Structural characterization and thermal stability of *Notothenia coriiceps* metallothionein¹

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Fish and mammalian metallothioneins (MTs) differ in the amino acid residues placed between their conserved cysteines. We have expressed the MT of an Antarctic fish, *Notothenia coriiceps*, and characterized it by means of multinuclear NMR spectroscopy. Overall, the architecture of the fish MT is very similar to that of mammalian MTs. However, NMR spectroscopy shows that the dynamic behaviour of the two domains is markedly different. With the aid of absorption and CD spectroscopies, we studied the conformational and electronic features of fish and mouse recombinant Cd-MT and the changes produced in these proteins by heating. When the temperature was increased from 20 to 90 °C, the Cd-thiolate chromophore absorbance at 254 nm of

mouse MT was not modified up to 60 °C, whereas the absorbance of fish MT decreased significantly starting from 30 °C. The CD spectra also changed quite considerably with temperature, with a gradual decrease of the positive band at 260 nm that was more pronounced for fish than for mouse MT. The differential effect of temperature on fish and mouse MTs may reflect a different stability of metal-thiolate clusters of the two proteins. Such a conclusion is also corroborated by results showing differences in metal mobility between fish and mouse Zn-MT.

Key words: absorbance spectroscopy, circular dichroism, NMR, temperature effect, zinc mobility.

INTRODUCTION

Among non-catalytic proteins, metallothionein (MT) occupies an outstanding position for its unique structure. Vertebrate MT is made of a single polypeptide chain characterized by a low molecular mass (about 6 kDa), high cysteine (30% of total residues in the molecule) and heavy metal contents, and the absence of aromatic residues and histidine [1–3]. The cysteine residues of vertebrate MT are arranged according to a substantially conserved pattern and are distributed in motifs consisting of CC, CXC and CXCC sequences [4]. All 20 cysteines bind seven metal atoms such that each metal atom is bound to four cysteine ligands forming metal-thiolate clusters [5]. These clusters are divided in two domains: the N-terminal β -domain constituting nine cysteines and three metal ions, and the C-terminal α -domain with eleven cysteines and four metal ions [6].

The possible function of MT is still a matter of debate: the induction by bivalent metal ions implies a detoxification role against heavy metals [7–10], whereas the ability to act as a scavenger of superoxide radicals suggests a participation in the defence against oxidative stress [11,12]. On the other hand, the remarkable stability of the metal complex ($K_a = 1.4 \times 10^{-13}$ M at pH 7 [6]) and the high kinetic lability resulting in a facile metal release suggest a zinc-donor role for MT [13,14].

MTs from various vertebrate classes have N-terminal sequences differing both in length and amino acid types; in addition, different MTs may present distinct amino acids types between the cysteine residues [15]. Intervening residues should

not affect the overall architecture of vertebrate MTs that, in the absence of well-defined secondary structures such as α -helices and β -strands, is mostly dictated by the structurally conserved domains that bind heavy metals [16–23].

With respect to mammalian MTs, piscine MTs present a number of distinctive differences in the primary structure, including the displacement of one of the 20 cysteines and a lower number of lysines juxtaposed to the cysteinyl residues [22]. These differences in primary structure may well affect dynamic properties of the protein, such as ion exchange, thermal stability and overall conformational flexibility that may finally result in a change in the related functions. We decided to investigate stability and reactivity of metal-thiolate clusters in fish and mouse MTs because a comparison of the physico-chemical features of MTs from homeothermic and poikilothermic organisms may shed light on the hitherto elusive function of MT. As representative of fish MT we chose that of an Antarctic fish, *Notothenia coriiceps*, subject of our previous studies. In order to make the comparison more stringent, both fish and mouse MTs were expressed in *Escherichia coli* in the presence of metal ions as glutathione S-transferase (GST) fusion proteins, and the MT moiety was recovered after cleavage with thrombin.

For all the MTs described so far, absorbance and CD data have revealed that optical properties depend mostly on the contribution of the metal-thiolate chromophore [23–25]. Early data obtained with CD and magnetic CD spectroscopies indicate that the metal-binding reaction is sensitive to temperature [26]. The conformational and electronic differences between fish and

Abbreviations used: MT, metallothionein; MT-I, MT isoform I; GST, glutathione S-transferase; PAR, 4-(2-pyridylazo)resorcinol; IPTG, isopropyl β -D-thiogalactoside; NOESY, nuclear Overhauser enhancement spectroscopy; NOE, nuclear Overhauser effect; LB, Luria–Bertani.

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The nucleotide sequence data reported for *Notothenia coriiceps* metallothionein will appear in the GenBank® Nucleotide Sequence Database under the accession number AJ006484. The nucleotide sequence data reported for mouse MT-I will appear in the SwissProt database under accession number P02802.

mouse recombinant Cd-MT have been studied by means of absorption and CD spectroscopies in the temperature range 25–95 °C. In addition, we have determined the difference in zinc mobility between the two MTs, by measuring *in vitro* zinc transfer from Zn-MT to 4-(2-pyridylazo)resorcinol (PAR). Marked differences in dynamics between the two domains of fish MT were also shown by natural-abundance ^{113}Cd – ^1H correlation NMR spectra.

EXPERIMENTAL

Materials

Fusion expression vector pGEX-4T-1 was purchased from Pharmacia Biotech (Uppsala, Sweden). *E. coli* cells strain BL21 (DE3) were from Novagen. Plasmid pGMA8 containing mouse MT-I (MT isoform I) cDNA [27] was kindly provided by Dr Binggen Ru (National Laboratory of Protein Engineering, College of Life Sciences, Peking University, Beijing, P.R. China). All other reagents used were of analytical grade.

Amplification, cloning and sequence analysis of recombinant fish MT

The *N. coriiceps* (Antarctic fish) MT cDNA coding a region of 183 bp was prepared by PCR with the 5'-end primer (5'-CCGAATTCACGATGGATCCTTGT-3') to add an *EcoRI* site upstream from the start codon ATG and with the 3'-end primer (5'-CCGTCGACTCACTGACAGCAGCT-3') to add an *SalI* site after the stop codon TGA. Plasmid pGEM/MT containing fish MT cDNA [28] was used as a template in the PCR reaction. Amplification was performed with 5 units of *Taq* DNA polymerase (Perkin-Elmer), 50 pmol of each the above primers and 0.2 mM dNTPs (final concentration). The mix was buffered with Perkin-Elmer PCR storage buffer (100 mM Tris/HCl, pH 8.3/500 mM KCl/15 mM MgCl_2 /0.01 % gelatine). Reaction was carried out in a DNA Thermocycler Express (Hybaid), with an initial denaturation step at 95 °C for 3 min followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and a final step at 72 °C for 15 min. The PCR fragment was gel-purified using a Genomed gel-extraction kit (Genomed, Bad Oeynhausen, Germany) and subcloned into a pCR2.1-TOPO vector using a Topo TA Cloning kit (Invitrogen, Leek, The Netherlands). *E. coli* cells were transformed with ligation mixture. Plasmid DNA was denatured and the MT coding region was sequenced bidirectionally by the dideoxynucleotide method [29] using the T7 sequencing kit (Pharmacia Biotech). The pCR2.1-TOPO vector, containing the MT coding region, was digested with *EcoRI* and *SalI* restriction enzymes. The resulting fragment was ligated into the corresponding sites of pGEX-4T-1, the GST-fusion expression vector. The resultant recombinant plasmid can produce a fusion protein of which the N-terminus is GST and the C-terminus is the fish MT. The sequence of the cloned MT cDNA in pGEX-4T-1 was again verified by sequencing in both directions using the pGEX primers (Pharmacia Biotech).

Growth of *E. coli* cells and expression of the recombinant fusion protein

E. coli strain BL21 (DE3) was transformed with recombinant plasmid DNA containing either fish or mouse MT cDNA to produce pGST-MT or pGMA8 cells. Transformed *E. coli* cells were grown in Luria–Bertani (LB) broth containing 100 $\mu\text{g}/\text{ml}$ ampicillin at 37 °C overnight. The overnight culture was diluted 100-fold using fresh LB broth plus ampicillin and incubation continued at 37 °C. The cells were grown until mid-exponential-growth phase (D_{590} , 0.6). At this point isopropyl β -D-

thiogalactoside (IPTG) and CdSO_4 were added to final concentrations of 0.1 mM and 0.2 mM respectively. For the preparation of Zn-MT, 0.2 mM ZnSO_4 was substituted for CdSO_4 . After incubation for a further 3 h at 37 °C, cells were pelleted by centrifugation at 7410 *g* for 20 min and washed twice in PBS (140 mM NaCl/2.7 mM KCl/10 mM Na_2HPO_4 /1.8 mM KH_2PO_4 , pH 7.3).

Purification of expressed MT proteins

The pelleted cells were resuspended in 5 % of the original volume of PBS containing 3 mM β -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 for 1 h to facilitate solubilization of proteins. The recombinant fusion proteins (GST-MT) were purified by affinity chromatography using a column of glutathione–Sephrose 4B (Pharmacia Biotech) equilibrated with PBS. The supernatant was applied to an affinity column and then washed extensively with equilibration buffer. The GST-MT was eluted with 10 mM glutathione in 50 mM Tris/HCl, pH 8.0. The fractions containing fusion protein were pooled and digested with thrombin and then fractionated on a column of Sephadex G-75 (45 $\times$ 1.5 cm) equilibrated with 0.02 M Tris/HCl buffer, pH 8.0. Column eluate was collected in 1 ml fractions and monitored for absorbance at 254 nm and cadmium content. The peak corresponding to recombinant MT was lyophilized. For use in NMR spectroscopy, the protein was dialysed against water before lyophilization.

SDS/PAGE, N-terminal amino acid sequence, MS analysis and metal determination

SDS/PAGE was carried out according to the method of Laemmli [30] using a 20 % polyacrylamide gel. Samples were dissolved in buffer with 5 % β -mercaptoethanol. Amino acid sequence analysis was carried out after the protein had been subjected to 20 cycles of Edman degradation in an Applied Biosystem, Mod. 785 A. Protein molecular mass was determined by electrospray ionization MS (Hewlett Packard, model 59987A-5989B). For the analysis the recombinant MT was dissolved in 50 % acetonitrile and 0.1 % trifluoroacetic acid at the final concentration of 0.5 $\mu\text{g}/\mu\text{l}$. Cadmium content was determined on an atomic absorption spectrophotometer (Perkin-Elmer, model 5100 PC Zeeman).

Absorption and CD spectroscopy

Absorption spectra of samples of recombinant MT in distilled water (0.03 mg/ml) were recorded in the range 220–320 nm, using a DU 640 spectrometer (Beckman) equipped with a temperature controller. CD spectroscopy was performed on homogeneous samples of recombinant mouse and fish MT (0.1 mg/ml in distilled water), in the temperature range 25–95 °C, using a J-710 spectropolarimeter (Jasco, Tokyo, Japan) equipped with the Neslab RTE-110 temperature-controlled liquid system (Neslab Instruments, Portsmouth, NH, U.S.A.) and calibrated with a standard solution of (+)-10-camphorsulphonic acid. Sealed cuvettes with 0.1 or 1.0 cm path lengths (Hellma, Jamaica, NJ, U.S.A.) were used in the far- and near-UV regions, respectively. Photomultiplier high voltage did not exceed 600 V in the spectral regions measured. Each spectrum was averaged three times and smoothed with Spectropolarimeter System software version 1.00 (Jasco). All measurements were performed under nitrogen flow. Before undergoing CD analyses, all samples were kept at the temperature being studied for 5 min. The results are expressed in terms of residue molar ellipticity. The percentages of secondary

Figure 1 Expression of recombinant MT fusion protein in *E. coli* cells

E. coli BL21 (DE3) cells transformed with recombinant plasmid pGST-MT were grown as described in the Experimental section and induced with 0.1 mM IPTG. Cell suspension (1 ml) was centrifuged, and the resulting pellet was solubilized in 150 μ l of sample buffer. A 20 μ l sample was analysed by SDS/PAGE. Lane 1, cells induced with IPTG. The arrow indicates the 34 kDa GST-MT fusion protein. Lane 2, control without IPTG. Lane 3, prestained molecular-mass standards (from the top: phosphorylase *b*, 103 kDa; BSA, 76 kDa; ovalbumin, 49 kDa; carbonic anhydrase, 33.2 kDa; soybean trypsin inhibitor, 28 kDa; lysozyme, 19.9 kDa).

structures were estimated according to the method of Yang et al. [31].

NMR spectroscopy

For NMR spectroscopy, 2 mM samples of recombinant *N. coriiceps* MT dissolved in either 95% H_2O /5% $^2\text{H}_2\text{O}$ or $^2\text{H}_2\text{O}$ at pH 7.0 under argon were used. Experiments were run on Bruker DRX-400 and DRX-600 spectrometers, at 288, 293 and 303 K. Data processing was performed with NMRPipe [32] and spectral analysis with NMRView [33]. A conventional set of two-dimensional spectra, according to the scheme of sequential assignment described by Wüthrich [34], was recorded: correlation

[35], TOCSY [36], nuclear Overhauser enhancement spectroscopy (NOESY) [37] and double-quantum [38] spectra. TOCSY spectra were collected with mixing times in the range 50–75 ms, using the DIPSI2-rc mixing scheme [39]. NOESY spectra were recorded with mixing times in the range 100–150 ms. Time-proportional phase incrementation (TPPI) was applied to achieve quadrature detection in the indirect dimension [40]. Water suppression was achieved either by presaturation or by using the WATERGATE pulse sequence [41]. Proton-detected natural-abundance ^{113}Cd - ^1H heteronuclear long-range correlation spectra were collected over a range of evolution times (15–30 ms), using gradient coherence selection, and were transformed in absolute value mode. The assignment of the Cd-thiolate clusters was based on the identification of scalar connectivities between Cd ions and cysteine β protons whenever the absence of heavy superpositions allowed unambiguous assignment, and on similarity with the analogous clusters of mammalian MTs in the remaining cases [16–21]. $^3J_{\text{NH}-\alpha}$ coupling constants were estimated with the method of Kim and Prestegard [42]; $^3J_{\alpha-\beta}$ coupling constants were measured by exclusive-correlation-spectroscopy experiments [43].

Kinetics of zinc release

Zinc release was determined on both fish and mouse Zn-MTs using the procedure described by Maret and Vallee [44] by measuring the zinc transfer from MT to PAR in the presence of reduced and oxidized glutathione.

RESULTS

Expression and characterization of *N. coriiceps* MT

IPTG induction of *E. coli* BL21 (DE3) cells transformed with the plasmid pGST-MT resulted in the production of a fusion protein with a mass of 34 kDa (Figure 1). The fusion protein was chromatographed on a glutathione-Sepharose 4B affinity column and cleaved with thrombin as described in the Experimental section. MT was separated from GST by gel-filtration chromatography on a Sephadex G-75 column and the purity was assessed

Figure 2 Molecular-mass determination by electrospray ionization MS of recombinant fish MT

The sample was prepared as described in the Experimental section. (A) Electrospray ionization mass spectrum of fish MT; (B) computer-generated deconvolution of the electrospray ionization MS peaks into a single peak at the correct protein mass.

Figure 3 Aligned sequences of recombinant fish and mouse MTs

Alignment was performed with the program GAP contained in the GCG Wisconsin package. Vertical bars indicate residue identity. The six amino acids in brackets were derived from thrombin cleavage.

Figure 4 Corresponding regions of the two-dimensional TOCSY and NOESY spectra of *N. coriiceps* Cd-MT

Both TOCSY and NOESY spectra were recorded at 293 K and 400 MHz, with mixing times of 70 and 150 ms, respectively.

by SDS/PAGE (results not shown). The yield of the purified MT was 5 mg/l of culture.

N. coriiceps MT shares 87% identity with other piscine MTs, and 63% with mammalian MTs. The sequence of the N-terminal region of the recombinant protein, carried out by Edman degradation, was Gly-Ser-Pro-Glu-Phe-Thr-Met-Asp-Pro-Cys-Glu-Cys-Ser-Lys-Ser. Apart from the first six residues, resulting from the cleavage of the C-terminus of GST with thrombin, the peptide N-terminal sequence coincided with that inferred from the nucleotide sequence (see Figure 3). The first six extra residues are also present in the recombinant mouse MT-I, which

was prepared with the same procedure used for fish MT. The molecular mass of the recombinant fish MT, determined by electrospray ionization MS, was 6637 Da (Figure 2), compared with the theoretical molecular mass of 6019 Da, owing to the contribution of the first six extra residues. Both mouse and fish recombinant MTs contained seven equivalent metal atoms per protein molecule (either Cd or Zn, depending on the metal added during the preparation). The full amino acid sequences of both *N. coriiceps* and mouse MTs, as deduced from the cDNA sequences, are shown in Figure 3. The two proteins differ in the position of a cysteine residue in the α -domain, and in the number

Figure 5 Natural-abundance two-dimensional ^{113}Cd - ^1H long-range correlation spectrum of *N. coriiceps* Cd-MT

The spectrum was collected at 303 K and 400 MHz proton Larmor frequency, with an evolution time for the heteronuclear coupling of 30 ms. The Cd resonances, labelled I–VII in order of increasing chemical shift, are grouped into the α - and β -domains. The bound cysteines are reported in brackets following the residue numbering of Figure 3: Cd I, 37, 41, 44 and 60; Cd II, 5, 7, 21 and 24; Cd III, 33, 34, 44 and 48; Cd IV, 34, 36, 37 and 50; Cd V, 15, 19, 24 and 29; Cd VI, 7, 13, 15 and 26; Cd VII, 50, 55, 59 and 60.

of CK motifs in the sequence. The fish MT has a cysteine at position 55, whereas in the mouse MT the corresponding cysteine is at position 57; in addition, the piscine MT contains four CK motifs in its sequence, compared with six in the mouse protein. Moreover, the high number of cysteine residues makes refolding of recombinant MTs important. Therefore, we deemed it necessary to characterize the three-dimensional structure of *N. coriiceps* MT in detail and undertook an NMR spectroscopy study. Figure 4 shows corresponding regions of the two-dimensional TOCSY and NOESY spectra of *N. coriiceps* MT. Residue type and

sequence-specific assignment of proton resonances were obtained by the standard protocol of Wüthrich [34]. The NOESY spectrum shows essentially intra-residue and sequential contacts. The most obvious feature of the spectrum is an extended network of sequential NH–NH cross peaks that rules out the presence of β -sheets. However, it is not possible to identify the patterns of cross peaks typical of α -helices. These results are consistent with known structures of mammalian MTs and were substantiated by a structure calculation based on nuclear Overhauser effect (NOE)-derived distance restraints and homonuclear J couplings (C. Capasso, S. D'Auria, V. Carginale, R. Scudiero, O. Crescenzi, D. Di Maro, P. A. Temussi and E. Parisi, unpublished work). Overall, the architecture of fish MT is very similar to that of mammalian MTs, i.e. it is characterized by two domains, α and β , differing markedly in structural definition. At the present stage of refinement the backbone root-mean-square deviations of the α - and β -domains are 1.4 and 2.7 Å, respectively, i.e. they differ by 90%: the corresponding differences for mammalian MTs range from 20 to 140% [17–21]. This result is a consequence of the fact that the number of NOESY cross peaks of measurable intensity for the β domain is always smaller than for the α -domain, and reflects the different flexibility of the protein chain in the two domains. In particular, for *N. coriiceps* MT the ratio of NOE-derived inter-residue distance restraints for the two domains is 0.44, which again lies within the range observed for mammalian MTs (0.27–0.67). However, a significant discrepancy between fish and mammalian MTs does become apparent from inspection of the natural-abundance two-dimensional ^{113}Cd - ^1H correlation spectra (Figure 5): the two domains of fish MT display correlation peaks strikingly different in apparent intensity, much more so than in mammalian MTs [19,21,45].

Spectral properties of fish and mouse MTs

We have investigated the effect of temperature on the physico-chemical properties of the two MTs and the results are compared. We have employed absorption and CD spectroscopy because these techniques allow unravelling changes in the metal-thiolate chromophore. UV spectra have been recorded for both fish and

Figure 6 Effect of increasing temperature on the UV spectral properties of Cd-MT

Spectra were recorded in the range 220–300 nm at the indicated temperatures. The high Cd-thiolate chromophore absorbance at 254 nm is plotted as a function of temperature. The results are representative of three independent sets of measurements for which the maximal difference in absorbance at each temperature was 0.12%. (A) Mouse MT; (B) *N. coriiceps* MT.

Figure 7 Effect of increasing temperature on the CD spectra of Cd-MT

CD spectra were recorded in the near-UV region (250–320 nm) at the indicated temperatures. The results are representative of three independent sets of measurements for which the maximal difference in molar ellipticity at each temperature was 0.15%. Broken lines, mouse MT; dotted lines, *N. coriiceps* MT.

mouse Cd-MTs in the 20–90 °C temperature range. The results in Figure 6 show that the absorbance at 254 nm, due to the charge-transfer interaction in the metal-thiolate complex, decreases by increasing the temperature of the sample. However, the absorbance of mouse MT is stable up to 60 °C (Figure 6A), whereas that of fish MT starts to decline steadily at 30 °C (Figure 6B). These results are supported by CD spectroscopy data. The effect of increasing temperature on the CD spectra of mouse and fish MTs is shown in Figure 7. As temperature increases from 25 to 95 °C, the CD spectra change quite considerably, with a gradual decrease in the positive band at 260 nm accompanied

also by a red shift. As noticed for the absorption spectra, the temperature-induced modifications of the CD spectrum are more pronounced for the fish MT than for its mammalian counterpart. When the temperature was lowered again to 25 °C, the original spectral properties were restored for both proteins (results not shown).

Zinc kinetics

The above results prompted us to investigate metal release in both fish and mouse MTs. The kinetics of zinc release from

Figure 8 Kinetics of zinc release from recombinant Zn-MT using the glutathione redox couple

Each assay contained 1.3 μ M MT in 0.2 M Tris/HCl, pH 7.4/100 μ M PAR/1.5 mM GSH/3 mM GSSG. Zinc release was followed by the formation of Zn(PAR)₂ at 500 nm. Each experimental point is the difference of the absorbances at 500 nm measured in the presence and absence of the redox couple. ■, Mouse MT; ●, *N. coriiceps* MT.

mouse and fish Zn-MTs induced by the glutathione redox couple was followed by measuring the formation of the Zn(PAR)₂ complex at 500 nm. The results in Figure 8 show that the stability of zinc binding is altered by interaction with the redox system; the fish MT, however, is more reactive than mouse MT.

DISCUSSION

Clustered metal-thiolate complexes are commonly present in invertebrate, vertebrate and plant MTs. The properties of these complexes confer on MTs spectroscopic features that are typical of this class of proteins. Consequently, the properties of absorbance and CD spectra may give useful information on the organization of metal ions with thiolate ligands.

In the present report, we described heterologous expression of fish MT in *E. coli* with a high-yield production of either Zn₇- or Cd₇-MT. We found that the fusion protein GST-MT contained a number of metal equivalents per molecule corresponding to that of native MT. The shape of the two domains as derived from the NMR analysis, albeit at low resolution, resembles closely those of mammalian MTs [16–21].

The percentages of secondary structures estimated by CD spectroscopy are consistent with an almost complete absence of α -helices and β -sheets, as indicated also by the NMR data. The presence of the six extra amino acid residues at the N-terminus of the recombinant proteins did not affect the features of the metal-thiolate clusters, as demonstrated by the fact that both recombinant mouse MT-I and native rabbit MT-I showed the same spectroscopic properties [27].

The results of the UV and CD analyses show that the optical properties of fish and mouse MT are sensitive to temperature. The shape of the absorption curve varies with temperature, but the change of the absorbance at 254 nm is more pronounced for the fish MT than for its mammalian counterpart. Such an effect may be attributed to changes in the dissociation constant

of the metal-thiolate complex. The CD spectra of heated MT were also modified drastically, with a progressive quenching of the wide positive band near 260 nm when the temperature was increased from 25 to 95 °C. Apparently, heating does not result in a modification of the far-UV CD spectra.

A number of studies suggest that the chiroptical features of MT, responsible of the conspicuous positive ellipticity at 260 nm, arise from the ligand-metal charge-transfer transitions of the metal-thiolate complex [23]. Such an optical activity has been attributed to the interaction of dissymmetrical co-ordinated chromophores at the level of the clusters [46]. Early studies showed that chemical modification of the lysines in rabbit MT resulted in modification of the CD spectra, suggesting an interaction of these residues with metal-thiolate clusters [47]. Usually, lysine residues are highly conserved in vertebrate MTs, but their distribution along the sequence varies in different species. Recent evidence, obtained with CD spectroscopy and NMR on a mutated recombinant MT, demonstrates that substitution of glutamate residues for lysines in three CK motifs in the α -domain resulted in a change of metal-thiolate interaction in both domains [48]. It is likely that the different temperature-induced modifications of the optical properties observed in fish and mammalian MTs may be due to the difference in lysine number in their sequences, and, in particular, to the lower number of CK motifs in fish MT. Another peculiarity in the α -domain typical of piscine MT is Cys-55 (see alignment in Figure 3), which is backshifted two positions to Cys-57 in mammalian MT. As a result of such a modification, the last CXCC motif in the mammalian MT sequence becomes CXXXCC in fish MT. An outstanding difference between fish and mammalian MTs, emerging from the present study, is the difference in dynamic behaviour between the two domains (α and β). As mentioned in the Results section, the structural flexibilities of the α - and β -domains of fish MT are quite different, both in terms of atomic root-mean-square deviations and numbers of measurable NOE contacts. Although these differences are higher than the average for mammalian MTs, they are still within the range of values reported [17–21]. However, a significant difference with respect to mammalian MTs emerges from the ¹¹³Cd-¹H NMR correlation experiments on *N. coriiceps* MT (Figure 5) that show a striking difference in intensity of the ¹¹³Cd-¹H correlations from the two domains. This feature is much less prominent, or not evident at all, in the heteronuclear correlation spectra of mammalian MTs reported in the literature [19,21,45]. A selective broadening of resonances from the β -domain could in part be the consequence of the larger structural flexibility; however, the fact that the observed broadenings specifically affect the heteronuclear spectra, whereas the homonuclear spectra are not significantly different from those of mammalian MTs, is best accounted for by an exchange phenomenon involving the metal ions within the β -domain, which seems to be operating in fish MT to a larger extent than in mammalian MTs.

Several lines of evidence show the important role of zinc in the structure and function of a large number of metalloproteins. The high kinetic reactivity and metal affinity of MTs are relevant for metal-transfer or -exchange reactions between MTs and other proteins, including enzymes and transcription factors. It has been reported that thionein may suppress transcription activation by zinc-finger factors whereas native MT is capable of restoring the transcriptional capacity [49]. Such an ability of MTs to modulate reversible zinc exchange depends on the structure and reactivity of the metal-thiolate clusters. Our data demonstrate that zinc mobility is higher for fish MT than for mouse MT, suggesting a difference in biological reactivity for the two proteins. It is interesting that the redox properties of MT are somehow

correlated with the heat stability of the metal-thiolate clusters. This suggests that differences in the MT amino acid sequence may affect cluster structure and their ability to exchange zinc. The propensity of different MTs to release or exchange zinc suggests the hypothesis that zinc-dependent processes may be differentially regulated in poikilothermic and homoeothermic organisms. However, for such a hypothesis to be supported, the molecular structure and the dynamic properties of MT should be investigated in other species as well. Such information would provide new perspectives on understanding the relationships between MT structure and zinc metabolism during evolution.

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