Comparative Luminescence of Rat Liver Cu-thionein and Its Chemically Synthesized α-Domain

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A peptide corresponding to the α-domain of rat liver metallothionein-2 was chemically synthesized employing the solid phase peptide synthesis technique. Its luminescence properties that depend on the coordinated Cu(I) have been studied using luminescence spectrometric titration in the presence of Cu(I). Unlike the intact metallothionein which has been converted into the Cu species, the emission and excitation spectra of the Cu-α-fragment showed a red shift by 20 nm and 65 nm, respectively, suggesting a more compact and stable luminophore in the α-domain. Saturation of Cu(I) coordination was reached in the presence of 6.5 mol eq Cu(I) when the α-fragment was used and 12 mol eq Cu(I) were specifically bound by the intact metallothionein. The emission bands were homogeneous and no decline of the cluster structure was observed when excessive Cu(I) was added after saturation. A rearrangement of the Cu-cluster in metallothionein during its formation seems to be plausible.

Introduction

Metallothioneins belong to a large family of low molecular-mass and cysteine-rich proteins found in vertebrates, invertebrates and microorganisms [1]. The vertebrate MTs contain 61 amino acids and various d^{10} metals including Cd(II), Zn(II) and Cu(I). Recently Cu-MTs were detected in bovine and human leucocytes and equine melanoma tissue [2, 3]. Apart from their possible detoxification function MTs play an important role in maintaining metal ion homeostasis *in vivo*. The structure of some mammalian MTs have been revealed. Seven Cd(II), Zn(II) or Co(II) are bound with 20 cysteines which are tetrahedrally arranged in two domains forming a M_4S_{11} cluster in the α-domain and a M_3S_9 cluster in the β-domain [4–7]. Unlike Cd, Zn-MTs the metal binding mode, stoichiometry and type of coordination of Cu-MTs are not known. From circular dichroism and luminescence titration of rabbit liver Zn-MT with Cu(I), Stillman *et al.* deduced a Cu_{12}-MT and a Cu_{20}-MT [8, 9].

Cu-MTs isolated from yeast, mammalian leucocytes, equine melanoma and *Neurospora crassa* show an orange-red luminescence in the 550–650 nm range in solution at room temperature [2, 3, 10–12]. Since small Cu(I) complexes of thiolates, for instance, β-mercaptoethanol, in solution do not emit at room temperature, the observed luminescence of Cu-MTs must be related to their compact cluster structure. This phenomenon may be convenient to study the Cu-coordination in Cu-MTs. It was assumed that the Cu/S ratios in different domains of Cu-MT are not identical. Thus, different luminophores are expected. Previously, an emission spectrum with two overlapping bands was recorded by Gasyna *et al.*, when Cu(I) was added to the solution of rabbit liver Zn-MT [9]. A detailed luminescence study employing a separated well characterized domain of Cu-MT seems to be an encouraging task. It was of interest to compare the luminescence characteristics of a separated Cu-domain with that of the intact Cu-MT and to examine whether or not the bands originate from the different luminophores of the domains. Nielson *et al.* have demonstrated [13, 14] that Cu(I) binds preferentially in the β-domain of rat liver apo-MT while Cd(II) in the α-domain. After proteolysis a Cu_6-β-fragment and a Cd_4-α-fragment were obtained. The α-domain coordinates 4 Cd(II), 6 Cu(I) or 6 Ag(I) while in the β-domain 3 Cd(II), 6 Cu(I) or 6 Ag(I) are bound, respectively. Peptides corresponding to the α- and β-domains of human liver MT-2 were

Abbreviations: MT, metallothionein; HPLC, high performance liquid chromatography.

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chemically synthesized using the fragment condensation method [15] and the solid phase method [16]. No luminescence spectral data have been reported so far for a chemically synthesized domain.

In this paper the α-fragment of rat liver MT-2 (corresponding to the C-terminal sequence 30–61) was synthesized using a newly developed solid phase technique. Its Cu-binding capacity and luminescence characteristics were examined and compared with those of the intact protein.

Materials and Methods

All chemicals were of analytical grade or better. The amino acids were obtained from Bachem (Heidelberg). All other chemicals were from Merck (Darmstadt). $[Cu(CH_3CN)_4]ClO_4$ was prepared using the method given in [17]. The synthesis of the α-fragment of rat liver MT-2 was carried out on polystyrenepolyoxethylene graft copolymer on a MilliGel 9050 continuous flow peptide synthesizer [18]. All amino acids were incorporated with the α-amino functions protected with the 9-fluorenylmethoxycarbonyl group. Side chain function groups were protected as follows: Ser and Thr as *t*-butyl ethers, Lys as the *t*-butyloxycarbonyl (Boc) derivative, Asp as a *t*-butyl ester, and Cys as the acetamidomethyl (Acm) ether taking into account that the peptide is cysteine-rich, containing 11 Cys out of a total of 32 amino acids. After the removal of the *t*-butyl and Boc groups using 80% trifluoroacetic acid in CH_2Cl_2 and in the presence of anisol the crude Acm-protected peptide was purified by fast protein liquid chromatography (Pharmacia) on a mono S column for cation exchange and a Sephadex G-25 column for desalting. Analytical HPLC was performed on a Nucleosil C_8 300 Å column (250 × 4.6 mm).

The Acm was removed from the cysteine residues of the peptide by $HgAc_2$ in 20 mM acetate buffer at pH 4 at 22 °C within 1 h [19]. Hg/S ratio was 2:1. Hg(II) was removed by a 48 h incubation of the solution with 30% β-mercaptoethanol which has no influence on the Cu-binding. The precipitate of the Hg compound was removed by centrifugation, and the supernatant was used directly for Cu-titration. Rat liver Cd, Zn-MT-2 was isolated from white Wistar rats previously treated with 3 mg Cd/kg body weight as $CdCl_2$, 4 times within one week. The purification involved gel filtration of the supernatant of the liver homogenate on Sephadex G-50, a repeated anion exchange chromatography on DEAE-Sephadex A-25 and desalting on Sephadex G-25. The protein was photometrically quantitated at 343 nm, ε = 7060 $M^{-1}cm^{-1}$, using 2′,2′-dithiodipyridine [20]. Hg was photometrically measured with dithizone [21]. All other metals were quantified on a Perkin-Elmer Zeeman/3030 atomic absorption spectrometer. Due to the known sensitivity of Cu(I) in aqueous solution to both oxidation and disproportionation, 50% aqueous CH_3CN (v/v), pH 6.5, was used as an appropriate solvent for all solutions in the course of Cu(I) titrations. The titrations were carried out under N_2. The luminescence emission and excitation spectra were recorded with 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. The excitation spectra were corrected for monochromator and Xe-lamp efficiency. An edge filter (440 nm) was used to suppress the second order emission of the excitation source and some background fluorescence of the CH_3CN solvent.

Results and Discussion

Synthesized Acm-protected peptide (**I**), the α-fragment of rat liver MT-2 corresponding to the C-terminal

30
NH_2–Lys–Lys–Ser–Cys(Acm)–Cys(Acm)–
35 40
Ser–Cys(Acm)–Cys(Acm)–Pro–Val–Gly–
45
Cys(Acm)–Ala–Lys–Cys(Acm)–Ser–Gln–
50
Gly–Cys(Acm)–Ile–Cys(Acm)–Lys–Glu–
55
Ala–Ser–Asp–Lys–Cys(Acm)–Ser–
60
Cys(Acm)–Cys(Acm)–Ala–COOH

(**I**) M_r = 4018 Daltons

sequence 30–61 shows a single peak on reverse phase HPLC after purification, with a retention time of 9.55 min (0–80% CH_3CN in 17 min). The peptide has a relative molecular mass of 4017 Dal-

tons, ascertained by its mass spectrum with a $(M + 2H)^{2+}$ signal at 2009.5 Daltons and a $(M + 3H)^{3+}$ signal at 1340.3 Daltons (Fig. 1). This relative molecular mass is exactly in agreement with the calculated value of the synthesized species. The purified rat liver MT-2 shows also a single peak on HPLC with a retention time of 12.65 min (0–80% CH_3CN in 30 min) and contains 4.9 Cd(II) and 2.2 Zn(II) per molecule. The luminescence spectrometric titration of the synthesized α-fragment with Cu(I) yielded a concomitant rise of the luminescence intensity at 630 nm (uncorrected) up to 6.5 mol eq Cu(I) (Fig. 2). The Cu(I)-dependent luminescence intensity and the homogeneous emission band allow the conclusion of the formation of a Cu_6S_{11} cluster. Likewise, the titration of intact rat liver Cd_5Zn_2-MT with Cu(I) reached a maximum of luminescence intensity at 610 nm (uncorrected) in the presence of 12 mol eq Cu(I). No overlapping emission bands as reported by Gasyna *et al.* [6] were observed. Both spectra of rat liver Cu-MT and the Cu-α-fragment exhibit a homogeneous emission band, provided that each of them has a homogeneous luminophore, although Cu-MT might have more than one domain. It was interesting to see that the emission and excitation bands of the Cu-α-fragment are red shifted by 20 nm and 65 nm, respectively, compared to those of the intact Cu-MT (Fig. 3). It may be suggested that the Cu–S luminophore formed in the separate α-fragment is different to the Cu–S luminophore in the α-domain located in the intact protein. According to the Jablonski diagram for luminescence [22], the triplet emitting states and the singlet excited states of the metal ligand charge transfer of the Cu-S luminophore in Cu-MT are more energetic compared to those in the Cu-α-fragment. This difference might be assigned to a rearrangement of the Cu clusters in MT during the titration. With the first 6 mol eq Cu added, a Cu-cluster was formed. When more than 6 Cu bind to the protein, the first cluster would be rearranged, and the energy states of the luminophore would be ready to form a new luminophore with more energetic excited and emitting states than the initial one. In contrast to Cd, Zn-MT, there are at present no structural data available which would favour the occurrence of two clusters in Cu-MT. The newly formed luminophore in Cu-MT might be arranged as one single cluster with a less com-

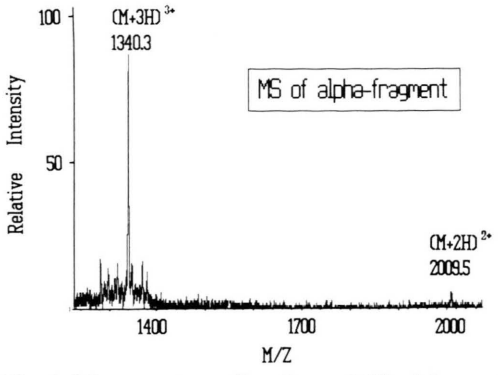

Fig. 1. Mass spectrum (Ion Spray MS) of the synthesized Acm-protected α-fragment of rat liver metallothionein-2.

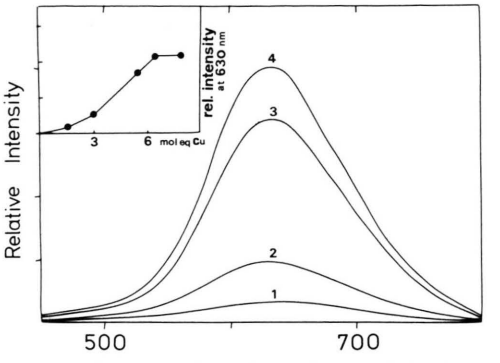

Fig. 2. Concentration dependence of the luminescence intensity of the peptide in the presence of rising concentrations of Cu(I) at 293 K. The concentration of the peptide was 7.5×10^{-5} M. Excitation was at 290 nm. The mol eq Cu(I) added for each trace: (1) 1.5; (2) 3.0; (3) 5.5; (4) 6.5 and 8.0.

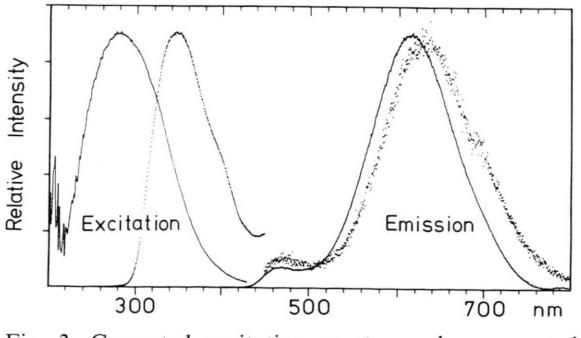

Fig. 3. Corrected excitation spectra and uncorrected emission spectra of $Cu_{6.5}$-α-fragment (·····) and Cu_{12}-MT-2 (——) at 293 K. For emission spectra excitation was at 290 nm; for excitation spectra emission intensity was measured at 610 nm.

pact structure. Alternatively, two or more clusters with identical luminophores might be formed and strongly interact with each other, so that the luminophore is more energetic.

A rapid loss of luminescence intensity of Cu-MT was reported by Gasyna *et al.* [5, 6], when more than 11 mol eq Cu(I) was added to the rabbit liver Zn-MT, and was believed to be a destruction of the formed Cu_{12}-cluster in MT by the excessive binding of Cu(I). A similar loss was also recorded by Byrd *et al.* [23], when apo-MTs from yeast were titrated using aqueous CuCl. This phenomenon was not observed in this work. After the maximum was reached, the luminescence intensities, both of the Cu_{12}-MT and the Cu_6-α-fragment, remain unchanged when excessive Cu(I) up to 24 and 8 mol eq were added, respectively. The earlier reported loss of luminescence intensity might be attributed to a strong quenching effect and/or disproportionation of Cu(I) in the aqueous solution and in the absence of suitable complexing agents known to be specific for Cu(I), as for example, 50% CH_3CN (v/v) which was used in the present study.

The comparison of the luminescence properties of Cu-MT with those of the separated α-domain provides an useful tool to study the contributions of individual domains to the thermodynamics and structure of metal binding to thionein.

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