-enriched and **b** cadmium-enriched media, and **c** comparison of the CD spectra of both preparations

contain isostructural Zn-MT and Cd-MT complexes. This statement is based on the well-accepted idea that similar CD fingerprints centered at the corresponding metal-specific wavelengths [i.e., at approximately 240 nm for Zn(II) and at approximately 250 nm for Cd(II)] suggest analogous chromophores and therefore similar 3D structures, whereas different CD envelopes inform about metal–MT complexes showing distinct 3D structures. As can be observed in Fig. 6a, the two types of preparations show comparable CD spectra that can lead to the above-mentioned conclusion. In contrast, Fig. 6b shows the spectroscopic CD fingerprints of the Zn-MT and Cd-MT bioproductions of *C. elegans* CeMT1 and CeMT2 isoforms [42], *M. edulis* MeMT [41], *Tetrahymena pyriformis* TpyMT [40], and *D. melanogaster* MtnA [30], which clearly differ among them.

In vivo versus in vitro conformed species

In vitro reconstitution of an apo form and/or replacement of zinc by cadmium in MTs are common procedures when 3D structural determination techniques that require high-purity samples, such as NMR or X-ray diffraction studies, are used. It is very important to highlight that when one uses these practices the in vivo folding of a single/particular MT cannot always be reproduced in vitro.

One striking example that illustrates the different folding of the in vivo constituted and the reconstituted in vitro species when binding the same metal ion comes from the recombinant MeMT-10-IV isoform (MeMT) of *M. edulis*. This protein has allowed us to show how, upon zinc coordination, the polypeptide folds in vivo into highly chiral and stable Zn_7–MeMT complexes, with an exceptional reluctance to fully substitute cadmium(II) for zinc(II). In vivo cadmium binding leads to homometallic Cd_7–MeMT complexes that, surprisingly, were shown to structurally differ from any of the in vitro prepared Cd_7–MeMT complexes (Fig. 7) [41]. These experimental data suggest that factors other than the affinity or specificity of a particular MT to bind a precise metal ion must take part in the folding of certain MTs.

Metal specificity of MT isoforms

For a long time, the general belief about the metal specificity of a particular MT and somehow inherently accepted among the MT community was again based on the experience accumulated with mammalian MT1/2 isoforms and yeast Cup1. Therefore, the assumption of all MTs with similar Cys content and distribution in their amino acid sequence to those of the mammalian MT1/2 behaving

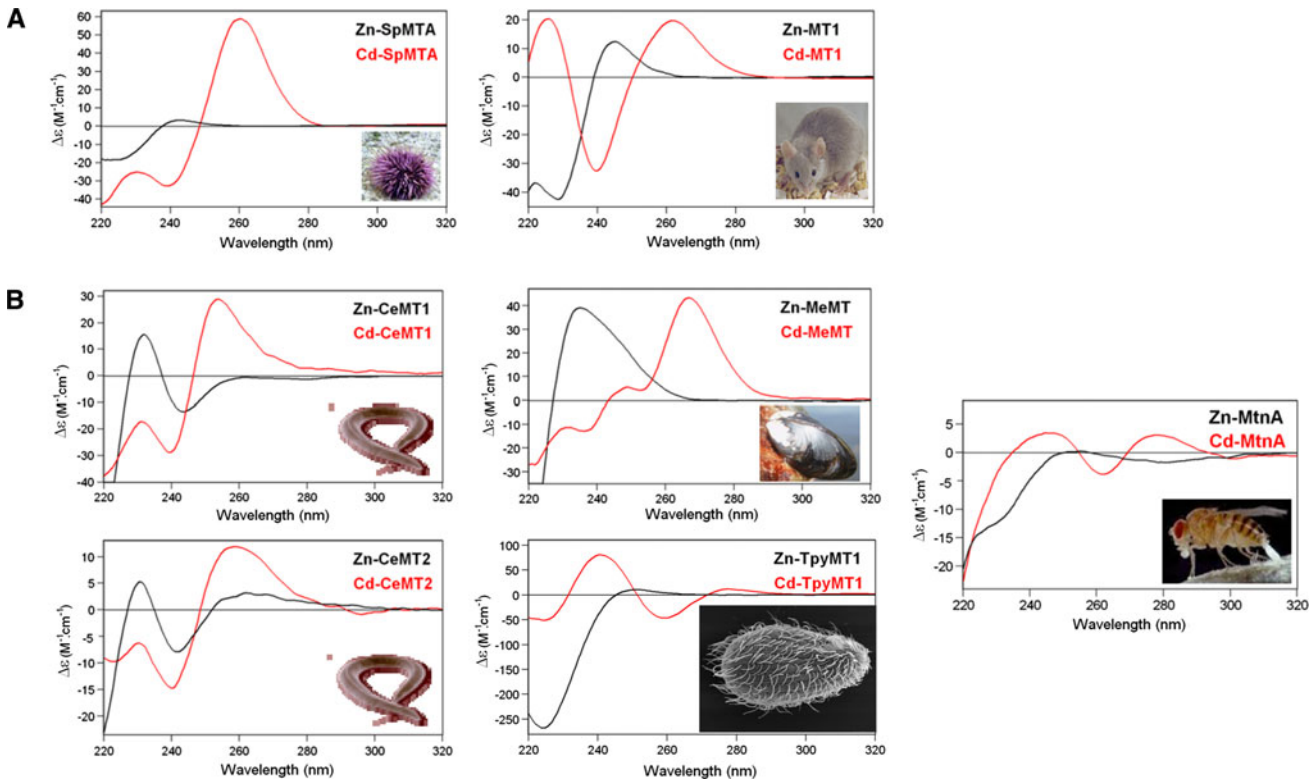


Fig. 6 Comparison of the CD spectra of the Zn-MT and Cd-MT in vivo preparations of **a** mouse MT1 and sea urchin SpMTA, which suggest isostructurality of their metal–MT complexes, and **b** *C. elegans* CeMT1 and CeMT2 isoforms, *M. edulis* MeMT, *T. pyriformis* TpyMT1, and *D. melanogaster* MtnA, whose respective CD fingerprints reveal structural differences between their zinc and cadmium complexes

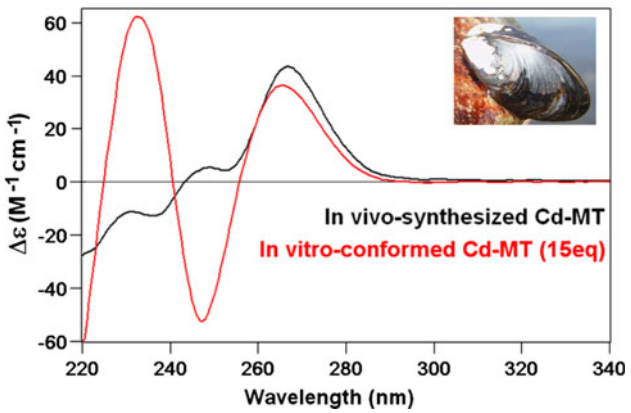


Fig. 7 Comparison of the CD spectra of the Cd_7–MeMT complexes. The differences between them are due to the in vivo (*black*) or in vitro (*red*) origin of the preparation

similarly and thus exhibiting similar metal specificity, mainly to divalent metal ions, is implicit in the literature. A similar reasoning was used for those MTs belonging to lower organisms and similar to the paradigmatic copper-binding yeast Cup1 isoform.

Nevertheless, the phylogenetic analysis and the characterization of the metal-binding abilities of the mouse MT4 isoform [39], which shows a high similarity with MT1 and MT2 (Table 3), provided enough arguments to reject the previous assumption. In fact, MT4 had already been suggested to display distinct physiological roles when compared with the other MT isoforms, mainly because of its specific expression pattern [71]. Our results, based on the biosynthesis of MT4, αMT4, and βMT4, demonstrated the much higher specificity of both the entire protein and its two independent fragments for Cu(I) than for MT1. Furthermore, MT4 was also shown to be less effective for Cd(II) binding than MT1. Consequently, the MT4 peptide was the first vertebrate MT to be reported to exhibit clear Cu(I) specificity, which suggests its Cu-thionein character. Subsequently, in 2004, we suggested that the metal specificity in MTs was interestingly driven by the noncoordinating amino acids, a fact that in some ways is difficult to understand from a strictly chemical point of view.

Our team has recently reported similar findings in this direction, which support the idea of the role of the noncoordinating amino acids in the determination of the MTs metal specificity [25]. The Roman snail, *H. pomatia*, has been shown to produce two MT isoforms [72] which exhibit differences in terms of tissue production, metal response synthesis, and, interestingly, distinct in vivo metal

Table 3 Comparison of the four major mouse MT isoforms

Cys are highlighted in yellow and the aromatic residue is in *purple*. Protein sequences were obtained from UniProt KB

specificity. One of these isoforms was identified as the cadmium isoform (HpCdMT) after its purification as Cd_6-HpCdMT from animals exposed to cadmium. The other isoform was obtained as a Cu_{12} species after a copper diet, and thus is considered the copper-specific isoform (HpCuMT). The most intriguing fact of the *H. pomatia* MT system is that both isoforms encompass 18 perfectly conserved Cys. The search for a plausible explanation of their divergent native metal load took into account their distinct tissue locations and the differences in the distribution of copper and cadmium in the organism; but the answer to this question came from recombinant studies. The biosynthesis of HpCdMT and HpCuMT in cultures enriched with their cognate metals produced the expected metal–MT complexes, i.e., Cd_6-HpCdMT and Zn_6-HpCdMT for the cadmium-specific isoform and Cu_{12}-HpCuMT for HpCuMT. Conversely, all attempts to biosynthesize the proteins with their noncognate metals always ended up with heterogeneous samples containing mixtures of metal–MT complexes, sometimes of a heteronuclear nature.

A further case illustrating the importance of the noncoordinating residues in metal specificity is found in the MT system of another terrestrial snail, *Cornu aspersum*, which produces up to three different isoforms [73]. The three isoforms share a similar Cys content, and a practically identical distribution of the Cys residues in their primary structure, but again each of them exhibits particular in vivo metal specificity either for Cu(I) or for Cd(II).

Overall, all the examples given support the proposal that a high similarity in number and position of the Cys residues does not guarantee two MT peptides behaving similarly towards a specific metal ion, or showing the same metal specificity, as the Cys residues are not the unique determinants of the metal-binding abilities of an MT. Therefore, it can be affirmed that metal–MT specificity comes from the primary structure of MTs and not from either their metal ion availability or their Cys content or position.

Role of His residues

It is well known that His residues can act as ligands in metalloproteins. However, in the case of MTs their participation was ignored for years because the initially known MTs lacked this residue. Concomitantly, with the increase in number of members of the MT family, the number of sequences including this amino acid was enlarged, and consequently the possibility of their contribution to metal binding became apparent. The first strong evidence of His participation in metal coordination came from a cyanobacterial MT (named SmtA), the NMR structure of which evidenced the direct participation of not one but two His imidazoles in the metal cluster of Zn_4-SmtA [74]. A similar finding was obtained in the recent determination of the 3D structure of wheat Zn_6-E_c-1, which interestingly shows an isolated and unprecedented $ZnCys_2His_2$ binding site [75].

Unfortunately, 3D structures are not available for metal complexes of all known MTs, and consequently there is a need for tools to provide evidence of His participation in these cases. The commonly used spectroscopic and spectrometric techniques have allowed us to devise some methods for the analysis of putative His participation in metal binding.

The two MT isoforms of the model nematode *C. elegans*, CeMT1 and CeMT2, exhibit similar metal-binding abilities [42] although they differ in the number of His residues present in their sequences: whereas CeMT1 contains four His and 19 Cys, CeMT2 has only one His and 18 Cys. Application of our methodological approach (vide infra) complemented with the design, synthesis, and study of some directed mutants (either of their presumed fragments or of the whole polypeptide) lacking His residues in their sequences allowed the conclusion that "the presence of histidine residues precludes these MTs behaving as Cu-thioneins, as would otherwise be according to their global protein sequence similarities." Interestingly, in this case the participation of the His residues was experimentally determined, by means of ESI–MS, through the study of the reaction of the distinct peptides with diethyl pyrocarbonate, which mainly reacts with available amino and imidazole groups. The analysis of the results suggested that only three of the four His of CeMT1 were participating in metal binding, whereas the unique His residue of CeMT2 was totally free to react with diethyl pyrocarbonate. As a

consequence, it was proposed that the role of His could not be devoted only to the coordination of the metal ions in MTs, but must also be devoted to the fine-tuning of their metal-binding specificity.

Another way in which His can have a role in MTs was proposed after the analysis of the binding features of chicken MT (ckMT) [38]. The results obtained not only provided evidence of the Cd–His coordination as responsible for pH-dependent CD absorptions at approximately 250 nm, but also suggested a novel intermolecular His-mediated dimerization process when the independent αckMT domain is exposed to excess Cd(II).

Additional ligands in the metal–MT complexes

It is widely accepted that Cys residues, present in higher amounts in MTs than in other proteins, are mainly responsible for the metal-binding abilities of MTs. These, together with His in some cases (vide infra) normally provide the sulfur and nitrogen donor atoms that metal ions require to satisfy their coordination environments. However, our investigation of a number of distinct MTs has led us, in some cases, to observe the participation of other inorganic ligands in the metal–MT complexes.

Chloride ligands

Although not very often, the participation of chloride ions as additional ligands in the metal–MT complexes has been reported. In Cd_7-MT2, it was associated with putative ATP binding regulation [76], and the observation of characteristic CD and UV–vis absorptions at approximately 240 nm allowed the participation of chloride ions in *D. melanogaster* MtnB [31] as well as in recombinant mouse MT4 [29] to be proposed. To shed light on the role of the chloride ions in the metal clusters of MTs, recombinant productions of mouse MT1 and MT4 isoforms in Zn(II)- and Cd(II)-supplemented media, as well as in vitro zinc/cadmium replacements in the presence and absence of chloride, were performed [77]. The results obtained confirmed the spectroscopic evidence (absorption at approximately 240 nm) for the participation of the chloride ions as additional ligands, suggesting that their contribution depends on each particular MT, and that it is mainly associated with the metal binding to MT1.

Evidence of S^{2-} as a third component of the metal–MT complexes

The participation of sulfide anions as additional ligands in MTs was first described in the class III, enzymatically synthesized, phytochelatin peptides [78] which produce the so-called crystallite particles. However, this remained unprecedented in the rest of the MTs until the evidence collected from the recombinant biosynthesis of *Q. suber* QsMT [35].

Here, and once again, our purpose to recombinantly produce MTs from very distinct organisms in diverse metal-loaded forms provided us with very interesting results, at the same time as giving us a lot of problems, until we unveiled the underlying reasons. Therefore, the synthesis of most of the analyzed MTs started providing a set of experimental data that were initially misunderstood. In their analysis we obtained (1) extremely low metal-to-protein ratios from inductively coupled plasma–atomic emission spectroscopy data, (2) unassignable peaks in their ESI–MS spectra, (3) unattributed absorption bands in CD and UV–vis spectra, and (4) extremely high reactivity towards 5,5′-dithiobis-2-nitrobenzoic acid. The clues provided by former studies conducted by Dameron et al. [79] with phytochelatins and a simple test tube reaction with $AgNO_3$ in acidic conditions enabled confirmation of the presence of labile S^{2-} ions in some of our preparations [80, 81].

Consideration of the participation of sulfide anions as additional ligands provided an explanation for all the bizarre results mentioned that were obtained when characterizing the cadmium-loaded forms of distinct MTs (Fig. 8) and provided us with a clear indication of their presence: the absorptions observed at approximately 280 nm in the CD and UV–vis spectra (Fig. 8a), which disappear with the acidification/reneutralization of the samples (Fig. 8b). Interestingly, the presence of these additional ligands was also observed in some zinc-loaded forms, but never in the Cu-MT preparations.

The initial reluctance of the scientific community to accept the presence of sulfide ligands in some metal–MT complexes was overcome when we were able to demonstrate that not only the recombinant but also the native preparations can contain these ligands, thus showing that their presence is not an artifact of the recombinant production [26]. Furthermore, the observation that the higher the Cu-thionein character (see later) of an MT, the higher its sulfide content in its cadmium-loaded forms has provided us with a further criterion for our proposal of classification of MTs [82].

Importance of the degree of oxygenation of the transformed *E. coli* cultures

Previous literature reports have shown that the synthesis of both MT isoforms of yeast, Cup1 and Crs5, depends on culture oxygenation. Specifically, anaerobiosis causes an intracellular copper accumulation sensed by Ace1, which triggers the expression of the MT target genes, whereas

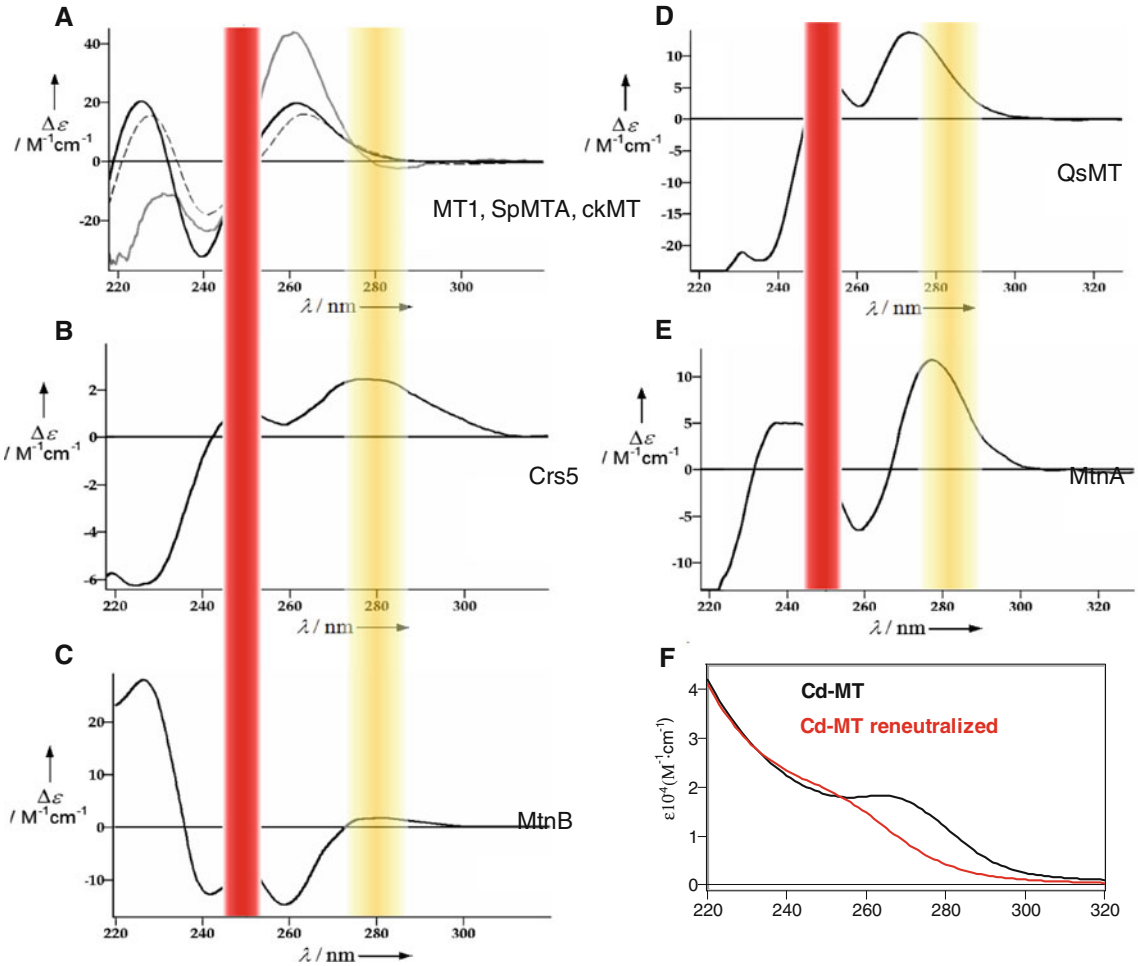


Fig. 8 a–e Representative CD spectra of distinct Cd–MT preparations from different organisms exhibiting the characteristic absorption features of the $Cd(SCys)_4$ chromophores at approximately 250 nm (*red*) and the absorptions attributed to the presence of $Cd_x(S^{2-})_y$–MT species at approximately 280 nm (*yellow*) [80]. **f** UV–vis spectra of a recombinantly produced Cd-MT species which contains sulfide ligands before (*black line*) and after (*red line*) an acidification and reneutralization cycle, showing the loss of the shoulder at approximately 280 nm attributed to the $Cd–S^{2-}$ chromophores

aerobiosis induces the loss of the cytoplasmic copper and thus the return of MT genes to their noninduced status [83]. It has also been shown that intracellular copper levels depend on the aerobic/anaerobic growth status, both in yeast [83] and in *E. coli* [84].

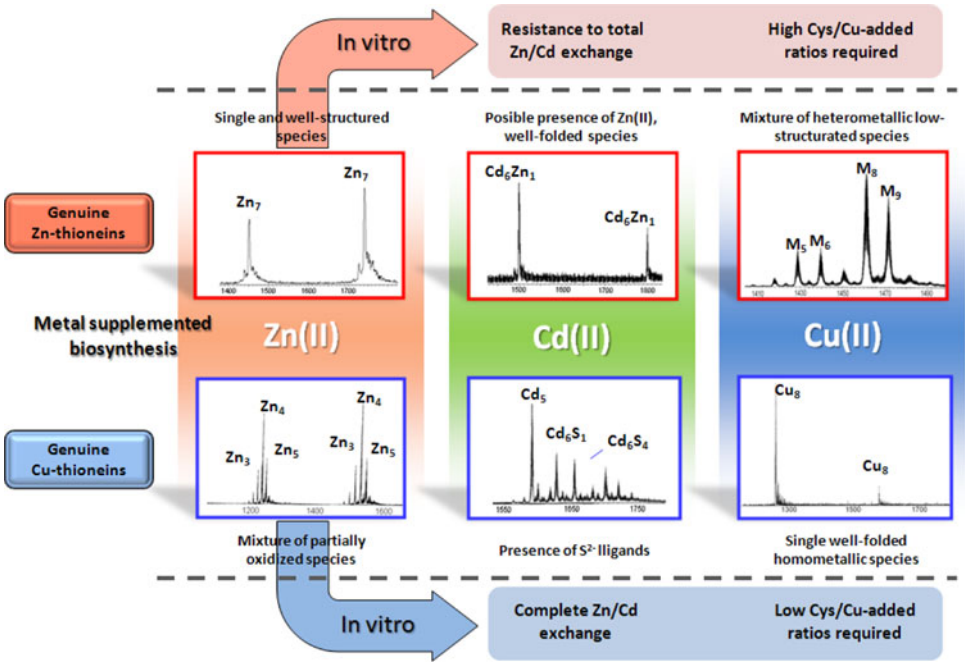
Interestingly, during the recombinant synthesis of Crs5 [39] in copper-supplemented media we realized that the homoometallic or heterometallic nature of the synthesized species was dependent on the aeration conditions of the cultures. When Crs5 was synthesized by cells kept at maximum aeration, heterometallic Zn,Cu-Crs5 complexes exhibiting the lowest copper-to-zinc ratio (2.8 Cu and 2.6 Zn per Crs5) were obtained. An intermediate scenario was provided by O_2-bubbling applied only during protein synthesis, which also yielded heterometallic Zn,Cu-Crs5 but with a higher copper-to-zinc ratio (5.5 Cu and 1.7 Zn per Crs5). Finally, small-scale cultures, where aeration was kept to a minimum, enabled homometallic Cu-Crs5 species to be recovered. Each of the samples recovered in the different situations exhibited particular and differentiated characteristics (CD fingerprints and metal speciation). The analysis of the different stages of a Cu(I) titration of Zn-Crs5 revealed a full correspondence between these three physiological situations and distinct steps of the zinc/copper replacement pathway, suggesting that the in vivo copper-binding abilities of an MT may depend on the aerobiosis/anaerobiosis status of the producing host cells and/or organs (either native or heterologous). Thus, a dual behavior of some MTs may reflect the aerobiotic/anaerobiotic environment in which they are synthesized. This oxygenation dependence on the nature of the metal–MT species biosynthesized has also been observed in other MTs [29, 37, 42] which exhibit a behavior analogous to that of Crs5, and led us to propose a step gradation of the metal preference among the recombinant MTs we have studied so far (see later).

Towards a comprehensive concept/idea of the behavior of MTs based on their Zn-thionein or Cu-thionein character

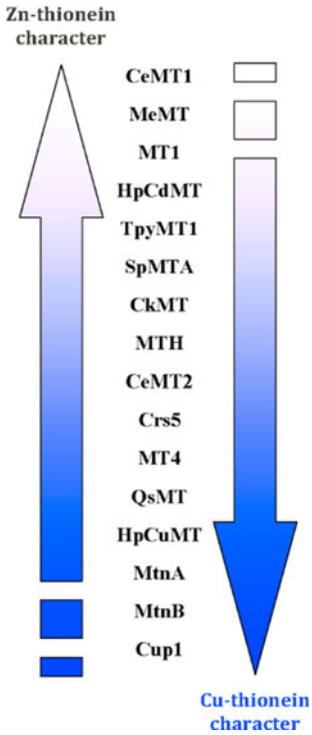
As mentioned before, MTs were first classified into three main classes on the basis of their sequence homology to horse MT1 [85]. A second classification, proposed by Kägi and coworkers [86], was based on taxonomic criteria. This classification led to as many MT families as taxons existing in the animal and vegetal kingdoms, without providing evidence of their putative functionality. About 10 years ago, we proposed a third type of classification of MTs [34] with the aim of introducing criteria related to their metal-binding abilities, i.e., molecular functionality grounds. This classification was based on the combined consideration of different results concerning the metal ion coordination performance of each MT peptide: (1) the metal–MT complex features when the proteins were recombinantly produced in zinc-, cadmium- and copper-supplemented media; (2) the analysis of protein sequence similarity; and (3) the type of metal ions inducing the corresponding MT gene in the native organism. Therefore, MTs were classified into two different groups: the so-called Zn-thioneins and Cu-thioneins. The final aim of this proposal rested on the idea that Zn(II) and Cu(I), the two ions physiologically bound to MTs, show dissimilar coordination preferences. The distinct coordination environments they will provoke, even for the same ligand (i.e., a specific MT), will drive the protein to adopt a particular folding, which on the basis of the structure–function relationship will conform the metal–MT aggregate for a distinct function depending on its load with Zn(II) or Cu(I).

The MTs we classified as Zn-thioneins (also Cd-thioneins) are characterized by the formation of homometallic species when they are produced in zinc- or cadmium-enriched media, exhibiting a high folding degree and mainly forming one prevalent complex with a specific and expected stoichiometry. However, when they are produced in copper-supplemented media, the Zn-thioneins give rise to the formation of several heteronuclear Zn,Cu-MT species with low folding and high degree of cysteinic thiol group oxidation—and subsequent disulfide bond formation—in which the metal content varies depending on the synthesis conditions. On the other hand, the MTs we denominated Cu-thioneins are characterized by yielding several coexisting metal–MT species when they are produced in zinc- or cadmium-enriched media, without yielding a single and predominant species, showing some diversity in folding and oxidation degree, and particularly those obtained from cadmium-enriched cultures presented a subpopulation of complexes including additional ligands (Cl^-, S^{2-}). The biosynthesis of Cu-thioneins in copper-supplemented cultures gives rise to the formation of homometallic Cu–MT complexes, which normally show a high degree of folding and low oxidation, forming one prevalent complex with the expected stoichiometry.

This initial proposal of dichotomic MT classification in Zn- or Cu-thioneins worked well until we sufficiently enlarged the number of MTs studied. The discovery of the presence of sulfide ligands in some Cd-MT preparations



Scheme 2 The characteristic features that, respectively, show the genuine Zn-thioneins and the genuine Cu-thioneins when recombinantly synthesized in different metal-supplemented media and when in in vitro metal-replacement studies the initially Zn(II)-loaded forms are treated with Cd(II) or Cu(I) solutions



Scheme 3 Gradation properties of the MTs from diverse organisms as regards their zinc- or copper-binding preferences ranging from genuine Zn-thionein to extreme Cu-thionein proteins, and indicating their opposite Zn-thionein versus Cu-thionein character

[80], the dependence of the homometallic or heterometallic nature of some biosynthesized Cu-MTs with the oxygenation degree of the cultures [39], and some other features arising from the in vitro metal binding studies made us fine-tune this previous scheme by proposing MTs should be classified on the basis of a step gradation between the two extreme situations: genuine Zn-thioneins and strict Cu-thioneins [82, 87]. Hence, we located all the MTs with behaviors between the two extreme cases by taking into account four different grounds: (1) the presence/absence of Zn(II) in the biosynthesized Cu-MT species, (2) the number of Cu(I) equivalents (Cys-to-Cu ratio) needed to in vitro reproduce the biosynthesized Cu-MT species, (3) the presence/absence of S^{2-} and/or Zn(II) in the biosynthesized Cd-MT species, and (4) the in vitro reluctance shown by the Zn-MT forms to totally replace Zn(II) by Cd(II) [82]. Therefore the Zn-thionein or Cu-thionein attributes of any considered MT, which are opposite each other, are established by taking account of the features displayed schematically in Scheme 2. Extreme Zn-thioneins render unique and well-folded Zn-MT species, retain, both in vitro and in vivo, Zn(II) in their Cd-MT preparations, and yield mixtures of heteronuclear Zn,Cu-MT species in presence of copper, which are easily reproduced in vitro by the addition of small amounts of Cu(I) to the Zn-MT forms. Conversely, genuine Cu-thioneins are synthesized as unique and homonuclear Cu-MT species in copper-rich media, contain sulfide ligands in their Cd-MT aggregates (the higher the Cu-thionein character of an MT, the higher the S^{2-} content of its cadmium complexes), and lead to mixtures of Zn-MT species when biosynthesized in the presence of Zn(II).

As shown in Scheme 3, the *C. elegans* CeMT1 isoform is the most genuine Zn-thionein we have studied, whereas the *S. cerevisiae* Cup1 isoform has been classified as the strictest Cu-thionein. Between these two extreme situations, 14 more MTs have been classified. These show intermediate behaviors, although they can be easily ordered on the basis of the four grounds mentioned before. For example, the mouse MT1 isoform has been classified close to genuine Zn-thioneins, whereas *D. melanogaster* MtnA and MtnB show a clear Cu-thionein character. The *Homarus americanus* MTH and the *C. elegans* MT2 isoforms have been positioned in the middle of the classification, with no clear preference for Zn(II) or Cu(I). Significantly, these MT isoforms appear in the root of phylogenetic trees, so it is logical to assume that they may reflect primeval, rather undifferentiated forms. In other cases, physiological requirements may have imposed a stricter metal specificity, and then paralogous isoform generation would have accounted for extremely specific MT isoforms [25].

Acknowledgments We wish to thank the Spanish Ministerio de Ciencia e Innovación for its continued financial support, and particularly for the current projects BIO2009-12513-C02-01 to S.A. and BIO2009-12513-C02-02 to M.C. The authors are members of the Grup de Recerca de la Generalitat de Catalunya, reference 2009SGR-1457, and the COST Action (EU) CM0603 on Free Radicals in Chemical Biology.

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