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MTO: the second member of a *Drosophila* dual copper-thionein system

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Abstract ***Drosophila*** **MTO metal binding features were analyzed for comparison with MTN, the paralogous** ***Drosophila*** **metallothionein, and to classify MTO as either zinc- or copper-thionein. This was achieved by a combination of in vivo, in vitro and in silico methodologies. All the results unambiguously classified MTO as a second** ***Drosophila*** **copper-thionein, putting** ***Drosophila*** **forward as the only metazoan in which any zinc-thionein has still to be reported. Interestingly, experimental data only showed minor differences in the coordinative behavior of both MTs, but provided a characteristic spectroscopic fingerprint, revealing the possible binding of chloride anions in certain metal-MTO aggregates.**

Key words: *Drosophila* metallothionein; Cu(I) binding; Cd(II) binding; Zn(II) binding; Molecular evolution; Recombinant synthesis

1. Introduction

Metallothioneins (MT) are low molecular weight, cysteine-rich proteins with an exceptional heavy-metal coordination capacity [1]. They have been reported in all animal phyla and in most fungi and plants, and are usually associated with protection against toxic metals and with storage/transport of physiological zinc and copper. Other functions, such as free radical scavenging or oxidative stress protection, have also been proposed [2].

Among MTs, copper-thioneins constitute an interesting example of evolutionary convergence [3]. The yeast *Saccharomyces cerevisiae* CUP1, a 53-amino acid peptide binding seven Cu(I) ions within a monodominial cluster [4], can be considered the archetypical copper-thionein, together with other fungal MTs, from *Neisseria crassa* [5] and *Agaricus bisporus* [6]. Transcription of their corresponding genes is positively regulated by high copper concentrations, remaining almost uninduced by increased zinc levels. Since fungal MTs were the only Cu-thioneins known for a long time, MT preference for copper was only associated with primitive eukaryotes and was thus largely undervalued in higher organisms. Nevertheless, the Cu-thionein subfamily is at present the object of increasing interest, as new representatives are being reported in several animal phyla, including higher Metazoa. Two main features are used to identify a Cu-thionein. One of these is a gene induction criterion: MT forms recovered specifically from Cu-treated organisms are considered Cu-thioneins, as with *Helix pomatia* (Mollusca/Gastropoda) [7], *Callinectes sapidus* (Arthropoda/Crustacea) [8] and *Tetrahymena pigmentosa* (Protozoa/Ciliophora) [9]. The other is a protein characterization criterion, based on the features of recombinant metal-MT species and on the analysis of amino acid sequences [3]. Following this approach, the mammalian β-MT domain [10] and MTN, one of the two *Drosophila* (Arthropoda/Insecta) MTs [11], have been identified as Cu-thioneins.

To date, except for *Drosophila*, all Metazoa whose MT system has been studied exhibit both Zn- and Cu-thionein forms. Therefore, the characterization of the metal preference of MTO, the possible Zn-thionein counterpart of MTN, was of great interest. Although very preliminary MTO sequence analysis associated MTO with CUP1 [11], MTN and MTO are such distant paralogues (only 26% amino acid identity) that a similar metal binding preference cannot be taken for granted. For example, the Zn- and Cu-thioneins from *C. sapidus* share sequence levels of up to 40% identity. Furthermore, MTN and MTO are encoded by two genes that exhibit highly different regulation profiles: distinct tissue and ontogenic patterns of expression and distinct response to metals as expression inducer agents [12–15], which might be related to different metal preferences and thus to differential physiological roles.

Mto encodes a 43-amino acid peptide including 12 Cys, previously purified from flies [16] and from recombinant yeast [17], but never functionally characterized. In this work, we evaluated the character of Zn-thionein or Cu-thionein of MTO, and we compared its metal binding abilities with those previously reported for MTN. The significance of the Cu-thionein character of both *Drosophila* metallothioneins is then discussed in the context of the state of the art of the *Drosophila* MT system, as an essential clue to understanding the possible MT physiological roles and the gene expression regulation mechanisms in this model organism.

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Abbreviations: CD, circular dichroism; ICP-AES, inductively coupled plasma atomic emission spectroscopy; ESI-MS, electrospray ionization mass spectrometry; MT, metallothionein; UV-vis, electronic absorption

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2. Materials and methods

2.1. cDNA cloning and recombinant protein preparation

Drosophila melanogaster MTO cDNA was kindly provided by Dr. M. Wegnez. It was PCR-amplified using as primers: 5′-CCCGGATCCATGGTTTGCAAG-3′ (upstream primer) and 5′-GCGCCCGTCGACTTATTTGTTGCT-3′ (downstream primer), which introduced a *Bam*HI site immediately before the ATG initiation codon and a *Sal*I site just after the stop codon. Thereafter, the PCR product was directionally inserted (*Bam*HI–*Sal*I) into the multiple cloning site of the pGEX-4T-1 plasmid (Amersham-Pharmacia), to generate the expression vector pGEX-*Mto*. DNA sequencing of the *Mto* coding fragment was performed with the Amersham Dye Terminator Cycle Sequencing Kit in an ABI 370 automatic sequencer. pGEX-*Mto* was transformed into BL-21 for over-expression of the recombinant protein [18]. Expression and purification of the metal-MTO complexes were performed as described for MTN [11,19]. Culture media were enriched with 300 μM $ZnCl_2$ or with 500 μM $CuSO_4$ for the synthesis of Zn- or Cu-MTO complexes, respectively. Aliquots of the Superdex75 FPLC fractions containing MTO in Tris–HCl buffer, or alternatively Tris–$HClO_4$ buffer (50 mM, pH 7), were analyzed in 15% SDS–PAGE Coomassie blue-stained gels. Samples were pooled, aliquoted and stored at −80°C under argon atmosphere until further use.

2.2. Metal-MTO species characterization and spectroscopic analyses of the Cd(II) and Cu(I) titration of Zn-MTO

The characterization of the recombinantly synthesized Zn-MTO and Cu-MTO complexes was achieved through inductively coupled plasma atomic emission spectroscopy (ICP-AES) and Ellman's method as described elsewhere [10]. The electronic absorption and circular dichroism (CD) measurements recorded in parallel during the titrations of Zn-MTO with either Cd(II) or Cu(I) salts were carried out and corrected, as described in [3] for Cd(II) and in [20] for Cu(I). The Zn-MTO cadmium binding ability was tested with either Tris–HCl or Tris–$HClO_4$ buffer, using either $CdCl_2$ or $Cd(ClO_4)_2$ as titrating agent, respectively. Exceptionally, the preparation of $Cd(ClO_4)_2$ required determination of the Cd^{2+} concentration by atomic absorption spectrometry, using a Perkin-Elmer 2100 apparatus.

2.3. Electrospray ionization mass spectrometry (ESI-MS) measurements of MTO-metal complexes

Molecular mass measurements were performed on a Fisons Platform II Instrument, equipped with MassLynx software and calibrated with horse heart myoglobin (0.1 mg/ml). Conditions for the Zn and Cd species were: 20 μl of protein solution injected at 30 μl/min; the use of a HPLC Uptisphere C_4 33 mm×2 mm×5 μm column to separate analytes; capillary counterelectrode voltage, 4.5 kV; lens counterelectrode voltage, 1.0 kV; cone potential, 60 V; source temperature, 120°C; *m/z* range, 800–1950; scanning rate, 3 s/scan; interscan delay, 0.3 s. The running buffer was an appropriate mixture of acetonitrile and 5 mM ammonium acetate/ammonia, pH 7.5. Conditions for the Cu species were: 20 μl of protein solution injected at 30 μl/min; capillary counterelectrode voltage, 4.5 kV; lens counterelectrode voltage, 0.5 kV; cone potential, 40 V; source temperature, 90°C; *m/z* range, 800–1950; scanning rate, 3 s/scan; interscan delay, 0.3 s. The running buffer was a 5:95 mixture of acetonitrile and 5 mM ammonium acetate/ammonia, pH 7.5. The molecular mass of the apo-forms was determined as for the Cu species, except that for apo-MTO obtained from Zn-MTO the carrier was a 5:95 mixture of acetonitrile and ammonium formate/ammonia, pH 2.5; source temperature, 120°C; and for apo-MTO obtained from Cu-MTO, the carrier was a 1:1 mixture of acetonitrile and trichloroacetic acid, pH 1.5. In both cases, the capillary counterelectrode voltage was 3.5 kV, the lens counterelectrode voltage 0.5 kV and the cone potential 40 V. Masses for the holo-species were calculated as described in [21].

2.4. Protein sequence and evolution analyses

Protein sequence alignments were performed using the Pileup utility enclosed in the GCG Package, v.10, [22]. Protein sequences were aligned by ClustalW, v1.75, using Blosum62 as distance matrix [23]. The ClustalW alignments were the input for calculating protein distances through Protdist, which uses the Dayhoff–Pam matrix distance. The corresponding phylogenetic trees were constructed using Fitch, which uses the Fitch–Margoliash tree-building algorithm [24]. Protdist and Fitch are included in the Phylip software package [25].

3. Results and discussion

3.1. cDNA cloning and preparation of recombinant metal-MTO aggregates

DNA sequencing of the major PCR product (156 bp) confirmed the *mto* coding region [15] and ruled out the presence of any mutagenic nucleotide substitution. SDS–PAGE of total protein extracts from pGEX-*Mto*-transformed BL-21 cells grown in Zn- and Cu-supplemented media showed the presence of a band of the GST-MTO expected size (30.6 kDa). Homogeneous metal-MTO preparations (final concentration of 5.3×10^{-5} M for Zn-MTO and 3.7×10^{-5} M for Cu-MTO) were obtained from fermenter cultures. Acidification of the Zn-MTO and Cu-MTO complexes yielded the apo-form, with a molecular mass in accordance with the theoretical value for the polypeptide (Table 1). This confirmed both the identity and the integrity of the recombinant protein.

3.2. Characterization of the divalent metal binding abilities of MTO

The behavior of MTO in front of divalent metal ions was established through detailed analysis of the in vivo synthesized Zn(II)-MTO and of the Cd(II)-MTO species obtained in vitro by Zn/Cd replacement in Zn-MTO.

ESI-MS analysis of the Zn-MTO preparations revealed the presence of three species: a major Zn_4-MTO and two minor Zn_5-MTO and Zn_3-MTO forms (Table 1). The degree of Zn-MTO oxidation ranged from 16 to 52%, depending on culture

Table 1
Molecular masses and metal (Zn or Cu) to protein ratios found for in vivo synthesized Zn-MTO and Cu-MTO, eluted in Tris–HCl buffer

Metal supplemented in culture media	MW_{exp} (apo-MTO)[a]	MW_{exp} (M-MTO)[b]	MW_{calc} [c]	M/MTO[d]	M/MTO[e]
M = Zn	4667.1 ± 0.6	4922.2 ± 0.4 (S)	4922.86	4	3.7
		4988.5 ± 1.9 (s)	4986.24	5	
		4858.3 ± 0.3 (s)	4859.48	3	
M = Cu	4667.6 ± 1.0	5231.2 ± 0.5 (e)	5232.29	9	7.4–8.1
		5170.1 ± 2.1 (e)	5169.74	8	
M = Cu[f]	4667.6 ± 1.0	5231.2 ± 0.5	5232.29	9	8.9

[a]Experimental molecular masses for the apo-proteins. MTO calculated mass is 4669.34.
[b]Experimental molecular masses for the Zn- and Cu-MTO complexes. (S) denotes a major species, (s) denotes a minor species and (e) denotes equimolarity.
[c]Calculated molecular masses for neutral species with loss of two protons/Zn bound and one proton/Cu bound.
[d]Zinc or copper per MTO molar ratio calculated from the mass difference between holo- and apo-protein.
[e]Zinc per MTO molar ratio calculated from the Zn content (ICP-AES) and cysteine thiolates (Ellman's method), and Cu per MTO molar ratio calculated from the copper and total sulfur content.
[f]Small-scale cultures.

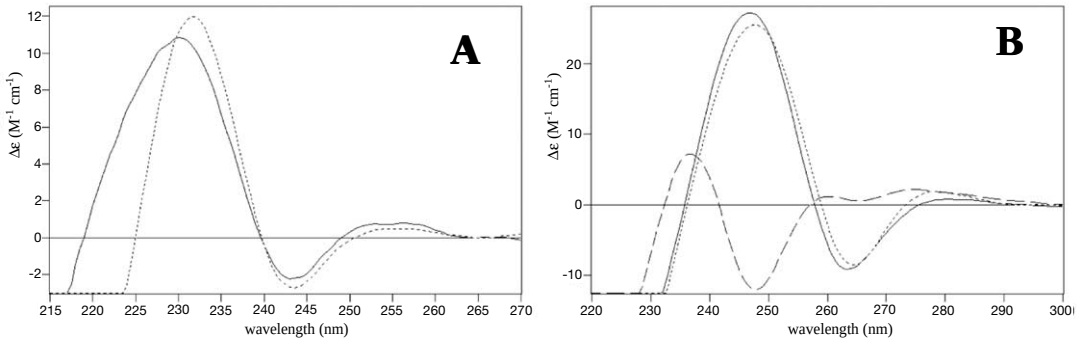

Fig. 1. A: CD spectra of recombinant Zn_4-MTO eluted in Tris–HCl (solid line) and in Tris–$HClO_4$ (dotted line). B: CD spectra of the Cd_4-MTO species in Tris–HCl buffer (solid line) and in Tris–$HClO_4$ (dotted line), and of the Cd_9-MTO aggregate (dashed line) obtained by Zn/Cd replacement of a 23.4 μM solution of Zn-MTO.

conditions [10]. These values agreed well with the ICP-AES data, indicating average ratios of Zn per reduced protein of 3.70 and 2.80, respectively. The weak intensity of the Zn-MTO CD spectrum (Fig. 1A) evidenced a low degree of chirality for the biosynthesized Zn-MTO aggregates in Tris–HCl, but still allowed identification of the Zn-thiolate chromophores by detection of an exciton coupling centered at ca. 240 nm. A new Gaussian-shaped band centered at ca. 230(+) nm was observed, with an intensity inversely related to the MTO oxidation degree. To investigate whether this absorption could be related to a non-standard zinc peptide chromophore, the spectroscopic features of Zn-MTO eluted in Tris–$HClO_4$ buffer were also analyzed (Fig. 1A). Since the absorption at 230 nm was red-shifted and narrowed, a possible role of the chloride anions in the folding of the Zn-MTO aggregates was inferred.

The in vitro formation of fully cadmium-loaded MTO as a result of Zn/Cd replacement in Zn-MTO proceeded in two stages, when using both $CdCl_2$ and $Cd(ClO_4)_2$ as titrating agent. The stoichiometric (ESI-MS) and spectroscopic (CD and UV-visible) data of the intermediate species are shown in Table 2 (detailed data are available upon request).

During the first step of the titration, although the Zn/Cd replacement increased the metal cluster organization, essentially the same mixture of species was found, i.e. $M(II)_4$-MTO (major) and $M(II)_5$-MTO (minor). Thus, the stoichiometric similarities and the parallelism of the CD spectra between the Zn- and Cd-MTO species pointed to an isomorphic Zn/Cd replacement. Consistently, $M(II)_4$-MTO should be considered the physiologically stable species, which can incorporate a fifth M(II) ion without significant alteration of the initial structure. In the second stage of the titration, the addition of Cd(II) caused an extensive rearrangement of the previous Cd_4- and Cd_5-MTO aggregates (Fig. 1B), which generated a final, unique Cd_9-MTO species. No intermediates were observed and no changes were introduced by further Cd(II) additions. In order to analyze the role of Cl^- ions in the formation of M(II)-MTO aggregates, the UV-vis difference spectra corresponding to the titrations of Zn-MTO with $CdCl_2$ or $Cd(ClO_4)_2$ were compared (Fig. 2). In addition to the red shift up to ca. 260 nm associated with the Zn/Cd substitution, an unprecedented absorption at ca. 240 nm was found for the binding of the fourth Cd(II) in the presence of chloride anions (Fig. 2A), absent when using $Cd(ClO_4)_2$ as titrating agent (Fig. 2B). Thus, the absorption at 240 nm would be in agreement with the presence of chloride ligands in the coordination environment of cadmium in MTO (i.e. $CdS_{4-x}Cl_x$), not observable for MTN (Fig. 2C). On the other hand, the apparent isostructural replacement of Zn by Cd in MTO called into question the possibility of assigning the 230-nm Gaussian band detected for Zn-MTO to $ZnS_{4-x}Cl_x$ chromophores. Unfortunately, ESI-MS of Cd- and Zn-MTO in Tris–HCl did not allow discrimination between the common ammonium adducts and possible species containing M(II)-Cl bonds. However, while the CD spectra of Zn-MTO show significant differences in the 220–240-nm region, depending on the elution buffer (Fig. 1A), those of Cd-MTO are very similar and thus indicative of structurally related metal–sulfur aggregates (Fig. 1B). Overall, the experimental results indicate the participation of Cl^- anions in both M(II)-MTO aggre-

Table 2
Analysis of the in vitro Zn/Cd replacement in Zn-MTO

Cd(II) eq./mol of Zn-MTO	Metal-MTO species obtained (ESI-MS and spectroscopic characterization)	Chiroptical features (CD spectroscopy, Fig. 1)
Step 1 (1–8)		
From 1 to 3	Mixture of all possible Zn_x,Cd_y-MTO aggregates ($x+y=4$), with predominance of those with $y=$ no. Cd(II) eq. added+1	Formation of Cd_4S chromophores
From 4 to 6	Mixture of species: Cd_4-MTO (S), Cd_5-MTO (s)	Development of a derivative-shaped profile (crossover point, 258 nm)
Up to 8	Equimolar mixture of species: Cd_4-MTO, Cd_5-MTO	Development of a Gaussian band at 245(+) nm
Step 2		
From 8 to 16	Final Cd_9-MTO, no intermediate species, no further transformation	Extensive rearrangement of the former Cd-thiolate clusters

(S) denotes a major species, (s) denotes a minor species.

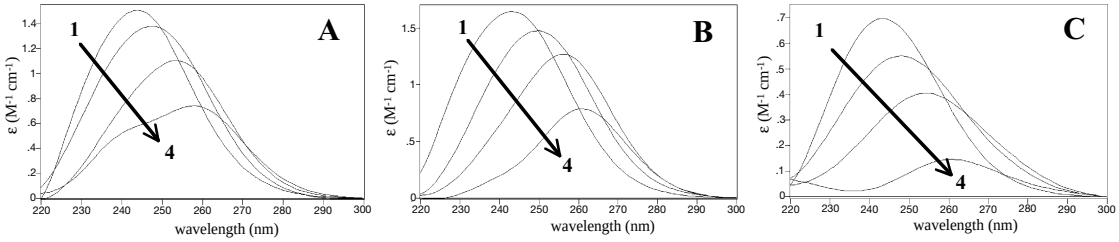

Fig. 2. Difference UV-vis absorption spectra corresponding to the titrations of recombinant (A) Zn-MTO in Tris–HCl buffer with $CdCl_2$ from 1 to 4 Cd(II) eq.; (B) Zn-MTO in Tris–$HClO_4$ buffer with $Cd(ClO_4)_2$ from 1 to 4 Cd(II) eq.; (C) Zn-MTN with $CdCl_2$ from 1 to 4 Cd(II) eq.

gates, M = Zn or Cd, but with higher structural significance in the Zn_4S_{12} than in the Cd_4S_{12} metal–cysteine clusters.

3.3. Characterization of the monovalent metal binding abilities of MTO

The analysis of the behavior of MTO in front of monovalent metal ions was achieved by characterization of the in vivo synthesized Cu-MTO aggregates and of the Cu-MTO species obtained in vitro by Zn/Cu replacement.

Mto-cDNA expression in *standard* copper-supplemented cultures yielded homometallic Cu-MTO species, with a total metal-per-sulfur content of 7.4–8.1 (ICP-AES results) and an approximate equimolar ratio of the Cu_8- and Cu_9-MTO species (ESI-MS analyses, Table 1). Small-scale synthesis yielded a unique Cu_9-MTO species according to ESI-MS, which corresponded well to an ICP-AES copper-to-MTO ratio of 8.9. Zn(II) absence in all Cu-MTO samples was systematically confirmed by ICP-AES. The high intensity and well-defined CD spectra of recombinant Cu-MTO (Fig. 3D) revealed a high metal cluster organization.

Zn/Cu exchange during titration of Zn-MTO with Cu(I) (Fig. 3) evolved in two distinct phases (Table 3), as for Zn/Cd replacement. At the end of the first step, 9 equivalents of Cu(I) added, Zn-MTO turned into the fully copper-loaded species Cu_8-MTO and Cu_9-MTO, which exhibited maximum chirality and was highly coincident with all the features of the in vivo synthesized Cu-MTO (Fig. 3D). The second step led to overloaded Cu_{15}- and Cu_{17}-MTO species.

3.4. Primary structure analysis of MTO, MTN and other Arthropoda MTs

The ClustalW-based analysis [3] of the MTO amino acid sequence included two *S. cerevisiae* proteins: CUP1 and CRS5, a MT-like peptide also associated with copper resis-

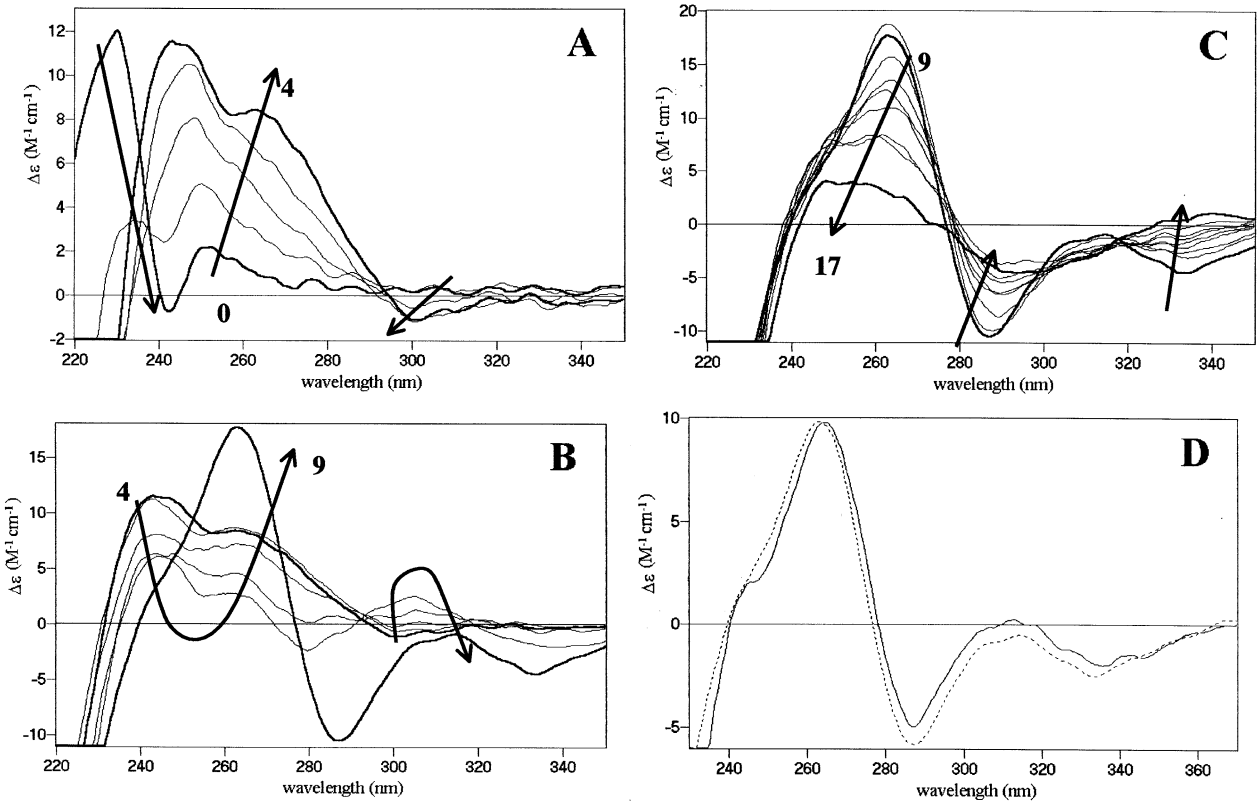

Fig. 3. CD spectra recorded during the titration of a 14.4 μM solution of Zn-MTO with Cu(I) at pH 7, shown in three stages: (A) from 0 to 4, (B) from 4 to 9, and (C) from 9 to 17 equivalents of Cu(I) added. Arrows indicate increasing Cu(I) equivalent amounts. Traces corresponding to the first and last spectrum of each stage are depicted in bold. D: Superimposition of the CD spectra of the recombinant Cu-MTO (solid line) and the Cu_9-MTO species generated in vitro by Zn/Cu replacement (dotted line).

Table 3
Analysis of the in vitro Zn/Cu replacement in Zn-MTO

Cu(I) eq./mol of Zn-MTO	Metal-MTO species obtained (ESI-MS and spectroscopic characterization)	Chiroptical features (CD spectroscopy, Fig. 3)
Step 1 (1–9)		
From 1 to 4	Cu_4,Zn_2-MTO (Chelex [26])	Formation of CuS chromophores
Up to 9	Cu_8-MTO, Cu_9-MTO	Red shift of the absorption indicative of Zn/Cu replacement
Step 2		
From 9 to 17	Overmetallated species[a]: Cu_{15}-MTO, Cu_{17}-MTO	Decrease in intensity of (+) and (−) bands: loss of the previous Cu–S chromophores

[a]Identified by CD results, non-detectable by ESI-MS, as repeatedly reported for homometallic Cu-MT aggregates of high nuclearity [26,27].

tance [28]. The generated alignment and the non-rooted neighbor-joining tree are shown in Fig. 4. MTO unquestionably came together with the known Cu-thionein sequences, with a significant bootstrap value of 999/1000 separating this cluster from that of the Arthropoda Zn-thioneins [3]. Strikingly, the two *Drosophila* MTs did not share the closest sequence similarity: the shortest Cu-thionein subset branch joined *Drosophila* MTN with *Callinectes* Cu-MT, while MTO was associated with both *S. cerevisiae* metallothioneins. This agrees with an MTN–MTO divergence preceding *Drosophila* differentiation, so that the branches from which MTO and MTN evolved could have split at an early stage of Arthropoda radiation. The opposite conclusion had previously been drawn [15], but only zinc-thioneins had been reported in Crustacea.

We unsuccessfully attempted to identify the sequence feature responsible for the clustering of Cu-thioneins. There are no Cys motifs or distribution patterns in Zn-thioneins that do not appear in one or other Cu-thionein, although it is true

A)

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                    10        20        30        40        50        60        70        80
A.astacus           -----MPGP--CCND-VCECAAG---GCKTG--CVCTS------CRCSPCDKCTS--GCKCPSKEE-C-AKTCSKPCECCP--
C.sapidusI          -----MPGP--CCND-KCVCQEG---GCKAG--CQCTS------CRCSPCQKCTS--GCKCATKEE-C-SKTCTKPCSCCPK-
C.sapidusII         -----MPDP--CCND-KCECKEG---ECKTG--CKCKS------CRCPPCDKCSS--ECKCTSKEE-C-SKTCSKPCSCCP--
C.maenas            -----MPDP--CCID-KCECKEG---GCKAG--CKCTS------CRCTPCEKCSS--GCKCTTKED-C-CKTCTKPCSCCP--
H.americanus        ------PGP--CCKD-KCECAEG---GCKTG--CKCTS------CRCAPCEKCTS--GCKCPSKDE-C-AKTCSKPCKCCP-
S.serrataII         ------PDP--CCND-KCDCKEG---ECKTG--CKCTS------CRCPPCEQCSS--GCKCANKED-C-RKTCSKPCSCCP--
S.serrataI          ------PGP--CCND-KCVCKEG---GCKEG--CQCTS------CRCSPCEKCSS--GCKCANKEE-C-SKTCSKACSCCPT-
P.leniusculus       -----MPGP--CCND-QCECAAG---GCKTG--CVCTS------CRCQPCDKCTS--GCKCPSKEE-C-AKTCSKPCRCCP--
P.potamios          ------PDP--CCAEGTCECEEG---KCKAG--CKCTS------CRCSPCEKCTS--ECECKSKEE-C-AKNCTKPCSCCP--
C.sapidus-Cu        -----MPCG--CGTS--CKCGSG---KCCCGSTCNCTTCPSKQSCSCND-GACGS--ACQCKTSCC-C-GADC--KCSPCPMK
Drosophila MTN      -----MPCP--CGSG--CKCAS-----QATKGS-CNCGS------DCKCG--GDKKS--ACGCSE--------------------
Drosophila MTO      -----MVCK--GCGT-NCQCSAQ---KCGDN-------------CACN--KDC----QCVCKNGP----KDQC---CSNK---
Yeast CUP1          MFSELINFQ--NEGH-ECQCQCG---SCKNNE--QCQK-----SCSCP--TGCNSDDKCPCGNKSEET-KKSC---CSGK---
Yeast CRS5          MTVKICDCEGECCKD-SCHCGSTCLPSCSGGEKCKCDHS-----TGSPQCKSCGE--KCKCETTCT-CEKSKCN--CEKC---
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B)

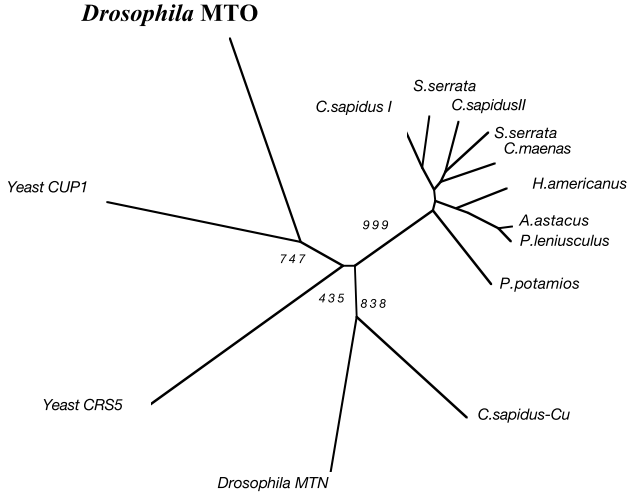

Fig. 4. A: ClustalW output of the multiple alignment of Arthropoda and yeast MT sequences, constructed using the Blosum62 weight matrix. The names of the organisms are: *Callinectes sapidus*, *Scylla serrata*, *Carcinus maenas*, *Astacus astacus*, *Pacifastacus leniusculus*, *Homarus americanus*, *Potamon potamios*, yeast (*Saccharomyces cerevisiae*) and *Drosophila melanogaster*. B: Consensus protein distance tree, constructed by the neighbor-joining method from the alignment shown in A, with a bootstrap value of 999. The mean bootstrap values for the longest tree branches are shown.

that no Cu-thionein shares the complete Cys arrangement with a Zn-thionein. Non-Cys residues also failed to reveal any regular pattern which which zinc or copper preference could be associated. MTO and MTN polypeptides differ not only in length (43 vs. 40 amino acids, respectively), but also in number of cysteines (12 vs. 10) and in their distribution. In MTN, Cys are arranged in five C-X-C motifs, while in MTO they are more randomly distributed, only eight of 12 arranged in C-X-C motifs. Taking the sequence similarities between MTO and CUP1 into account, it is difficult to explain the eight/nine Cu(I) ions bound to recombinant MTO if the two last C-terminal Cys were devoid of binding capacity, as has been postulated for the two consecutive Cys of the C-terminal tail of CUP1 by nuclear magnetic resonance studies [29,30].

4. Conclusions

Both the experimental and in silico results presented in this work unambiguously classify MTO as a second *Drosophila* Cu-thionein, according to the phylogenetic analysis method proposed in [3]. This signifies the unprecedented fact of an animal organism for which no Zn-thionein form has yet been reported. The in vivo and in vitro criteria used to assess the Cu-thionein character of MTO are the following. First, the high degree of folding and stability of Cu-MTO vs. the weak chirality and oxidation susceptibility of Zn-MTO, indicative of a low preference of MTO for Zn and of an intrinsic instability of Zn-MTO in intracellular environments, similar to those reported for MTN [11]. Second, the recovery of homometallic Cu-MTO species from recombinant synthesis on Cu-supplemented media. Particularly, although in vitro Zn/Cu replacement in Zn-MTO led to the formation of intermediate (the heterometallic Cu_4,Zn_2-MTO) and overloaded (Cu_{15}- and Cu_{17}-MTO) species, the structural and stoichiometric similarities between the biosynthesized Cu-MTO aggregates and those resulting from the addition of 9 Cu(I) to Zn-MTO pointed to Cu_9-MTO as the species produced in intracellular Cu-rich environments. It is worth noting here that the tree clustering of copper-thioneins identified by two independent criteria – copper gene inducibility for CUP1 [31] and *Callinectes* Cu-MT [8], and metal coordination studies for *Drosophila* MTN [11] and MTO (this work) – validates the protein sequence similarity approach and the coherence of the thus defined copper-thionein subfamily.

The proposed Zn-thionein/Cu-thionein classification concerns the evolutionary origin of the MTs, as well as the primordial *molecular function* of the chelating peptide, which, in the case of Cu-thioneins, would imply an intrinsic ability to form homometallic, biologically stable, copper aggregates. However, the *biological role* of MTs should be considered from a broader perspective than that of the optimal molecular function, since, by definition, MTs are proteins which do not exhibit specificity for metal chelation. Thus, the classification of a given MT as Cu-thionein does not imply its inability to react towards metals ions other than copper, especially in metal stress conditions. The case of *Drosophila* MTs, and particularly MTO, could yield a good example of the duality of an MT's metal binding behavior, if physiological or metal poisoning situations are considered.

In physiological conditions, the two metal ions to be taken into account are Cu(I) and Zn(II). From the results presented here, the optimal reactivity of MTO towards copper vs. the poor behavior towards Zn is clear. Therefore, the MTO Cu-thionein nature has to be considered when trying to understand its normal biological role and its basal gene regulation patterns. It has recently been reported that a Cu-MT complex in the midgut *copper cells* could be involved in *Drosophila* digestive acid secretion [32], and the putative demands imposed by copper-oxygen transporters (hemocyanins) in insects are well known [33]. With respect to gene regulation, although *Mto* and *Mtn* follow different temporal and tissue patterns, it remains true that both genes exhibit the highest expression levels after copper induction, while remaining poorly induced by zinc [12–17]. In fact, the analysis of *Mto* and *Mtn* molecular control mechanisms reveals a puzzling situation: a transcription factor homologous to mammalian MTF-1 [34], dMTF, has been reported, which binds upstream of *Mto* and *Mtn* to several MRE boxes upon zinc coordination, while MT synthesis in flies as a response to this metal remains very poor [35], as would be expected when considering the copper-thionein character of the encoded peptides.

In metal stress conditions, it is worth considering the behavior of MTO towards cadmium, as a paradigm of a xenobiotic divalent metal ion. Previous results clearly showed the involvement of MTO in *Drosophila* Cd resistance [15], and *Mto* inducibility by cadmium, although lower than by copper, is far more pronounced than by zinc [12–17]. In fact, our results are in full agreement with the hypothesis that MTO, although a Cu-thionein by origin, plays an important role in Cd detoxification in *Drosophila*. First, the degree of folding of the *canonical* Cd-MTO species (Cd_4- and Cd_5-MTO) is considerably higher than that of Zn-MTO, which would ensure a good stability of the cadmium-MTO aggregates in intracellular environments. Second, under excessive Cd(II) concentrations, we show that MTO can rearrange its peptide chain to accommodate extra metal ions, yielding an equally stable, overmetallated aggregate, namely Cd_9-MTO. And finally, it is worth noting that MTN and MTO exhibit only a few differences in metal binding behavior, although the spectroscopic results have provided evidence for the possible binding of chloride anions in the M_4-MTO, M = Zn(II), Cd(II), aggregates. However, the contribution of the chloride ions in M-MTO complexes has been found to be less significant for M = Cd(II) than for M = Zn(II). This would point to the MTO polypeptide's higher capacity for association with cadmium than with zinc, a metal ion that would require extra ligands to maintain the corresponding MTO aggregate.

Further study is needed to reveal whether the coexistence of MTN and MTO is maintained by differential gene expression programs or by any of the slight differences in metal coordination highlighted in this work. Moreover, the search for new MT forms by in silico *Drosophila* genome screening and the elucidation of the MT gene transcriptional activation pathways ensure that the *Drosophila* MT system is an intriguing model for which many questions still remain unanswered.

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References

[1] Kägi, J.H.R. (1993) in: Metallothionein III. Biological Roles and Implications (Suzuki, K.T. Imura N. and Kimura, M., Eds.), pp. 29–55, Birkhäuser Verlag, Basel.
[2] Fisher, E.H. and Davie, E.W. (1998) Proc. Natl. Acad. Sci. USA 95, 3333–3334.
[3] Valls, M., Bofill, R., González-Duarte, R., González-Duarte, P., Capdevila, M. and Atrian, S. (2001) J. Biol. Chem. 276, 32835–32843.
[4] Bertini, I., Hartman, H.-J., Klein, T., Liu, G., Luchinat, C. and Weser, U. (2000) Eur. J. Biochem. 267, 1008–1018.
[5] Lerch, K. (1980) Nature 284, 368–370.
[6] Münger, K. and Lerch, K. (1985) Biochemistry 24, 6751–6756.
[7] Dallinger, R., Berger, B., Hunziker, P. and Kägi, J.H.R. (1997) Nature 388, 237–238.
[8] Syring, R.A., Hoexum-Brouwer, T. and Brouwer, M. (2000) Comp. Biochem. Physiol. C 125, 325–332.
[9] Santovito, G., Irato, P., Palermo, S., Boldrin, F., Sack, R., Hunziker, P. and Piccinni, E. (2001) Protist 152, 219–229.
[10] Cols, N., Romero-Isart, N., Bofill, R., Capdevila, M., González-Duarte, P., González-Duarte, R. and Atrian, S. (1999) Protein Eng. 12, 265–269.
[11] Valls, M., Bofill, R., Romero-Isart, N., González-Duarte, R., Abián, J., Carrascal, M., González-Duarte, P., Capdevila, M. and Atrian, S. (2000) FEBS Lett. 467, 189–194.
[12] Latowski-Perry, D., Otto, E. and Maroni, G. (1985) J. Biol. Chem. 260, 1527–1530.
[13] Durliat, M., Bonneton, F., Boissonneau, E., André, M. and Wegnez, M. (1995) Biometals 8, 339–351.
[14] Maroni, G., Wise, J., Young, J.E. and Otto, E. (1987) Genetics 117, 739–744.
[15] Mokdad, R., Debec, A. and Wegnez, M. (1987) Proc. Natl. Acad. Sci. USA 84, 2658–2662.
[16] Silar, P., Theodore, L., Mokdad, R., Erraiss, N.E., Cadic, A. and Wegnez, M. (1990) J. Mol. Biol. 215, 217–224.
[17] Silar, P. and Wegnez, M. (1990) FEBS Lett. 269, 273–276.
[18] Studier, F.W. and Moffatt, B.A. (1986) J. Mol. Biol. 189, 113–130.
[19] Capdevila, M., Cols, N., Romero-Isart, N., González-Duarte, R., Atrian, S. and González-Duarte, P. (1997) Cell. Mol. Life Sci. 53, 681–688.
[20] Bofill, R., Palacios, O., Capdevila, M., Cols, N., González-Duarte, R., Atrian, S. and González-Duarte, P. (1999) J. Inorg. Biochem. 73, 57–64.
[21] Fabris, D., Zaia, J., Hathout, Y. and Fenselau, C. (1996) J. Am. Chem. Soc. 118, 12242–12243.
[22] GCG (1994) Program Manual for the Wisconsin Package, v. 10, Wisconsin Genetics Computer Group, Madison, WI.
[23] Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) Nucleic Acids Res. 22, 4673–4680.
[24] Fitch, W.M. and Margoliash, E. (1967) Science 155, 279–284.
[25] Felsenstein, J. (1989) Cladistics 5, 164–166.
[26] Bofill, R., Capdevila, M., Cols, N., Atrian, S. and González-Duarte, P. (2001) J. Biol. Inorg. Chem. 6, 405–417.
[27] Zaia, J., Fabris, D., Wei, D., Karpel, R.L. and Fenselau, C. (1998) Protein Sci. 7, 2398–2404.
[28] Culotta, V.C., Howard, W.R. and Liu, X.F. (1994) J. Biol. Chem. 269, 25295–25302.
[29] Narula, S.S., Winge, D.R. and Armitage, I.M. (1993) Biochemistry 32, 6773–6787.
[30] Peterson, C.W., Narula, S.S. and Armitage, I.M. (1996) FEBS Lett. 379, 85–93.
[31] Butt, T.R., Sternberg, E.J., Gorman, J.A., Clark, P., Hamer, D., Rosenberg, M. and Crooke, S.T. (1984) Proc. Natl. Acad. Sci. USA 81, 3332–3336.
[32] McNulty, M., Pujlung, M., Jefford, G. and Dubreuil, R.R. (2001) Cell Tissue Res. 304, 383–389.
[33] Brouwer, M., Syring, R. and Hoexum-Brouwer, T. (2002) J. Inorg. Biochem. 88, 228–239.
[34] Heuchel, R., Radtke, F., Georgiev, O., Stark, G., Aguet, M. and Schaffner, W. (1994) EMBO J. 13, 2870–2875.
[35] Zhang, B., Egli, D., Georgiev, O. and Schaffner, W. (2001) Mol. Cell. Biol. 21, 4505–4514.