# Metallothioneins: unparalleled diversity in structures and functions for metal ion homeostasis and more†

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Metallothioneins have been the subject of intense study for five decades, and have greatly inspired the development of bio-analytical methodologies including multi-dimensional and multi-nuclear NMR. With further advancements in molecular biology, protein science, and instrumental techniques, recent years have seen a renaissance of research into metallothioneins. The current report focuses on *in vitro* studies of so-called class II metallothioneins from a variety of phyla, highlighting the diversity of metallothioneins in terms of structure, biological functions, and molecular functions such as metal ion specificity, thermodynamic stabilities, and kinetic reactivity. We are still far from being able to predict any of these properties, and further efforts will be required to generate the knowledge that will enable a better understanding of what governs the biological and chemical properties of these unusual and intriguing small proteins.



## 1 Introduction

Metallothioneins (MTs) are ubiquitous, intriguing low-molecular weight proteins that usually reside inside cells, but in higher organisms can also occur in extracellular media such as blood plasma and cerebrospinal fluid. They are aptly named, as their sulfur content is very high, and because they are the proteins with the overall highest metal ion binding capacity. Typically, the combined metal and sulfur content of fully metallated metallothioneins ranges between *ca.* 10–20% of total protein mass.

This high density of metal and sulfur is achieved by the incorporation of characteristic metal–thiolate "clusters". Metallothioneins can in principle bind a variety of metal ions, but the most important *in vivo* binding partners are Zn(II), Cu(I) and Cd(II).

Since the discovery of the first metallothionein in 1957 by Margoshes and Vallee,[1] 11709 publications on metallothioneins have appeared, 3002 since 2003 alone. There are also a number of good recent reviews available,[2] and an entire volume of the series "Metal Ions in Life Sciences" has recently been dedicated to metallothioneins.[3] The overwhelming majority of MT literature has focused on mammalian MTs, both in terms of physiological functions as well as of molecular mechanisms including molecular structures. There appears to be a perception that the knowledge accumulated for vertebrate MTs is transferable to other MTs; however, the relatively few in-depth studies available for MTs from other phyla have in each case revealed that the diversity in primary structures leads to surprising and unexpected molecular properties.

The current report will therefore attempt to take a different angle from previous reviews on metallothioneins. We will place

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particular emphasis on the remarkable structural diversity in this heterogeneous protein superfamily, highlighting the wealth of knowledge that is still awaiting discovery for so-called "class II" metallothioneins, which comprise all proteinaceous MTs that have no discernible sequence similarity to equine renal MT,[4] and constitute the majority of family members. Metallothioneins have featured repeatedly as model compounds for pioneering biophysical method development, and continue to do so. For the purpose of this review, we will also summarise advances in selected biophysical techniques, focusing on the "chemistry" of metallothioneins, in particular their preparation, the bioanalytical characterisation of the isolated proteins, their structures and their reactions. We will not explicitly consider genetics, functional studies, or *in vivo* methods, and for these topics the reader is referred to the various reviews mentioned throughout the text.

## 2 Structural diversity

### 2.1 Taxonomic distribution and diversity of metallothionein primary structures

The criteria that define a protein or peptide as a metallothionein are: (i) low molecular weight (<10 kDa), (ii) high metal and sulfur content (>10%), (iii) spectroscopic features typical of M–S bonds (iv) absence or scarcity of aromatic amino acids.[5] Criteria (ii) and (iii) pose a problem for metallothioneins that have not been isolated, as primary sequences cannot inform on metal content or spectroscopy. Therefore, for the sake of caution, proteins that fulfil several criteria, but for which no *in vitro* data is available, are often called metallothionein-like proteins.

At the time of writing of this report, 2332 sequences for metallothioneins and metallothionein-like proteins were available in the nucleotide sequence database at NCBI.[6] The ubiquitous presence of these proteins across major eukaryotic phyla is illustrated by their extensive taxonomic coverage (Fig. 1), and a further 320 nucleotide sequences for bacterial metallothioneins (see Section 6.2) are available. A non-comprehensive compilation of protein sequences (Table 1) highlights that the metallothionein superfamily is a collection of diverse proteins with unclear phylogenetic relationships.

The diversity in primary structures implies diversity in 3D structures, molecular properties, and biological functions. The need to correlate these properties has led to the development and refinement of classification systems for metallothioneins. The earliest definition and classification scheme to be proposed[4] had a "vertebrate-centric" viewpoint; more recent attempts prompted by the ever-increasing number and diversity of new metallothionein sequences have aimed to use phylogenetic relationships and justifiable similarities and differences in protein and/or gene structure.[7] The classification system proposed by Binz and Kägi defines families and subfamilies, with no discernible phylogenetic relationship between families, but within families. Unfortunately, this careful classification scheme is not coherently reflected in the literature or current bioinformatic databases.

Despite their ambiguous/unresolved evolutionary origins, metallothionein sequences from different (super)kingdoms share common characteristics, which may be clearly observed in Table 1. Typically, metallothionein sequences contain a large proportion of Cys residues (ranging from 15–30%), which are arranged in characteristic CxC and CxxC motifs. Direct juxtaposition of Cys residues as CC or even CCC motifs also occurs, but these features are not found in all metallothioneins. A high prevalence for small amino acids such as Gly and Ala is also common, which allows the protein backbone to adopt otherwise unfavourable tight turns. Aromatic and bulky hydrophobic residues such as Tyr, Trp, Phe, Leu and Ile are scarce within Cys-rich domains; this too can be understood on a structural basis. In other proteins, such residues tend to be buried in the interior of the protein and provide structural stability through hydrophobic interactions. In metallothioneins, this role is performed by the metal–thiolate clusters themselves. It is this interplay between cluster formation and protein folding that endows metallothioneins with their unique metal binding properties, which are characterised by high thermodynamic stability combined with kinetic lability.[2]

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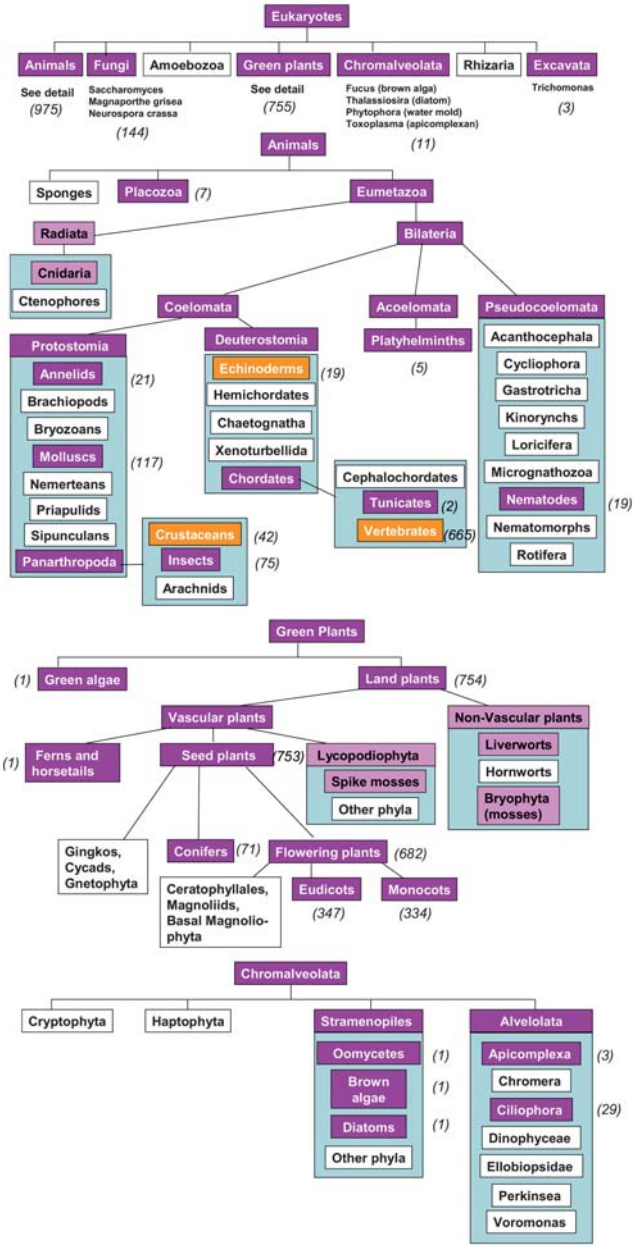


**Fig. 1** Availability of sequence and structural data for eukaryotic metallothioneins. Boxes coloured in magenta signify availability of sequence data at either nucleotide or protein level, the number in brackets refers to the number of sequences deposited in the "nucleotide" database at NCBI. Boxes shaded in pink refer to availability of additional sequence data in dbEST, which contains 3567 mRNA sequences annotated as metallothioneins. Orange boxes highlight availability of 3D structural data.

### 2.2 Overview of metallothionein 3D structures

Whilst sequence coverage, as discussed in Section 2.1 is quite advanced, only a fraction of possible folds for metallothioneins has been determined experimentally, as is summarised in Table 2 and Fig. 2.

In total, only 32 entries in the Protein Data Bank (PDB) refer to metallothioneins.[8–14] Of these, 15 are for vertebrate MTs (Fig. 2A), and these all adopt very similar folds, and the associated proteins display relatively similar *in vitro* properties as well. Eight further entries pertain to echinoderm (Fig. 2B) and crustacean MTs (Fig. 2C), which are also relatively closely related to vertebrate MTs. Six further structures refer to the *Saccharomyces cerevisiae* Cup1 protein (Fig. 2D). The remaining three entries are for *Neurospora crassa* MT (for a structure without metal ions; not shown), cyanobacterial $Zn_4$SmtA (Fig. 2E), and one domain of the wheat $E_C$-I/II protein (partially modelled; Fig. 2F). Clearly, the sequences and structures for the three MTs shown on the right hand side in Fig. 2 are highly divergent from the other 29 entries. In addition, each of these three metallothioneins displays a number of unexpected and unprecedented features which critically impact on the dynamic behaviour of the proteins, as will be discussed for SmtA and $E_C$-I/II in Section 6.

### 2.3 Metal–thiolate clusters

With the exception of bacterial MTs which contain a β-bridge, a β-hairpin, and an α-helix that form a treble-clef zinc finger fold,[16] other known MT structures contain few secondary structure elements. This correlates with the fact that for most metallothioneins that have been studied, folding is dominated by metal binding, and an ordered structure is only achieved in the presence of bound metal ions. Very little is known about the mechanisms for metal-induced protein folding in general, and in the case of MTs, the presence of clusters adds to the complexity of this process. Most remarkably, despite the plethora of possible permutations of combining up to 21 thiolates with up to 7 divalent or 12 monovalent metal ions, usually only one of these configurations is realised. Experimental evidence so far has pointed to the fact that the one configuration observed corresponds to that which is the thermodynamically most stable, as properly folded metallothioneins can usually be reconstituted from the apo-forms *in vitro*. Note, however, that very recent work has questioned this paradigm.[15]

So far, broadly two types of clusters have been reported for metallothioneins with bound Zn(II) and Cd(II), with both metals adopting tetrahedral coordination spheres. The basic unit for these clusters is $M_3Cys_9$, with the three metal ions and three bridging cysteine sulfurs forming a six-membered ring. The remaining six thiolates coordinate one metal each, and each metal has two of these "terminal" Cys residues. Such rings are found in the β domains of mammalian MTs, in one domain of the sea urchin MT, and in both domains of crustacean MTs (Fig. 2). The $M_4Cys_{11}$ cluster found in the α domains of mammalian and sea urchin MT contains two fused six-membered rings, which share two bonds. This gives a structure with five bridging and six terminal Cys residues, and two types of metal ions: two with two terminal and two bridging thiolates, and two with one terminal and three bridging Cys. This structure can, in principle, be derived from the structures of synthetic $M_4(S–R)_{10}$ clusters[2] by breaking one of the bridges in the adamantane cage adopted by these compounds. Note, however, that fused six-membered rings observed in MTs adopt either distorted boat or chair conformations, whereas those in adamantane-like structures have chair conformations only. Simplistically, many clusters in MTs are structurally related to hexagonal ZnS (wurtzite), and those in synthetic metal thiolates to cubic zinc blende.[16]

 

**Table 1** Diversity of primary structures of metallothioneins from different species. The table contains MT sequences for which structures are available (see Table 2), plus sequences for which biophysical data exists, plus further MTs specifically mentioned in the text (see Section 6.4), and a selection of sequences from different phyla. The eukaryotic sequences are grouped according to the scheme shown in Fig. 1

| Species | Name/*in vivo* metal | Sequence | Number of Cys residues | Experimentally confirmed metal stoichiometry |
|---|---|---|---|---|
| **Animals – Bilateria** | | | | |
| *Homo sapiens* | MT-2A | MDPNCSCAAGDSCTCAGSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCICKGASDKCSCCA | 20 | 7 M(II), or 12 M(I) |
| *Homo sapiens* | n.a. (Zn) | MDPNCSCATGVSCACTGSCKCKECKCTSCKKSCCSCCPVGCAKCAHGCVCEGALEKCNCYA | 20 | |
| *Homo sapiens* | MT-1M | MDPCECSKSGTCNCGGSCTCTNCSCKSCKKSCCPCCPSGCTKCASGCVCKGKTCDTSCCQ | 20 | |
| Fish (*Notothenia coriiceps*) | MTA | MDPCECSKSGTCNCGGSCTCTNCSCKSCKKSCCPCCPSGCTKCASGCVCKGKTCDTSCCQ | 20 | 7 M(II) |
| Reptile (*Chalcides chalcides*) | n.a. | MDPQDCSCNTGGTCTCAGSCKCKNCKCTSCKKSCCSCCPAGCDNCAKGCVCKEPLSGKCSCCH | 20 | |
| Bird (chicken) | — | MDPQDCTCAAGDSCSCAGSCKCKNCRCRSCRKSCCSCCPAGCNNCAKGCVCKEPASSKCSCCH | 20 | 7 M(II), 10–12 Cu(I) |
| Sea urchin | — | MPDVKCVCCKEGKECACFGQDCCKTGECCKDGTCCGICTNAACKCANGCKCGSGCSCTEGNCAC | 20 | 7 M(II) |
| Blue crab | CdMT-1 | MPGPCCNDKCVCQEGGCKAGCQCTSCRCSPCQKCTSGCKCATKEECSKTCTKPCSCCPK | 18 | 6 M(II) |
| Blue crab | CuMT-2 | MPCGCGTSCKCGSGKCCCGSTCNCTTCPSKQSCSCNDGACGSACQCKTSCCCGADCKCSPCPMK | 21 | |
| Insect (*Orchesella cincta*) | OcMT (Cd) | MSSTQGSASEAIRNCLCCGENCKCGGAEGKSPTCCKEKKCCGGGATQTASCCTCCGPDCVCKDGASLPCCANKTCCK | 19 | 7 or 8 Cd(II) |
| Insect (*Drosophila melanogaster*) | MtnA (Cu) | MPCPCGSGCKCASQATKGSCNCGSDCKCGGDKKSACGCSE | 10 | 4–5 Zn(II), 8–9 Cu(I) |
| | MtnB (Cd) | MVCKGCGTNCQCSAQKCGDNCACNKDCQCVCKNGPKDQCCSNK | 12 | 3–4 Zn(II), 8 Cu(I) |
| | MtnC | MVCKGCGTNCKCQDTKCGDNCACNQDCKCVCKNGPKDQCCKSK | 12 | |
| | MtnD | MGCKACGTNCQCSATKCGDNCACSQQCQCSCKNGPKDKCCSTKN | 12 | |
| Mussel (*Mytilus galloprovincialis*) | MT-10 (Cd) | MPAPCNCIESNVCICGTGCSGEGCRCGDACKCSGADCKCSGCKVVCKCSGSCACEAGCTGPSTCRCAPGCSCK | 21 | 7 Cd(II) |
| Snail (*Helix pomatia*) | MT-Cd | SGKGKGEKCTSACRSEPCQCGSKCQCGEGCTCAACKTCNCTSDGCKCGKECTGPDSCKCGSSCSCK | 18 | |
| | MT-Cu | --SGRGKNCGGACNSNPCSCGNDCKCGAGCNCDRCSSOHCSNDDCKCGSQCTGSGSCKCGSACGCK | 18 | |
| Earthworm (*Eisenia foetidea*) | MT-Cd | DTQCCGKSTCAREGSTCCCTNCRCLKSECLPGCKKLCCADAEKGKCGNAGCKCGAACKCSAGSCAAGCKKGCCGD | 21 | |
| Nematode (*Caenorhabditis elegans*) | MTL-1 | MACKCDCKNKQCKCGDKCECSGDKCCEKYCCEEASEKKCCPAGCKGDCKCANCHCAEQKQCGDKTHQHQGTAAAH | 19 | 7 M(II) |
| | MTL-2 | MVCKCDCKNQNCSCNTGTKDCDCSDAKCCEQYCCPTASEKKCCKSGCAGGCKCANCECAQAAH | 18 | 6 M(II) |
| Flatworm (*Schistosoma*) | Cd-MT | MPSIGPELRCYQCNSLTQPHCADYFDNRTFPLVACPNDGRNYSRCVKMIQEMYLDGKWTRRYYRDCAVTGVIGAEDGRW CIDRLGTYRVKVRYCNCNNKNGCACKEYGESCMKTVFNKCCGSNVCQLIGPFNGKCVRCIQTGYSCLHNGECCSRNCWL FRCCRALGEKCSKTVFDRCCGDTVCHLSSPFHGKCVKCLKEGTLCVSDKNCCSHKCNIGKCTKEKHHY | 34 | |
| **Animals – Radiata** | | | | |
| Coral (*Acropora cervicornis*) | Cu | SPCNCIEIAPCNCIELHSVEKCPGRKNVPTFILVRGIFALCEPATCKCKYQRSTKCDSSC | 10 | |
| **Animals – Placozoa** | | | | |
| *Trichoplax adhaerens* | n.a. | MDPPCNCAETGDCQCPSNCACTNCKCAPNCRLCGGKTCKCAENISSCVCTTCICTDCKCPKGCKN | 19 | |

**Table 1** (*Contd.*)

| Species | Name/*in vivo* metal | Sequence | Number of Cys residues | Experimentally confirmed metal stoichiometry |
|---|---|---|---|---|
| **Fungi** | | | | |
| Yeast | Cup1 | QNEGHECQCQCGSCKNNEQCQKSCSCPTGCNSDDKCPCGN | 10 | 8 Cu(I) |
| | CRS5 | MTVKICDCEGECCKDSCHCGSTCLPSCSGGEKCKCDHSTGSPQCKSCGEKCKCETTCTCEKSKCNCEKC | 19 | 5–7 Zn(II), 10–12 Cu(I), 6–8 Cd(II) |
| *Candida albicans* | — | MACSAAQCVCAQKSTCSCGKQPALKCNCSKASVENVVPSSNDACACGKRNKSSCTCGANAICDGTRDGETDFTNLK | 12 | |
| | | MSKFELVNYASGCSCGADCKCASETECKCASKK | 6 | |
| Fission yeast | Zym1 (Zn) | MEHTTQCKSKQGKPCDCQSKCGCQDCKESCGCKSSAVDNCKCSSCKCASK | 12 | |
| *Neurospora crassa* | — | GDCGCSGASSCNCGSGCSCSNCGSK | 7 | |
| *Magnaporthe grisea* | MMT1 | MCGDNCTCGASCSCSSCGTHGK | 6 | 2 Zn(II) |
| *Heliscus lugdunensis* | Cd | SPCTCSTCNCAGACBSCSCTSCSH | 8 | |
| **Green plants** | | | | |
| *Arabidopsis thaliana* | MT-1A | MADSNCGCGSSCKCGDSCSCEKNYNKECDNCSCGSNCSCGSNCNC | 13 | See Section 5.3 |
| | MT-1C | MAGSNCGCGSSCKCGDSCSCEKNYNKECDNCSCGSNCSCGSSCNC | 13 | " |
| | MT-2A | MSCCGGNCGCGSGCKCGNGCGGCKMYPDLGFSGETTTTETFVLGVAPAMKNQYEASGESNNAENDACKCGSDCKCDPCTCK | 14 | " |
| | MT-2B | MSCCGGSCGCGSACKCGNGCGGCKRYPDLENTATETLVLGVAPAMNSQYEASGETFVAENDACKCGSDCKCNPCTCK | 14 | " |
| | MT-3 | MADSNCGCGSSCKCGDSCSCEKNYNKECDNCSCGSNCSCGSNCNC | 10 | " |
| | MT-4A | MADTGKGSSVAGCNDSCGCPSPCPGGNSCRCRMREASAGDQGHMVCPCGEHCGCNPCNCPKTQTQTSAKGCTCGEGCTCASCAT | 17 | " |
| | MT-4B | MADTGKGSASASCNDRCGCPSPCPGGESCRCKMMSEASGGDQEHNTCPCGEHCGCNPCNCPKTQTQTSAKGCTCGEGCTCATCAA | 17 | " |
| Wheat | $E_C$-I/II | GCDDKCGCAVPCPGGTGCRCTSARSGAAAGEHTTCGCGEHCGCNPCACGREGTPSGRANRRANCSCGAACNCASCGSATA | 17 | |
| *Xerophyta humilis* | n.a. | MADVKKCDETCGCPVPCSLDTACKCSVESGNAASRHATCTCGEHCSCNPCSCGKVPIGVQAGKGSCSCGSGCNCEKCSC | 18 | |
| Douglas fir | n.a. | MASTCTRGAECGCGETCACVDCKCGAGSTATPSDQTSGGNAYCKCGENCSCNPCNCSKSDQTASGKSYCKCGENCACETCSCSRAQM | 19 | |
| *Selaginella moellendorfii* | n.a. | MARTEGCGCATPCPCGDACKCGMDKEKSSAPGAETSSSFCSCGEKCSCDPCSCSKVSASGDGFCKCGSECKCDKCSCAKSVVARA | 18 | |
| *Azolla filiculoides* | AzMT2 | MSCCGGNCGCGSGCRCGKRSFDETAFDAPMEVEVTVFGNEGSNCGFDSSCGFESDAQATSQNGCSCGSNCTCNPCRC | 14 | |
| **Chromalveolata** | | | | |
| *Fucus vesiculosus* | — | MAGTGCKIWEDCKCGAACSCGDSCTCGTVKKGTTSRAGAGCPCGPKCKCTGQGSCNCVKDDCCGCGK | 16 | 6 Cd(II), 5 As(III) |
| *Thalassiosira pseudonana* CCMP1335 | — | MNANANSESGKKRSINENDTVPTTTTNNNPTNTTCKCSKSRCIKLYCDCPHGGNLCNSLCNCTDCKNTTEFREEREWKMK EVLKLNPKAFSEDSDKFNTKRQRMSRGNGCACPSSHCLKKYCSCFGADAGCTDKCSCNDCE | 18 | |
| *Tetrahymena pyriformis* | MT1 (Cd) | MDKVNNNCCCGENAKPCCTDPNSGCCCVSETNNCCKSDKKECCTGTGEGCKCTGCKCCEPAKSGCCCGDKAKACCTDPNS GCCCSSKTNKCCDSTNKTECKTCECCK | 31 | 9–11 Zn(II), 11 Cd(II) |

**Table 1** (*Contd.*)

| Species | Name/*in vivo* metal | Sequence | Number of Cys residues | Experimentally confirmed metal stoichiometry |
|---|---|---|---|---|
| *Tetrahymena thermophila* | MTT1 (Cd) | MDKVNSCCCGVNAKPCCTDPNSGCCCVSKTDNCCKSDTKECCTGTGEGCKCVNCKCCKPQANCCCGVNAKPCCFDPNSGC CCVSKTNNCCKSDTKECCTGTGEGCKCTSCQCCCKPVQQGCCCGDKAKACCTDPNSGCCCSNKANKCCDATSKQECQTCQC CK | 48 | |
| *Tetrahymena thermophila* | MTT2 (Cu) | MDTQTQTKVTVGCSCNPCKCQPLCKCGTTAACNCQPCENCDPCSCNPCKCGVTESCGCNPCKCAECKCGSHTEKTSACKC NPCACNPCNCGSTSNCKCNPCKCAECKC | 32 | |
| **Excavata** | | | | |
| *Trichomonas vaginalis* | n.a. | MSSQKTCDCNKQNAGKSQWFKCQCCPGCKCGSNCHCTKDNKCSPDCHCGEGCNCNEGCYCNEGCKCGSNCHCTKDNKCSP DCHCGEGCHCNEGCKCGSNCHCTKDNKCSPDCHCGEGCNCNEGCYCNEGCKCGSNCHCTKDNKCSPDCHCGEGCNCNEGC YCNEGCKCGSNCHCTKDNKCSPDCHCGEGCHCNEGCKCGSNCHCTKDNKCSPDCHCGEGCNCNEGCYCNEGCKCGSNCHC TKDNKCSPDCHCGEGCNCNEGCYCNEGCKCGSNCHCTKDSKCSPDCHCGEGCNCNEGCKCGADCHCNN | 91 | |
| **Bacteria** | | | | |
| *Synechococcus* PCC7942 | SmtA | MTSTTLVKCACEPCLCNVDPSKAIDRNGLYYCSEACADGHTGGSKGCGHTGCNCHG | 9 | 4 M(II) |
| *Synechococcus* CC9311 | Sync_1081 | MTVTVVKCACSSCTCEVSSSSAISRNGHSYCSDACASGHRNNEPCHDAAGACGCNCGS | 10 | |
| | Sync_2379 | MATSNQVCACDPCSCAVSVESAVQKDGKVYCSQPCADGHSGSDECCKSCDCC | 11 | |
| | Sync_2426 | MTTNLVRCDCPPCTCSIEEATAAMYGNKLFCSEACATAHINQEPSNSAEHTECSCGC | 9 | |
| | Sync_0853 | MNEVLLLCDCSLCKRSVEESRSIRIGGQHFCSESCAKGHPNMEPCDGERDGCNCGIAELELLLAAAD | 8 | |
| *Pseudomonas aeruginosa* | — | MNSETCACPKCTCQPGADAVER-DGQHYCCAACAGGHPQGEPCRDADCPCGGTTRPQVAEDRQLDDALKETFPASDPISP | 10 | 3–4 M(II) |
| *Mycobacterium tuberculosis* | MymT | MRVIRMTNYEAGTLLTCSHEGCGCRVRIEVPCHCAGAGDAYRCTCGDELAPVK | 7 | 6 Cu(I) |
| *Streptomyces lividans* | — | MRTASVAHGEGARLAAAVSLVLASAKVPDHVADGDGLLARRPVALAARRIAGGWDAAGARGGGAVGFDTAVLVDAVDRQGGIE ALAGPGTPLLTVTEPTETATAAAFAHTAHLAGRPHNAVPLAEAGRLFGRLAHLLDAVEDREADAASGAWNPLTATGTPLAEAR RLADDAVHGVRLALREAEFTDGRLVHRLLVHELGSSVDRAFGTVSCAHGSHPYAPPGAPGGPGGPTPPEPPRRDRRGLLAGCA VWLGLACTCQMCCGTFNDPWSGQRREGLCSQCDCGNCCDCCDCCSNCCGDDGCGCGCDGCDCGCSC | 24 | |

**Table 2** Summary of published 3D structures for metallothioneins[a]

| Metallothionein | PDB code | Experimental details | Metals | Ref. |
|---|---|---|---|---|
| Rat MT-2 | 1mrt (α) and 2mrt (β) | NMR, min. av. | $Cd_3$(β), $Cd_4$(α) | 10 |
| | 4mt2 | X-ray | $Cd_5Zn_2MT$ | 8*a* |
| Mouse MT-1 | 1dfs (α) and 1dft (β) | NMR, min. av. | $Cd_4$(α), $Cd_3$ (β) | 8*b* |
| Mouse MT-3 | 1ji9 (α) | NMR, min. av. | Cd4 (α) | 8*c* |
| Rabbit-MT-2A | 1mrb (α) and 2mrb (β) | NMR, min. av. | $Cd_4$(α), $Cd_3$ (β) | 8*d* |
| Human MT-2 | 1mhu (α) and 2mhu (β) | NMR, min. av. | $Cd_4$(α), $Cd_3$ (β) | 11 |
| Human MT-3 | 2f5h | NMR, min. av. (α) | $Cd_4$(α), $Cd_3$ (β) | 13 |
| | 2fj4 | Ensemble | " | " |
| | 2fj5 | Ensemble, isolated α | " | " |
| *Notothenia coriiceps* MT | 1m0g (α) and 1m0j (β) | NMR, min. av. | $Cd_4$(α), $Cd_3$ (β) | 8*e* |
| *Strongylocentrotus purpuratus* MTA | 1qjk (α) and 1qil (β) | NMR, min. av. | $Cd_4$(α), $Cd_3$ (β) | 8*f* |
| *Callinectes sapidus* MT-1 | 1dmc (α), 1dme (β) | NMR, min. av. | $Cd_3$ (α and β) | 8*g* |
| | 1dmd (α), 1dmf (β) | Ensembles | " | " |
| Lobster MT-1 | 1j5l (β), 1j5m (β, synthetic) | Isolated β domain | $Cd_3$ | 8*h* |
| Wheat $E_C$-I/II | 2kak | C-term. domain | $Zn_4$ | 14 |
| Yeast Cu-MT | 1aoo | NMR, min. av. | $Ag(I)_7$ | 8*i* |
| | 1aqq | Ensemble | " | " |
| | 1aqr | NMR, min. av. | $Cu(I)_7$ | 8*i* |
| | 1aqs | Ensemble | " | " |
| | 1fmy | NMR, min. av. | $Cu(I)_7$; metals not shown | 8*j* |
| | 1rju | X-ray; engineered | $Cu(I)_8$ | 9 |
| *Neurospora crassa* | 1t2y | NMR; full length, min. av. | Metals not shown | 8*k* |
| *Synechococcus* PCC 7942 SmtA | 1jjd | NMR, min. av. | $Zn_4$SmtA | 12 |

[a] Min. av.: minimised average structure.

The only cluster structure available for Cu(I)-binding metallothioneins is that of yeast Cup1.[9] The $M_8Cys_{10}$ cluster contains Cys residues that bridge either two or three Cu(I) ions, but no terminal Cys residues are present. Several fused six-membered rings are formed, but in contrast to the Zn(II) and Cd(II) clusters, they share only one bond. Six Cu(I) have trigonal planar coordination geometry, and two are coordinated in a linear/digonal fashion. Trigonal coordination for Cu(I) as well as fused six-membered rings are also found in the mineral chalcocite, the $Cu_2S$ form stable at room temperature.

We will demonstrate in Section 6 that novel metallothioneins may display metal-binding motifs and cluster structures that deviate from those described above.

### 2.4 Challenges in metallothionein 3D structure determination

It is generally accepted that the 3D structures of metallothioneins are largely governed by the bound metal ions, and their arrangement in turn is governed by the spacing of the metal ligands, together with some contributions from non-coordinating residues. Thus, if the rules for cluster formation and folding in MTs were fully understood, it should in principle be possible to predict the 3D structures of MTs from sequence information. However, our very limited knowledge base on 3D structures is insufficient to develop an understanding of these rules, and the current collection also falls woefully short of providing a comprehensive basis for the prediction of structures by homology modelling.

Furthermore, as will be discussed in Section 6, novel structures may be correlated with novel molecular properties, and a truly comprehensive understanding of the reactivity and mode of action of metallothioneins is impossible without knowledge of their actual structures. The importance of structure determination is further illustrated by the finding that reports that deal with the molecular properties of non-vertebrate metallothioneins are littered with assumptions and misconceptions, starting with unfounded assertions about the number of domains present. For example, despite mounting evidence to the contrary (see Section 6), there is a widespread perception that eukaryotic MTs mostly adopt a 2-domain structure. Even in cases where this is true, it has occurred that the domain boundaries have been wrongly assigned, using inappropriate sequence alignments. It is also often assumed that MTs contain either α- ($M_4Cys_{11}$) or β-type ($M_3Cys_9$) clusters, and the possibility for alternative binding modes is scarcely considered.

Comparing the number of metallothionein structures to the number of zinc finger structures deposited in the PDB (currently 2915 entries), and considering the ubiquitous distribution of metallothioneins, this staggering lack of fold coverage raises the question why only so few MT structures have been determined.

The most severe obstacle is probably the fact that it is often not easy to determine a metallothionein structure, even if sufficient quantities can be obtained. Due to the relatively high flexibility of their protein backbone, MTs are usually difficult to crystallise, and only two out of the 32 entries in the PDB are X-ray structures. For example, Weser and colleagues had tried for three decades to obtain a conclusive structure for yeast Cup1, finally succeeding in 2005.[9] This leaves NMR spectroscopy for structure determination, and indeed, rat liver metallothionein was one for the first proteins that had its structure determined by NMR spectroscopy.[10] In his article "The way to NMR structures of proteins",[17] Kurt Wüthrich recalls that "metallothionein had

 

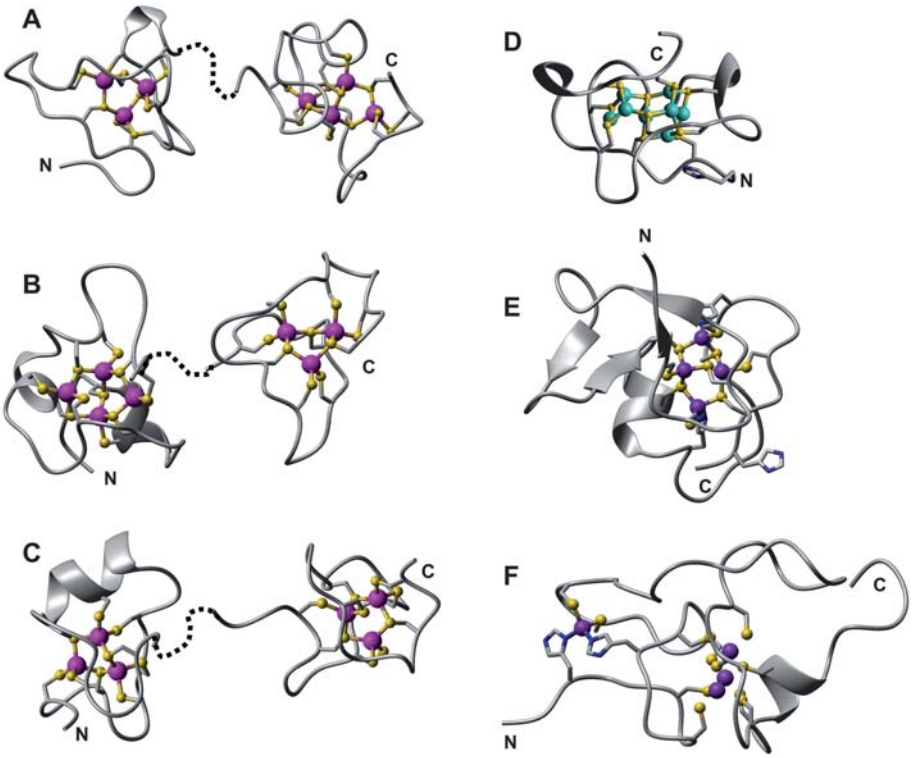


**Fig. 2** Representative 3D structures of metallothioneins. (A) Rat liver $Cd_7$MT-2 (PDB 1mrt and 2mrt; determined by NMR spectroscopy, and mapped onto the X-ray structure of the same protein). (B) Sea urchin *Strongylocentrotus purpureus* $Cd_7$MTA (PDB 1qil and 1qik; NMR). (C) Blue crab *Callinectes sapidus* $Cd_6$MT-1 (PDB 1dmd and 1dmc; NMR). The mutual orientation of the two domains as drawn in (B) and (C) is arbitrary, as this information could not be obtained by NMR structure determination. (D) Engineered yeast *Saccharomyces cerevisiae* $Cu_8$Cup1 (PDB 1rju; X-ray). (E) Cyanobacterial *Synechococus* PCC 7942 $Zn_4$SmtA (PDB 1jjd; NMR). (F) Domain II of wheat (*Triticum aestivum*) $E_C$-I (PDB 2kak; NMR) with $Zn_3Cys_9$ cluster and isolated $Cys_2His_2$ site. Sulfur atoms are drawn in yellow, cadmium in magenta, zinc in purple, copper in cyan, and nitrogens in blue. Structures A–C are of class I MTs, and D–F are the only structures for class II MTs available that include metal ions. All three class II MTs break with one or more previously accepted, and occasionally still perpetuated, paradigm: all three contain His residues (either coordinating or non-coordinating), and D and E are single-domain proteins. E displays extensive secondary structure elements which form a treble-clef zinc-finger fold.

been a tough challenge for all of us involved". Unfortunately, nothing much has changed since the 1980s. On the contrary, it appears that many non-vertebrate metallothioneins are even more refractory to attempts to elucidate their structures by either NMR spectroscopy or crystallography. The particular challenges associated with using NMR spectroscopy for structure elucidation start with the fact that many MTs have a high glycine content, and these residues provide very little structural information. A further complication lies in the low variability in the primary sequence, which makes conclusive sequential assignment more difficult. Most crucially, information from conventional protein NMR spectroscopy ($^1$H, $^{13}$C, $^{15}$N) is usually not sufficient to locate the positions of Cys sidechains, and therefore, an unambiguous assignment of metal–ligand connectivities, and hence cluster structure, is not possible by using these data alone. These connectivity patterns have to be determined by further non-standard experiments (described in Section 5.4.1), which may require the replacement of the native metal ion by an NMR-active nucleus. For example, Cu(I) can be replaced by $^{109}$Ag(I), and Zn(II) by $^{111}$Cd(II) or $^{113}$Cd(II). However, this replacement with non-native metal ions can create problems, as their coordination chemistry and ionic size are not identical, and hence, the replacement may or may not be isostructural. In each case, it is therefore necessary to (i) get a good idea about the metal ions bound *in vivo*, (ii) select the appropriate spectroscopic probe, and (iii) test whether this leads to isostructural replacement of the native metal ion(s).[18]

Finally, it has been argued that all structural studies on MTs have been focusing on fully reduced, fully metallated MTs – but that these are actually not the physiologically predominant species.[19] Since partially metallated metallothioneins are likely to be unfolded to some degree, they are unlikely to be amenable to structural studies by either NMR spectroscopy or X-ray crystallography. The only method giving access to these species in a 3D structural context is molecular modelling. Relatively little serious work has so far been carried out in the area of modelling apo- and partially metallated MTs, with the notable exception of Stillman's recent work.[20]

## 3 Functional diversity

Most reviews on metallothioneins state that their function is still a matter of debate. One reason for this contention is that the enigmatic unified function for "metallothioneins" simply does not exist. Instead, the members of this protein superfamily are not only diverse in terms of structure, but also in terms of

function. In addition, a single metallothionein may indeed fulfil more than one function. That at least some metallothioneins are "multi-purpose proteins"[21] follows from analysis of their promoter sequences, which contain response elements for glucocorticoids, oxidative stress, and metal stress.[22]

Furthermore, many organisms contain multiple genes for metallothioneins, for example, *Homo sapiens* has at least 17 different isoforms,[2] the mouse has four isoforms,[2] the fruit fly also has four,[23] the nematode *Caenorhabditis elegans* has only two,[24] and thale cress (*Arabidopsis thaliana*) has seven expressed isoforms.[25] Different isoforms are expressed in different tissues and under different conditions; hence, it can be expected that their physiological functions are different as well, and in some cases, this has been confirmed experimentally.[2,23–25]

Metallothioneins as a response to cadmium toxicity in a variety of organisms still constitute an active research area,[26] but in mammals, MTs have also been attributed with anti-inflammatory, anti-apoptotic, antioxidant, proliferative, and angiogenic activities, and are also involved in resistance to chemotherapy.[27] They have been implicated with a range of diseases and conditions, including diabetes,[28] neurodegenerative diseases such as Alzheimer's,[29] ageing and immune response including immunosenescence,[30] and cancer.[27]

The physiological essentiality of metallothioneins in eukaryotes under non-stressed conditions has been questioned, owing to the assertion that MT knock-out strains did not display severe phenotypes. In some cases, the lack of a phenotype may be due to the presence of genes for multiple isoforms, which may provide a level of contingency by substituting each other. On the other hand, MT-null mice suffer from obesity, impaired spatial learning, and enhanced seizures and neurodegeneration,[31] conditions which probably would be considered as "severe" if they were found in humans. Further evidence for more profound functions of MTs comes from the finding that MT expression in eukaryotes is also regulated in dependence on developmental stage. For example, both mammalian and plant embryos have been shown to have high levels of MT mRNA.[32] Other proliferating tissues, including malignant tumours, often also display increased MT expression, although the role of MTs in cancer is complicated and not well understood.

The wide-ranging repertoire of metallothioneins is intimately linked both with their metal-binding as well as their redox chemistry. With respect to metal binding, their involvement in zinc and copper homeostasis is crucial. The importance of zinc in immunity, signalling, cancer, and ageing is becoming increasingly appreciated,[33] and Zn dyshomeostasis has been identified as an integral component of neuronal injury.[30] Copper also clearly is implicated in a variety of neurodegenerative diseases.[34] Significantly, metallothioneins act as neuroprotectants.[35]

Relatively recent work has highlighted that metallothioneins may exist *in vivo* not only in their metallated, but also in partially metallated, or even their apo forms.[19] Furthermore, MTs are thought to be able to exist *in vivo* in different redox states, mediated *via* disulfide-bond formation of cysteine residues. This enables MTs on the one hand to respond to oxidative stress, by effectively acting as antioxidants, and on the other hand, this provides a link between the redox state of the cell and fluctuations in zinc concentrations.[36] It is important to note that owing to their increased electron affinity, metal-bound thiolates are much harder to oxidise than free thiols or thiolates. The redox link can therefore in principle work in both directions: the zinc status determines the facility with which MTs can become oxidised, and the redox state of the cell determines how much Zn(II) can be bound to MT – effectively, MTs are thought of as cellular mediators of zinc signals.[36]

Whilst a large number of studies have been carried out regarding the biological implications of metallothioneins in man and mammals, much less functional data is available on metallothioneins in organisms other than mammals, although the "-omics" era has begun to fill this gap. Amongst the most intriguing set of metallothioneins are those found in plants.[37] Recent work has begun to dissect the functional diversity of the seven MTs of *Arabidopsis thaliana*. Intriguingly, the four subclasses of plant MTs display differential responses to copper and zinc.[25] More intriguingly, the zinc-binding type 4 plant MTs are not induced by metal ions at all, but are regulated by hormones, and are particularly abundant in reproductive organs, including seeds[32] and pollen.[38]

The overarching conclusion from the evidence so far is that metallothioneins are much more than just "metal sponges" which deal with occasional excesses, but that at least in certain organisms, including vertebrates and plants, they play an integral role in the trafficking and homeostasis of essential metal ions. Excitingly, computational modelling studies of zinc homeostasis in neurons have proposed that metallothioneins are likely to act as part of a high-affinity "zinc muffler" that shuttles cytosolic zinc to so-called "deep stores" (mitochondria, endoplasmatic reticulum and the Golgi apparatus), especially under conditions that increase MT expression.[39]

## 4 *In vitro* metal specificity

An issue that has become much more prevalent in recent years concerns the specificity of metal binding properties of metallothioneins. Respective *in vitro* studies are inspired by the observation that different metallothioneins are differentially induced by different metal ions, *e.g.* in snails,[40] earthworms,[41] and plants.[25]

Early studies of vertebrate MT-1 and MT-2 demonstrated the "promiscuity" of these proteins. Complexes with Co, Fe, Ni, Hg, Ag, Pb, Pt, and Bi have been prepared and studied spectroscopically. However, more recent *in vitro* and *in vivo* work has revealed that many MTs are more "discerning" than initially thought.

*A priori*, it could be argued that a biological system can achieve specificity by tight regulation of gene expression, and that therefore the expressed metal homeostasis protein itself does not have to be particularly specific. In practice, the picture that is emerging for metal ion trafficking is one that combines metal-responsive transcription, access, and allostery with the relative *in vitro* specificity of expressed proteins.[42] We would like to highlight an interesting hypothesis put forward by Capdevila and Atrian,[43] who propose that it may be possible to draw conclusions about metal specificity from how "well-behaved" a protein is in terms of well-defined metal binding stoichiometry as evidenced by clean, simple electrospray mass spectra.

From the description of the cluster structures found for divalent and monovalent metal ions in Section 2.3 it follows that

 

coordination geometry and stoichiometry are fundamentally different for these. Hence, also bearing in mind that the structures are dominated by the metal clusters, it can be deduced for those metallothioneins that bind either, that their structures with mono- and divalent metal ions may be substantially different. This has recently been demonstrated experimentally in a study on vertebrate MT-1 domains.[44] In this case, stable protein folds were obtained for either Zn(II)- or Cu(I)-bound species, but it was also clear that the folds differed dramatically. In other cases, stable folds may only be obtained with the "correct" metal ion (see Section 6). Vertebrate MT-3 may serve to illustrate this concept: attempts to reconstitute MT-3 in a homometallic fashion with either Zn(II) or Cd(II) lead to (i) ill-defined speciation as evidenced by mass spectrometry and (ii) high conformational flexibility in the β domain that makes structure determination impossible. We believe that it is relevant that the form isolated from brain is $Cu_4Zn_3$ or $Cu_4Zn_4$ MT-3, with a $Cu_4$ cluster in the β domain.[45] It can be anticipated that the β domain may fold differently in the presence of the "native" Cu(I), but to our knowledge, no structural data is available for the $Cu_4$β domain of MT-3.

Ultimately, specificity as well as functional diversity is encoded in the primary sequence, and we have only just begun to study the subtleties that govern this structure–function relationship.

## 5 Advances in experimental bioanalytical approaches – metallothionein (bio)chemistry and bioinorganic chemistry

The last comprehensive collection of methods for the study of metallothioneins dates back to a volume of "Methods in Enzymology" published in 1991.[46] This collection is still authoritative, but contains relatively little or no information on recombinant expression, NMR spectroscopy, and mass spectrometry, the most important techniques used in our lab for the *in vitro* study of novel MTs. A range of further techniques, for example fluorimetry,[47] and various spectroscopies[20] have also greatly contributed to the advancement of our understanding of MT structures and function, and are reviewed elsewhere.

### 5.1 Recombinant expression and purification of metallothioneins

Metallothioneins can in principle be isolated from natural sources; however, apart from ethical issues, this often results not only in relatively small quantities, but also in heterogeneous preparations with several isoforms present, which can render conclusive interpretation of measurements difficult.[20] A further problem that has been encountered frequently is the isolation of proteolytic fragments, as reported for MTs from plants,[37,48,49] the springtail *Orchesella cincta*,[50] and the earthworm *Eisenia foetida*.[51] In some cases, it was not clear whether this was due to proteolytic degradation during the purification procedure, or whether the observed proteolysis also occurs normally *in vivo*. In the case of *E. foetida*, the utmost care had been taken to avoid proteolysis during purification, but still, only truncated domain I was observed, and not even fragments corresponding to the putative domain II were detected by MS or amino acid analysis, which strongly suggests that proteolysis had occurred *in vivo*.[51] In the case of the springtail MT, cleavage was only observed for the protein isolated from native sources, but not the recombinantly expressed protein.[50]

The majority of problems with isolation and purification described above can be overcome by recombinant expression, which also offers a range of other advantages, such as the facile and affordable production of $^{15}N$- and $^{13}C$-labelled proteins for NMR studies. Furthermore, production of single domains which help to reduce complexity,[52] as well as of mutated proteins, which help to understand the role of individual residues, is possible. For example, the role of individual histidine residues in the cyanobacterial SmtA were studied by generating His-to-Arg mutants,[53] which displayed dramatic changes to the metal stoichiometry and overall affinity of the proteins. A more subtle approach involves the replacement of coordinating side chains with other coordinating residues, and Cys-to-His mutants of mammalian MTs have been generated.[54,55] Functional proteins which retained their metal-binding capacity were obtained; in fact, the relative affinity towards Zn(II), compared to Cd(II), increased in the β domain by the introduction of His residues. Conversely, the mutation of the two coordinating His residues in the cyanobacterial MT SmtA to Cys residues established that this replacement results in metal-loaded proteins, but that it impacts negatively on protein folding, metal binding affinity and kinetic lability, and sensitivity towards oxidation.[56]

A range of studies employing genetically engineered MT sequences have revealed that the reactivity can subtly, and sometimes quite dramatically, be controlled by non-coordinating residues. The most striking examples are found in comparisons of the mammalian neuronal growth inhibitor MT-3 with its non-active counterparts MT-1 and MT-2.[57,58] Crucially, the mutant proteins can be studied both *in vitro* and *in vivo*, and much can be learned from the correlation of these data.

Despite these advantages, recombinant heterologous expression generates a new set of problems. The salient question concerns the equivalency of the recombinant product with the natural protein. Since metallothioneins are not enzymes, there are no straightforward assays to ascertain that a "functional" product has been obtained. Secondly, if the natural protein has never been isolated and no relevant functional data is available, the question of "which metal?" becomes prevalent. Typically, expression cultures for metallothioneins are supplemented with the metal ion that the researcher wishes to find bound in the complete product. This stabilises the newly synthesised protein, but there is a need to carefully make the most appropriate choice. One issue with heterologous expression is our persisting lack of knowledge on the mechanisms for metal (in particular zinc) incorporation into newly synthesised proteins. In this context, it is noteworthy that recombinant metallothioneins from a variety of species expressed as fusion proteins in *E. coli* contained inorganic sulfide ($S^{2-}$) in their clusters, as evidenced by species with unusually high masses in ESI-MS (electrospray ionisation mass spectrometry) spectra, differences in ICP-AES (inductively-coupled plasma atomic emission spectroscopy) results under neutral and acidic conditions, quantitative amino acid analysis, and direct detection of inorganic sulfide.[59] This appeared to be a particular issue for cadmium-containing preparations. In the absence of the native proteins isolated as *in vivo* metal-loaded species from natural sources, the question

remains whether the sulfide ions are physiologically relevant in their native environment, or whether their presence is an artefact from the recombinant expression, peculiar to *E. coli*, and due to differences in metabolism between bacteria and eukaryotes. Irrespective of the answer to this question, it is very important that researchers who make use of recombinant systems are aware of this issue, and take appropriate measures to deal with it. For example, pure homometallic species can, in principle, be generated by a method developed by Vašák;[60] however, it is important to perform checks that the protein has indeed properly refolded.

The use of fusion proteins, or tags, may help with expression and purification, but they are not without problems. Certain tags and purification procedures can lead to the isolation of metal-depleted species or other complications. For example, fusions with glutathione-S-transferase have repeatedly furnished metal ion:protein ratios that were too low, for example in the case of bacterial[61] and plant metallothioneins,[62] although other labs have harnessed this system successfully.[43] In studies concerning various plant MTs, the metal:protein ratios were determined from independently measured protein and metal concentrations, and thus it is difficult to pinpoint whether the low ratios are due to inherent inaccuracies in protein concentration determinations, or reflect accurate values for species that have been metal-depleted by the purification process. In the case of the cyanobacterial metallothionein SmtA, the metal:protein ratio was determined by simultaneous metal and sulfur ICP-OES (inductively-coupled plasma optical emission spectroscopy), which is the most accurate method for this purpose. This established that the preparation was indeed metal-depleted,[61] and close inspection of 2D $^{1}$H NMR data allowed the detection of an additional species which corresponds to $Zn_1$SmtA. The reason for metal loss during purification remains unresolved, but following these observations, our lab has been using expression systems preferentially without tags.

A further problem introduced by using fusion proteins is the tag itself. To obtain a meaningful protein for subsequent biophysical studies, the removal of the tag is required, which adds a further step in the workflow that can generate additional complications. This has been illustrated by recent reports on recombinant wheat $E_C$-I,[63] in which the cleavage of an intein-tagged metallothionein led to the observation of covalent adducts with Tris and dithiothreitol. Both of these adducts render interpretation especially of MS data complicated, and have also led to other problems. The formation of an adduct with Tris can be avoided by choosing another buffer, and the level of the dithiothreitol adduct can be minimised by prolonged reaction times for hydrolysis, but such actions can only be taken once the problem has been recognised.

Even after cleavage of the tag, the resulting proteins contain additional extra amino acid residues at one terminus. For large proteins, this may not have a large impact, but for small proteins like metallothioneins, for which backbone dynamics are absolutely pivotal for their function,[2] adding residues to the native sequence becomes a concern. The impact of ligand-free termini on structure and dynamics of MTs has been demonstrated for yeast Cup1, the structure of which could only be correctly determined by X-ray crystallography after removal of the first four and last 13 residues.[9]

We conclude that expression of metallothioneins is best carried out by using the untagged full-length sequence. Since the size of metallothioneins is so different from the majority of cellular proteins, it is most straightforward to achieve a good level of purification by gel filtration chromatography, which can be polished by anion exchange chromatography. Both methods allow keeping the protein metallated during purification, which, at least in the case of zinc-binding MTs, should ensure minimal loss because of oxidation. A precaution that should be taken is the exclusion of copper contamination, which is a particular problem with biological-grade Tris buffer. Since the affinity of copper for metallothioneins tends to exceed that of either zinc or cadmium, irrespective of the issues discussed in Section 4, its presence in purification buffers, even at low levels, leads to the formation of mixed species.

### 5.2 Analytical chemistry of metallothioneins *in vitro*

Some metallothioneins have been and still are popular model metalloproteins for analytical method development. They frequently feature in reports and reviews on (metallo-)proteomics and coupled or hyphenated techniques.[64] Considering the pioneering role of these MTs as model proteins for a host of instrumental analytical methods, it is somewhat astonishing that so few other MTs have been appropriately characterised so far. The primary questions that need to be answered once a novel MT has been isolated are: (i) is it the correct protein, (ii) what is its concentration, and (iii) which and how many metal ions are bound?

Question (i) is best answered using mass spectrometric methods, which will be discussed in more detail in Section 5.3. Reliable quantification is possibly one of the biggest issues for proteins that cannot be quantified by their enzymatic activity, or in cases which require absolute concentration data, for example if question (iii) needs to be answered. Conventional protein assays tend to be not accurate enough for the reliable determination of metal ion:protein stoichiometries. For metallothioneins, this dilemma can be resolved in two ways, both of which rely on the abundant Cys residues. The most reliable method is the simultaneous determination of the sulfur and metal ratio by ICP-OES/AES or ICP-MS, providing the measurements are carried out in acidified solutions.[59] If such instrumentation is not available, it is also possible to determine the concentration of thiols with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), after incubation with ethylenediamine tetraacetic acid (EDTA), which reacts with bound metals and renders the Cys thiols readily reactive towards DTNB.

The second set of questions important in studies of metalloproteins concerns their affinity towards the relevant metal ion(s). This issue is less trivial than it may seem at first, and is particularly complicated in the case of metallothioneins, because they bind multiple metal ions, and not necessarily with the same affinity. On the contrary, recent work has highlighted that zinc ions in recombinant human MT-2 are bound with at least three distinguishable dissociation constants that vary over a range of four orders of magnitude, from nano- to low picomolar.[65] The data also suggest that $Zn_5$MT and $Zn_6$MT are the predominant species under normal physiological conditions. It should be noted that a yet more recent report suggests that the weakest

 

binding site is only present in $Zn_7$MT that has undergone a cycle of acidification and refolding, and that native mammalian MT has an average apparent stability constant for Zn(II) in the range of $2 \times 10^{11}$ $M^{-1}$, with two distinguishable constants.[15]

In the age of systems biology, the generation of such accurate quantitative data has become particularly important, to allow a meaningful computational modelling of zinc fluctuations and their effects,[39] but the controversy described above is a stark illustration of experimental difficulties encountered in this endeavour, and emphasises the impact of sample preparation.

A further important issue that does not receive sufficient attention is how the stability constant(s) are defined, and their dependence on experimental conditions including pH and ionic strength. The importance of ionic strength is illustrated by the dramatic decrease by about two orders of magnitude (from $5 \times 10^{10}$ $M^{-1}$ to $4.3 \times 10^{8}$ $M^{-1}$)[66] in the affinity of wheat $E_C$-I/II towards zinc upon increasing the ionic strength from 4 to 105 mM.

The final set of challenges arises in the context of studying the reactivity of metallothioneins. Clearly, considering the roles of MTs *in vivo*, there is a requirement to elucidate their biologically relevant reactions, and to understand the mechanisms of these reactions. They include metal uptake[20,67] and release,[68] metal transfer between proteins,[29,69] metal exchange,[70,71] and thiolate-centred redox reactions,[72] including those with reactive oxygen species and nitric oxide. As emphasised by several experts,[65,69] these studies should not be restricted to fully metallated species, but should focus on the species expected *in vivo*.

Many of the traditional "bioinorganic" tools are not applicable to the "spectroscopically silent" $d^{10}$ metal ions preferred by metallothioneins. They are, however, amenable to optical spectroscopies, reviewed elsewhere,[67] and much of the work concerning metallothionein reactivity has employed thiolate-to-metal charge-transfer bands to follow reactions. For Zn(II) (220 nm), this is relatively close to the absorbance maximum for peptide bonds (215 nm), which can be problematic in terms of sensitivity and data interpretation, and many published studies therefore have made use of Cd(II) or Co(II). The advantage of UV-Vis and, to a lesser extent, CD spectroscopy is their speed which allows following relatively rapid reactions, especially if stopped-flow techniques are employed. Nevertheless, the loading with either Zn(II) or Cd(II) and folding of mammalian MT domains at neutral pH was still too fast (complete within 4 ms) to be followed by stopped-flow spectrophotometry.[73]

A further problem with electronic absorption spectroscopies and various other methods is their very limited "resolution": they can only show an overall picture, and it is difficult to deduce which species are present in solution at a given time point. We will discuss below how ESI-MS has opened novel avenues in this respect. Finally, optical spectroscopies alone also do not allow conclusions regarding whether different sites are reacting with different rates, and which sites are more reactive than others. The method that offers the most promise here is NMR spectroscopy, which will be discussed in Section 5.4.

For kinetic studies, both NMR and MS suffer from the fact that they are slow methods, thus, in order to build up a full picture, a combination of techniques is required, and importantly, also structural information is needed to help interpret the findings of spectroscopic and MS data.[74]

### 5.3 Mass spectrometry: Speciation and metal movements

The most significant progress in the *in vitro* study of metallothioneins in the past decade probably concerns the increasing use of mass spectrometry, and in particular Electrospray Ionisation Mass Spectrometry (ESI-MS), to characterise the composition of metallothioneins, pioneered by Catherine Fenselau,[75] and recently reviewed in ref. 67.

In principle, the observation of metallated metallothioneins by Matrix-Assisted-Laser Desorption Ionisation Mass Spectrometry (MALDI-MS) is also possible, but commonly used sample preparation methods involve the use of acidic matrices, relatively high amounts of trifluoroacetic acid, as well as organic solvents. Consequently, the predominantly observed species are denatured apo-MTs, with minor amounts of partially-metallated species, as for example reported for the MT from the aquatic fungus *Heliscus lugdunensis* investigated in ref. 76. Hence, the overwhelming majority of MS investigations of metallothioneins have employed ESI-MS, which can be easily adapted to study proteins under near-native conditions.

ESI-MS is a soft ionisation technique, capable of bringing very large charged molecules into the gas phase, without breaking covalent bonds. Under suitable experimental conditions, it is also possible to maintain non-covalent interactions, such as metal–ligand coordinative bonds. The successful use of ESI-MS for native (metallo)proteins is based on the tenet that the species observed in the gas phase are related to the speciation in solution, a principle that is still hotly debated.[77] It is clear that the gaseous environment experienced by proteins after electrospray ionisation impacts on non-covalent interactions, and hence protein structure, on timescales ranging from $10^{-12}$ to $10^{2}$ s.[77] Therefore, careful experimental design is critical to the success of this method. Initially electrostatic interactions are promoted in the gas phase, whilst hydrophobic interactions are weakened. Since the overall stability constants of the metal-MT complexes are very high, and the structure and folding stability of MTs is generally dominated by the metal–ligand bonds, with a significant electrostatic component, they are ideally suited for analysis by ESI-MS. In turn, mass spectrometric methods are ideally suited to study complexes of $d^{10}$ metal ions such as Zn(II) and Cu(I), as they do not rely on any spectroscopic features.

In theory, high resolution MS should allow the distinction between Zn(II) (expected mass increase Zn(II)–$2H^+$: $65.4 - 2 = 63.4$ Da) or Cu(I) (expected mass increase $63.5 - 1 = 62.5$ Da), but in practice, an independent determination by elemental analysis of the metal ions present is strongly recommended. In our work, we routinely use ICP-OES, to corroborate the results of ESI-MS experiments (Fig. 3). In our experience, metal-to-protein stoichiometries determined by these two methods usually agree within <10%, lending further support to the idea that gas-phase speciation corresponds to solution speciation.

Sample preparation and conditions are possibly the most critical parameters that govern the outcome of an ESI-MS experiment. First and foremost, and contrary to common conditions of MS experiments, the pH of the sample solution for the holo protein must be kept in the neutral range, to avoid demetallation by competition with protons. Secondly, as with all ESI-MS experiments, any non-volatile compounds (salts, buffers, detergents) must be rigorously excluded from the sample

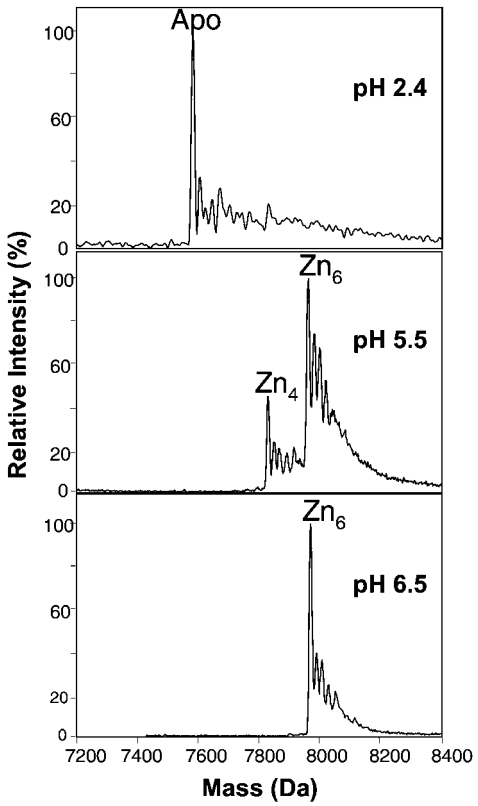


**Fig. 3** Electrospray ionisation mass spectrometry of wheat $E_C$-I/II as isolated from wheat germs (10 mM ammonium acetate, 10% MeOH).[66] Top: At strongly acidic pH, the apo form is the predominant species. From the observed neutral apo-mass (7581.4 Da, identical to the expected neutral mass), the identity of the protein can be confirmed. Bottom: At *ca.* neutral pH, only $Zn_6E_C$-I/II is observed (7961.0 ± 0.9 Da, consistent with the $Zn_6$-$E_C$-I/II species with a theoretical neutral mass of 7581.4 + (6 × 65.4) − 12 = 7961.8 Da). Middle: Lowering the pH to 5.5 leads to the cooperative loss of two zinc ions.

solution. This can be achieved by either dialysis or chromatographic methods, taking care that bound metal ions are not removed as well. If metallated species are generated by reconstitution with an excess of metal ions, the specific removal of any excess is important, as otherwise, the formation and observation of non-specific adducts is possible.[78] Furthermore, the addition of an organic modifier, such as methanol, can enhance ionisation efficiency, but again care must be taken that the organic solvent does not denature the protein, which would lead to metal loss. Finally, instrumental parameters need to be optimised in such a way that the ionised species do not acquire too much energy, to avoid activation and cleavage of non-covalent bonds. These parameters may differ slightly from protein to protein, and are of course instrument-specific. Experimental conditions are available in the manuscripts cited in this section.

The more important contribution of ESI-MS towards understanding metallothioneins goes of course far beyond simple characterization of isolated proteins. It is the only method that allows the direct observation of individual differentially and/or partially metallated species of metallothioneins under different conditions or during metal uptake,[67,79] metal exchange (Fig. 4),[70] or metal release and transfer reactions (Fig. 3 and 5).[74,80]

Demetallation of metallothioneins can be achieved by lowering the pH (Fig. 3) or the addition of chelators with higher stability constants, including other proteins. Fig. 5[74] shows ESI-MS spectra of a mutant bacterial metallothionein during the reaction with EDTA. The progressive formation of a $Zn_1$ species can be clearly seen. Surprisingly, in this reaction, very little apo-protein was observed. Closer inspection of raw MS data allowed the detection of protein fragments, which are likely to have been generated by either proteolysis or inadvertent in-source fragmentation, with the apo-metallothionein being more prone to degradation or fragmentation (Fig. 5). This particular example may serve as an illustration of caveats and limitations of quantitative kinetics by ESI-MS. Further caveats are our lack of knowledge on ionisation efficiencies for the different species, and the relatively long time it takes to bring a sample from solution into the mass spectrometer; therefore, very fast reactions cannot directly be followed and only the endpoints at equilibrium are detected. Nevertheless, a stopped-flow ESI-MS method has recently been developed and successfully employed to quantitatively monitor metallation reactions with As(III) of mammalian MTs.[67]

The issue whether metal binding in metallothionein clusters is cooperative or not has been addressed numerous times throughout the past five decades. ESI-MS has been used to prove both cooperative[79] and non-cooperative[78,81] binding. Such experiments involve the titration of the apo-protein with the metal ion of choice, equilibration, and observation of the species formed. Cooperative binding is characterised by the observation of full or empty clusters only, with no evidence for partially metallated clusters, whereas non-cooperative binding is reflected in the observation of the entire range of possible species.

Species observed by ESI-MS may also give hints as to whether metal ions are replaced isostructurally or not.[71,82] For example, the analysis of charge state distributions has been used to argue against isostructural replacement of Zn(II) by Cd(II) in mammalian MT-3, as a clear trend to higher charge states for the $Cd_7$ species, compared to the $Zn_7$ species, was observed.[82] Higher charge states are thought to arise from partial unfolding, which provides statistically more protonation sites. The influence of the charges that are introduced by the metal ions is an issue that has so far not been satisfactorily addressed.

Another unique facility offered by ESI-MS is the straightforward observation of mixtures of proteins and their metal speciation. For example, an experiment involving mixtures of mammalian MT-2 and MT-3 has demonstrated that the metal affinity of MT-3 is lower than that of MT-2, and that MT-3 shows higher variability in metal stoichiometries.[83]

Finally, metallothioneins have also served as convenient model systems for mass spectrometric method development, *e.g.* for top-down proteomics,[84] nanospray-MS and collision-induced ionisation,[85] and ion mobility-mass spectrometry.[86] It is also worth noting that the oxidation state of metallothioneins *in vivo* can be determined by proteomics methods, which has revealed that disulfide bonds exist *in vivo*, and that their proportion increases under oxidative stress.[87]

### 5.4 NMR spectroscopy: Access to structure and dynamics

Owing to their small size and their discovery as cadmium-binding proteins, metallothioneins have played an important role in the

 

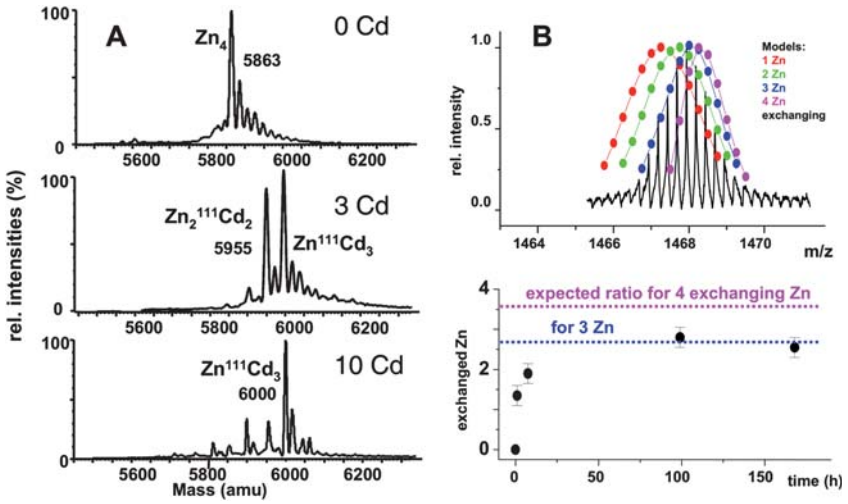


**Fig. 4** Using ESI-MS to monitor speciation and metal exchange reactions. (A) Incubation of $Zn_4$SmtA at neutral pH with substoichiometric and excess Cd(II). The formation of heterometallic species, all with four metal ions bound, can be discerned. The only reaction product with excess Cd(II) is $ZnCd_3$SmtA (20 μM protein, 10 mM ammonium acetate, pH 7.0, 10% methanol). (B) High resolution FT-ICR-MS reveals that the reluctance to exchange the final metal ion has kinetic, not thermodynamic reasons: The +4 charge state of natural abundance $Zn_4$SmtA after equilibration for 99 h with 10 mol. equiv of $^{67}ZnCl_2$ has an isotopic composition that closely matches a theoretical model for three exchanged zinc ions (blue model) and is not compatible with four exchanging zinc ions (magenta). The self-exchange reaction in SmtA is relatively slow and can be monitored by ESI-MS in a time-dependent manner (10 μM protein, 10 mM ammonium acetate, pH 7.0, 30% methanol).[70]

development of multidimensional $^1$H as well as multinuclear and $^{113}$Cd/$^{111}$Cd NMR spectroscopic methods.[17,88] Early studies were aided by their high solubility; up to 8 mM samples have been employed in some studies. In the following, we will focus on advances in $^{113}$Cd/$^{111}$Cd NMR spectroscopy, followed by an appreciation of the use of NMR spectroscopy for studying dynamics.

**5.4.1 Advances in Cd NMR spectroscopy.** The usefulness of $^{113}$Cd or $^{111}$Cd nuclei for NMR stems from a combination of several beneficial magnetic properties, which are exhibited by both of these nuclei. The bulk of work has been carried out with $^{113}$Cd, but it should be noted that the magnetic properties, including chemical shift ranges, of $^{111}$Cd are very similar to those of $^{113}$Cd. Both spin-½ nuclei have a similar sensitivity as $^{13}$C, and also resonate at similar frequencies (*ca.* 106 and 111 MHz, respectively, on a 500 MHz/11.7 T spectrometer). Furthermore, they are highly sensitive to the electronic (de)shielding that is provided by their immediate environment, leading to a chemical shift range of about 900 ppm, with oxygen donors being the most deshielding, followed by nitrogen and then sulfur ligands.[88] Thus, it is possible to draw conclusions on the ligand set from $^{111/113}$Cd NMR chemical shifts. Crucially for MT research, small differences in coordination geometry also influence the chemical shift, and therefore, the 7 Cd ions in fully Cd-substituted mammalian metallothioneins can be fully resolved in 1D spectra.

Finally, the $^3J$ coupling constants between $^{111/113}$Cd and $^1$H lie in a useful range to allow 2D heteronuclear experiments. These facts have ultimately enabled the first correct structure determination of a metallothionein. The principal steps for the elucidation of the structures of metallothioneins by NMR spectroscopy have been developed in the mid-1980s,[17] and have been refined over the years in various ways.

For a full structure determination, knowledge on the linkages between individual Cd ions and Cys (or other) residues is required, which can be obtained by studying the 3-bond scalar coupling between $^1$H and $^{113}$Cd (or $^{111}$Cd) nuclei. This manifests itself in $^1$H homonuclear experiments, as shown in Fig. 6A for a geminal H,H crosspeak in a 2D DQF-COSY (Double-Quantum-Filtered Correlation Spectroscopy) spectrum. Estimates for $^3J$ coupling constants can be extracted from such 2D $^1$H spectra, and like other $^3J$ bond couplings, they follow a Karplus relationship ($^3J = 36\cos^2\phi - 13\cos\phi + 1$).[89] This allows the deduction of torsional (dihedral) angles around the H–C–S–Cd bond (Fig. 6B), which can in principle be used in structure calculations. Determination of the full cluster structure including assignment of defined S–Cd bonds requires both $^1$H homonuclear and [$^1$H,$^{111/113}$Cd] heteronuclear experiments (for a recent comprehensive review on heteronuclear NMR spectroscopy for metal detection, see ref. 90), ideally performed on the same sample. The $^1$H homonuclear experiments (usually 2D DQF-COSY, 2D TOCSY (Total Correlation Spectroscopy) and 2D NOESY (Nuclear Overhauser Spectroscopy)) allow the sequential assignment of all protons, including the methylene protons of individual cysteines, the chemical shifts of which are required to fully interpret the 2D heteronuclear experiments.

It follows from the Karplus relationship that the $^3J$ coupling constants vary over a wide range (0–50 Hz and more). Unfortunately, most heteronuclear 2D NMR experiments can only be optimised for a small range of coupling constants, and as a consequence, it is usually necessary to acquire several 2D spectra for different coupling constants. A time-saving solution was introduced in 2000, in the form of the accordion HMQC (heteronuclear multiple quantum coherence spectroscopy).[91]

A further complication is presented by the fact that the chemical shifts of the cysteine methylene protons are very similar

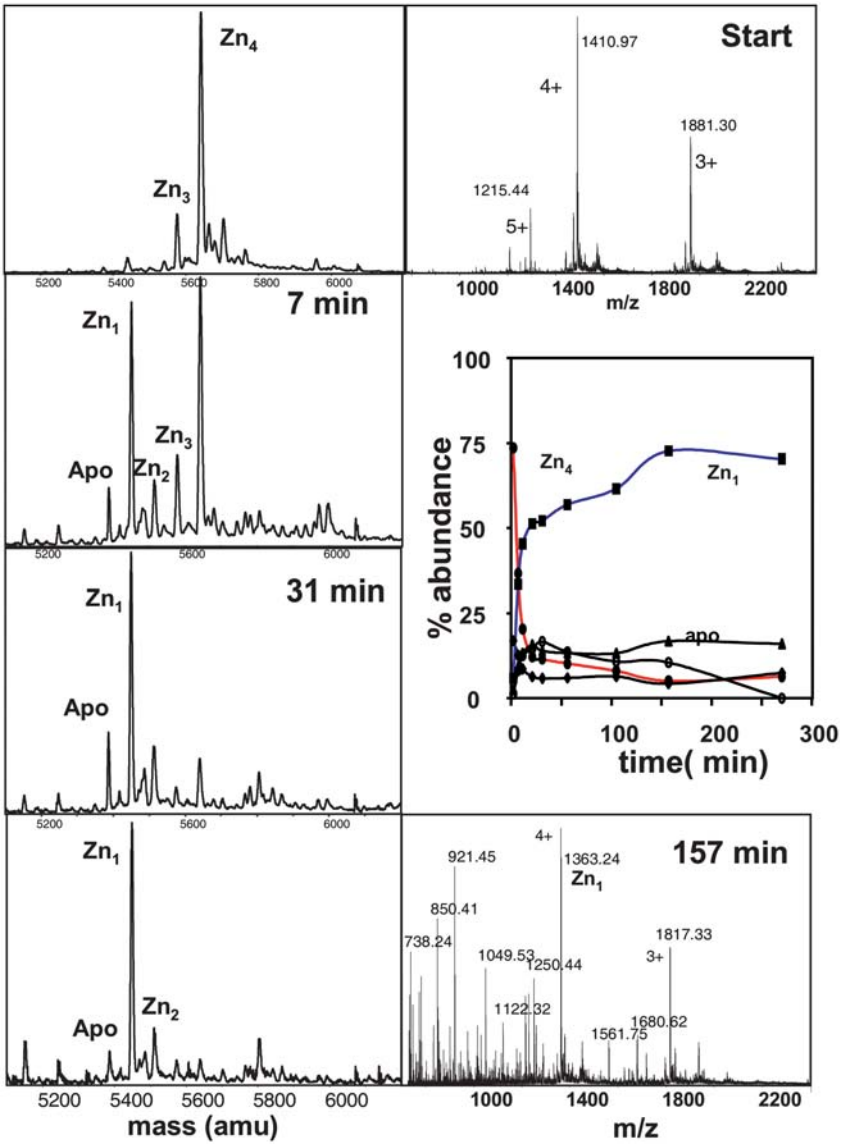


**Fig. 5** Metal release studied by ESI-MS (15 μM protein, 10 mM ammonium acetate, pH 7.4, 10% methanol).[74] The left hand side shows deconvoluted MS spectra of the H40C mutant of SmtA during the course of reaction with equimolar amounts of EDTA (400 μM protein, 1600 μM EDTA). Raw MS data at the start and after 157 min are shown to the right-hand side. The emergence of peptidic fragments can clearly be discerned, and accounts for the low percentages of apo-protein observed. A semi-quantitative plot of speciation during the course of the reaction is also shown. The abundances of species are plotted as percentages of total intact protein. The $Zn_4$ species is rapidly lost, whereas the $Zn_1$ species reacts much more slowly.

for different residues, leading to ambiguities in assignment in the heteronuclear spectra. This problem can be solved by combining hetero- and homonuclear experiments in one, for example in a [$^1$H,$^{111}$Cd] HSQC-TOCSY (Heteronuclear Single Quantum Coherence – Total Correlation Spectroscopy) spectrum (Fig. 7).[18,92] This experiment links Cd nuclei not only to the methylene protons, but also to backbone CH$\alpha$ and NH protons, the chemical shifts of which are much more dispersed, and the combination of them is usually even unique. A slight drawback of this technique is the inherently lower sensitivity of this experiment compared to ordinary HSQC spectra, which is partially offset by $^1$H decoupling.

In addition, $^1$H,$^{113}$Cd HMQC spectra have been used for the detection of NH–S–Cd hydrogen bonds, which have been exploited as additional constraints in structure determinations.[92] Finally, not only cysteine, but also histidine residues coordinated to Cd can be detected by 2D [$^1$H,$^{111/113}$Cd] heterocorrelated experiments (Fig. 7), even though the coupling constants tend to be very small, as the torsional angle for the H–C–N–Cd bond is, due to the aromatic nature of imidazole, close to 0.

Despite the major advances that have been made by developing $^{111/113}$Cd NMR spectroscopy, the universal use of these techniques for metallothioneins suffers from a number of limitations. First and foremost, not all metallothioneins are "designed" to bind cadmium. This is of course a particular problem for Cu(I)-specific MTs, because Cu(I) requires a completely different coordination geometry than Cd(II). Ag(I), which also has two NMR-active nuclei ($^{107}$Ag and $^{109}$Ag), is

 

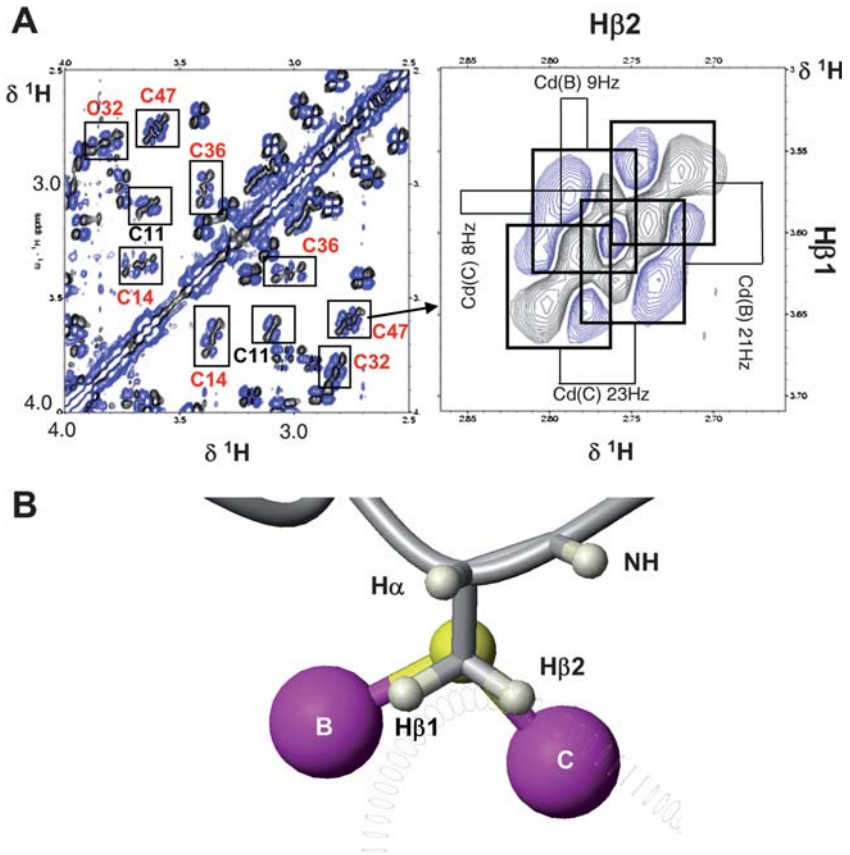


**Fig. 6** Observation of 3-bond [$^{1}$H,$^{111}$Cd] couplings for cysteine methylene protons in a 2D double-quantum-filtered COSY NMR spectrum of $^{111}Cd_4$SmtA. (A) Left: Methylene proton region. The boxes indicate geminal cross-peaks for Cys methylene protons. Bridging Cys are highlighted in red. Right: Both protons of the bridging Cys47 are coupling to two $^{111}$Cd(II) nuclei. The bold boxes illustrate the boundaries of the four resulting crosspeaks. Estimates for 3-bond coupling constants ($^{3}J$), and hence $^{111}$Cd–S–Cβ–H torsional angles, can be derived in the indicated way. (B) Close-up view of Cys47 along the Cβ–S bond, showing the torsional angles between $CH_2$ protons and $^{111}Cd^{2+}$ ions. Cd(B)–S–Cβ–Hβ1 and Cd(C)–S–Cβ–Hβ2 are in an almost eclipsed conformation (torsional angle close to 0°); this is consistent with $^{3}J$ coupling constants around 20–25 Hz. Cd(B)–S–Cβ–Hβ2 and Cd(C)–S–Cβ–Hβ1 show angles around 110°; this is consistent with $^{3}J$ coupling constants around 7–12 Hz.

thought to be a better probe for Cu(I), but Ag(I) has a much higher preference for linear coordination than Cu(I), which is often found in trigonal planar geometry. Further problems may arise from the difference in ionic radii.

In contrast, the perception that Cd(II) is a good structural probe for Zn(II) is widespread. We would caution that this tenet has to be established for each individual protein, because it may or may not hold true, as we will illustrate in Section 6 with two proteins under study in our lab.

A method that can aid the identification of coordinating His residues in metallothioneins or other proteins, and does not rely on $^{111/113}$Cd NMR, utilises [$^{1}$H,$^{15}$N] HSQC spectra. The chemical shifts of the two aromatic imidazole $^{15}$N nuclei, and their coupling pattern with protons are highly dependent on their protonation or metallation state.[93] Fig. 8 shows a [$^{1}$H,$^{15}$N] HSQC spectrum optimised for a coupling constant of 30 Hz for the wheat $Zn_6E_C$-I/II protein.[94] One nitrogen in each His residue has a chemical shift in a region typical for metal-coordinated imidazole N (*ca.* 205–225 ppm). Importantly, from the observed coupling pattern, it is possible to deduce whether the metal ion is bound to the Nδ1 or Nε2 nitrogen. The advantage of this method is the fact that it can be applied to any diamagnetic metal ion, even if it is not NMR-active itself. However, the method is not unambiguous; similar chemical shifts may also be observed for strongly hydrogen-bonded, or partially protonated imidazole nitrogens, and further experiments to corroborate conclusions are required.

**5.4.2 Dynamics by NMR.** The true potential of NMR spectroscopy lies of course not only in providing structural, but also dynamic information. The timescales that can be explored by various NMR techniques range from picoseconds to days. The dynamic nature of the protein part of metallothioneins can be studied by conventional $^{15}$N NMR backbone dynamics experiments, and these are now routinely employed during the course of conventional NMR structure determinations.[13,14]

The kinetic lability of bound Cd(II) nuclei has been studied by $^{113}$Cd saturation transfer experiments.[58] Briefly, the experiment involves irradiation of one $^{113}$Cd nucleus, and observation of the other $^{113}$Cd nuclei. If significant exchange occurs between these nuclei during the time-scale of the NMR experiment, a reduction in signal intensity is observed. In the case of a mutated mouse MT-1 β-domain, reductions in intensity of up to 94% were observed, indicating a highly dynamic system.[58] This

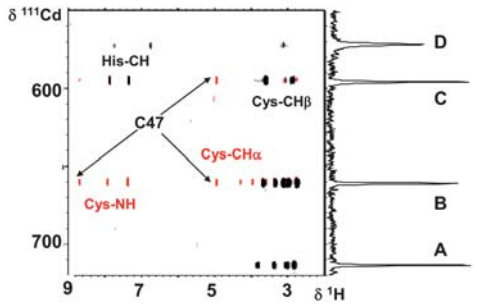


**Fig. 7** Heteronuclear NMR techniques can help to elucidate the structures of metallothioneins. The figure shows 2D [$^{1}$H,$^{111}$Cd] HSQC (Heteronuclear Single Quantum Coherence; in black) and HSQC-TOCSY (Heteronuclear Single Quantum Coherence – Total Correlation Spectroscopy; in red) spectra of $^{111}Cd_4$SmtA, optimised for a 3-bond coupling constant of 25 Hz. Selected HSQC-TOCSY crosspeaks of Cys47 CHα and NH protons with Cd(B) and Cd(C) are highlighted (see Fig. 6B), confirming the bridging nature of the residue as suggested by the data shown in Fig. 6A. The increased information content and diminished ambiguity of HSQC-TOCSY spectra is evident. The figure also illustrates the importance of narrow linewidths for $^{111}$Cd signals: very little crosspeak intensity is seen for peak D, which in 1D spectra (shown on right, recorded at 308 K) shows an increased linewidth compared to the other three peaks.

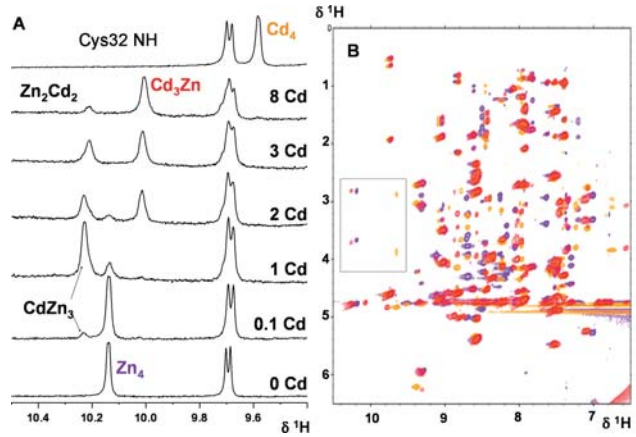


**Fig. 9** Isostructural replacement of Zn(II) by Cd(II) in SmtA and effect on backbone amide resonance of Cys32 (A) and overall fold (B), as monitored by $^{1}$H NMR. (A) 1D $^{1}$H resonances of Cys32 NH during titration of $Zn_4$SmtA with Cd(II). The assignment of peaks to various species is based on comparison with 1D $^{111}$Cd spectra (not shown). (B) Fingerprint region of 2D $^{1}$H TOCSY spectra of $Zn_4$SmtA (purple), $^{111}Cd_3$ZnSmtA (red, the endpoint of exchange reaction with excess Cd(II) at neutral pH, see Fig. 4), and $Cd_4$SmtA (orange). In all three cases, the 2D spectra indicate that homogeneous preparations have been obtained. Slight differences in chemical shifts between (A) and (B) are due to differences in experimental conditions between (A) and (B), such as pH and temperature, to which NH backbone protons are relatively sensitive.

combination of site-directed mutagenesis with elegant NMR methods has revealed that the cluster dynamics, and hence the reactivity of bound metal ions, in mammalian MTs are determined by thermodynamics and kinetics of protein folding and protein structural flexibility, and are not dominated by the properties of metal–ligand bonds.

Various further NMR techniques have been employed to study the reactivity of metallothioneins and to monitor reactions. $^{1}$H and $^{15}$N NMR spectroscopy have been used to study the consequences of metal release or exchange on the overall protein fold.[18,74,95] The effects of replacing Zn(II) with Cu(I) in mouse MT-1 domains, monitored by 2D NOESY and TOCSY NMR, including a determination of the protein fold for the mixed species, revealed that substantial structural re-arrangements in both domains are required to accommodate these different ions.[44]

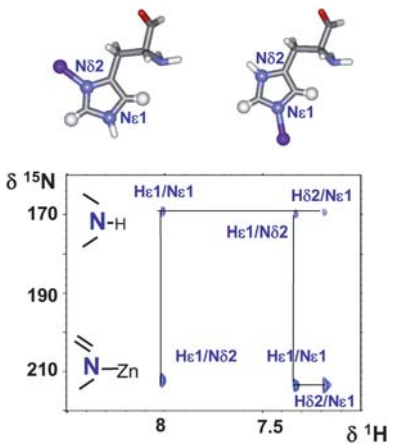


**Fig. 8** [$^{1}$H,$^{15}$N] HSQC spectrum optimised for a 2-bond $^{1}$H,$^{15}$N coupling constant of 30 Hz. The region shown reports on protons and nitrogens of histidine imidazole side-chains. The chemical shifts of the nitrogen nuclei are sensitive towards protonation, hydrogen bonding, and metal coordination. The chemical shifts of His32 and His40 in wheat $E_C$-I/II are compatible with Zn(II) coordination. Most significantly, the observed crosspeak patterns allow the determination of the tautomeric state of His residues.

The $^{1}$H NMR spectra shown in Fig. 9 demonstrate that even under conditions of "isostructural" replacement, the spatial requirements of Zn(II) and Cd(II) are perceptibly different, and that the replacement of Zn(II) with Cd(II) elicits structural consequences throughout the protein.[18]

## 6 Metallothioneins with novel properties

### 6.1 Biophysical studies of novel MTs from animals

Due to some of the advances described above, this decade has seen, beside the extension of studies on vertebrate MTs to MT-3[2,45,57,58] and MT-4,[96] a considerable increase in the *in vitro* characterisation of novel metallothioneins. The groups of Capdevila and Atrian have been playing an important role in this new push, and since 2000 have studied, amongst others, the four metallothioneins from the fruit fly,[23] Crs5 from yeast,[97] the mussel *Mytilus edulis*,[98] *Tetrahymena pyriformis*[99] and *Caenorhabditis elegans*,[100] MT-2 from the cork oak, and the vertebrate MT-4[101] and chicken MT-1.[102] Metal-binding features for 16 different MTs are summarised in ref. 43.

Intriguingly, the study of the four *Drosophila* MTs has demonstrated that the strongest inducer of transcription is also the metal ion preferentially bound, with MtnA responding to and binding Cu(I), and MtnB responding to and binding Cd(II), although the latter had previously been characterised as

 

a Cu(I)-thionein.[23] Similar conclusions apply to the *Tetrahymena pyriformis* MT1, which plays a role in Cd-detoxification, and only yields defined preparations of the form $M_{11}$MT1 in the presence of Cd(II). Expression in the presence of Zn(II) or copper yielded heterogeneous, partially metallated and oxidised species.[99] MT-10-IV from the mussel *Mytilus edulis* appears to have a role in essential Zn(II) homeostasis, and *in vitro* studies have demonstrated that at least one site in this protein has a pronounced preference for Zn(II) over either Cu(I) or Cd(II). The fully exchanged $Cd(II)_7$ and $Cu(I)_{12}$ species exhibited dramatically different structures than the $Zn(II)_7$ species, as judged from CD spectroscopic envelopes.[98] Another intriguing feature of this protein concerns the fact that the Cd(II) complexes formed *in vivo* (in *E. coli*) and *in vitro* are not equivalent, overthrowing one of the paradigms in MT research.

The related metallothionein MT-10 from *Mytilus galloprovincialis* was studied in its $^{113}Cd_7$ form (formed by supplementing growth media with 0.2 mM $^{113}CdCl_2$) by $^1$H, $^{15}$N, and $^{113}$Cd NMR spectroscopy.[103] The $^1$H spectra showed high dispersion of NH resonances, suggesting a well-folded protein, and backbone dynamics were slower than for other MTs. The $^{113}$Cd NMR resonances of this protein are also highly dispersed over 210 ppm (673.3 down to 463.5 ppm), suggesting that the coordination environment is significantly different from other MTs. Indeed, in the putative N-terminal α domain, which contains 12 Cys residues, one of the five bridges is severed and replaced with two terminal cysteines, giving rise to a completely novel cluster structure. This new example containing a $M_4Cys_{12}$ cluster extends the portfolio of cluster structures. The series $M_4Cys_{10}$ (adamantane structure, not observed in metallothioneins), $M_4Cys_{11}$, and now $M_4Cys_{12}$ is reminiscent of the closo-, arachno-, and nido-borane structures, and begs the question whether certain trends in reactivity may be expected. Since terminal Cys residues are inherently more reactive than bridging ones, it may be anticipated that the overall reactivity, both in terms of metal- and sulfur-centered reactions, of the $M_4Cys_{12}$ clusters may be increased. This study also presents a further cautionary tale, illustrating the unpredictability of metallothionein cluster structures, as a model suggested by the same authors earlier bore no resemblance to the one determined.

### 6.2 Bacterial metallothioneins

For a comprehensive review on bacterial metallothioneins up until 2007, see ref. 104. Until 2008, the only experimentally confirmed bacterial metallothioneins (pfam02069, InterPro: IPR000518) all belonged to the BmtA family,[61] with SmtA from the freshwater bacterium *Synechococcus* PCC7942 as the prototype.[12] Homologues of SmtA have been identified in the genomes of about half the sequenced cyanobacteria,[105] in a few α and γ proteobacteria, and in one firmicute, *Staphylococcus epidermidis*.

There has been a recent addition to the portfolio of bacterial MTs, in the form of the copper-binding MymT from *Mycobacterium tuberculosis*.[106] The expression of MymT is induced by Cu, Cd, nitric oxide or superoxide, and the synthetic protein has been shown by ESI-MS to bind up to six copper ions, whereas spectra in the presence of zinc showed low quality. This small (4.9 kDa) protein had not previously been annotated in any of the *Mycobacterium* genomes, and was only discovered during a chemical genetics screen. It is possible that further diverse bacterial metallothioneins exist, that have not yet been discovered. An example for such a "cryptic" metallothionein may be present in the actinomycete *Streptomyces coelicolor*, which contains a putative expression regulator with a 18-Cys domain that resembles metallothioneins (Table 1), but the protein has not been isolated. No MTs have so far been described in archaea.

The *smt* operon in *Synechococcus* PCC 7942 responds strongly to Zn, and the expression products, the metallothionein SmtA and the transcriptional repressor SmtB, are thought to operate as mainly Zn-binding proteins *in vivo*. SmtA folds very well as the $Zn_4$ species (Fig. 9). It folds equally well as the $Cd_3Zn$ or $Cd_4$ species (Fig. 9), and from a detailed evaluation and comparison of the 2D $^1$H data for both homometallic species, including a full structure determination for $Cd_4$SmtA, we were able to conclude that the replacement is isostructural, although the larger size of Cd(II) requires a slight expansion of the cluster (Fig. 10).[18]

SmtA is currently the metallothionein with the highest content of secondary structure; 20 out of a total of 55 residues are part of a "treble-clef" zinc finger fold.[16] The peculiar combination of a metallothionein cluster with a zinc finger fold has intriguing consequences for the metal dynamics of SmtA.[70,74] The three "metallothionein-like" zinc ions (B, C, D; Fig. 10) react considerably faster than the "zinc finger" one (A) in metal transfer reactions from SmtA; this is reflected in the observation of partially metallated $Zn_1$SmtA species in ESI-MS spectra during the reaction with EDTA (as shown for the H40C mutant in Fig. 5). The preservation of the zinc finger fold was established by running 1D and 2D NMR experiments during the reaction (not shown).[74]

The zinc finger fold also plays an important role in the metal exchange dynamics of SmtA, which was studied by 1D $^{111}$Cd and $^1$H NMR (Fig. 9)[12] as well as high-resolution ESI-FT-ICR mass spectrometry (Fig. 4). The combination of these techniques established unequivocally that in the intact 4-metal cluster, the zinc finger site A is inert towards exchange.[70]

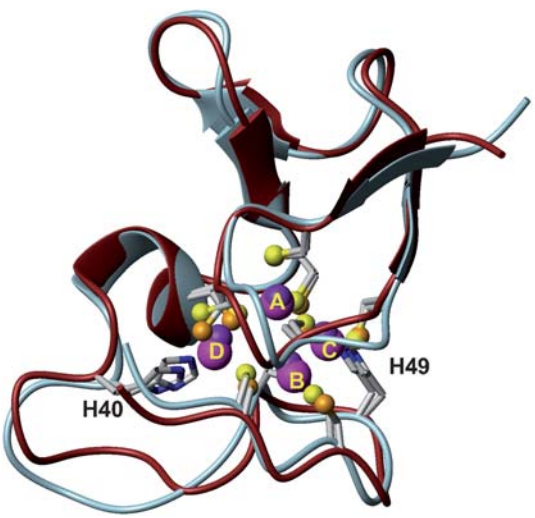


**Fig. 10** Comparison of structures for homometallic $Zn_4$SmtA (blue backbone, purple Zn(II), and yellow sulfurs) and $Cd_4$SmtA (red backbone, magenta Cd(II), and orange sulfurs). The maintenance of the zinc finger fold is evident, as is the effect of the larger size of Cd(II).

SmtA was also the first metallothionein for which metal coordination by histidine residues was established.[12,53] The crucial role for protein as well as metal cluster stability was discovered by studying His-to-Cys mutants.[56,70] His40 (Fig. 10) is essential for stabilising the fold, and His49 provides a redox inert site for metal release. Intriguingly, the loop comprising His49 is the most variable region in the sequences of BmtAs (Table 1).[56] Studies on a small number of other BmtAs have revealed that these variations have major impacts on metal binding properties.[61,104] Several marine *Synechococcus* strains appear to produce not only one, but several BmtA isoforms,[105] with the coastal strain CC9311 containing genes for no fewer than four different putative MTs (Table 1).

### 6.3 Plant metallothioneins

The existence of metallothioneins in plants has long been debated, mostly due to misconceptions about the function of MTs as well as experimental difficulties with their isolation. Today, there are over 400 plant MT sequences deposited in Uniprot, and many more sequences can be retrieved from the NCBI nucleotide and expressed sequence tags (dbEST) databases (see Fig. 1). Metallothioneins occur in flowering plants, conifers, spike mosses, liverworts, mosses, ferns and green algae. Numerous functional studies testify to their extensive expression, as reviewed elsewhere.[3,37,48,107] An MT from the brown alga *Fucus vesiculosus* (Bladder wrack), which is not a green plant, but a stramenopile, has also been isolated and characterised.[108]

For plant MTs, four sub-families have been defined so far, referred to as types 1 to 4 (Table 1). The diversity between these four types is so pronounced that they cannot be considered as isoforms, although it is likely that a phylogenetic relationship exists between the four types. Most higher plants appear to express representatives for each type in a tissue- and developmental stage-dependent manner.[107] It should be noted that the sequences from ferns, the green alga *Ostreococcus*, and (as is to be expected) the Bladder wrack do not belong to any of the defined subfamilies.

One salient characteristic feature of plant MTs is the presence of extended stretches that are devoid of Cys residues. These are often referred to as "linkers", suggesting that these stretches connect individual domains with individual metal–thiolate clusters. Freisinger's group has studied a representative of each type by a variety of methods.[37,109] Most significantly, and contrary to widespread pre-conceptions, for all three types 1, 2 and 3, the available evidence points towards a one-domain structure, in which the "linker" forms a hairpin. A similar suggestion has been made for MT-2 from the cork oak,[52] and indeed in an early study on MT-1 from pea.[49] Consistent metal stoichiometries have been determined recently for all four types:[37,109] type 1 MTs, exemplified by chickpea MT, appear to bind 4–5 divalent or 6 or 9 Cu(I) ions; type 2, exemplified by chickpea MT-2, binds 5 divalent or 8 Cu(I) ions; type 3, exemplified by banana MT-3, binds 3–4 divalent ions, with no information on Cu(I) binding available; and type 4 MTs, as measured for wheat $E_C$-I/II, bind 6 zinc ions.[66]

Overall, most progress has been made in the case of the type 4 metallothionein $E_C$-I/II from wheat. $E_C$-I/II folds very well as the $Zn_6$ species isolated from natural sources (Fig. 11A) and from recombinant expression. Involvement of the two conserved His residues in metal binding, as well as their spatial proximity, was suggested in 2007,[66] based on pH titrations monitored by atomic absorption spectroscopy (Fig. 11B), 1D $^1$H NMR (Fig. 11C) and 2D NOESY data (not shown). The backbone fold of full-length $E_C$-I/II was calculated from $^1$H and $^{15}$N NMR data in late 2007[110] and presented during 2008 on various meetings.[94] The low-resolution structure generated at that time, supported by [$^1$H,$^{15}$N] HSQC spectra (Fig. 8) allowed the conclusion that unprecedentedly $E_C$-I/II contains an isolated zinc site formed by His32, His40, Cys46, and Cys48 (see also ref. 14). Remarkably, it is also the very first site in a metallothionein with a $Cys_2His_2$ ligand set. Furthermore, it is also clear that the protein forms two domains, and that the N-terminal domain contains another unprecedented feature for a metallothionein, namely, a $Zn_2Cys_6$ cluster, otherwise only reported for fungal transcription factors.

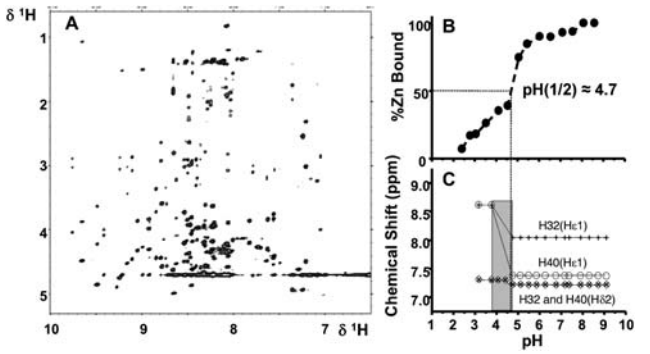


**Fig. 11** Properties of a type 4 plant metallothionein. (A) Fingerprint region of a 2D $^1$H TOCSY NMR spectrum at 900 MHz of native $Zn_6E_C$-I/II as isolated from wheat germs, indicating a well-folded protein.[66] (B) and (C) Zinc loss, as monitored by atomic absorption spectroscopy (B), and histidine protonation, as monitored by $^1$H NMR (C), are concerted, hinting at involvement of the two His residues in metal binding. The data also allow an estimate for the pH of half-dissociation, *ca.* 4.7 (in 10 mM ammonium acetate), which is in the range expected for Zn-metallothioneins.

However, repeated attempts by us[66] and others[14] to generate a cadmium-bound species that could be studied comprehensively by NMR spectroscopy have so far been thwarted by the corruption of protein folding in the C-terminal domain by the incorporation of Cd(II). Our interpretation of this failure is that this embryo-specific protein has evolved as a highly Zn-specific metallothionein that has a role in Zn distribution during development and differentiation, and that its metal-dependent protein dynamics may aid in excluding Cd from Zn sites in the developing plant embryo or seedling.[66] We also note that cadmium-loaded wheat $E_C$-I/II shows a behaviour that is reminiscent of that of the $Cd_3\beta$ domain of mammalian MT-3.[2] Further studies to elucidate the cluster structure in the C-terminal domain will be required to establish whether the remaining nine cysteine residues and three metal ions behave as expected or not.

### 6.4 The emergence of histidines as metal-coordinating residues in metallothioneins

The implications of His coordination in divalent-metal binding metallothioneins are extensively discussed in ref. 111. The first

 

conclusive example for Zn(II) and Cd(II) coordination by His residues in an MT was afforded by SmtA from the cyanobacterium *Synechococcus* PCC7942 as described above.[53] Meanwhile, histidine residues have been found in a variety of metallothionein sequences, including those from other bacteria,[105,111] yeast Crs5[97] and Cup1, Zym1 from fission yeast,[112] MTL-1 and MTL-2 from *Caenorhabditis elegans*,[24] and many plant metallothioneins (Table 1), especially the fruit-specific isoforms of MT-3.[111] In addition, and contrary to a widespread misconception, histidines do also occur in vertebrate MTs, for example in at least two human isoforms (MT-1M and the not yet named Trembl entry Q8TDC4, which appears to be involved in monocyte metabolism), chicken and other avian MT-1,[102] and various reptilian MTs (Table 1).[113]

So far, with the exception of SmtA and $E_C$, relatively little conclusive information on whether these His residues participate in metal binding is available. In those few cases where data on metal affinity and specificity is available, a relative reduction in Cd(II) binding *versus* Zn(II) binding upon Cys-to-His replacement has been established.[54,55] Based on optical spectroscopic evidence, the likelihood of His coordination has been discussed for MT-3 from banana[37] and for yeast Crs5.[97] A recent report on the two *C. elegans* metallothioneins[100] has suggested that the three additional His residues in mtl-1 are involved in the binding of an additional metal ion, and the findings reported have qualitatively confirmed expected trends.

## 7 Conclusions

Research activity in the field of metallothioneins has recently experienced a renaissance, due to mounting evidence for the biological importance of MTs and ongoing advances in experimental approaches. It is clearly not possible to predict structure, metal specificity, affinity and stoichiometry, and the kinetics of metal uptake and release for uncharacterised MTs; therefore, for a true qualitative and quantitative understanding, and also in terms of a "systems biology" approach, these data need to be generated.

Conversely, the biophysical and bioinorganic–chemical measurements described in the current report are important, but they can only be truly meaningful if they are integrated with *in vivo* research. The powerful combination of *in vitro* and *in vivo* data will enable better experimental design for both chemistry and biology angles, and advance our understanding of metal handling in biological systems.

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