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ORIGINAL ARTICLE

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A distinct Cu_4-thiolate cluster of human metallothionein-3 is located in the N-terminal domain

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Abstract Metallothionein-3 (MT-3), also known as neuronal growth inhibitory factor, is a metalloprotein expressed almost exclusively in the brain. Isolated MT-3 contains four Cu(I) and three Zn(II) ions organized in homometallic metal-thiolate clusters located in two independent protein domains. In this work a Cu(I) binding to metal-free MT-3 has been studied, aiming at the better understanding of the domain specificity for this metal ion. The cluster formation was followed by electronic absorption, circular dichroism, and by luminescence spectroscopy at room temperature and 77 K. The stepwise incorporation of Cu(I) into recombinant human apo-MT-3 revealed the cooperative formation of two Cu_4S_9 clusters in succession, formed in both protein domains, i.e. Cu_4- and Cu_8-MT-3. Further binding of four Cu(I) caused an expansion of these Cu(I) cores, leading to fully metal-loaded Cu_{12}-MT-3 containing Cu_6S_9 and Cu_6S_{11} clusters in the β- and α-domains of the protein, respectively. The location of the preferentially formed Cu_4 cluster in the protein was established by immunochemistry. Using domain-specific antibodies, in combination with limited tryptic digestion of a partially metal-occupied Cu_4-MT-3, we could demonstrate that the Cu_4S_9 cluster is located in the N-terminal β-domain of the protein that contains a total of nine cysteine ligands. Electronic supplementary material to this paper, comprising Table S1 (amino acid sequences of peptides used in immunization) and Fig. S1 (luminescence spectra of Cu(I) titrated apo-MT-3), can be obtained by using the Springer Link server located at http://dx.doi.org/10.1007/s00775-002-0339-1.

Keywords Metallothionein-3 · Copper · Copper cluster · Metal specificity

Abbreviations *AP*: alkaline phosphatase · *MT*: metallothionein · *TBS*: Tris-buffered saline · *TFA*: trifluoroacetic acid

Introduction

Metallothionein-3 (MT-3), also termed growth inhibitory factor, is a member of the zinc- and copper-binding family of metallothioneins. This protein is expressed almost exclusively in the central nervous system and possesses neuroinhibitory properties that make it unique from the widely expressed mammalian MT-1/MT-2 isoforms [1]. The amino acid sequence of human MT-3 (68 amino acids) exhibits about 70% sequence identity with those of mammalian MT-1 and MT-2 isoforms (61–62 amino acids), including the preserved array of 20 cysteines [1].

Growth inhibitory activity in neuronal cell cultures has been reported for isolated Cu_4,Zn_3-MT-3 as well as for recombinant Zn_7-MT-3 [1, 2]. Other studies have established that the bioactivity is confined to the N-terminal β-domain of MT-3, i.e. Zn_3-β(1–31) [2, 3], and that this activity is abolished by the mutation of the conserved C(6)-P-C-P(9) motif to that found in MT-1/MT-2 [2, 4].

With the aim of gaining an insight into the mode of action of MT-3 at the molecular level, we have conducted structural and biological studies on isolated Cu_4,Zn_3-MT-3 and recombinant $M(II)_7$-MT-3. The structural studies of native Cu_4,Zn_3-MT-3 by means of various spectroscopic techniques, including Cu and Zn *K*-edge EXAFS, established the presence of Cu_4- and Zn_3-thiolate clusters located in two independent protein domains, with Cu(I) ions being primarily trigonally and

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Zn(II) tetrahedrally coordinated. From these and other spectroscopic studies, the presence of a Cu(I) cluster in the β-domain (1–31) of MT-3 has been inferred [5, 6, 7]. The structural characterization of recombinant human Zn_7- and Cd_7-MT-3 by spectroscopy revealed the presence of a $M_3(CysS)_9$ and a $M_4(CysS)_{11}$ cluster localized in two mutually interacting α- and β-domains, respectively. A detailed ^{113}Cd NMR investigation of $^{113}Cd_7$-MT-3 revealed the existence of unusual dynamic processes connected with the formation of both conformational and configurational cluster substates [8]. The latter can be visualized as major structural fluctuations due to temporarily breaking and reforming of the metal-thiolate bonds. Recent studies showed that the mutation of the unique C(6)-P-C-P(9) motif in MT-3 to C(6)-S-C-A(9) of human MT-2 not only abolished the biological activity, but also profoundly affected the formation of configurational cluster substates [4].

Since no comparative biological studies between the isolated Cu_4,Zn_3-MT-3 and recombinant Zn_7-MT-3 metalloforms exist, further studies of the former metalloform may be of importance for a better understanding of the MT-3 function. However, owing to the inherent difficulties of isolating large amounts of this factor from the brain, we plan to use recombinant MT-3 into which both Cu(I) and Zn(II) ions will be incorporated in vitro. For this purpose we have developed the following strategy: (1) accumulate sufficient structural/spectroscopic information on isolated Cu_4,Zn_3-MT-3 [6, 9, 10] and (2) examine the metal binding properties of both synthetic domains of MT-3, i.e. the α-domain (32–68) and the β-domain (1–31), with mono- and divalent metal ions [5, 11]. With this information at hand the Cu(I) and Zn(II) ions will be incorporated into the metal-free MT-3. Because of the very high affinity of Cu(I) toward thiolate ligands, their correct incorporation and the localization of the anticipated Cu_4 cluster in the protein structure was considered crucial for the generation of Cu_4,Zn_3-MT-3 and further characterization of this metalloform.

In the present studies a method for selective Cu(I) incorporation into both protein domains of apo-MT-3 has been developed. The Cu(I) binding has been followed by a combination of various spectroscopic techniques, including electronic absorption, circular dichroism (CD), and luminescence spectroscopy at room temperature and 77 K. Using domain-specific antibodies, in combination with limited tryptic digestion of a partially metal-occupied Cu_4-MT-3, we could demonstrate that the preferentially formed Cu_4 cluster is located in the N-terminal β-domain of the protein.

Material and methods

Expression and purification of MT-3

Human Zn_7-MT-3 was expressed in *Escherichia coli* and purified as described [8]. The apo-form of MT-3 was generated by the method of Vašák [12]. The concentration of apoprotein was determined spectrophotometrically ($\epsilon_{220} = 53{,}000\ M^{-1}\ cm^{-1}$) and the free cysteine content (20 cysteine residues/apoprotein) was assessed by thiol reaction with 2,2′-dithiopyridine in 0.2 M sodium acetate/1 mM EDTA (pH 4), using $\epsilon_{343} = 7600\ M^{-1}\ cm^{-1}$ [13].

Cu(I) titration of MT-3

All solutions used in the preparation of Cu(I)-MT-3 complexes were rendered oxygen-free by three freeze-pump-thaw cycles on a vacuum line and all preparation steps were carried out in a nitrogen-purged glovebox. The metal content of the freshly prepared $[Cu(MeCN)_4]ClO_4$ stock solution (5 mM HCl/20% MeCN) was determined by atomic absorption spectroscopy (IL Video 12). After addition of increasing Cu(I) equivalents to apo-MT-3 (10 mM HCl/5% MeCN) the pH was raised to 8.0 with 0.5 M Tris base. Each titration step was performed at least in duplicate.

Spectroscopic measurements

Absorption spectra were recorded on a Cary 3 spectrometer. CD measurements were made using a Jasco spectropolarimeter (model J-715). The absorption and CD spectra were collected with protein concentrations of 15 μM. The latter are expressed as molar circular-dichroitic absorption ($\Delta\epsilon$), in units of $M^{-1}\ cm^{-1}$. The final CD spectra were smoothed using the fast Fourier transformation algorithm of the Microcal Origin 5.0 software. Luminescence spectra were obtained on a SPEX fluorolog spectrofluorometer using 4.5 nm excitation and emission bandwidth. The excitation wavelength was 300 nm. For low-temperature measurements (77 K) the fluorometer was fitted with a 1934C phosphorimeter accessory using 45° detection geometry. Luminescence spectra were recorded at 77 K on the microcrystalline frozen samples using a 50 μs delay and a 0.3 ms acquisition window and were smoothed using the algorithm mentioned above. The samples were placed into 2 mm diameter quartz tubes and immersed in a cylindrical quartz Dewar vessel filled with liquid nitrogen.

Preparation of antibodies against human MT-3

Specific antibodies against the α- and β-domain of human MT-3 (hMT-3) were produced in rabbits as described by Uchida et al. [1]. The peptides I and II (see Supplementary material, Table S1), linked to keyhole limpet hemocyanine, were used in the immunization of New Zealand white rabbits. Both peptides were synthesized on an ABI 433 peptide synthesizer by standard Fmoc chemistry using conditions according to the recommendations of the manufacturer and purified as follows. The crude peptides were dissolved in 0.1% trifluoroacetic acid (TFA)/5% acetonitrile (MeCN) and applied to a semipreparative C_{18} RP-HPLC column (Brownlee Aquapore ODS300, 20 μm, 250×10 mm) using 0.1% TFA as solvent A and 0.08% TFA/84% MeCN as solvent B. The correctness of the synthesis was checked by ESI-MS spectrometry (Sciex API III$^+$).

Immunochemical analysis

In all immunochemical experiments the unpurified antisera were used. The antiserum obtained against peptide I, interacting with the β-domain of the protein, is designated as anti-hMT-3β. Similarly, that against peptide II, interacting with the α-domain, is designated as anti-hMT-3α. For dot blot analysis a slightly modified method by Mizzen et al. [14] was used. Briefly, following the direct application of 1.5 μL of trypsin-treated samples to the nitrocellulose membrane, the membrane was incubated in 2.5% glutaraldehyde for 1 h. After thorough washing with phosphate buffer (8.1 mM Na_2HPO_4, 1.2 mM KH_2PO_4, 2.7 mM KCl, pH 7.4) and addition of 50 mM monoethanolamine in the last washing step, the

membrane was blocked with 3% nonfat dry-milk dissolved in Tris-buffered saline (TBS) for 2 h at room temperature. The membranes were then incubated overnight with primary antibodies (anti-hMT-3β or anti-hMT-3α) diluted in 3% nonfat dry-milk in TBS. Subsequently, the membranes were washed in TBS containing 0.05% Tween-20 and incubated for 4 h with alkaline phosphatase (AP) linked secondary antibody [goat-anti-rabbit IgG (Bio-Rad)] placed in 3% nonfat dry-milk in TBS. Before color development using the AP conjugate substrate kit (Bio-Rad), the membranes were washed twice in TBS containing 0.05% Tween-20 and once without Tween-20.

Enzymatic digestion of Cu(I)-MT-3

Partially metal-loaded Cu_4-MT-3 was generated as described above. Then 2 mL of this sample were concentrated to a final volume of 100 µL and trypsin (Cooper) was added in a ratio of 1:20 (w/w). After 2 h incubation at 37 °C the reaction was stopped by addition of 10 µL of 1 M DTT, 10% SDS, and 10 µL of 1% TFA.

Results and discussion

At present, only one three-dimensional structure of a Cu(I)-thiolate cluster in proteins exists. This is the NMR structure of $Cu(I)_7$-MT from yeast, determined as its $^{109}Ag(I)$ derivative [15]. However, a recent NMR re-investigation of the protein fold in $Cu(I)_7$-MT together with molecular modeling has put the isostructural replacement of Cu(I) by Ag(I) in question [16]. In the past, the structural features of Cu(I)-thiolate clusters in proteins, e.g. transcription factors Ace1 and Amt1, MTs, and related proteins, have been studied by various spectroscopic techniques including electronic absorption, CD, luminescence, XAS, and EXAFS [17, 18, 19, 20, 21, 22, 23]. The former three techniques have also been used in the present studies.

In order to examine the binding capacity of apo-MT-3 with Cu(I) ions, the complexes formed with differing mole equivalents of copper were spectroscopically characterized. The absorption spectra of Cu(I)-MT-3 are characterized by the appearance of metal-dependent features starting at about 380 nm and extending into the far-UV region. The broad absorption envelope shows a prominent shoulder at 262 nm and a weak shoulder at about 300 nm (Fig. 1). The intensity of the 262 nm shoulder increases more or less linearly with increasing Cu(I) equivalents, up to around eight Cu(I) equivalents. Further incremental addition of four Cu(I) equivalents led to an unresolved spectral envelope with a substantially increased absorption in the low-energy region. Above this titration point, no dramatic changes of the spectral profile occurred. As revealed by the inset to Fig. 1, where a 262 nm/290 nm ratio is plotted as a function of Cu(I) bound, the spectral changes are consistent with the formation of two Cu(I) complexes, i.e. Cu_8- and Cu_{12}-MT-3.

Examination of the corresponding CD spectra reveals well-resolved CD bands at (+)260 nm, (+)295 nm, and at about (+)350 nm with the first four Cu(I) equivalents added (Fig. 2). All CD profiles show rather similar shape, with the intensity increasing linearly with Cu(I) added. The CD profiles obtained in the titration with the first four Cu(I) equivalents are rather similar, but no isodichroic points are observed (Fig. 2A). This is due to the fact that, like MT-1/MT-2, apo-MT-3 lacks absorption features above 250 nm since aromatic amino acids and histidine are absent in its amino acid sequence. The plot of the intensity at 280 nm as a function of Cu(I) equivalents increases linearly up to about four Cu(I) equivalents, followed by an intensity drop with an additional four Cu(I) equivalents bound (Fig. 2A, inset). Moreover, additional Cu(I) binding to Cu_8-MT-3 results in the formation of new Cu(I) complexes (see below). The CD spectra clearly indicate the formation of two distinct complexes with the first eight Cu(I) equivalents. We interpret the CD behavior as being consistent with the cooperative formation of two Cu_4 clusters in succession. On going from four to eight Cu(I) equivalents, the occurrence of isodichroic points at 273 nm and 343 nm supports this conclusion (Fig. 2B). In previous mass spectrometric studies the cooperative formation of a Cu_4 cluster in MT-3 at early titration steps has also

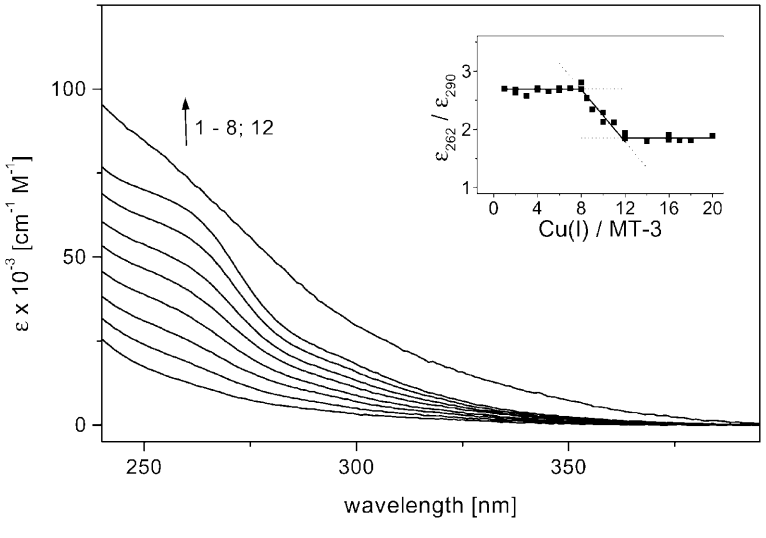

Fig. 1. Electronic absorption spectra of human apo-MT-3 recorded as a function of increasing Cu(I) equivalents at pH 8. *Inset*: dependence of the molar absorbance ratio 262 nm/290 nm on the mole equivalents of Cu(I) added to apo-MT-3. For details regarding the sample preparation and the determination of apo-MT-3/metal ratios, see Materials and methods

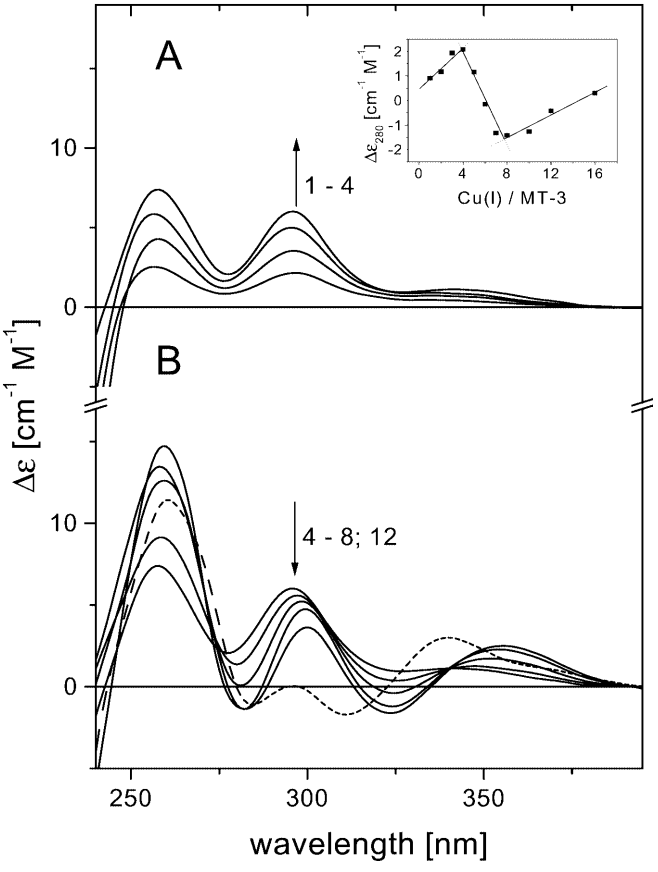

Fig. 2A, B. Circular dichroism spectra of human apo-MT-3 recorded as a function of increasing Cu(I) equivalents at pH 8. *Arrows* indicate increasing Cu(I) equivalents present in the individual samples. *Dashed line* in **B** represents the spectrum of Cu_{12}-MT-3. *Inset*: effect of mole equivalents of Cu(I) added to apo-MT-3 on the molar circular dichroitic absorption at 280 nm

been suggested [7]. A representative example of a Cu(I)/MT-3 complex with more than eight Cu(I) bound is the CD spectrum of Cu_{12}-MT-3, which shows a substantially different CD profile mainly in the low-energy region. However, in contrast to the electronic absorption studies, there is no distinct breakpoint for the formation of a distinct Cu_{12}-MT-3 complex in the CD titration studies (Fig. 2A, inset). Taken together, all spectroscopic data indicate the formation of both Cu_8- and Cu_{12}-MT-3 forms. It may be noted that mammalian MT-1/MT-2 can be loaded with up to six Cu(I) ions in each protein domain [19, 24, 25, 26].

Further structural information about the Cu(I)-MT-3 complexes formed relies on the knowledge of the spectral origin of the absorption and CD features of Cu(I)-thiolate complexes. Such a detailed analysis exists for mammalian Cu(I)-MT-1 [21]. Thus, while the bands in the high-energy region (below 280 nm) originate predominantly from CysS-Cu(I) LMCT transitions, the features in the low-energy region with very weak molar absorptivities, but rather strong CD bands, originate from formally spin-forbidden 3d→4s metal cluster-centered transitions brought about by Cu(I)-Cu(I) interactions in polynuclear Cu(I) complexes. Therefore, the presence of similar low-energy features in Cu_8- and Cu_{12}-MT-3 indicates the existence of Cu(I) polyhedra ([21] and references therein). Since the high-energy region originates from LMCT transitions, the intensity of the first LMCT band at about 262 nm should reflect the number of thiolate ligands involved in metal binding. With 12 Cu(I) bound, all 20 cysteine residues are involved in metal binding ($\epsilon_{262} \approx 75{,}100\ M^{-1}\ cm^{-1}$). Using the molar absorptivity of ca. 3750 $M^{-1}\ cm^{-1}$ per cysteine thiolate at 262 nm, the number of cysteine ligands involved in metal binding can be estimated. The magnitude of the 262 nm absorption of $\epsilon = 31{,}000\ M^{-1}\ cm^{-1}$ for the first Cu_4-thiolate cluster suggests that about 8–9 thiolates participate in metal binding and hence the existence of a $Cu_4(CysS)_{8-9}$ cluster. Using the same argument, between 8–9 thiolates are also involved in the coordination of Cu(I) ions in the second Cu_4 cluster ($\epsilon_{262} \approx 62{,}000\ M^{-1}\ cm^{-1}$ for Cu_8-MT-3). In view of these results and previous Cu(I) binding studies on individual protein domains, it is reasonable to assume that each domain harbors one $Cu_4(CysS)_{8-9}$ cluster. Consequently, the additional binding of four Cu(I) equivalents will give rise to Cu_{12}-MT-3 possessing two Cu_6 clusters, i.e. Cu_6S_9 and Cu_6S_{11}, a process accompanied by a substantial structural rearrangement of the original $Cu_4(CysS)_{8-9}$ clusters. This is illustrated by dramatic changes of the cluster-centered transitions (above 280 nm) in the CD spectrum of Cu_{12}-MT-3 (Fig. 2B).

A characteristic property of Cu(I) complexes is their luminescence. In our studies, luminescence at room temperature and at 77 K were used in the characterization of Cu(I)-MT-3 complexes. The representative room temperature luminescence spectra of Cu(I)-MT-3 with increasing metal content are presented in Fig. S1 (see Supplementary material). The spectra show a broad band characterized by a maximum at about 610 nm and a small shoulder at about 700 nm. The intensity of the emission bands reaches a maximum with 12 Cu(I) bound. The subsequent decrease of the band intensity with 14 Cu(I) equivalents is due most likely to a dynamic quenching by the solvent, brought about by more accessible Cu(I) centers above the Cu(I)/apoMT ratio of 12. These results provide independent support for the formation of the Cu_{12}-MT-3 form.

In contrast to room temperature behavior, the spectra of Cu_4 and Cu_8 cluster species at 77 K reveal one emission band at a different position (420 nm) (Fig. 3). Conversely, in the case of Cu_{12}-MT-3, two weak bands at 420 and about 600 nm occur. The high- as well as the low-energy emission decayed in all Cu(I) titration steps according to a single exponential function with lifetimes (τ) of approximately 30 and 100 μs, respectively. Both the large Stokes shift (defined as the energy difference between the excitation at 300 nm and the position of the emission bands) and the lifetimes suggest that both emissions are spin forbidden; hence, the relevant excited states have triplet spin multiplicity. In the previous detailed studies of the mammalian Cu(I)-MT complexes,

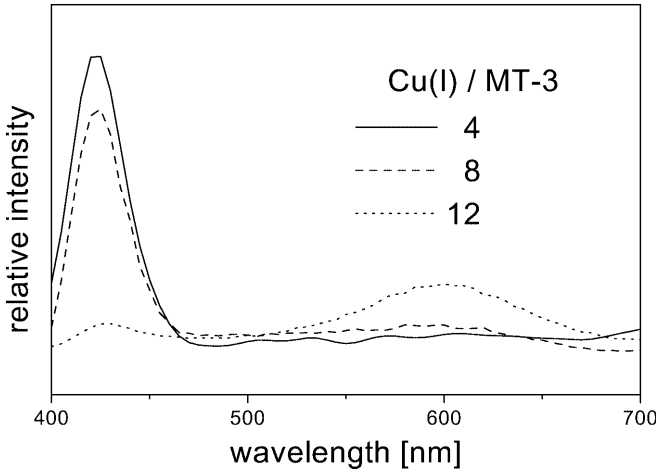

Fig. 3. Representative luminescence spectra of human Cu-MT-3 complexes at 77 K; excitation at 300 nm. The spectra are normalized by the total area under the emission envelope

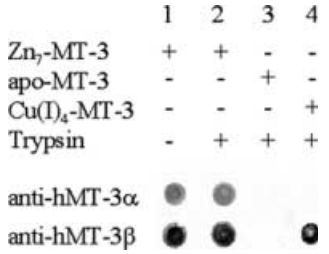

Fig. 4. Immunochemical detection of various MT-3 forms by dot blot analysis using anti-hMT-3α and anti-hMT-3β. *Lane 1*: Zn_7-MT-3; *lane 2*: Zn_7-MT-3 and trypsin; *lane 3*: apo-MT-3 and trypsin; *lane 4*: Cu_4-MT-3 and trypsin. For details see Materials and methods

closely similar low- and high-energy emission bands with similar lifetimes have been reported [21]. These emissions have been assigned to two distinct triplet excited state manifolds of $Cu(I)_4$-thiolate clusters [21, 27]. The strong lower energy emission at 600 nm was assigned to a triplet LMCT excited state and the weaker higher energy band at 420 nm to a triplet cluster-centered (^{3}CC) excited state, being of mixed LMCT/d-s character [21]. The observation of a single emission band for Cu_8-MT-3 at 600 nm or 420 nm, depending on the temperature (see above), is striking. However, a similar temperature behavior of emission bands has been reported for a number of inorganic Cu(I) clusters. Thus, in studies of the temperature dependence of the emission spectrum of Cu_4I_4(4-phenylpyridine) in toluene at 295 K, a single low-energy band at about 700 nm has been seen, whereas at 195 K a single high-energy band at about 510 nm has been discerned. This and similar observations with a number of Cu(I) halide clusters led to the term "luminescent thermochromism". Although the origin of this effect is not fully understood, important contributions appear to be changes in the relative intensity and the position of both emission bands with temperature. In addition, which of these two emission bands originate from the cluster center and which from charge-transfer excited states is at present also not clear ([28] and references therein).

In view of the fact that isolated Cu_4,Zn_3-MT-3 possesses homometallic Cu(I) and Zn(II) clusters [6], a domain specificity for these metal ions must exist. Although the presented spectroscopic data provide important insights into the structural and spectroscopic properties of the Cu(I) complexes formed with apo-MT-3, no clear indication regarding the location of the first preferentially formed $Cu_4(CysS)_{8-9}$ cluster was obtained. The latter cluster most probably reflects that found in the isolated protein. For the mammalian MT-1 and MT-2 isoforms it is well established that while apo-proteins are easily accessible to enzymatic digestion, the metal-occupied proteins are resistant to proteases [29]. Providing that the presence of the first $Cu_4(CysS)_{8-9}$ cluster protects a MT-3 domain against enzymatic digestion, then immunostaining using domain-specific antibodies should establish the location of the Cu_4 cluster. To identify the metal-protected part of the protein, we used polyclonal antibodies raised against specific peptides of the α- and β-domains of MT-3, i.e. anti-hMT-3α and anti-hMT-3β, respectively (see Table S1). Hence, Cu_4-MT-3 and, as a control, Zn-MT-3 and apo-MT-3, were subjected to proteolytic digestion with trypsin that cleaves after Lys and Arg residues. Subsequently, dot blot analysis using anti-hMT-3α and anti-hMT-3β was performed. The immunological results are summarized in Fig. 4, where each lane represents an individual experiment. Both anti-hMT-3α and anti-hMT-3β show immunoreactivity with Zn_7-MT-3 in the absence and in the presence of trypsin, respectively, thus demonstrating the resistance of metal-loaded Zn_7-MT-3 to trypsin digestion (lanes 1 and 2). In contrast, both antibodies did not recognize apo-MT-3 exposed to trypsin (lane 3). However, exposure of partially metal-loaded Cu_4-MT-3 to trypsin resulted in immunoreactivity only with anti-hMT-3β. This result indicates that the first $Cu_4(CysS)_{8-9}$ cluster is located in the N-terminal β-domain of the protein (residues 1–31), containing a total of nine cysteine residues. The simplest crystallographically defined inorganic model where similar Cu(I)/thiolate stoichiometry exists is that of the Cu(I)-thiourea complex, i.e. $Cu_4[SC(NH_2)_2]_9^{4+}$. This structure shows an adamantane-like Cu_4S_9 core with one trigonal planar copper and three very distorted tetrahedral copper species [30].

In conclusion, the formation of distinct Cu_8- and Cu_{12}-MT-3 species containing $Cu(I)_4$- and Cu_6-thiolate clusters, respectively, has been demonstrated. In the Cu_8-MT-3 form, two similar Cu_4S_{8-9} clusters are formed cooperatively and in succession, whereby the first cluster is confined to the β-domain of the protein. These results indicate a higher affinity of the β-domain toward Cu(I) ions. Although a similar finding has been reported for copper-containing MT-1/MT-2, in this case the involvement of all nine cysteine residues of the β-domain

in metal binding required six Cu(I), i.e. a Cu_6S_9 cluster [19, 24, 25]. The obtained data on Cu(I)-MT-3 are essential for the in vitro generation of the native-like Cu_4,Zn_3-MT-3 form through selective metal incorporation into recombinant apo-MT-3. However, the presence of three Zn(II) ions in the α-domain of MT-3 would be inconsistent with our previous studies using divalent metal ions, revealing the formation of $M^{II}{}_4(CysS)_{11}$ (see above). Further studies aimed at generation of the native-like MT-3 form are currently in progress in our laboratory.

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