Structural and functional studies of vertebrate metallothioneins: cross-talk between domains in the absence of physical contact

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In previous studies, we showed that the chemical and dynamic properties of fish and mouse MTs (metallothioneins) present a number of distinctive differences linked to their primary structures, and that phylogenetic relationships of mammal and fish MTs correlate with their three-dimensional structures. The different behaviours of MTs may also be linked to the interaction between their two domains. In the present study, we have compared the physicochemical properties of the isolated recombinant domains constituting Notothenia coriiceps and mouse MTs, and compared them with those of the corresponding whole MTs. NMR spectra of the separated domains of N. coriiceps are almost superimposable on those of the parent MT, suggesting an apparent lack of interaction between the two domains in the protein. However, certain dynamic and physicochemical features of the isolated domains are unlike those of the whole protein. In particular, the temperature-induced changes in the chiroptical properties, thiol reactivity of the Zn-MT domains and the Zn2+/Cd2+ rate of exchange are different for the two domains and with respect to the whole protein. Taken together, these results provide a strong argument in favour of the interaction of the two domains in the MT molecule, in spite of the elusive evidence provided by the structural analyses.

Key words: circular dichroism, metallothionein, metal mobility, NMR, thiol reactivity.

INTRODUCTION

Zinc and copper are physiologically important nutrients for animals [1] and plants [2], providing essential structural and catalytic functions to a large number of proteins and enzymes [3]. Zinc also plays a crucial role in the regulation of gene expression, especially via transcription factors known as zinc-finger proteins [4,5].

A large body of evidence suggests that cellular zinc homoeostasis is regulated at one or more stages by MTs (metallothioneins) [6–9]. These are low-molecular-mass proteins made of approx. 60 amino acids, 20 of which are cysteine residues whose thiol groups form, along with metal atoms, two structural elements known as metal–thiolate clusters [9,10]. MT thiol groups are highly reactive in spite of their remarkable thermodynamic stability [11–13]. Indeed, metal ions not only undergo exchange phenomena within and between clusters, but they also participate in metal-transfer reactions from MT to apoproteins [14–16].

Although MTs may serve in the disposition and detoxification of environmental cadmium and superoxide radicals [17], increasing evidence suggests their involvement in the redistribution of cellular zinc [18,19]. At first glance, the primary structure of vertebrate MTs reveals a high percentage of similarity among species [7,9,20]. However, even a few non-conserved amino acid substitutions may affect the structural flexibility of MTs, influencing markedly the metal-binding properties. This is the case of the two distinct functional and structural MTs present in the snail, one binding cadmium and the other copper, which differ in a limited number of amino acid substitutions involving residues that are placed between the conserved cysteine residues [21,22].

In two recent papers, we compared the mouse MT with the Antarctic fish Notothenia coriiceps MT; the results showed that the structures of the metal–thiolate clusters of these MTs are differentially modified by temperature [12,23]. In addition, we demonstrated a different reactivity of the two MTs, with a zinc mobility higher for fish MT than for its mammalian counterpart [12,23].

In keeping with these results, recent work carried out in our laboratory shows that the three-dimensional structure of fish MT, determined by homonuclear and heteronuclear NMR spectroscopy, presents a number of distinctive features with respect to mammalian MT [24]. Although the overall architecture is apparently very similar for the two proteins, the misalignment of the ninth cysteine residue of the α-domain of fish MT markedly modifies the orientation of a loop of four residues with respect to the corresponding loop present in mammalian MT. In addition, NMR spectroscopy reveals that the dynamic behaviour of the two domains is markedly different. A broadening of the resonances from the β-domain specifically affecting the heteronuclear spectra suggests the existence of an exchange phenomenon of the metal ions in the β-domain, more evident in fish MT than in mammalian MT. These results, together with our previous observations that fish MTs are less hydrophobic and hence more flexible than mammal MTs [25], led us to postulate the existence of biological differences between the two proteins.

A study carried out by other authors on the two separated domains of human MT showed that the α- and β-domains differ markedly in terms of chemical reactivity and metal binding capacity [14,15]. However, so far it has not been investigated whether these differences apply to other MTs as well. In the present

Abbreviations used: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GST, glutathione S-transferase; LB, Luria–Bertani; MALDI–TOF, matrix-assisted laser-desorption ionization–time-of-flight; MT, metallothionein; NOE, nuclear Overhauser effect; PAR, 4-(2-pyridylazo)resorcinol.

Mus musculus and Notothenia coriiceps metallothioneins are available in Swiss-Prot Protein Database under the accession numbers P02802 and P62339 respectively.

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paper, we have investigated the properties of the two domains constituting the *N. coriiceps* MT, with the specific aim to verify whether the differences detected between fish and mouse MTs exist also when comparing the isolated domains. In the present paper, we have examined the properties of the isolated domains of *N. coriiceps* and mouse MTs in order to establish their specific influence on the physicochemical and biological differences observed in the whole MTs.

**EXPERIMENTAL**

**Materials**

Fusion expression vector pGEX-4T-1 was purchased from Amersham Biosciences. *Escherichia coli* strain BL-21 (DE3) was from Novagen. Plasmid pGMA8 [26] containing mouse MT-1 (MT isoform I) was kindly provided by Dr Binggen Ru (National Laboratory of Protein Engineering, College of Life Sciences, Peking University, Beijing, People’s Republic of China). Other analytical grade reagents were from Sigma Chemical Co.

**Amplification, cloning and sequence analysis of α- and β-domain-coding regions of recombinant fish and mouse MTs**

DNA fragments coding for α- and β-domains of fish and mouse MTs were prepared by PCR using the full-length fish MT cDNA and mouse MT-I cDNA, previously cloned in the plasmid pGST-MT [12] and pGMA8 [26] respectively. DNA fragments encoding peptides Lys31–Gln61 and Lys31–Ala61 were amplified to obtain the α-domain-coding regions of fish and mouse MTs respectively. The fish and mouse upstream primers (5′-CCGAATTCACGATGTCG-3′ and 5′-CCGAATTCACGATGTCG-3′) were designed to generate an ATG initiation codon before Lys31 that was preceded by an EcoRI restriction site. The fish and mouse downstream primers were 5′-CCGTCGACTCACTGACAGCAGCTGTC-3′ and 5′-CCGTCGACTCACTGACAGCAGCTGTC-3′, reproducing the stop codon of the MT gene followed by a Sall restriction site.

The fish and mouse β-domains extend from Met1 to Lys30. The fish and mouse upstream primers were 5′-CCGAATTCACGATGTCG-3′ and 5′-CCGAATTCACGATGTCG-3′ respectively, and the downstream primers were 5′-CCGTCACTCACTGACAGCAGCTGTC-3′ and 5′-CCGTCACTCACTGACAGCAGCTGTC-3′ respectively. The upstream primers contained an EcoRI restriction site just before the start of the coding region. In the downstream primers, a stop codon was added just after Lys30, followed by an adjacent Sall restriction site. Plasmids pGST-MT and pGMA8 were used as templates in PCRs. Amplification was performed with 5 units of Taq DNA polymerase (PerkinElmer), 50 pmol of each of the above primers and 0.2 mM dNTPs (final concentration). The mix was buffered with PerkinElmer PCR storage buffer (100 mM Tris/HCl, pH 8.3, 500 mM KCl, 15 mM MgCl2 and 0.1% gelatin). PCRs were carried out in a DNA Thermocycler Express (Hybaid), with an initial denaturation step at 95 °C for 3 min, 30 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and a final step at 72 °C for 15 min. All the PCR fragments were gel-purified using a Genomex gel-extraction kit and subcloned in a pCR2.1-TOPO vector using a Topo TA Cloning kit (Invitrogen). *E. coli* cells were transformed with a ligation mixture. Plasmid DNA was digested with EcoRI and Sall restriction enzymes, and the resulting fragments were ligated into the corresponding sites of the GST (glutathione S-transferase) fusion expression vector pGEX-4T-1. The resultant recombinant plasmids can produce a fusion protein in which the N-terminus is GST and the C-terminus is either fish or mouse MT domains. The sequences of all MT cDNA domains cloned in pGEX-4T-1 were again verified by sequencing in both directions using the pGEX primers (Amersham Biosciences).

**Expression and purification of recombinant fusion domains**

To overexpress fish and mouse MT domains, the recombinant constructs were introduced into the protease-deficient strain *E. coli* BL-21 (DE3) to produce the plasmids pGST-βMT and pGST-αMT for fish MT domains, and pGMA-βMT and pGMA-αMT for mouse MT domains. Transformed *E. coli* cells were grown in LB (Luria–Bertani) broth containing 100 μg/ml ampicillin at 37 °C overnight. The overnight culture was diluted 100-fold using fresh LB broth with added ampicillin, and cells were grown until mid-exponential phase (OD600 of 0.6). At this point, IPTG (isopropyl β-D-thiogalactoside) and CdSO4 were added to a final concentration of 0.1 mM and 0.2 mM respectively. For the preparation of Zn-MT, 0.2 mM ZnSO4 substituted for CdSO4. After incubation for a further 3 h at 37 °C, cells were pelleted by centrifugation at 7400 g for 20 min and washed twice in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4, pH 7.3).

The pelleted cells were resuspended in 5% of the original volume of PBS containing 3 mM 2-mercaptoethanol and lysed by mild sonication at 4°C. Triton X-100 was added to a final concentration of 1%, and the suspension was mixed gently at room temperature (25°C) for 1 h to facilitate solubilization of proteins. The recombinant fusion protein was purified by affinity chromatography on a column of glutathione–Sepharose 4B (Amersham Biosciences) and cleaved with thrombin to obtain fish or mouse MT domains. Isolated MT domains were purified further by gel-filtration chromatography on a column of Sephadex G-75 [23]. The fractions containing MT domains were pooled and freeze-dried.

**N-terminal sequence analysis, MS and metal determination**

N-terminal sequence analysis was carried out by Edman degradation using an Applied Biosystem sequencer. Mass spectra were obtained on a Perseptive Biosystem Instrument Voyager-DE MALDI–TOF (matrix-assisted laser-desorption ionization–time-of-flight) mass spectrometer: the fish and mouse MT domains (60 pmol/μl) were diluted 2-fold with the cinnamic acid matrix, and the sample was dried on a stainless steel plate. The data from 50 laser shots were acquired and averaged to produce the spectra shown in Figure 1. Insulin (5700 Da) was used for external calibration. Cadmium content was determined by atomic absorption spectrophotometry on a PerkinElmer apparatus, model 5100 PC Zeeman.

**Absorption and CD spectroscopy**

Absorption spectra and CD spectroscopy of samples of recombinant α- and β-domains were performed as described previously [23]. The protein concentration was 0.03 mg/ml for absorbance spectroscopy and 0.1 mg/ml for CD.

**Thiol reactivity of the zinc domains**

The thiol reactivity of the fish and mouse Zn-MT domains was assayed spectrophotometrically at 412 nm and 25 °C with Ellman’s reagent DTNB [5,5′-dithiobis(2-nitrobenzoic acid)], using the procedure described by Jiang et al. [14]. The reaction was carried
out in 0.2 M Tris/HCl, pH 7.4, with 10 µM protein and 4 µM DTNB. To calculate the amount (in nmol) of cysteine, a five-point calibration curve was determined using cysteine as standard.

**Kinetics of zinc release**

Zinc release was determined on both fish and mouse Zn-MT domains using the procedure described by Maret and Vallee [28] by measuring the zinc transfer from MT to PAR [4-(2-pyridylazo)resorcinol] in the absence or in the presence of reduced and oxidized glutathione.

**Kinetics of Zn/Cd substitution in α- and β-domains**

The cadmium substitution in fish and mouse Zn-domains was measured by monitoring spectrophotometrically the absorbance at 254 nm. Cadmium (21 µM) was incubated with zinc domains (2 µM) in 0.2 M Tris/HCl, pH 7.4, and the formation of the Cd-thiolate complex was followed at 254 nm. All data were analysed as first-order reactions by plotting \( \ln(A_\infty - A) \) against time.

**NMR spectroscopy**

NMR measurements were performed using 1.2 mM samples of each domain in \( \text{H}_2\text{O}/\text{D}_2\text{O} (19:1) \) at pH 7.0 under nitrogen. Experiments were run on Bruker DRX-400 at 293 and 303 K. Data processing was performed with NMRPipe [29] and spectral analysis with NMRView [30]. A conventional set of two-dimensional spectra, COSY [31], TOCSY [32] and NOESY [33], was used for sequential assignment according to the scheme described by Wüthrich [34]. TOCSY spectra were collected with mixing times in the range 50–75 ms, using the DIPSI2-rc mixing scheme [35]. NOESY spectra were recorded with mixing times in the range 100–150 ms. TPPI (time-proportional phase incrementation) was applied to achieve quadrature detection in the indirect dimension [36]. Water suppression was achieved either by presaturation or by using the Watergate pulse sequence [37].

**RESULTS**

**Characterization of domain peptides of fish and mouse MTs**

Isolated α- and β-domains of fish and mouse MTs prepared as described in the Experimental section were analysed by MALDI-TOF MS to ascertain whether their sizes are consistent with those of the domains in the whole MT molecules. The data in Figure 1 show that the molecular masses correspond to the theoretical values calculated from the amino acid sequences of the two peptides deduced from that of the respective cDNAs. The metal/protein ratio was 4±0.2 and 3±0.1 equivalents of either cadmium or zinc per mol for α- and β-domains respectively.
Comparison of corresponding regions of the TOCSY spectra of (A) N. coriiceps MT, (B) αMT domain, and (C) βMT domain. All the spectra were recorded at 400 MHz at 293 K, with a mixing time of 65 ms.

NMR spectroscopy

We have compared the NMR spectra of the isolated domains with the corresponding spectra of the same domain in the whole protein. It could be predicted that even minor direct interactions between the two domains can affect the chemical shifts of protons of either domain, since the dependence of chemical shifts, even on small local environmental changes, is highly non-linear. Figure 2 shows the NH-α fingerprint region from the TOCSY spectra of the isolated α- and β-domains and of the whole metallothionein. It can be appreciated that corresponding resonances occupy virtually the same position, a clear proof of complete lack of correlation between the α- and the β-domains of fish MT over NMR timescales.

Effect of α- and β-domain interaction on metallothionein CD spectra

In order to investigate the contribution of the isolated domains on the dichroic properties of fish MT, we have compared the CD spectra of the whole MT with the spectra of α- and β-domains. From the data reported in Figure 3, it is clear that the CD spectrum of the α-domain is similar to that of MT: the molar ellipticity at 260 nm of the α-domain accounts for approx. 80% of the molar ellipticity of the whole molecule measured at the same wavelength. The chiroptical properties of the β-domain are much less pronounced, and the molar ellipticity contributes only 20% to the total. Nevertheless, the molar ellipticity of the whole MT turns out to be approximately the sum of the contributions of the two isolated domains.

Modifications of spectral properties with temperature

The electronic spectra of Cd–thionein have an absorption maximum at 265 nm, with a shoulder at 254 nm typical of a charge-transfer complex. Cd–thionein exhibits an extrinsic Cotton effect at 260 nm, mostly due to asymmetric interactions of thiol groups with metal ions. Our previous studies showed changes in the chiroptical properties of Cd–thionein induced by temperature that were attributed to an altered conformation of the metal–thiolate clusters.

We reckon that the knowledge of temperature-induced modifications of both absorption and CD spectra of isolated MT domains may unravel unexpected differences with respect to the whole molecule. In addition, a comparison of the spectral properties of both α- and β-domains of distinct MTs may help us to understand the contribution of the two domains to the establishment of such differences. Therefore both Cd4-α-domains and Cd3-β-domains of fish and mouse MTs were monitored for absorbance at 254 nm in temperature ranges between 25 and 90°C. The results in Figure 4 show that a steady decline in absorbance occurs with the increase of the temperature; however, the α-domain of fish MT appears to be more thermosensitive than that of mouse (Figure 4A). The behaviour of the isolated α-domains during heating does not mirror that of the respective whole MTs [23]. As shown by the data summarized in Table 1, the absorbance of the mouse MT is stable up to 60°C, whereas the absorbance of the isolated α-domain starts to decline at approx. 30°C (see also Figure 4A). The decrease in absorbance is approx. 2% between 25 and 60°C, and approx. 4% between 60 and 90°C. Similarly, the isolated α-domain of fish MT is more sensitive to heating with respect to the whole fish MT: the loss of absorbance in the range 25–90°C is approx. 20%, whereas that
of the whole MT is 6.6%. The isolated β-domains of both fish and mouse MTs display a similar behaviour in the temperature range 25–90°C (Figure 4B).

As shown in Figure 5 and summarized in Table 1, the chiroptical properties of the Cd₄-α-domain of fish (Figure 5A) and mouse (Figure 5B) are affected by temperature to a larger extent than the whole MT molecules are (Table 1). The spectral profiles of fish and mouse α-domains are restored when the temperature is brought back to 25°C. The CD spectra of the isolated Cd₄-β-domains of both MTs display a remarkable instability with temperature (Figures 5C and 5D, and Table 1): the Cotton effect at 260 nm is completely lost at 50°C for both β-domains, and these are incapable of recovering the pristine conformation once the initial thermal conditions have been re-established. It is noteworthy that the profiles of the CD spectra of the two β-domains are modified in a different fashion by heating, suggesting that the two proteins acquire different intermediate conformations during unfolding.

**Reactivity of thiol groups with DTNB**

We have demonstrated previously that fish and mouse MTs display a different reactivity of their thiol groups [24]. In particular, the number of reactive cysteine residues is lower in fish MT than in mouse MT (see inset in Figure 6A). The titration experiments carried out with DTNB on the isolated α-domains show that the mouse protein has a higher number of reactive thiol groups than its fish counterpart (Figure 6A). This result is consistent with the structural differences between fish and mouse MTs [24], showing that the surface of fish MT differs from mouse MT in the number and distribution of charged residues. In particular, the negative charges of the fish α-domain appear concentrated around the mouth of a channel opened by the different orientation of a loop formed by four amino acid residues. As a consequence, the interaction of the negatively charged DTNB with the α-domain of fish MT is hindered by the localization of the negative charges on the protein. On the other hand, the β-domains of both fish and mouse MTs apparently contain an almost equal number of titratable cysteine residues (Figure 6B). It should be noted, however, that the sum of the reactive thiol groups present in the α- and β-domains exceeds the number of the thiol groups titrated in the whole MTs (see inset in Figure 6A), suggesting that the isolated domains are more reactive than when they are assembled in the whole protein.

**Zinc transfer from Zn²⁺-MT domains to PAR**

Both Zn₄-α-domain and Zn₄-β-domain from fish and mouse MTs were compared for their ability to transfer zinc to PAR. The results show that the amount of zinc transferred is higher for both the α- and β-domain (Figures 7A and 7B) of fish MT than for the corresponding domains of mammalian MT. From the shape of the curves reported in Figure 7, it appears that the kinetics is biphasic for both domains.

**Kinetics of Zn/Cd substitution in α- and β-domains**

In a recent investigation aimed at studying the relationship between chemical structure and functional properties of fish and mouse MTs, we found that two MTs display a different attitude in the Zn²⁺/Cd²⁺ exchange [24]. The results showed that fish MT has a better exchange capability than mouse MT, probably...
100  C. Capasso and others

**Figure 5  Effects of increasing temperature on the CD spectra of fish and mouse MT domains**

CD spectra were recorded in the near UV region (250–320 nm) in the 20–90 °C temperature range. The results are representative of three independent sets of measurements for which the maximal difference in molar ellipticity at each temperature was 0.12 %. (A) Fish Cd4-α-domain. (B) Mouse Cd4-α-domain. (C) Fish Cd3-β-domain. (D) Mouse Cd3-β-domain. Mol. ellip., molar ellipticity [(deg · cm² · dmol⁻¹) × 10⁻³].

because of the net negative charge of the former. In the present study, we have determined the Zn²⁺/Cd²⁺ substitution rate for both fish and mouse Zn₄-α-domain and Zn₃-β-domain. The results reported in Figure 8(A) show that the amount of metal exchanged was comparable for both fish and mouse α-domains, and the exchange occurred at the same rate. The substitution rate for the fish β-domain was higher than that of the mouse β-domain (Figure 8B). At the end of the exchange reaction, a sample aliquot was dialysed and used for cadmium determination by atomic absorption spectrometry. The amount of cadmium bound to α-domains was 3.0 ± 0.1 mol/mol of protein, whereas the amount of metal bound to β-domains was 2.0 ± 0.08. These results suggest that in MT the detoxification of toxic heavy metals occurs with efficiency and at a rate different from that of the isolated domains, thus providing an additional evidence of their interaction in the whole MT molecule.

**Reactivity of MT domains with the glutathione redox couple**

In a previous comparison between fish and mouse MTs, we found that the redox reactivity of fish MT is higher than that of its mouse counterpart [23]. In the present study, we have measured the reactivity of the isolated Zn₄-α-domain and Zn₃-β-domain of both fish and mouse MTs, using the redox couple GSH/GSSG and monitoring the absorption of the Zn–(PAR)₂ complex at 500 nm. The results in Figure 9 show that, in the presence of the redox couple, the reactivity of the Zn₄-α-domain of fish MT is higher than that of the respective mouse domain. Conversely, under the same conditions, the Zn₃-β-domain of fish MT is more reactive than the mouse domain. Notice the different reactivity between the α- and β-domain of the mouse MT with respect to the comparable reactivity of the fish domains.

**DISCUSSION**

In a comparative study on MTs of different piscine and mammalian species, we showed that protein hydropathy (a quantity inversely proportional to flexibility) significantly correlates with the optimal temperature of the organisms [25]. In keeping with this observation, the chemical and dynamic features of *N. coriiceps* and mouse MT were found to be markedly different, confirming a different conformational flexibility of the two proteins [23,24]. Although the presence of the more flexible MT in poikilotherms and of the more rigid counterpart in homoeotherms may suggest an adaptive scenario, phylogenetic comparative methods such as contrast analysis unravel a non-significant correlation between MT hydropathy and organismal temperature after the phylogenetic component had been removed [38]. Hence, the clustering of MTs according to their flexibility may be interpreted as a consequence of their descent from a common ancestry and not as an adaptation to environmental conditions.

The aim of the present work was to clarify the existence of interactions between MT domains and their role in determining the dynamic properties of different MTs. The observation of stable interdomain interactions by NMR relies on the possibility of detecting a number of NOEs (nuclear Overhauser effects) between pairs of protons belonging to different domains, or at least chemical shift perturbations, which can be induced even by small local environmental changes. We have not observed NOEs, but they may be so weak as to be virtually undetectable amongst the stronger intraresidue NOEs in crowded two-dimensional spectra. The fact that the NMR spectra of the isolated domains of fish MT are almost superimposable on those of the whole molecule confirms the apparent lack of interaction between the two domains in the protein. However, it is important to keep in mind that NMR
The thiol reactivity of the fish and mouse Zn-MT domains was assayed spectrophotometrically at 412 nm and 25°C with Ellman's reagent (DTNB). The reaction was carried out in 0.2 M Tris/HCl, pH 7.4, with 10 µM protein and 4 µM DTNB. The inset shows the thiol reactivity of whole fish and mouse Zn2⁺-MTs.

The present study demonstrates that it is possible to gain a better understanding of domain interactions by integrating structural data with functional data. From the analysis of the spectral properties, the α-domain of fish MT appears to be more sensitive to temperature than that of mouse MT, whereas the β-domains of fish and mouse MTs display comparable sensitivity to temperature. Similarly, the temperature-induced changes in the chiroptical properties of the α-domain of fish MT are more pronounced than for the corresponding domain of mouse MT. In contrast, the β-domains of both fish and mouse MTs undergo irreversible chiroptical modifications in response to the temperature increase. It is clear that the spectral modifications induced by temperature in both fish and mouse whole MTs are less evident than in the isolated domains, suggesting a stabilizing effect derived by the interaction of the two domains when they are linked in the whole molecule.

The study of the thiol reactivity of the isolated domains shows that the reactivity of the α-domain with DTNB reflects the behaviour of the whole MT. Indeed, SH reactivity in the α-domain is higher for mouse MT than for fish MT, whereas the β-domain is equally reactive in both MTs. As mouse MT is more reactive than fish MT, we must conclude that the contribution of the α-domain is determinant. In contrast, in the reaction of zinc transfer measured with PAR, the two isolated domains of both mouse and fish MT behave like the entire protein, with the fish moieties being more reactive than their mouse counterparts.

Previous studies have shown that both fish MT and mouse MT substitute Cd2⁺ for Zn2⁺ at the same rate; however, the number of Zn2⁺ equivalents exchanged per mol of protein is higher in fish MT than in mouse MT [24]. Conversely, the final number of metal equivalents exchanged is the same for both fish and mouse α- and β-domains, although the rate of exchange is different for the two domains: indeed, in the β-domain of fish MT, the exchange occurs at a higher rate than in the mouse β-domain. This result provides additional evidence of the complexity of inter-domain interactions in the entire MT, with the α-domain exerting a stabilizing effect and the β-domain having a weakening effect on the metal–thiol interactions.

We have shown previously that fish MT is more reactive than its mouse counterpart in the presence of the redox couple GSH/GSSG [23]. The isolated domains, however, display a different oxidation pattern when tested under the same conditions. Indeed, the α-domain of fish MT is more reactive than its mouse counterpart,
Cadmium exchange in fish and mouse Zn 2+ -domains was measured following spectrophotometrically the absorbance at 254 nm. Cadmium (21 μM) was incubated with zinc domains (2 μM) in 0.2 M Tris/HCl, pH 7.4, and the formation of the Cd–thiolate complex was monitored by measuring the change in absorbance at 254 nm. In the insets, the data were analysed as first-order reactions by plotting ln(A∞−A) against time.

while the opposite is true for the β-domain. Maret and Vallee [28] proposed a mechanism for thiolate oxidation in MT, involving both CC and CXXC, and the juxtaposed thiols in the tertiary structure of MT. However, as pointed out by the same authors, the reactivity of thiol groups is strongly influenced by steric factors whose effects cannot be predicted easily because of the non-linearity of the underlying phenomena. The different redox activity of thiol groups between the α- and β-domains, and between isolated domains and respective MTs is evidence of the interactions governing the complex network of metal–thiolate clusters in MT. Electrostatic interactions have been indicated as possible factors capable of modulating pKₐ and redox potential of proteins containing CXXC motifs [39].

What is the contribution of each domain to MT flexibility and reactivity? The structural similarity of the two domains suggests that the MT gene evolved by duplication of an ancestral gene, coding for a polypeptide folding like one of the two domains, a hypothesis supported by the existence of truncated MTs occurring in some invertebrates [40]. The evolution of an MT molecule made of two structurally different domains may have important functional consequences, with the more stable α-domain involved in metal detoxification, and the more labile β-domain more likely to act in oligo-element homeostasis. The study of the reconstructed ancestral MT sequences in vertebrates suggests that, at the early stages of evolution, the MT could exist as a very flexible molecule [25]. The different flexibility distinguishing fish and mammal MTs is linked to the evolution of the α-domain: presumably, the β-domain remained flexible and, consequently, relatively unstable, whereas the α-domain underwent genetic modifications, gaining a higher stability. An important contribution to the stability of the α-domain of mammal MT may derive from the different positioning of the ninth cysteine residue, although other amino acid substitutions may be relevant as well, in particular those interesting charged residues.

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