Synthesis of a nonacosapeptide (β-fragment) corresponding to the N-terminal sequence 1–29 of human liver metallothionein II and its heavy metal-binding properties

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A nonacosapeptide (β-fragment) corresponding to the N-terminal sequence 1–29 of human liver metallothionein II was synthesized by the fragment condensation method. The Cd-binding ability of the β-fragment was much stronger than that of cysteine as thionein and synthetic α-fragment corresponding to the C-terminal sequence 30–61 of human liver metallothionein II. Both the α- and β-fragments bound preferentially to Cu ions rather than Cd ions.

Human liver metallothionein II *N-terminal nonacosapeptide* *Chemical synthesis* *Heavy metal-binding*

1. INTRODUCTION

Metallothioneins are a class of low-M_r, cysteine-rich metal-binding proteins. Otvos and Armitage [1] reported that rabbit metallothionein includes two separate metal clusters, one containing 4 Cd^{2+} (cluster A) and the other containing 3 (cluster B). Recently, Winge and Miklossy [2] isolated cluster A after proteolytic (subtilisin) digestion of rat MT, identified it as the C-terminal dotriacontapeptide (position 30–61) and designated it α-fragment. However, they failed to isolate the N-terminal polypeptide segment (cluster B, β-fragment) due to its degradation into a small peptide by subtilisin. Nielson and Winge [3] isolated Cu-binding N-terminal peptide after subtilisin digestion, which consisted of the N-terminal untriacontapeptide (position 1–31). They indicated that Cd ions bound to the α-domain preferentially and Cu ions to the β-domain. This paper deals with the synthesis of a nonacosapeptide corresponding to the N-terminal sequence 1–29 of human liver metallothionein II (hMT II) [4] and its heavy metal-binding properties.

Abbreviations: MT, metallothionein; *n*-BuOH, *n*-butanol; Z, benzyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; MBzl, *p*-methoxybenzyl; DMF, *N,N*-dimethylformamide; AcOH, acetic acid; AcOEt, ethyl acetate

2. MATERIALS AND METHODS

A nonacosapeptide was synthesized by the fragment condensation method. According to the route shown in fig.1, 5 peptide fragments (1–5) were prepared stepwise or by fragment condensation. Each peptide fragment was condensed successively by the azide method [5] to minimize racemization. The protected peptides were purified by reprecipitation from DMF/MeOH and/or column chromatography on Sephadex LH-60. The final deprotection was performed by the HF method using thioanisole and *m*-cresol as scavengers [6]. The product obtained as the HF salt was converted to the acetate form by treatment with Amberlite IRA 45 (acetate form). After reduction with dithiothreitol and mercaptoethanol, the peptide was purified by gel filtration on Sephadex G-15 using 3% AcOH as an eluant.

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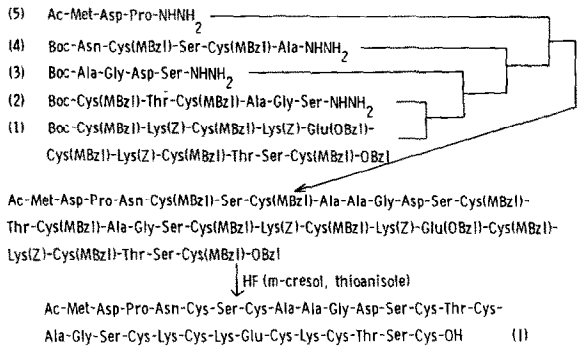

Fig.1. Synthetic route to N-terminal nonacosapeptide (β-fragment) of human liver metallothionein II (I).

$[\alpha]_D^{28}$ $-84.6°$ (c = 0.1, 3% AcOH), TLC, R_f 0.41 (n-BuOH:pyridine:AcOH:AcOEt = 4:1:1:2), 0.80 (n-BuOH:pyridine:AcOH:H_2O = 1:1:1:1) (ninhydrin test, H_2PtCl_6-KI test and nitroprusside test positive). Full details of the synthesis and characterization of this peptide will be published elsewhere (Y. Okada et al., in preparation).

The binding abilities of the peptide with heavy metals were studied by the ultraviolet (UV) absorption method for Cd and the gel-filtration method of peptide-metal complex for Cd, Zn, and Cu as reported [7].

3. RESULTS AND DISCUSSION

The free SH content of the synthetic nonacosapeptide (I) was 8.7/peptide as calculated from the value for the SH content determined by the Ellman method [8] and the average recovery of amino acid. The amino acid ratios in acid hydrolysate were in good agreement with the expected values as shown in table 1. This peptide was mixed with Zn^{2+} to form Zn-peptide complex, which was purified by gel filtration on Sephadex G-75 as reported in [7]. The amino acid ratios in acid hydrolysate of the Zn–β-fragment (I) complex so obtained are listed in table 1. Met residue was partially converted to Met sulfoxide during acid hydrolysis. From the value for the Zn content and the average recovery of amino acid, it was found that the Zn content of this complex was 2.7/peptide. Since Zn-peptide is easily replaced with Cd^{2+} [9] and Hg^{2+} [10], the Zn-peptide can still act as a heavy metal detoxifying agent.

The UV absorption of the peptide (I) and Cd-peptide (I) complex was quite similar to that of metal-free human MT and human MT, respectively [11]. A broad absorption shoulder at 250 nm of the Cd-peptide is due to Cd-thiolate formation [11]. Since the extinction at 250 nm is proportional to the bound Cd content [11,12], the absorption can be used as an indicator of the content of bound metal ions. The Cd^{2+}-binding activity of the synthetic β-fragment (I) was examined. Cd^{2+}-mercaptide formation was measured by following the increase in absorbance at 250 nm, and the results are shown in fig.2 in comparison with those for thionein, synthetic α-fragment [13] and cysteine. The binding ability of the β-fragment was much stronger than that of cysteine as thionein and synthetic α-fragment. The β-fragment (I) was saturated with Cd^{2+} at a concentration of 45–50 μM, corresponding to 2.7–3.0 gatom/mol peptide. On the other hand, the synthetic α-fragment was saturated with Cd^{2+} at a concentration of 50–55 μM, corresponding to 3.6–3.9 gatom/mol peptide [13]. These values are in good agreement with the theoretically expected values.

It has been reported that Cu ions bound preferentially to the β-domain and this order of cluster formation by Cu was opposite to that observed with Cd ions [3]. The β-fragment (I) was

Table 1

Amino acid compositions of β-fragment (I), Zn– and Cu–β-fragment complexes (residues/molecule)

Residue	β-fragment (I)	Zn–β-fragment	Cu–β-fragment
Asp (3)	3.34	3.27	3.15
Thr (2)[a]	1.87	1.71	1.76
Ser (4)[a]	3.43	3.85	3.67
Glu (1)	1.00	0.80	1.01
Pro (1)	1.10	0.90	0.88
Gly (2)	2.30	2.10	2.00
Ala (3)	3.03	3.02	3.07
Cys (9)	8.50[b]	–[c]	–[c]
Met (1)	0.80	0.52[d]	–[e]
Lys (3)	2.83	3.00	3.00

[a] Values are from 20 h hydrolysate
[b] Cys was determined after performic acid oxidation
[c] Cys was not determined
[d,e] Met was partially and completely oxidized to form Met sulfoxide, respectively, during acid hydrolysis

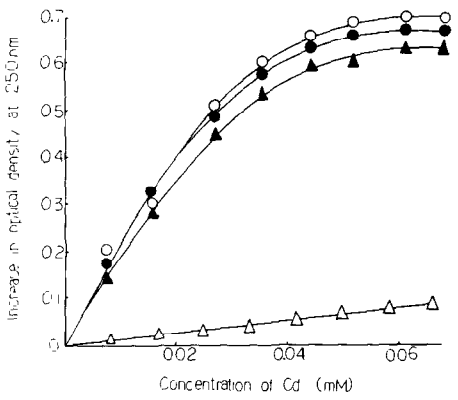

Fig.2. Binding ability of β-fragment (I) with Cd^{2+}. Peptide: 0.15 mM as SH, 3 ml in Tris-HCl (10 mM, pH 7.0). (●—●) β-fragment (I), (○—○) α-fragment [13], (▲—▲) rat thionein, (△—△) cysteine.

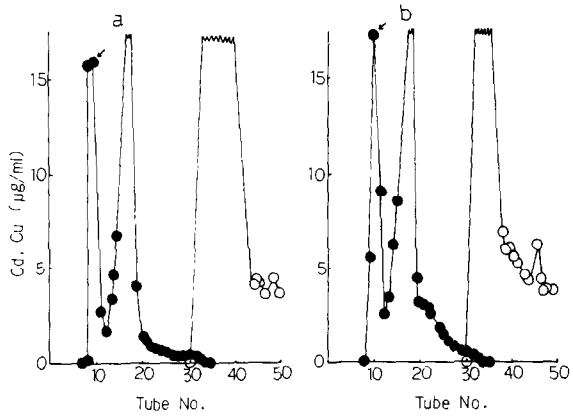

Fig.3. (a) Gel filtration of Cu–β-fragment complex on Sephadex G-10, (b) Cu–α-fragment complex. Aliquots of the elution fractions were examined by measuring the metal content by atomic absorption spectrometry. (●—●) Cu, (○—○) Cd.

mixed with an excess amount of Cu^{2+} and Cd^{2+} at the same time, the molarities of which were equal to each other, and the resultant metal complex was separated by gel filtration on Sephadex G-10. The eluted material was examined by measuring the Cu and Cd contents by atomic absorption spectrometry. The chromatographic pattern is shown in fig.3a. The Cu–β-fragment complex was eluted first, followed by blue-colored Cu^{2+} and then Cd^{2+}. The amino acid ratios in acid hydrolysate of the Cu–β-fragment complex so obtained are listed in table 1. These ratios are in good agreement with the expected values except for Met, which was converted to Met sulfoxide presumably due to the presence of Cu^{2+} [14]. The data indicated that only Cu ions bound preferentially to the peptide (I) and not Cd^{2+}. The values for the Cu content and the average recovery of amino acids or the SH content determined by the Ellman method suggested that the ratio of Cu to peptide was 3:1. Nielson and Winge [3] reported that the β-domain bound to 6 Cu ions and the α-domain to 5–6 Cu ions. The low value for the Cu content in our experiments might be due to Cu^{2+}, which is known to catalyze oxidation of the thiol group to form a disulfide bond [14].

The synthetic α-fragment [13] was also mixed with Cu^{2+} and Cd^{2+} and the resultant metal complex was isolated in the same manner as described above. The chromatographic pattern is shown in fig.3b. The first peak corresponds to Cu–α-fragment complex. The ratio of Cu to peptide was 4:1. This low value for the Cu content might be due to the same reason as described above. From these results, it appeared that the α-domain and β-domain could not discriminate between Cu^{2+} and Cd^{2+} when sufficient amounts of Cu and Cd were present at the same time. Since the affinity of Cu ions for the thiol group is stronger than that of Cd ions, both fragments bind to Cu ions.

The MT of *Neurospora crassa* [15] is a 25-residue polypeptide, which contains 7 Cys residues, and is homologous to the β-domain of mammalian MTs. Metal ions bind to *N. crassa* MT to form a single cluster [15]. The reason why further evolved MTs have two metal clusters is not yet understood. Detailed studies on the heavy metal-binding properties of the synthetic α- and β-fragments of MT should help to resolve the above question and clarify the intrinsic role of MT.

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