Circular Dichroism, Luminescence, and Electronic Absorption of Copper Binding Sites in Metallothionein and Its Chemically Synthesized α and β Domains

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The stoichiometry and molecular architecture of Cu–thionein is unknown. Thus, the oligonuclear copper-binding sites of chemically synthesized α and β fragments employing continuous flow solid phase peptide synthesis technique have been studied and compared with those of the apo-MT, intact Cd_5Zn_2-MT and its substituted form Cd_7-MT. The Cu(I)-binding species can be unambiguously identified as Cu_6-α, Cu_6-β, Cd_5Cu_3-MT and Cu_{12}-MT, attributable to their circular dichroism (CD), electronic absorption (UV), and, above all, luminescence emission properties.

Metallothionein (MT)[1] belongs to a class of low molecular mass metal binding proteins which is known to be rich in both cysteine and d^{10} metals. MTs have been found in almost all tissues, as well as in microorganisms and in vertebrates.[2] In general, mammalian MTs contain 61 amino acids and preferentially Zn(II), Cu(I), or Cd(II). The detection of Cu-MT in bovine and human leucocytes[3] and equine melanoma tissue[4] suggests that MT might play an important role in maintaining metal ion homeostasis in vivo apart from its possible detoxification function. The structure of rat liver Cd,Zn-MT has been established using X-ray diffraction[5] and 2D-NMR[6,7] techniques. Seven IIB metals are tetrahedrally coordinated with cysteines and arranged in two clusters, a M_4S_{11}-cluster in the α domain and a M_3S_9-cluster in the β domain. Unlike its Cd,Zn-analogues, only limited structural and stoichiometric data are available on mammalian Cu-MT, attributable to the failure to obtain single crystals of the protein and the large electric quadrupole moment of Cu isotopes (^{63}Cu = –0.211, ^{65}Cu = –0.195 barn)[8] for NMR measurement.[9]

Contradictory reports on the Cu-binding stoichiometry in mammalian MT are known. Rat liver MT induced by $CuSO_4$ contained 9–11 Cu(I)s.[10] Proteolytic studies by Nielson and Winge[11] showed that 11 or more Cu(I)s were necessary to prevent apo-MT from proteolytic digestion by subtilisin, suggesting that mammalian MT binds approximately 12 Cu(I)s. From metal replacement reactions of rabbit liver Zn-MT with Cu(I) detected by CD and luminescence emission, Cu_{12}-MT and Cu_{20}-MT species were deduced.[12] It is attractive to assume that mammalian Cu-MT has also a two-domain structure in a manner similar to that of Cd,Zn-MT. A Cu_6-β domain was obtained using proteolytic digestion of a substoichiometrically reconstituted apo-MT with six Cu(I)s,[11] while the proteolysis of a partially Cd(II) loaded apo-MT resulted in a Cd_4-α domain.[13] This strongly suggested a domain-specific metal binding mechanism. The β domain fills prior to Cu binding in the α domain, and, just the reverse, the α domain fills prior to Cd binding in the β domain. Metal dependent proteolytic studies of these domains revealed that both α and β domain bind six Cu(I)s.[14] The extended X-ray absorption fine structure analysis of the Cu-β domain supports a structure with six trigonally coordinated Cu(I)s.[15] Unfortunately, proteolysis proceeds usually in an uncontrolled manner. The protecting mechanism of the metals bound to MT against enzymic digestion is unknown. Saturation-transfer ^{113}Cd NMR spectroscopy of rabbit liver MT showed that Cd is bound more dynamically to the β domain than to the α domain in the protein.[16,17] Rapid exchange of ^{113}Cd within the β domain via intermolecular interactions was detected, while ^{113}Cd in the α domain remained static. Metal-chelating agents, such as EDTA, can selectively remove Cd or Zn from the β domain of Cd,Zn-MT[13,18] or Cd_7-MT.[19] In the presence of EDTA, holo-MT can also be proteolytically digested, resulting in the Cd_4-α domain. From these data, a dynamically controlled mechanism, instead of the domain-specific metal binding one, is also possible to explain the results from partial proteolysis studies.

To obtain unequivocal evidence of both the existence of two clusters and the mode of their formation in Cu-MT, systematic spectroscopic studies on chemically synthesized and well-defined separate α and β domains seemd to be most promising. In an earlier study in our laboratory, the α fragment of rat liver MT-2 was successfully synthesized using a continuous flow solid phase peptide synthesis technique.[20] Cu-binding studies using luminescence emission revealed the coordination of six Cu(I)s to this domain.[20] The synthesis of α and β fragments of human liver MT-2,[21,22] *Neurospora crassa* MT[21] as well as a modified

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(1) The abbreviations used are as follows: MT, metallothionein; HPLC, high performance liquid chromatography; FPLC, fast protein liquid chromatography; Acm, acetamidomethyl; DMF, dimethylformamide.

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Table I. Protocol of the Solid Phase Synthesis of the β Fragment of Rat Liver Metallothionein-2 on a MilliGen 9050 Continuous Flow Peptide Synthesizer Employing a Graft Copolymer Polystyrene–Polyoxethylene as the Solid Support

No.	Step	Flow Rate [ml/min]	Time [min:sec]
1	Washing with DMF	10	3:00
2	Deprotection with 20% piperidine in DMF	10	4:00
3	Washing with DMF	20	3:00
4	Dosing Fmoc-amino acid	0	0:10
5	Coupling in Recycle	20	15:00

metalloselenonein[23] employing either a solid phase or a solution fragment condensation technique were also reported. Unlike the Cd,Zn-binding studies, the Cu-binding experiments of the α[21] and β[22] fragments of human liver MT-2 using Cu(I) and Cu(II) yielded no conclusive results on the exact stoichiometry of the Cu-binding, attributable to the oxidation of Cu(I) and the cysteine residue.

A newly modified continuous flow solid phase peptide synthesis technique was devised and employed to synthesize both α and β fragments of rat liver MT-2. Their Cu(I)-binding properties were compared with those of intact, demetaled, and reconstituted MTs employing comparative spectrometric methods including circular dichroism, luminescence emission, and electronic absorption.

Materials and Methods

The purification of intact rat liver Cd_5Zn_2-MT-2 and the chemical synthesis of its α fragment corresponding to the C-terminal sequence (Lys30–Ala61) were performed as previously described.[20] The β fragment, the N-terminal sequence (acetyl-Met1–Lys31), was also chemically synthesized on a MilliGen 9050 continuous flow solid phase peptide synthesizer. A graft copolymer, polystyrene–polyoxyethylene, with 0.21 mmol/mg substitution was employed as the solid support. All amino acids were incoporated with 9-fluorenylmethoxycarbonyl (Fmoc) at the α amino group, and the side-chain function groups were protected as follows: *O-tert*-butylaspartic acid, *S*-Acm–cysteine, *N*-(*tert*-butyloxycarbonyl)lysine, *O-tert*-butylserine and *O-tert*-butylthreonine. The synthesis procedure was programmed as shown in Table I. The solid support and all side-chain protecting groups except for Acm, which was chosen to effectively prevent cysteine from possible oxidation throughout the synthesis and purification process, were separated from the peptide using trifluoroacetic acid with 20% anisol as cation scavenger. The purity of the crude Acm-protected peptide was examined on a reverse phase column (Nucleosil C_8 300 Å, 250 × 4.6) in a SYCAM HPLC System. Due to the frequently observed erratic determination of cysteine and other amino acids in the course of the usual amino acid analysis, a recently developed mass spectroscopy technique (MS), i.e. ion spray MS, was employed to identify the synthesized peptide. The measurement was performed on a SCIEX mass spectrometer.

To remove Acm from the cysteine, the peptide was incubated for 1.5 h with Hg(II) acetate in a 20 mM acetate buffer, pH 4.0 at 20 °C. The Hg/Cys ratio was 2. The obtained Hg–peptide was stripped from excessive Hg(II) by gel filtration (Sephadex G-25, 1.5 × 70 cm). The Hg(II) derivative was dissolved in the above buffer containing 25% mercaptoethanol to remove Hg(II). After 48 h of incubation, the apo-peptide was purified on a cation exchange FPLC column (mono S, HR 5/5), detected at 200–300 nm with a 2140 diode array rapid spectral detector (Pharmacia). To ascertain anaerobic separation conditions the following measures were taken: all solutions were deoxygenated and saturated with argon (O_2 impurity $\leq$ 0.001%); the FPLC system including fraction collection was operated under argon. The elution position of the apopeptide was determined both by their absorption spectra recorded by diode array detector and by thiol content titration using the method of Grassetti.[24] After being desalted using the above gel filtration the apopeptide fractions were stored under argon at 77 K.

All chemicals used were of analytical grade quality or better. The Fmoc-protected amino acids were purchased from Nova (Heidelberg) and all other chemicals from Merck (Darmstadt). $[Cu(CH_3CN)_4]ClO_4$ was prepared using the method of Hemmerich and Sigwart.[25] Concentrations of α fragment (11 Cys), β fragment (9 Cys), and intact MT (20 Cys), were assayed spectrophotometrically by thiol titration,[24] using 2′,2′-dithiopyridine at 343 nm, $\epsilon = 7060\ M^{-1}\ cm^{-1}$. Concentrations of Cu, Cd, and Zn were quantitated on a Perkin-Elmer Zeeman/3030 atomic absorption spectrometer. Hg concentration was determined on a Perkin-Elmer 1100 atomic absorption spectrometer equipped with a MHS-10 mercury/hydride system. Due to the known sensitivity of Cu(I) in aqueous solution to both oxidation and disproportionation, a suitable solvent system seems to be important for the Cu(I) titration. In earlier work we have reported that CH_3CN in the protein solutions could effectively keep Cu(I) stable during the titration even in the presence of excessive Cu(I).[19] For the preparation of the protein solutions, a 50 mM acetate buffer with 25% CH_3CN (v/v) was used. The solutions with a final protein concentration of 10 μM were titrated with the stock $[Cu(CH_3CN)_4]ClO_4$ solution (9.1 mM) in 50% (v/v) CH_3CN at 22 °C. Titrations of Cd_5Zn_2-MT and reconstituted Cd_7-MT were performed at pH 6.5. For the titration of apo-α, β, mixed α and β fragments, and apo-MT pH 2.5 was chosen taking into account the following considerations: (1) Cu(I) can bind to thionein at low pH down to pH 1.5 and Cu-MTs have nearly doubled quantum yields at pH 2.5 as those at a pH above 3.5.[26] (2) It is well-known that a protonated thiol group in the apo-protein is much more stable to resist oxidation at low pH than that of a dissociated one at high pH. (3) At pH 2.5 Cd_5Zn_2-MT is entirely demetaled and behaves spectroscopically just exactly the same as the purified apo-MT.[27] Early pH-studies on the reconstitution of Cd, Zn-MT showed no difference in the cluster formation pathway until the pH rose above 7.[2] We have also compared the Cu(I) binding properties of purified apo-MT at pH 6.5 and pH 2.5. No significant difference was observed in the features of the changes both in their chiroptical properties in the charge-transfer range (220–350 nm) and in their emission spectra, except for the quantum yields.[27] In previous works, the metal titrations of apo-MT were always carried out as follows: Metal ions were added to the protein solution at low pH.[28-30] After the solution was adjusted with concentrated base buffer to neutral pH the spectrum was recorded. This means, for each addition of metal a new protein solution was needed, leading to a limited reproducibility. Our titrations at pH 2.5 are continuous, convenient, and effective to prevent both thiolate groups and Cu(I) from oxidation. All titrations were carried out at least in doublicate. The reproducibility of each single point was better than ±4.5%.

CD and UV spectra were recorded on a JASCO J-720 Spectropolarimeter at 22 °C, controlled with a EPSON PC AX2 computer using a J-700 programme. The sample chamber was kept under N_2 to avoid the disturbance of ozone induced by the Xenon lamp. Luminescence emission spectra, excited at 290 nm, were recorded at 22 °C on a modified fluorimeter (Spex) equipped with a 450-W Xe arc lamp, two double monochromators (band width 1.5 nm/mm), and a photon counting system with digital data processing. An edge filter (480 nm) was used to suppress the second-order emission of the excitation source and some background fluorescence of the CH_3CN solvent.

Results

Synthesis. A synthesized Acm-protected β fragment of rat liver MT-2, precipitated by ether, showed a purity of 80%. After FPLC cation exchange chromatography and desalting the purity rose to 95% (Figure 1, inset). The peptide has a relative molecular mass of 3862.2, calculated from the 2-, 3-, and 4-fold charged molecular signals with m/z values of 1933.0, 1288.4, and 966.8, respectively, in its mass spectrum (Figure 1), according to eq 1. M_n is the m/z value of a n-fold charged molecular signal. This mass is exactly in agreement with the theoretical value of 3863.2, calculated on the basis of the peptide sequence. After removal

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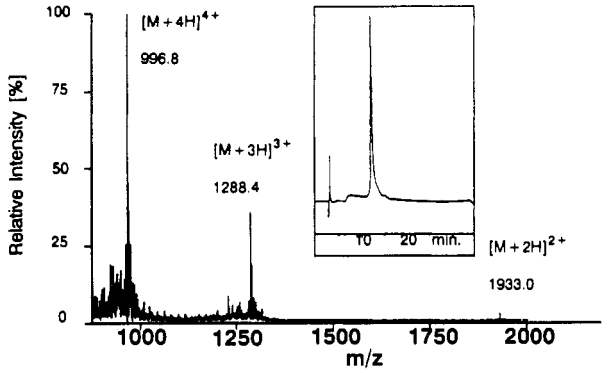

Figure 1. Ion-spray mass spectrum of solid phase synthesized β fragment of rat liver metallothionein-2. The peptide was Acm-protected. The calculated M_r = 3863.3, based on its primary structure. The found M_r = 3862.3. The inset shows the reverse phase HPLC, which was performed on a Nucleosil C_8 300-Å column (250 × 4.6 mm), 0–65% CH_3CN over 35 min. The retention time of the peptide is 11.6 min, detected at 215 nm.

$$M_r = n(M_n - 1) \quad (1)$$

of Acm and Hg(II) the apo-peptides were shown to be homogeneous on a mono S column. The Hg(II) concentration was lower than 1 nM in a 10 μM peptide solution.

Design of Copper Binding Experiments. Two facts encourage the use of CD spectroscopy in the MT study: (1) mammalian MTs contain neither aromatic amino acids nor disulfide bonds, known to have CD contributions in the range of metal thiolate charge-transfer transitions (230–400 nm); (2) the molecules of MTs are large enough to build their secondary structure and clusters, and, at the same time, small enough to avoid possible compensations of the CD contributions of chromophores. Previous spectroscopic studies on Cd(II), Zn(II), Hg(II), and Ag(I) binding properties in MTs have shown that CD spectroscopy is a useful method to monitor the metal binding situation in MT.[28-30] Apart from chiroptical properties, the unusual luminescence of Cu-MTs in solution at room temperature is considered to presuppose not only the charge-transfer transition of the Cu–S bonds but also the formation of a tight Cu–S cluster, which is compact enough to shield effectively the luminophores from the strong quenching effect of water molecules. UV electronic absorption of Cu-MT around 260 nm depends only on the net increase of Cu–S bonds and is a good indicator for the formation of new Cu–S bonds.

In this work a series of thioneins and fragments were titrated with Cu(I) and the changes of their CD, luminescence, and absorption spectra were recorded and analyzed in order to elucidate as to how Cu(I) binds to the native apo-MT. Separate apo-β and apo-α fragments served to characterize the existence of the possible domains in mammalian Cu-MT and the eventual domain-specificity of Cu-binding to the protein. Intact Cd_5Zn_2-MT was used as a model, where Cu-MT is formed by replacement of the initial metals with Cu(I), while reconstituted Cd_7-MT served as a reference for the intact species to find out the Zn contribution during the replacement experiment. A mixture of α and β fragments in a ratio of 1:1 was used to simulate a system where the linkage of the two domains is broken giving rise to eventual interdomain interaction.

Apo-β Fragment. Parts A–C of Figure 2 show the emission, CD, and absorption spectra respectively recorded during the titration of apo-β fragment with Cu(I) in 1 mol equiv aliquots. The apo-β fragment has a less pronounced CD feature (Figure 2B, line 0). There are no signals above 240 nm. The intensive negative band at 205 nm is attributed to the n–π^* transitions of the amide bonds of the polypeptide, whose form suggests a predominant random coil state with some β-sheet and β-turn and perhaps also a little α-helix structure. Upon the addition of Cu(I), a derivative-like envelope (+) 262 nm and (–) 285 nm develops isodichroically, the absorption at 265 nm increases, and the solution begins to emit with a homogenous luminescence band centered at 595 nm. Parts a–c of Figure 2 show the changes in intensities of emission, CD, and absorption at several definite wavelengths. The emission intensity at 595 nm develops linearly with the increasing amount of Cu(I) and reaches a clearly defined maximum with six coppers, indicating the formation of a Cu_6S_9 cluster. Also, the absorption of 265 nm increases concomitantly, but less linearly. It is quite interesting that the increase is slightly diminished following the addition of three coppers and the maximum intensity is reached with five coppers, instead of six coppers. We interprete this as follows: It is well-known that the increase of the absorption at 265 nm is solely attributable to the charge-transfer transitions of the newly formed Cu–S bond, more exactly, to the net increase of the number of Cu–S bonds. Cu(I) in its thiolate complexes is usually coordinated tetrahedrally or trigonally. Initially, Cu(I) is coordinated in the β-fragment involving four Cu–S bonds for each added Cu, attributable to excessive free thiol groups. The exact molecular architecture of the 4-fold coordinate thiolate and/or bridging sulfurs in this Cu_6 unit awaits its precise structural elucidation.

The CD spectra develop in a very interesting manner. The Cotton bands (+) 262 nm and (–) 285 nm reach their maxima with only three and four coppers, respectively. The two to three coppers added after reaching the maxima are bound to the fragment, forming a Cu_6S_9 cluster as indicated by the changes in both the emission and electronic absorption spectra; however no changes in the range of the Cu–S charge-transfer transition are seen using circular dichroism. These Cu-binding sites appear to be CD-silent. In contrast, the CD intensity at 240 nm remains unchanged in the presence of up to three coppers. It increases in the course of the addition of four to seven coppers, and the spectrum blue-shifts. Since both charge-transfer transitions of the Cu–S bonds and n–π^* transitions of the polypeptide chain are CD active in this wavelength range, the change of CD properties needs further assignment.

Apo-α Fragment. The spectral data recorded in the course of the Cu(I) titration of apo-α peptide are of considerable interest (Figure 3). Upon the addition of six coppers, a well-defined saturation of Cu-binding in this fragment, following the proportionally increased emission intensity at 615 nm and absorption at 265 nm is seen (Figure 3B,C,E). In contrast to this phenomenon, the CD spectrum of the apo-α fragment between 240 and 265 nm changes in an uncontrolled manner (Figure 3A,D). A negative envelope at 285 nm, similar to that in the Cu-β fragment, is induced by the first Cu. It declines with the second Cu, then the intensity rises concomitantly up to five coppers, and declines again when a further Cu is added. Much to our surprise, the negative band of apo-α at 222 nm, which is characterized by the secondary structure of the peptide-chain, changes progressively with the increasing molar equivalents of copper and reaches a minimum with six coppers.

Apo-MT. The CD and electronic absorption spectra recorded during the Cu titration of apo-MT are shown in Figure 4. At pH 2.5, rat liver Cd_5Zn_2-MT has a very similar CD spectrum (Figure 4A, line 0) compared to those of apo-α and apo-β (line 0 in Figures 2B and 3A), certifying a total demetalation of the protein at this pH. The emission intensity at 610 nm as a function of Cu concentration is shown in Figure 5 (line O- - -O). Several remarkable features are observed in the changes in the spectra of apo-MT: (1) The maximum intensities of all three spectra, CD, UV (Figure 4), and emission (Figure 5), change in a quite similar manner and they all reach saturation in the presence of twelve coppers. (2) Unlike the CD data recorded in the Cu titrations of apo-α, and apo-β, only one asymmetric positive envelope around 260 nm arises upon the progressive Cu addition to apo-MT. It intensifies linearly up to twelve coppers, with only

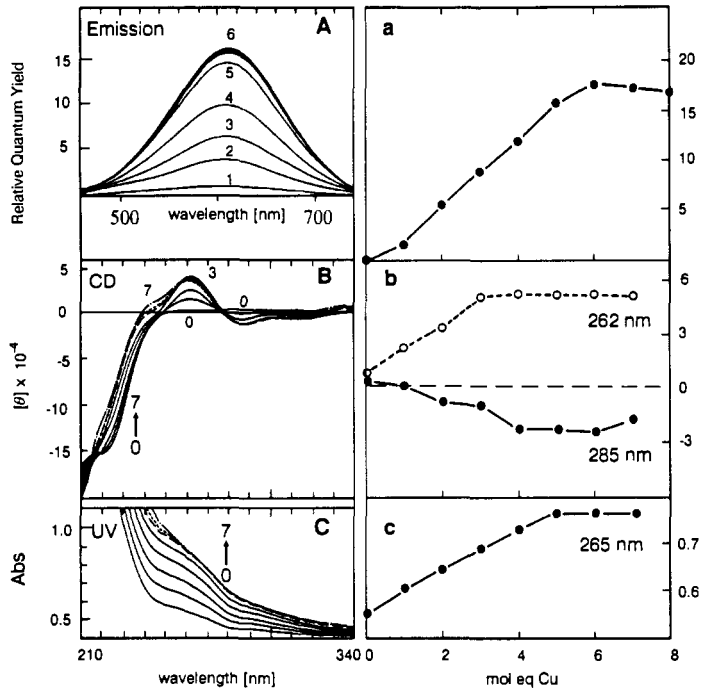

Figure 2. Spectral data of β fragment titrated with Cu(I): (A) emission; (B) circular dichroism; (C) electronic absorption. The spectra were recorded at 22 °C. A 10 μM protein solution with 25% CH_3CN, pH 2.5, was titrated with 9.1 mM $[Cu(CH_3CN)_4]ClO_4$ in 50% CH_3CN. At each step of the titration the concentration of Cu(I) was increased by 10 μM (1 mol equiv to protein). The numbers in parts A–C represent the molar equivalents of Cu(I). Incubation time: 3 min. Recording time: 5 min for emission spectra and 4 min for CD spectra. The same spectrum was recorded when the scanning was repeated. Parts a–c show the Cu(I)-induced contributions to emission intensity at 595 nm (the maximum wavelength), ellipticities at 262 and 285 nm, and absorbance at 265 nm. [θ] refers to the molar protein concentration. The reproducibilities of each single point in double repeated titrations are better than ±4.5%.

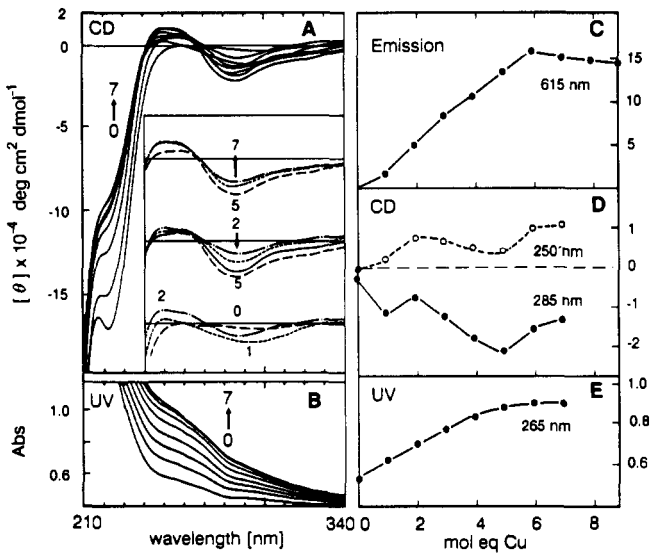

Figure 3. Spectral data of α fragment titrated with Cu(I). Same conditions as in Figure 2. The inset in A shows the development in the CD spectrum of the peptide in three parts: 0–2, 2–5, and 5–7 mol equiv of Cu(I). Emission spectra are not shown. They are similar to those of the β fragment except for the maximum wavelength.

a minor change in its symmetry. The negative band at 285 nm in the Cu-α and Cu-β fragment is absent in intact Cu-MT. (3) The intensity of luminescence emission centered at 610 nm develops in two well-defined stages, one to six and seven to twelve coppers. Each Cu added in the second stage has a 3-fold high quantum yield compared to those added in the initial stage of the titration, suggesting a cooperative cluster formation.

Cd_5Zn_2-MT and Reconstituted Cd_7-MT. In earlier works, the replacements of metals in rabbit liver Zn_7-MT and Cd_7-MT with Cu(I) were examined by both CD and luminescence emission spectrometry.[12,31] Several Cu species, including Cu_6, Cu_{12}, and Cu_{20}, were suggested. Upon the addition of 12–20 coppers an unusual scavenging of luminescence was observed. The titrations were carried out in aqueous solutions omitting CH_3CN. The limited specificity of the data and the uncontrolled luminescence were believed to be due to the possible disproportionation of Cu(I) into Cu(II) and Cu(0) with the subsequent oxidation of thiolate groups.[30]

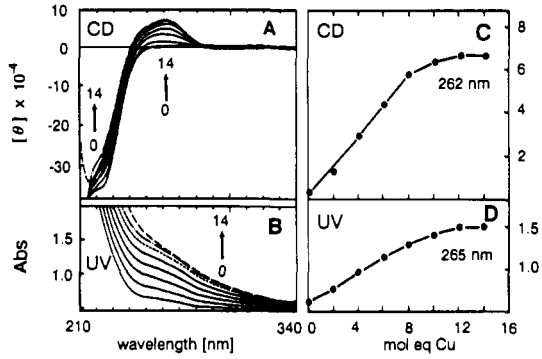

Figure 4. Circular dichroism and electronic absorption of apo-MT titrated with Cu(I). Same conditions as in Figure 2. A selection of spectra is shown (i.e. at 0, 2, 4, 6, 8, 10, 12, and 14 mol equiv of Cu).

Our earlier chiroptically controlled Zn replacement studies[31] by Cd(II) were improved in that intact Cd_5Zn_2-MT was converted into Cd_7-MT at pH 6.5 by quantitative substitution of Zn(II) by two Cd(II)s monitored by the increase in absorption at 254 nm. No significant change in the chiroptical properties were noticed when the conversion into Cd_7-MT was complete. It was concluded that two out of the seven Cd(II)s did not contribute to the chiroptical properties. CD silence of some of the Cd(II)s in fully loaded cadmium thionein was also observed by Stillman and Zelazowski.[29]

Either Cd-MT species was titrated with Cu(I) in the same solvent system used for apo-MT, however pH 6.5 was chosen

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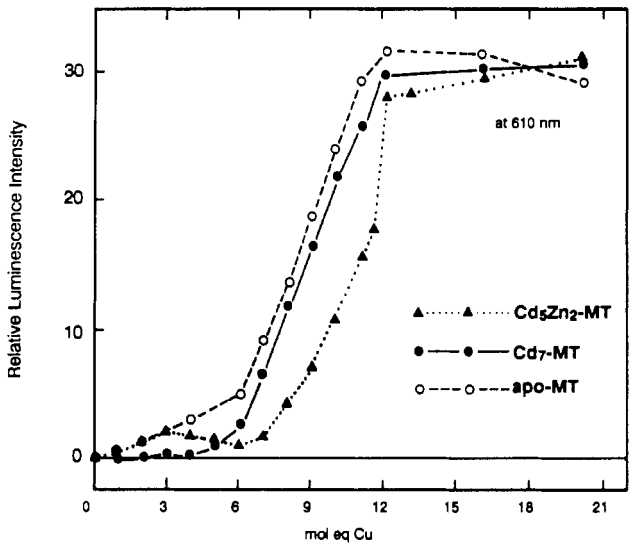

Figure 5. Cu(I)-induced changes in emission intensity of apo-MT, native Cd_5Zn_2-MT, and reconstituted Cd_7-MT at 610 nm as a function of molar ratio of Cu to MT. Cd_5Zn_2-MT and Cd_7-MT were titrated at pH 6.5; apo-MT was titrated at pH 2.5. Other conditions were the same as in Figure 2. Note the different pattern of the three species during the addition of the first six coppers.

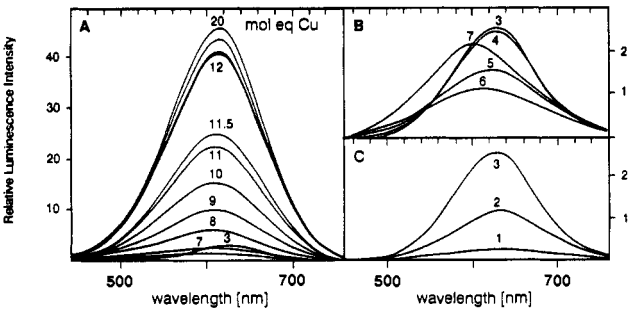

Figure 6. Development of emission spectrum of native Cd_5Zn_2-MT during the Cu titration (A). B and C show details of the changes in both intensity and band position during the addition of the first seven coppers.

compared to the pH 2.5 used for the apo-MT species. During the Cu titrations of both species the luminescence spectra were recorded and the changes in the emission intensities at 610 nm are compared with that of apo-MT (Figure 5). At first sight, the three contour diagrams seem to be similar, however, there are some interesting distinct differences in the course of the addition of the first six coppers. In Cd_7-MT no luminescence was recorded until six coppers were added. Titration of homogeneous Zn_7-thioneins resembled very much the Cu(I) titrated apo MT attributable to the very weak binding of Zn(II) to the thiolate residues.[12,32] The emission spectra of Cd_5Zn_2-MT with increasing concentration of Cu are depicted in three parts (Figure 6). The scale of the y-axis in Figure 6B,C is enlarged to show more clearly the fine changes of the emission spectra in both intensity and emission energy. Upon the addition of Cu an emission envelope centered at 630 nm is developed, and a maximum is reached with three coppers. In the presence of four to six coppers, the intensity at 630 nm diminishes and the band blue-shifts to 610 nm. The new developed emission band at 610 nm intensifies dramatically concomitant with the added Cu and reaches a maximum at 12 coppers. In comparing these data with those in the first stage of Cu-binding (one to six coppers) in Cd_7-MT, where no luminescence was observed, it is suggested that the Cu_3 luminophore formed in Cd_5Zn_2-MT is the derivate of the Cu-substitution of two zincs in the β domain. The emitting states of this luminophore exhibit a lower energy than those in Cu_{12}-MT. This was attributed to its 20-nm red-shifted emission band.

A collapse of luminophores in Cu-MTs induced by excessive Cu(I) was repeatedly reported.[12,26,32] This phenomenon was not observed in all of our experiments. Cd_5Zn_2-MT, Cd_7-MT, and apo-MT were titrated up to 20, while α and β domain up to 8 mol equiv of Cu. The luminophores remain stable. We believe that a suitable solvent system, such as 25% CH_3CN used in the present study, is necessary for the Cu(I) titration to maintain Cu(I) as a stable acetonitrile complex. Uncontrolled disproportionation of Cu(I) into Cu(II) and Cu(0) is avoided.

Parts A–F of Figure 7 summarize the CD and UV spectral data of Cd_5Zn_2-MT during the metal substitution. Saturation was reached in the presence of 12–14 coppers, attributable to the changes in the CD intensity at 240, 262, and 290 nm. The CD spectra obtained by adding 12–14 coppers to the intact Cd_5Zn_2-MT at pH 6.5 are very similar to that of Cu_{12}-MT from titrated apo-MT at pH 2.5. Distinct changes in chiroptical properties were also observed at three and six coppers. Similar pattern in CD and electronic absorption changes were recorded with reconstituted Cd_7-MT at pH 6.5; however, the kink at three coppers in Figure 7F was not seen.

Mixed α and β Fragments. A solution containing 10 nmol of the α and β fragment, respectively, in 1 mL was titrated with Cu and the spectra were recorded (Figures 8A–E). The absorption intensity at 265 nm rises progressively with the added Cu and reaches its maximum in the presence of 12 coppers. The CD properties show a two-phase development. In the first phase employing one to eight coppers, a symmetric envelope (+) 262 and (–) 285 nm with a isodichroic point at 278 nm develops as a linear function depending on the amount of Cu added. In the second phase using 9–14 coppers, the negative band at 285 nm levels off, while the positive band blue-shifts from 262 to 255 nm with a small loss in intensity and becomes more asymmetric with a shoulder arising at 245 nm. The emission intensity of the solution at 605 nm increases also in two steps, being, however, quite the reverse as observed using apo-MT, in an uncooperative manner (Figure 8). The quantum yield per Cu in the first stage (up to eight coppers) is 2.5-fold greater compared to that in the second stage up to 14 coppers.

Discussion

Stoichiometry of Cu Binding in MTs. On the basis of the absence of Cotton bands and a very weak UV electronic absorption in the range above 240 nm as well as the absence of luminescence near 600 nm in the case of apo-species, spectroscopic changes in these regions reflect directly both formation of S→Cu charge-transfer chromophores and cluster formation. The Cu-MT species are identified from the developments and saturations in CD, luminescence, and UV intensities in the course of Cu titration (Table II). A fairly good agreement in Cu-binding stoichiometry was seen using three different spectrometric methods with two exceptions (CD of β fragment; emission of mixed α and β fragments). It is clearly demonstrated that either the α or β fragment binds six Cu(I)s, while intact MT is saturated with 12 Cu(I)s, regardless of whether or not it was previously saturated with Cd(II) or completely demetaled. This substantiates the early work of Nielson and Winge,[14] where the ratios of Cu to protein in either domain obtained by partial enzymic digestion were determined using metal dependent proteolysis. No Cu_{20} species in MT[12] were deduced.

Assignment of the Spectral Characteristics of Cu-MT. Although a Cu-β domain was obtained by proteolysis of MT which was partially loaded with Cu(I),[11] there are no structural data available which support the occurrence of two copper clusters in mammalian Cu-MT. Cu_{12}-MT reconstituted from apo-MT, intact Cd_5Zn_2-MT, or Cd_7-MT has the same CD feature in terms of charge-transfer transitions, which is characterized by an

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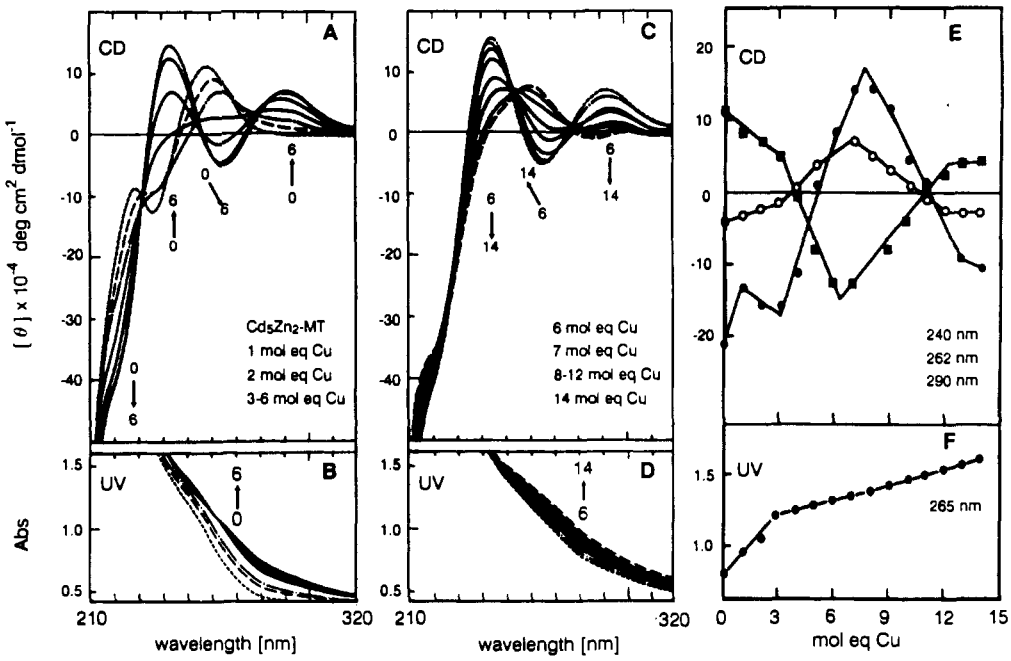

Figure 7. Circular dichroism and electronic absorption of native Cd_5Zn_2-MT titrated with Cu(I). Parts A–D show the spectra in two parts: 0–6 and 6–14 mol equiv of Cu. Parts E and F show the changes in ellipticity at 240, 262, and 290 nm and absorbance at 265 nm.

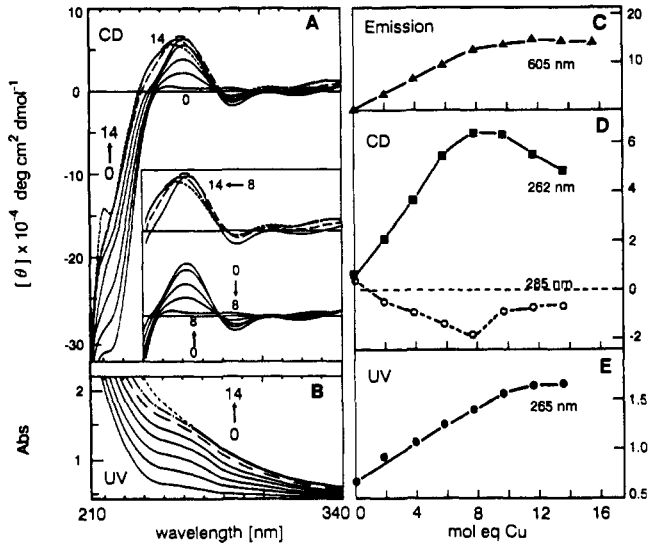

Figure 8. Spectral data of mixed α and β fragments titrated with Cu(I). A protein solution containing 10 μM α and 10 μM β fragment was titrated at pH 2.5 with Cu(I) in 1 mol equiv aliquots. The inset in part A shows the CD spectra in two parts: 0–8 and 8–14 coppers.

Table II. Copper(I) Binding Stoichiometries in Metallothioneins and Their Chemically Synthesized Domains Determined Using Three Spectrometric Methods[a]

MTs / Methods	apo-α domain	apo-β domain	apo-MT	Cd_5Zn_2-MT	Cd_7-MT	mixed $\alpha+\beta$
	R_{sat} (mol eq Cu(I)/mol protein)					
Emission	6	6	12	12	12	8-14
CD	5-6	3-4	12 (6)	12-14 (3, 6)	12-14 (6)	12-14 (8)
UV	6	5	12	>12	>12	12

[a] R_{sat} is the Cu(I):protein ratio at a saturation point of copper(I) binding. The values in brackets are those of the transient species.

asymmetric Cotton band centered at 263 nm with a shoulder at 245 nm (Figures 4A and 7C). Cu_6-α and Cu_6-β domains have quite different CD spectra (Figure 9B). In order to identify the contributions of an oligonuclear Cu-binding site in either domain, provided that it really exists, to the overall CD properties of Cu-MT, a CD spectrum of Cu_{12}-MT with two domains was simulated using mathematical addition of the CD contributions of Cu_6-α and Cu_6-β domains and compared with the spectrum of intact Cu_{12}-MT obtained with apo-MT. As expected, the simulated spectrum completely matches that of Cu_{12}-MT in the range 220–270 nm (Figure 9A). There is only one obvious difference; the weak double negative band between 270 and 325 nm in the simulated spectrum is absent in the spectrum of the intact Cu_{12}-MT. This band was attributed to the interaction between the Lys residues at the breaking terminals (Lys30–Lys31 at the N-terminal of the α domain; Lys30 at the C-terminal of the β domain) and the Cu–S chromophores in the separate domains with the following justifications: (1) The amino group of the Lys residue has a relative high affinity to both Cu(II) and Cu(I). Mixed coordinated copper such as N–Cu–S is frequently found in metalloproteins. (2) The terminal positions of the Lys residues allow such interaction spatially. (3) The CD feature of the separate domains, especially the β domain, bears a close resemblance with those of yeast Cu-MT and *Neurospora crassa*

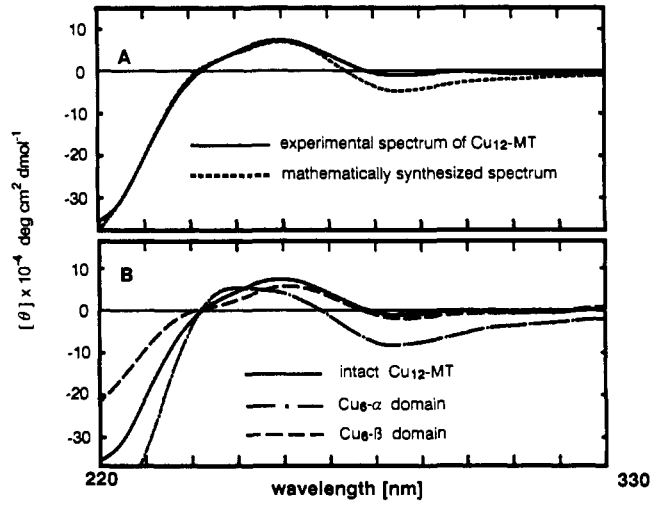

Figure 9. Comparison of the mathematically simulated circular dichroism spectrum of Cu_{12}-MT including $\alpha + \beta$ domains with that of Cu_{12}-MT from apo-MT. The simulated spectrum was obtained by a mathematic addition of the ellipticities of Cu_6-α and Cu_6-β at every single wavelength, using the data processing program J-700 installated in an EPSON PX-2 computer. B: Circular dichroism spectra of the intact Cu_{12}-MT and the separate Cu_6-α and Cu_6-β domains.

Cu-MT,[21,33] both of which have a C-terminal Lys residue (Lys53 in the yeast Cu-MT and Lys25 in the other). Moreover, both of them are apparently in one single cluster[34,35] just as in the β domain. Thus, we conclude that the full matching of the spectrum of Cu_{12}-MT with that of the mathematically synthesized species is direct proof of the consideration that mammalian Cu-MT consists of two domains, namely the α domain with a Cu_6S_{11} cluster and the β domain with a Cu_6S_9 cluster. The comparison shows also that the main Cotton band of Cu_{12}-MT centered at 263 nm is predominantly characterized by the β domain, while the shoulder at 245 nm arises from the α domain.

The mathematically simulated spectrum of Cu_{12}-MT excludes any interdomain interactions. The matching of the spectrum with that of genuine species emphasizes that such interaction does not occur in mammalian Cu-MT. This is in full agreement with the interpretation of the proton-decoupled ^{113}Cd NMR spectra of MT.[36] ^{113}Cd atoms in the separated Cd_4-α fragment have chemical shifts similar to those of cluster A in the intact MT. This suggests the same interaction as observed as in the present Cu-binding studies using comparative CD spectroscopy. By way of contrast, mixed α and β fragments titrated with Cu(I) show strong interdomain interactions as deduced from the unusual development of the luminescence spectrum (Figure 8C). Quenching of the luminescence occurred in the second stage of titration and could be derived from a bridging of the domains by Cu.

All Cu species exhibit a homogeneous luminescence emission band, of different emission energies. No overlapping bands[32] were observed. The band centers were found at 595, 610, 615, and 630 nm (uncorrected, under similar conditions) for Cu_6-β, Cu_{12}-MT, Cu_6-α, and Cd_5Cu_3-MT, respectively.

Pathway of Cluster Formation in MT. We have shown that separate Cu_6-α and Cu_6-β domains differ from each other in their CD properties, both in band symmetry and in intensity. Their emission energies are shifted by 20 nm from each other. Provided Cu binds to MT in a domain-specific manner, i.e. to the β domain as suggested by Nielson and Winge,[11] this should be seen spectroscopically during the course of titration. This would predict that the changes in the spectroscopic features of apo-MT during the addition of the first six coppers should be similar to those of the apo-β fragment while those for the last six coppers are similar to those of the apo-α fragment and/or apo-MT. A red shift of the luminescence emission band center from 595 nm of the Cu_6-β domain to 615 nm of the Cu_6-α domain or 610 nm of the Cu_{12}-MT should be seen when apo-MT is titrated. Apart from this prediction the development of both CD and emission spectra does not support such a domain-specific Cu-binding mechanism. Upon addition of the first Cu to apo-MT, the characteristic CD envelope centered at 263 nm with a shoulder at 245 nm appears, and its intensities in this range increase concomitantly up to 12 coppers with only a minimal change in its symmetry. Furthermore, the luminescence emission parallels the chiroptical properties and the expected red shift is not observed. In fact, a Cu_6-MT species was formed, attributable to a two-stage increase of emission intensity at 610 nm with a kink at six coppers. However, the relative quantum yield of the Cu_6-MT does not correspond to that of Cu_6-β domain which has even a larger quantum yield than the Cu_6-α domain.[27] The Cu_6-β domain obtained in early proteolytic experiment[11,13] was the consequence of an enzymatic digestion process that occurred in partially metalated MT. By way of contrast, spectroscopic studies on the metal binding during titration of a dilute protein solution offer a structural insight into the protein as metals bind to it without any thermodynamically controlled chemistry. We try to interprete these contradictory results with a dynamically controlled rearrangement mechanism. The first six coppers bind to MT in a random manner in both domains. Owing to the somewhat diminished compact cluster structure and/or the intensive interaction between the luminophores and solvent, this Cu_6-MT species emits less pronounced upon excitation. However, the Cu(I) bound to the α domain is perhaps more dynamic than that bound to the β domain, facilitating total digestion of the α fragment by subtilisin. This is in contrast to the Cd(II) in Cd_7-MT, where labile metal binding has been assigned in the β domain.[16,17] A rearrangement of Cu binding occurs during the proteolysis and results in the Cu_6-β domain. Further added Cu causes a cooperative formation of both the α and the β domain and a dramatic intensification of the emission band is noticed. Since the protecting mechanism of metals bound in MT against enzymatic proteolysis is unknown, further results on the observed dynamics will be awaited with great interest.

Domain-specific Cu binding does occur in the first stage of titration of intact Cd_5Zn_2-MT, deduced by the changes in its UV and emission properties compared with those of Cd_7-MT. The first three coppers added replace the two zincs located in the β domain and form a heterometallic Cu_3Cd_5 cluster which shows a weak red-shifted luminescence at 630 nm compared to those of Cu_{12}-MT (610 nm), Cu_6-α (615 nm), and Cu_6-β (595 nm). As the protein appears to control Zn and Cu homeostasis in both adult and neonatal tissues, a fast exchange of Zn and Cu may be of importance in Cu transport and transient species; for example, Cu_3Cd_5-MT may serve as a specific Cu carrier.

CD-Silent Metal Binding and Unspecific Metal Binding in MT. Circular dichroism has proven to be very sensitive for structural changes in MT metal binding sites. CD-silent Cu binding in yeast Cu-MT was reported in a previous study.[33] When two labile coppers were removed from intact Cu_8-MT using Cu(I) chelators no CD change was recorded in the range of charge-transfer transitions. A similar phenomenon was also observed in the present spectrometric titrations. The well-defined saturation of Cu binding to the β fragment detected by luminescence indicates the clear formation of a Cu_6-β domain; however, the last two incoming coppers induce no dichroic changes in the range 245–300 nm (Figure 2). The same was also observed when intact Cd_5Zn_2-MT was converted into Cd_7-MT. Two cadmiums replace the zinc in the protein, resulting in the increase of UV absorption at 255 nm, but no significant change in the CD feature of Cd_5Zn_2-MT is seen. This is also consistent with the independent observation that the Cd_4 α domain dominates the chiroptical properties of Cd_7-MT.[29] The mechanism about such CD-silent metal binding is unknown. It is quite intriguing to realize that two metals always are involved in this phenomenon. It might be attributed to a compensation of positive and negative Cotton extrema introduced by a simultaneous binding of two metal ions to the protein.

Excessive Cu can unspecifically bind to MT. Unlike the Cd binding in intact Cd,Zn-MT, the CD changes induced by such unspecific Cu binding are limited in the wavelength range <235 nm only (Figures 2B, 3A, and 4A). This suggests that there is only a very weak influence of such Cu binding on the Cu–S chromophores of the oligonuclear centers since the major CD contribution below 235 nm is induced by the n–π^* transitions of the polypeptide chains.

In conclusion, the presence of CD-silent metals and the unspecific metal binding obscure sometimes the analysis of CD data. Therefore, it is always advisable to use combined spectrometric methods to elucidate metal binding stoichiometry in metalloproteins.

Conclusion

Mammalian Cu-MT contains two oligonuclear metal binding sites, a Cu_6S_{11} cluster in the α domain and a Cu_6S_9 cluster in the β domain.

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Cu(I) binds to apo-MT in a random fashion at pH 2.5. No preferential occupation of either domain is seen. Of course, other modes at neutral pH cannot be fully excluded.

The characteristic CD feature of mammalian Cu_{12}-MT is an asymmetric positive Cotton band centered at 263 nm with a shoulder at 245 nm. The main band at 263 nm is dominated by the chromophores of the β domain, while the shoulder is attributed to the chromophores of the α domain.

The luminescence emission energy of the Cu_6-α domain, the Cu_6-β domain, Cu_{12}-MT, and Cd_5Cu_3-MT, respectively, is different. The bands are homogeneous and centered at 615, 595, 610, and 630 nm each (uncorrected).

There are two CD-silent Cu(I)-binding sites in the separate β domain. Likewise, two CD-silent Cd(II) chromophores are observed in substituted Cd_7-MT.

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