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Unique Properties of Cd-Binding Peptides Induced in Fission Yeast, *Schizosaccharomyces pombe*

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Metallothioneins, a class of low molecular weight cysteine-rich proteins that bind heavy metal ions, have been found in various eucaryotic organisms. When fission yeasts are grown in the presence of high concentration of $CdCl_2$, large amounts of Cd-binding peptides (Cd-BP1 and Cd-BP2) are synthesized. Cd-BP1 (MW 4000) contains 4 mole of small unit peptide (cadystin, MW 771), 6 mole of Cd^{2+}, and 1 mole of the labile sulfide; on the other hand, Cd-BP2 (MW 1800) contains 2 mole of cadystin and 2 mole of Cd^{2+}.

While Cd-BP2 shows similarities to mammalian Cd-thioneins in UV and CD spectra, Cd-BP1 has a characteristic shoulder at 265 nm in the UV absorption spectrum and shows two marked Cotton bands at 257 nm (negative) and 275 nm (positive). These characteristics of Cd-BP1 are not found in the other Cd-thioneins. When Cd-BP1 is acidified (pH 2.0) and successively neutralized, a shoulder of 265 nm in the UV spectrum and a Cotton band at 275 nm disappear, and the molecular weight changes from 4000 to 1800, with simultaneous loss of the labile sulfide. While the reconstituted complex without labile sulfide showed the characteristics of Cd-BP2, the reconstituted complex in the presence of labile sulfide indicated partial reconstitution of Cd-BP1. The UV and CD spectra differences between reconstituted and native Cd-BP1 suggest the requirement for some additional molecular architecture including another peptide-Cd^{2+} interaction.

Induction of cadystin synthesis is almost exclusive for Cd, but an exception is a small amount of cadystin also induced by the higher concentration of $CuCl_2$ (2.5 mM). The UV spectrum of the natural Cu–cadystin complex was similar to that of Cd-BP1. Since Cu(I)-replaced Cu–cadystin complex prepared *in vitro* also shows the Cd-BP1-type UV spectrum, it is suggested that the copper in the natural Cu–cadystin complex is present in the cuprous form, or that labile sulfide is also contained in the natureal Cu–cadystin complex. On the basis of these findings the models for Cd-BP1 and Cd-BP2 are proposed.

Introduction

Metallothioneins are a class of low molecular weight, cysteine-rich proteins binding heavy metal ions and have been found in various organisms (*1,2*). Protective effects of the thioneins against acute effects of heavy metal toxicity on the activity of the certain SH-containing enzymes (*3*), on the RNA synthesis with isolated nuclei (*4*), and on the other functions (*1,5*) have been discussed. On exposure of eucaryotic organisms to heavy metals, such as Cd, Zn, and Cu, the thioneins are inductively synthesized and bind to heavy metals for detoxification.

In microorganisms, *Saccharomyces cereviciae* and *Neurospora crassa* synthesize much smaller proteins on the exposure to Cu, and these small proteins still indicated the properties of thioneins. When fission yeast *Schizosaccharomyces pombe* is grown in the presence of high concentration of $CdCl_2$, large amounts of Cd-binding peptides, Cd-BP1 and Cd-BP2, are inductively synthesized. These peptides are composed of the unit peptide (cadystin), Cd and the labile sulfide, and are much smaller than mammalian Cd-thioneins. However, these small peptides have shown some characteristics similar to those of the mammalian metallothionein, for example, high metal content, no aromatic amino acid or histidine, high cysteine content, and optical features characteristic of metal thiolate. In fact, these are the same characteristics described in the definition of metallothionein (*1,2*).

Experimental

Induction of Cd-BPs

The fission yeast strain *Schizosaccharomyces pombe* L972 (h^-) was a gift from Dr. K. Yoshida of The Biological

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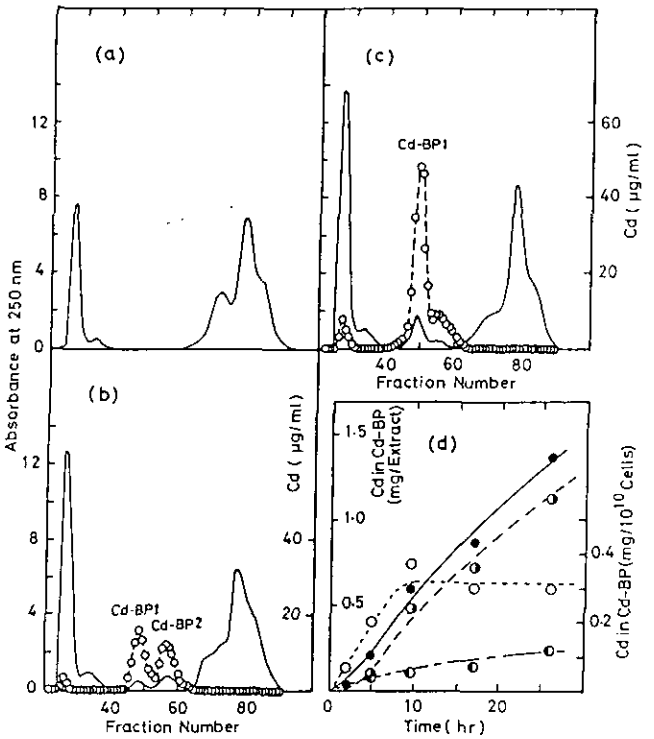

FIGURE 1. Induction of Cd-BP analyzedby Sephadex gel filtration. Extracts of the fission yeast grown in Cd-medium were analyzed on a Sephadex G-50 SF column (1.6 × 53 cm). Each fraction contained 1.5 mL of eluate. Extracts were obtained from cells of 500 mL cultures (*a*) without Cd^{2+}, (*b*) with 1 mM Cd^{2+} for 5 hr and (*c*) with Cd^{2+} for 9.5 hr. Absorbance at 250 nm (——) and Cd concentration in each fraction were determined and expressed as normalized values per 10^{10} cells. Increases of Cd-BPs in extracts from 500 mL culture cells with time (*d*) were expressed as their Cd amounts: (●) Cd-BP in total cell extract; (○) Cd-BP1 in the total cell extracts; (○) Cd-BP2 in the total cell extract; (○) Cd-BP per 10^{10} cells.

Institute, Nagoya University, Nagoya. An overnight culture of the fission yeast in YEPD medium (1% yeast extract, 2% polypeptone, and 2% dextrose) was diluted from fresh YEPD medium and incubated at 29°C with vigorous aeration. When $CdCl_2$ was added to the medium at lower than 1 mM concentration, the growth rate of fission yeast decreased somewhat, but continued at least 20 hr after Cd^{2+} addition. After an appropriate incubation time, 500 mL culture was harvested and washed three times with 0.05 M Tris-Cl (pH 7.6)–0.1 M KCl. Pelleted cells were homogenized at 4°C with a mortar and pestle with three times their weight of acid-washed quartz sand, and extracted with 10 mL of 0.05 M Tris-Cl (pH 7.6)–0.1 M KCl. The extract was centrifuged at 17,000*g* for 20 min at 4°C, and 3 mL of the supernatant was analyzed on a Sephadex G-50 SF column (1.6 × 53 cm). Two peaks of Cd-binding peptides (Cd-BP1 and Cd-BP2) in the low molecular weight region appeared and increased with time; these peaks were not observed in the extract of cells grown without Cd^{2+} (*6*). Cd content and absorption at 250 nm, which probably originated

Table 1. Amino acid compositions of Cd-BP1 and Cd-BP2.

	Relative molar quantities[a]		
Amino acid	Oxidized BP1[b]	*S*-Carboxy-methylated BP1[b]	Oxidized BP2[c]
Glu[d]	3.0	3.0	3.0
Cys	2.8	2.7	2.7
Gly	0.9	1.0	1.2

[a] Relative molar quantities of other amino acids are less than 0.1. NH_2-terminal amino acid is Glu in both BPs, determined by the dansylation procedure.

[b] The values represent the average of samples hydrolyzed for 24 and 72 hr.

[c] The samples were hydrolyzed for 24 hr.

[d] Ammonia detected was only 0.3 mole/3 mole Glu.

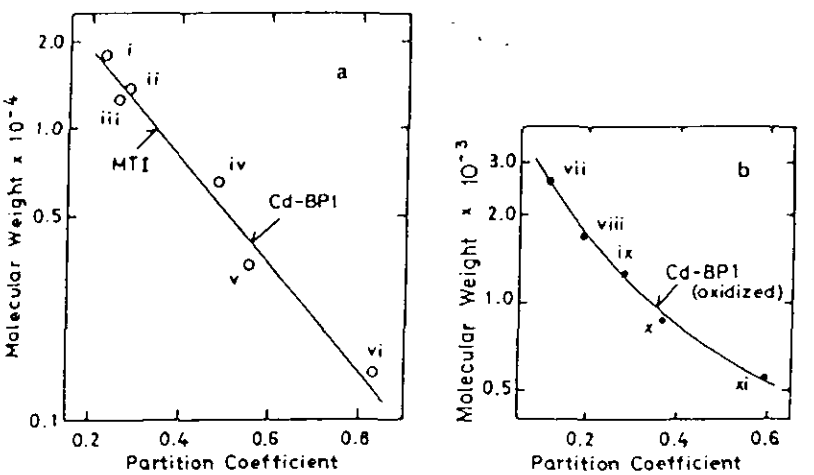

FIGURE 2. Molecular weight estimations of Cd-BP1. (*a*) Sephadex G-50 gel filtration. The column (1.6 × 53 cm) was equilibrated and eluted with 0.05 M Tris-Cl (pH 7.6)–0.1 M KCl. Fractions of 1.5 mL were collected. (*b*) Bio-Gel P4 gel filtration in 6 M guanidine-HCl; fractions of 0.7 mL were collected. Two or three marker proteins were loaded simultaneously with Blue Dextran 2,000 for comparison. Marker proteins and peptides are (i) myoglobin (Mr 17,000), (ii) RNase A (Mr 13,700), (iii) cytochrome c (Mr 12,400), (iv) pancreatic trypsin inhibitor (Mr 6500), (v) calcitonin (Mr 3400), (vi) bacitracin (Mr 1422), (vii) A-chain of insulin (Mr 2531), (viii) neurotensin (Mr 1673), (ix) physalaemin (Mr 1265), (x) serum thymic factor (Mr 859), and (xi) leucine-enkephalin (Mr 557). MT-I is Cd-thionein I from mouse. Cd-BP1 in (*b*) was oxidized by performic acid.

Table 2. Acid-labile sulfide in Cd-BP1, Cd-BP2, and mouse metallothionein I and II.

Preparation	Content: Cd-BP1, Cd-BP2, MT-I[a] or MT-II nmole[a,b]	Content: Labile sulfide, nmole[c] Experimental	Content: Labile sulfide, nmole[c] Corrected[d]	Molar ratio labile sulfide/Cd-BP1, Cd-BP2, MT-I, or MT-II Experimental	Molar ratio labile sulfide/Cd-BP1, Cd-BP2, MT-I, or MT-II Corrected[d]
Cd-BP1 (1)	29.5	22.1	27.3	0.75	0.93
(2)	28.6	22.6	27.9	0.79	0.98
(3)	27.5	23.7	29.3	0.86	1.06
Cd-BP2 (1)	53.8	0	0	0	0
(2)	51.8	0.47	0.58	0.01	0.01
MT-I	28.6	0.51	0.63	0.02	0.02
MT-II	29.7	0.74	0.91	0.02	0.03

[a] MT-I and MT-II represent mouse (C57BL/6J) metallothioneins I and II, respectively. These were purified according to Tsunoo et al. (*12*).
[b] The amounts of Cd-BP1 and Cd-BP2 were determined with molar extinction coefficients at 250 nn of ϵ_{250} = 24,800 and ϵ_{250} = 11,400 (*13*), respectively. The amounts of mouse metallothioneins I and II were determined from absorbance at 250 nm using the data of Tsunno et al. (*12*).
[c] Acid-labile sulfide was determined according to King and Morris (*14*) except for 14 μg of Cd^{2}+ was added to each standard Na_2S reaction mixture.
[d] The efficiency of labile sulfide detection was 0.81 in the presence of Cd^{2}+.

from the mercaptide bonds with Cd, in the region of Cd-BPs increased with time, indicating accumulation of these peptides. The amount of Cd-BPs per cell, in the case of 1 mM $CdCl_2$ induction, increased for 10 hr, then remained constant (Fig. 1). This means that it takes about 10 hr to synthesize the saturating amount of Cd-BPs in the cell with 1 mM $CdCl_2$ induction. This is consistent with the time taken to get the maximum Cd uptake per cell, implying cellular Cd uptake is allowed as long as Cd-BP synthesis continues.

Constituents of Cd-BPs

To prepare enough amounts of Cd-BPs for characterization of physical and chemical properties, purification steps by Sephadex G-50 SF gel filtration and by KCl gradient elution from DE52 column were employed. Homogeneity of purified Cd-BPs was verified by paper electrophoresis at pH 6.5 of performic acid-oxidized Cd-BP1 and Cd-BP2 (*6*) and amino acid analysis of Cd-BPs (Table 1). Molecular weight estimation on a Sephadex G-50 SF column with protein markers (*7*) indicated 4000 for Cd-BP1 and 1800 for Cd-BP2 (*6,8*). The molecular weight of the performic acid-oxidized Cd-BP1 was estimated to be 950 by Bio-Gel P4 gel filtration in 6 M guanidine-HCl (*6*) (Fig. 2). The latter result indicates that the molecular weight of the unit peptide comprised in Cd-BPs is about 800. This molecular weight coincides closely with the minimum molecular weight, 771, calculated from the results of amino acid analysis (Table 1). Since Cd-BP1 and Cd-BP2 have the same amino acid composition and the same

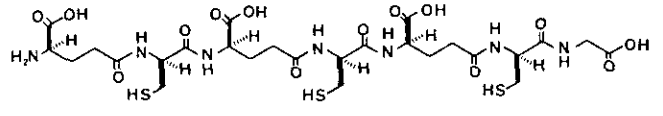

FIGURE 3. Structure of cadystin. The unit peptide, cadystin, of cadmium-binding peptides occurring in fission yeast, *S. pombe* was determined to have structure H-γ-Glu-Cys-γ-Glu-Cys-γ-Glu-Cys-Gly-OH; all Glu and Cys being L-form.

Table 3. Constituents of Cd-BP1 and Cd-BP2 from fission yeast.

	Cadystin (Glu 3, Cys 3, Gly), mole	Acid-labile sulfide, mole	Cd, g-atom	Molecular weight
Cd-BP1	4	1	6	4000
Cd-BP2	2	0	2	1800

amino-terminal amino acid, Glu, we presumed that Cd-BP1 and Cd-BP2 consist of the same peptides, which were named later as cadystin (*9*) and the amino acid sequence was also determined as γ-Glu-Cys-γ-Glu-Cys-γ-Glu-Cys-Gly (*10*) (Fig. 3). Thus, it is assumed that 4 mole of unit peptide (cadystin) forms Cd-BP1 with 6 g-atoms of Cd and 2 mole of cadystin forms Cd-BP2 with 2 g-atoms of Cd per mole, respectively. Cd-BP1 also contains 1 mole of the acid-labile sulfide per mole (*11*), and on the other hand Cd-BP2 contains no labile sulfide (Table 2). These findings on the constituents of Cd-BP1 and Cd-BP2 are summarized in Table 3.

Characteristics of Cd-BPs

Cd^{2+} can be dissociated by acid treatment (pH 2) from Cd-BPs and can be bound again to the peptides by neutralization in the presence of excess Cd^{2+}. While the reconstituted complex in the presence of labile sulfide indicated partial reconstitution of Cd-BP1 (*11*), the reconstituted complex without labile sulfide showed characteristics of Cd-BP2. As shown in Figure 4, Cd-peptide complex in the presence of labile sulfide gained on Cd-BP1 in the molecular weight, and contained Cd^{2+} and the labile sulfide.

The UV spectrum of Cd-BP1 shows a distinct shoulder at 265 nm and differs from those of Cd-thioneins from mouse and other sources, whereas the reconstituted complex in the absence of labile sulfide shows a spectrum similar to that of mouse Cd-thionein, especially with respect to the ratio of A_{250} to A_{280} as shown in Figure 5, which is the same UV spectrum as that of Cd-BP2. On

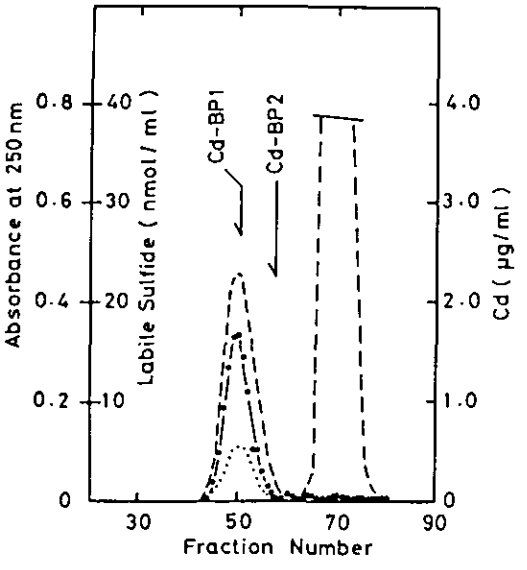

FIGURE 4. Partial reconstitution of Cd-BP1: (...) absorbance at 250 nm (--) Cd concentration; (●) labile sulfide. Cd-BP2 (53.8 nmole) in 3 mL of 0.05 M Tris-Cl(pH 7.6)–0.1 M KCl was mixed with 3 μL of 0.1 M Na_2S (final 0.1 mM) and then with 15 μL of 0.1 M $CdCl_2$ (final 0.5 mM). After 10 min standing at room temperature, the centrifuged supernatant (10,000*g*, 15 min, 4°C) was applied onto a column of Sephadex G-50 SF(1.6 × 53 cm) equilibrated with the buffer described above, and 1.5-mL fractions were collected. The amount of labile sulfide eluted at the position of Cd-BP1 was about half of the Na_2S added to the reaction mixture. The rest of the sulfide in the mixture was probably precipitated as CdS and removed from the reaction mixture by the centrifugation. In the labile sulfide determination, 2μg of $CdCl_2$ was added to each standard Na_2S solution, because the peak fraction (Nos. 48–52) submitted to the analysis contained about 2 μg of $CdCl_2$. Further corrections were not made. Cd concentrations were determined by atomic absorption spectrometry.

the other hand, the UV spectrum of the Cd-peptide complex reconstituted in the presence of acid-labile sulfide differs from that of Cd-BP2 or mouse Cd-thionein (Fig. 6), and also from that of Cd-BP1. The content of labile sulfide per unit peptide was about 4-fold that of Cd-BP1 and indicates that this Cd-peptide complex is quite different from Cd-BP1 in the configuration and in the mercaptide bond formation. These observations indicate that the conversion of Cd-BP1 to Cd-BP2 *in vitro* can be achieved completely by the release of labile sulfide and that the reverse reaction is still incomplete (*11*).

In the CD spectrum of Cd-BP1 (Fig. 7), negative bands at 235 nm and 257 nm and positive bands at 215 nm and 275 nm are observed. The reconstituted complex in the absence of labile sulfide also shows bands at 215 nm and 257 nm as shown in Figure 7*a* but has lost the band at 275 nm. Since the bands at 257 nm and also at 275 nm in Cd-BP1 disappear completely when Cd-binding bonds in Cd-BP1 are broken by acid treatment (pH 2), by EDTA addition to 1 mM, by dithiothreitol addition to 25 mM, or by pCMB (*p*-chloromercury benzoate) titration, these Cotton effects must be attributed to the asymmetric interaction of the unit peptide with Cd^{2+}. The extrinsic Cotton effects at longer wavelength probably show that metal ions bind to specific sites in Cd-BP1 and Cd-BP2 (*15*). On the other hand, Cd-thionein from mouse (Fig. 7*b*) and other sources (*15–17*) have a positive Cotton band at 257 nm and no band at longer wavelengths. The appearance of a positive or a negative band at the same wavelength would result from differences in the microenvironment around the Cd-mercaptide bonds (e.g., left-handed and right-handed asymmetry). The CD band at 275 nm of Cd-BP1 suggests the presence of some additional molecular architecture, including another peptide-

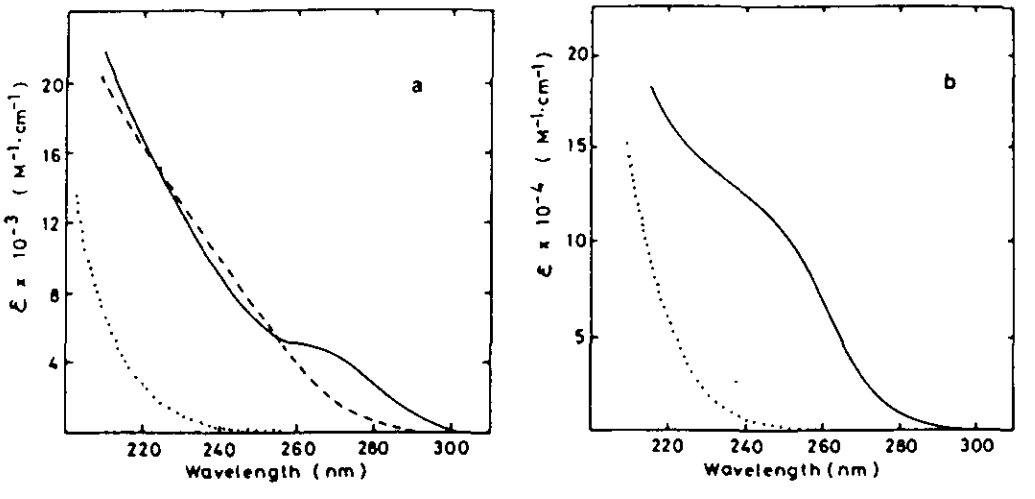

FIGURE 5. UV absorption spectra of Cd-BP1 from fission yeast and Cd-thionein I from mouse. (*a*) Cd-BP1 was 0.193, or 0.097 mg/mL in 5 mM Tris-Cl(pH 7.6)–10 mM KCl–0.2 mM $CdCl_2$ (——). The pH of the solution was lowered with HCl (···) and then neutralized to pH 7.6 with NaOH (---). (*b*) Cd-thionein I prepared from mouse was reduced with 1 mM dithiothreitol and acidified. This apothionein was loaded with sufficient amount of Cd^{2+}. Cd-thionein I was 0.0148 mg/mL in 5 mM Tris-Cl(pH 7.6)–10 mM KCl–0.2 mM $CdCl_2$ (——). The pH of this solution was lowered to 2 with HCl (···).

Cd^{2+} interaction. The occurrence of acid-labile sulfide in Cd-BP1 indicated that the presence of Cd and labile sulfide cluster beside mercaptide bonds of cysteinyl residues as assumed in the case of [2Fe-2S] ferredoxin (*18–20*). The CD band at 275 nm of Cd-BP1 could be derived from the Cd-S cluster as described here, or from the participation of glutamic acid residues in tetramerization of cadystin in the presence of Cd^{2+} as indicated in the case of the storage form of bovine insulin (*21*).

From differences in molecular weight, contents of Cd per unit peptide (and also SH/Cd ratio), and UV and CD spectra, it is concluded that Cd-BP1 and Cd-BP2 bind Cd^{2+} in different forms, that is, one species of peptide binds Cd^{2+} in two kinds of molecular forms (*6,8,9*).

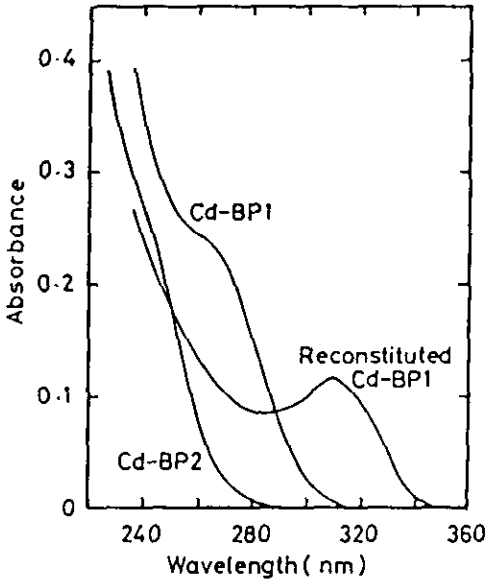

FIGURE 6. UV absorption spectra of Cd-BP1, Cd-BP2, and partially reconstituted Cd-BP1. Cd-BP1 and Cd-BP2 were used at 34 μg peptide/mL (44 μM unit peptide) and 25 μg peptide/mL (32 μM unit peptide) in 0.05 M Tris-Cl(pH7.6)–0.1 M KCl, respectively. The partially reconstituted Cd-BP1 was obtained by the procedure described in the legend to Fig. 4 except that the amount of starting Cd-BP2 was 135 nmole. The peak fraction (no. 51, approximately 35 μM unit peptide) of the eluate from the Sephadex G-50 SF column was submitted to UV spectrum recording.

Induction and Complex Formation of Cadystin with Other Metal Ions

Induction of cadystin synthesis is almost exclusive with Cd, but as the exception a small amount of cadystin is inductively synthesized by the addition of higher concentration of $CuCl_2$ (2.5 mM). The molecular weight of Cu-cadystin complex was estimated to be 3000, because the eluting position from Sephadex G-50 SF column was just behind Cd-BP1 and ahead of Cd-BP2 (Fig. 8). The UV spectrum of this natural Cu-cadystin complex was similar to that of Cd-BP1 (Fig. 9). The reconstitution of the Cu-cadystin complex *in vitro* from free cadystin and Cu(I) or Cu(II) ion was tried, but the complex could not be observed in the analyses by Bio-Gel P2 column. However, Cd in Cd-BP1 was replaced *in vitro* by Cu(I) or Cu(II) when an excess amount of Cu(I) or Cu(II) was mixed without previous dissociation of Cd^{2+} from Cd-BP1. That is, Cu(I) or Cu(II) ions replaced Cd-BP1 *in vitro*, but could not make complex from free cadystin and

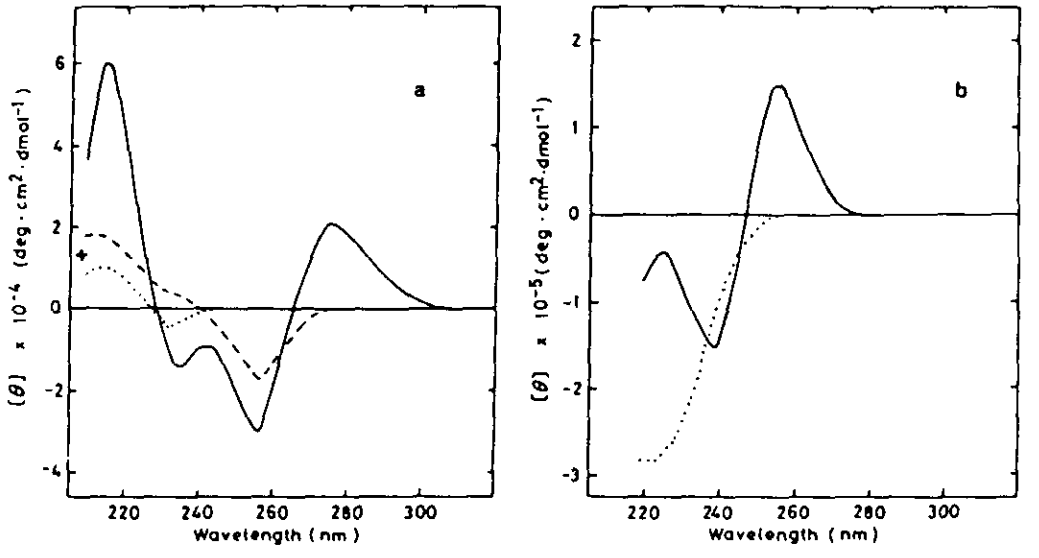

FIGURE 7. Cd spectra of Cd-BP1 from fission yeast and Cd-thionein I from mouse. (*a*) The concentration of Cd-BP1 was 0.193 mg/mL in 5 mM Tris-Cl(pH 7.6)–10 mM KCl–0.2 mM $CdCl_2$ (— —). The pH of this solution was adjusted to 2 with HCl (···), and then neutralized to pH 7.6 with NaOH (—). (*b*) The concentration of Cd-thionein I was 0.148 mg/mL in 5 mM Tris-Cl(pH 7.6)–10 mM KCl–0.2 mM $CdCl_2$ (— —). The pH of this solution was adjusted to 2 with HCl (···). [θ] is the molar ellipticity coefficient of the unit peptide (cadystin) for Cd-BP1 or of the peptide chain for Cd-thionein I from mouse. Light path of quartz cell was 0.5 cm.

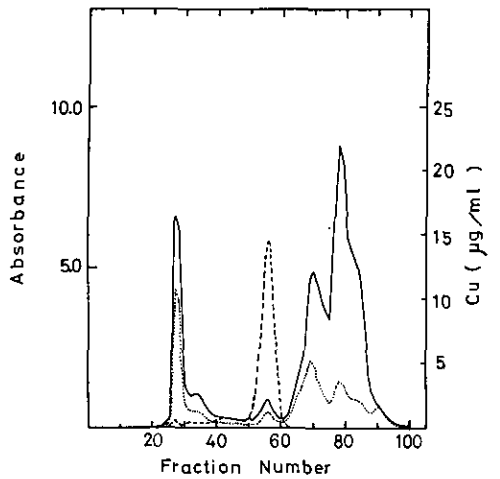

FIGURE 8. Induction of Cu-binding compound analyzed by Sephadex gel filtration. $CuCl_2$ was added to the medium at 2.5 mM and culture continued for 17 hr. An extract was obtained from 500 mL culture cell as described previously in the legend to Fig. 1 (*6*). Absorbance at 250 nm (— —) and at 280 nm (···), and Cu concentration (---) in each fraction were determined and expressed as normalized values per 10^{10} cells. A small amount of Zn^{2+} was found in Cu-binding compound (not shown in this figure).

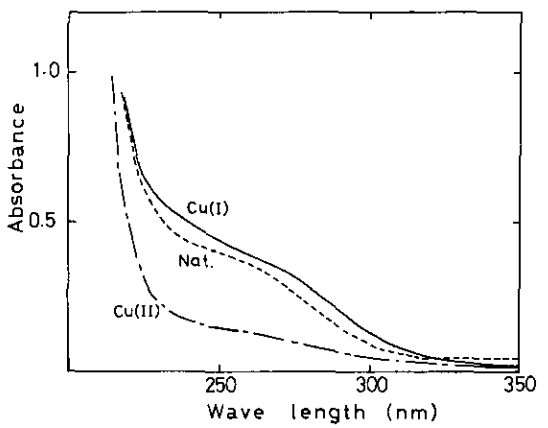

FIGURE 9. UV absorption spectra of Cu-binding compounds. UV spectra were recorded for the natural Cu-binding compound (---), Cu(I)-replaced complex (— —), and Cu(II)-replaced complex (–·–) in 5 mM Tris-Cl (pH 7.6)–10 mM KCl at about 0.1 mg cadystin/*mL each*.

Cu ions. Cu(I)- or Cu(II)-replaced complexes were isolated by use of a Bio-Gel P2 or Sephadex G-10 column, and the UV spectra were determined (Fig. 9). While UV spectrum of the Cu(I)-replaced complex was similar to that of the natural Cu–cadystin complex, the spectrum of the Cu(II)-replaced complex was similar to that of Cd-BP2. UV spectra of these complexes imply that the natural Cu–cadystin complex is very likely formed with Cu(I). The mechanism of replacement with Cu ions and the reason of the failure of complex formation from free cadystin and Cu ions are still undissolved.

Formation of complexes with cadystin *in vitro* with other heavy metal ions was also tried. Among them, Zn(II) and Co(II) could form stable complex, respectively.

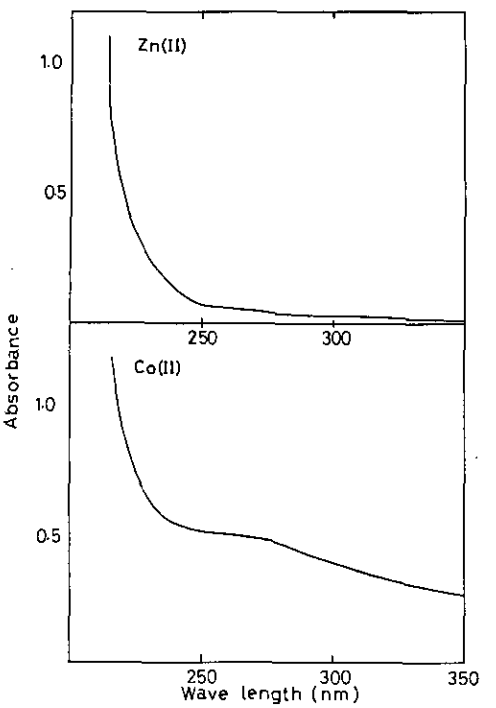

FIGURE 10. UV absorption spectra of reconstituted metal–cadystin complexes: Zn(II) and Co(II). UV spectra of about 0.02 mg/mL each complex were determined in 5 mM Tris-Cl(pH 7.6)–10 mM after reconstitution from cadystin and free metal ions and subsequent isolation by Bio-Gel P2 column.

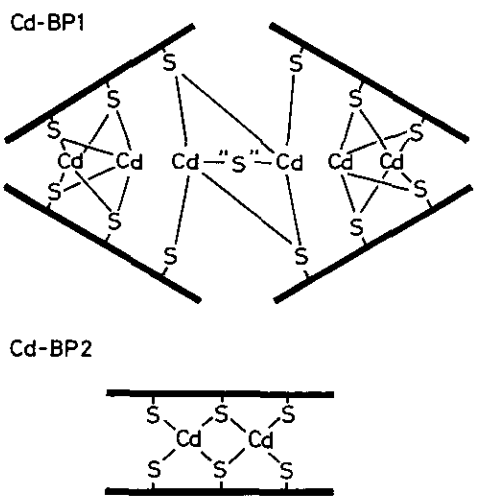

FIGURE 11. Proposed models for Cd-BP1 and Cd-BP2 from fission yeast, *S. pombe*. The unit peptide (cadystin, γ-Glu-Cys-γ-Glu-Cys-γ-Glu-Cys-Gly) is expressed as the bold bar. Sulfhydryl groups of cysteine residues in cadystin bind Cd atoms in cooperation with labile sulfide (expressed as "S") in the case of Cd-BP1, increasing the molar ratio of Cd/cysteine.

The Zn(II)–cadystin complex showed the same UV spectrum as that of Cd-BP2, on the other hand, the Co(II)-cadystin complex had a unique spectrum (Fig. 10), indicating a somewhat different structure from the Zn(II) complex and Cd-BP2. These observations indicated that

cadystin can form complex with other heavy metals by the mercaptide bonds and/or other unknown binding.

Concluding Remarks

Cd-BPs induced in fission yeast differ from Cu-thionein in *Saccharomyces cerevisiae* or *Neurospora crassa*, and also from Cd-thioneins in mammals in many respects including the subunit structure, the occurrence of labile sulfide as a constituent of Cd-BP1, and a new mode of metal binding in Cd-BP1, which is more efficient than that in Cd-thioneins in mammals (1.5 mole Cd^{2+}/3 mole Cys vs. 1 mole Cd^{2+}/3 mole Cys). The 275 nm positive band in Cd spectrum of Cd-BP1 must have some kind of relation with this different mode of metal binding.

The lack of free Cys residues and disulfide bridge and the absorption shoulder at 250 nm of Cd-BPs are indicative of the existence of Cd-thiolate bonds. On the basis of these findings it is proposed that the models for Cd-binding peptides from fission yeast are as shown in Figure 11. Cd-BPs fulfill the characteristics for metallothioneins (*1*), that is, they are low molecular weight peptides with high affinity to heavy metals, and contain the unique amino acid composition (high content of Cys, no aromatic amino acid nor histidine), and show characteristic extrinsic Cotton effects.

Since it is comparatively easy to synthesize chemically a small peptide like cadystin, the application of cadystin in detoxification for the acute poisoning of heavy metals has been considered (*13*).

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