Biochemical Pharmacology, Vol. 29, pp. 689–692.
Pergamon Press Ltd. 1980. Printed in Great Britain.

ISOLATION AND CHARACTERIZATION OF METALLOTHIONEIN DIMERS

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(*Received* 23 *July* 1979; *accepted* 27 *September* 1979)

Abstract—The dimeric forms of metallothionein were isolated from livers of rabbits which accumulated cadmium in large quantities. The dimers were separated into three isoproteins, both on a DEAE Sephadex A-25 column and on a gel permeation column. Reduction of each dimer with mercaptoethanol yielded monomeric metallothionein-I, an equivalent mixture of metallothionein-I and -II, and metallothionein-II. The three isoproteins were identified as a dimer of metallothionein-I, a dimer of metallothionein-I and -II, and a dimer of metallothionein-II, respectively.

The presence of cadmium-binding proteins other than metallothionein in liver [1, 2] and kidney [3] which have accumulated cadmium in large quantities has been established. As metallothionein is assumed to be a defensive protein for the toxicity of cadmium [4], and the cadmium-binding proteins other than metallothionein increase in liver and kidney when tissue starts to show some signs of intoxication [3], those cadmium-binding proteins may have some connection with toxicity of cadmium.

Furthermore, a 30,000 mol. wt testicular Cd-binding protein was indicated as a possible link to Cd-induced testicular injury [5]. Although some comments as to a higher molecular weight cadmium-binding protein, such as polymeric forms of metallothionein [2], have been reported, there have been no definitive characterizations.

The present paper deals with the isolation and characterization of one of the high molecular weight cadmium-binding proteins. At the same time, the present paper shows an application of a newly developed analytical method for metalloprotein [6].

MATERIALS AND METHODS

Isolation of metallothionein dimers. Cadmium chloride was injected subcutaneously into female rabbits (body wt 2.5–2.8 kg; 0.5 mg Cd/kg body wt; 27 injections during 5 weeks). The animals were killed 2 days after the last injection and the livers were homogenized in three volumes of 0.1 M Tris–HCl buffer solution (pH 7.4) containing 0.25 M glucose, using a teflon homogenizer, and the homogenate was centrifuged at 105,000 *g* for 90 min at 4°. The supernatant was applied to a preparative Sephadex G-75 column (5 × 80 cm) and eluted with 10 mM Tris–HCl buffer solution (pH 8.6). The eluate between $Ve/Vo = 1.4$ and 1.8 was combined and concentrated on a Diaflo UM-10 membrane.

Reduction of metallothionein dimers. Reduction of metallothionein dimers was performed by adding neat mercaptoethanol (10–50 μl) to a solution containing metallothionein dimers (2–20 μg Cd/0.2–2 ml of 50 mM Tris–HCl buffer solution, pH 8.6) at room temperature. The reaction mixture was applied to a Sephadex G-75 column or a gel permeation column after adding cadmium chloride (10–50 μg Cd) to fill all binding sites in metallothionein with cadmium.

High speed liquid chromatograph equipped with a gel permeation column for separation and atomic absorption spectrophotometer for metal analysis. A high speed liquid chromatograph (Toyo Soda 803, Toyo Soda Co., Tokyo, Japan) was equipped with a gel permeation column (Toyo Soda TSK GEL SW 3000, 21.5 mm I. D. × 600 mm) and 50 mM Tris–HCl buffer solution (pH 8.6) containing 0.1% sodium azide was used as an eluting buffer solution (flow rate 3.7 ml/min). The eluate was directly introduced into a flame atomic absorption spectrophotometer (Hitachi 508) and cadmium atomic absorption was continuously monitored.

RESULTS AND DISCUSSION

Cadmium in the liver supernatant obtained from animals which did not accumulate the metal in large quantities is known to be almost confined to the metallothionein fraction ($Ve/Vo = 1.8$–2.2), along with a small amount of cadmium in the high molecular weight fraction (near the void volume of the column). On the other hand, another cadmium peak has been noted to be increased in liver and kidney supernatants obtained from animals which had accumulated cadmium in high quantity, the elution volume being between that of the metallothionein and the high molecular weight fractions.

Figure 1 shows a typical Sephadex G-75 elution profile for liver supernatant obtained from animals repeatedly injected with cadmium ion. The cadmium peak in question was observed at $Ve/Vo = 1.4$–1.8. The molecular weight of the cadmium-binding protein was initially estimated to be less than 30,000 daltons because the protein was eluted at a slower rate than that of the zinc- and copper-containing superoxide dismutase peak ($Ve/Vo = 1.4$–1.6) (32,600 daltons) [7]. Although the molecular

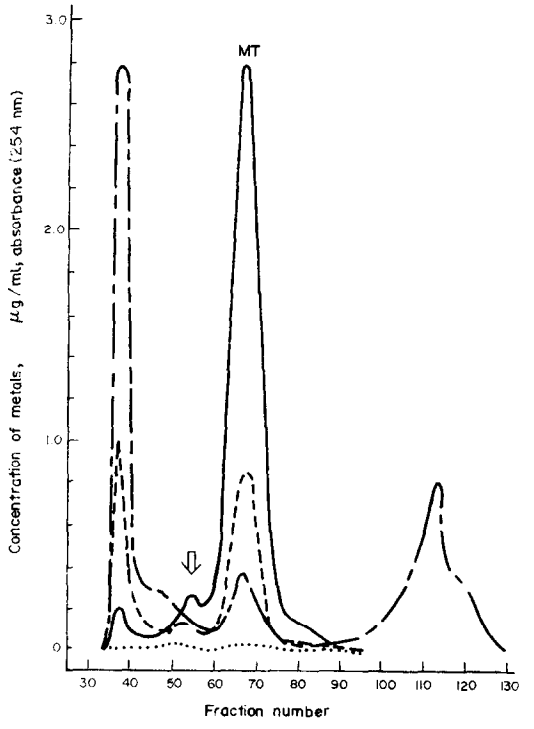

Fig. 1. Sephadex G-75 elution profile of liver supernatant obtained from rabbits injected with cadmium chloride. Liver supernatant (3 ml) was applied to a Sephadex G-75 column (2.6 × 90 cm) and eluted with 1 mM Tris–HCl buffer solution. Five milliliter fractions were collected. MT indicates the metallothionein fraction and the arrow indicates the metallothionein dimer fraction. ———, Cd; — — —, Zn; – – –, Cu; ———, absorbance at 254 nm.

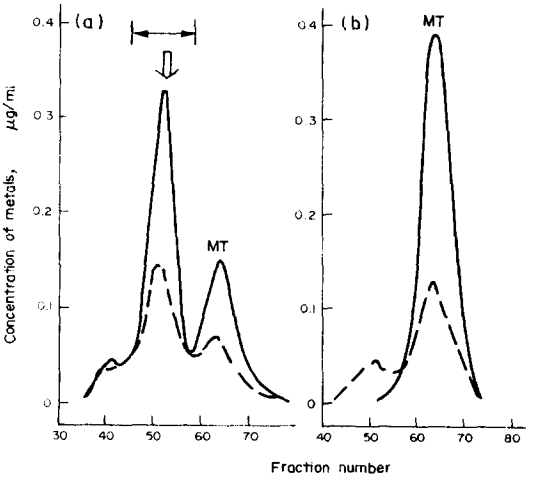

Fig. 2. Sephadex G-75 elution profiles of metallothionein and its dimer fraction before (a) and after reduction with mercaptoethanol (b). Panel a: The concentrated dimer fraction (2 ml) (*Ve/Vo* = 1.4–1.8 in Fig. 1) was applied to a Sephadex G-75 column as indicated in Fig. 1. Panel b: The concentrated dimer solution (2 ml) was reduced with mercaptoethanol (100 μl) and the solution was gel filtered in the same way after adding cadmium chloride (20 μg Cd^{2+}). MT indicates the metallothionein fraction and the arrow indicates the metallothionein dimer fraction. ———, Cd; – – –, Zn.

weight of metallothionein is less than 7000 daltons, an estimated molecular weight based on a gel filtration column has been around 14,000 daltons [7]. Therefore, if the cadmium peak at *Ve/Vo* = 1.4–1.8 is related to metallothionein, the molecular weight is assumed to be about 14,000 daltons.

Figure 2a shows a Sephadex G-75 elution profile for the cadmium-binding protein fraction obtained from a preparative column (5 × 80 cm). The figure shows a relative elution volume for the cadmium-binding protein and metallothionein.

Reduction of the cadmium-binding protein-containing solution (Fig. 2a) with mercaptoethanol shifted the cadmium peaks other than the metallothionein peak to the metallothionein fraction, as shown in Fig. 2b. The result indicated that the cadmium-binding protein at *Ve/Vo* = 1.4–1.8 possibly consists of metallothionein dimers.

Figure 3 shows a DEAE Sephadex A-25 elution profile for the cadmium-binding protein fraction (between the arrows in Fig. 2a). The cadmium-binding protein was separated into three major cadmium-binding peaks according to the respective isoelectric points.

Figure 4 illustrates a gel permeation chromatogram monitored by cadmium atomic absorption for the cadmium-binding protein fraction (the same sample used for a gel filtration column as shown in Fig. 2a). The direct connection of a high speed liquid chromatograph equipped with a Toyo Soda TSK GEL SW 3000 column to an atomic absorption spectrophotometer has been shown to be a powerful analytical method for metallothionein [6]. Liver metallothionein is separated into two isometallothioneins when the column is eluted with an alkaline buffer solution; namely, metallothionein-I and -II were eluted at retention times of 41.8 and 39.7 min, respectively, as shown in Fig. 4a.

Figure 4a shows the elution profile of a solution containing the higher molecular weight cadmium-binding protein and metallothionein which corresponds to Fig. 2a. Three cadmium peaks other than the two cadmium peaks corresponding to monomeric isometallothioneins were observed. The higher molecular weight cadmium-binding protein (collected between the arrows in Fig. 2) was separated mainly into three cadmium peaks (Fig. 4b). The three cadmium peaks were correlated to the three main cadmium peaks on an anion exchange column (Fig. 3) by applying each cadmium-binding protein isolated from an anion exchange column to the gel permeation column. The three cadmium-binding proteins were eluted on the gel permeation column at a reverse elution rate to a DEAE Sephadex A-25 column, as also observed for monomeric metallothioneins [6], indicating that the gel material of the gel permeation column has not only gel chromatographic but also cation exchange chromatographic properties.

Reduction of the individual cadmium-binding proteins (I–I, I–II and II–II) with mercaptoethanol gave metallothionein-I, an equivalent mixture of metallothionein-I and -II, and metallothionein-II, respectively, for the gel permeation chromatograms (monitored by cadmium atomic absorption), as typically illustrated in Fig. 4c. The three cadmium-binding

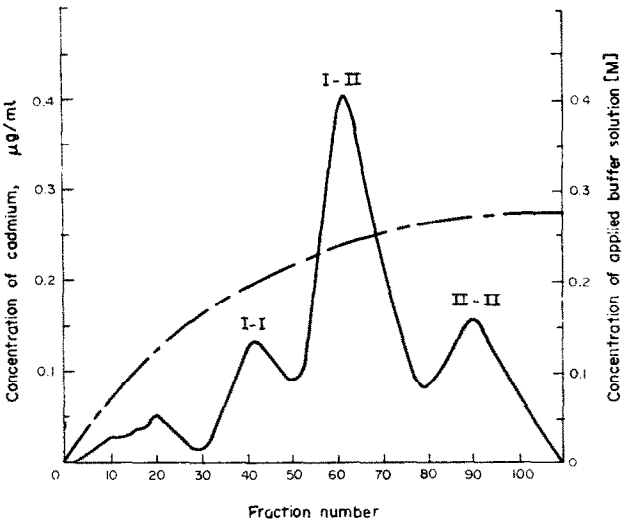

Fig. 3. DEAE Sephadex A-25 elution profile of metallothionein dimer fraction. The metallathionein dimer fraction (between the arrows in Fig. 2a) was applied to a DEAE Sephadex A-25 column (1.5 × 18 cm). The column was washed with 1 mM Tris–HCl buffer solution (pH 8.6, 25 ml) and then eluted with a concentration gradient of Tris–HCl buffer solution (pH 8.6) between 1 mM (100 ml) and 300 mM (300 ml). Fractions (2.7 ml) were collected. —, Cd; – – –, concentration of applied buffer solution. I–I, I–II and II–II indicate dimers of metallothionein-I, -I and -II, and -II, respectively.

proteins were thus confirmed to be three kinds of metallothionein dimers, being dimerized through intermolecular sulfide bond formation between monomeric isometallothioneins.

Application of a solution containing the dimers and mercaptoethanol to the gel permeation column without filling all binding sites with cadmium ion (without adding extra cadmium ion) resulted in unresolved or broad cadmium peaks according to the added amount of mercaptoethanol. This is probably due to competition of cadmium to the binding sites between thioneins and mercaptoethanol, and unoccupied binding sites of different mercapto groups and/or different numbers of mercapto groups in thioneins may cause different isoelectric points and/or conformations to metallothioneins.

Summarizing the result, the cadmium peak at Ve/Vo = 1.4–1.8 on a Sephadex G-75 column is due to the dimeric forms of metallothioneins through intermolecular sulfide bond formation and these are a mixture of three kinds of dimers; namely, a dimer of metallothionein-I, a dimer of metallothionein-I and -II, and a dimer of metallothionein-II. The dimers are reductively cleaved to monomers by mercaptoethanol.

Iodine oxidation and air oxidation of metallothi-

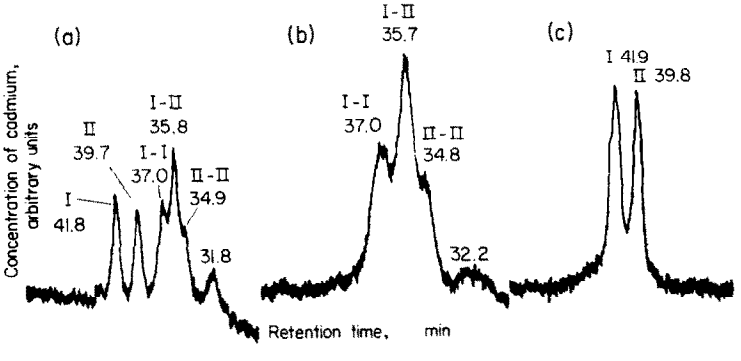

Fig. 4. Gel permeation chromatograms of metallothionein and its dimer fraction as monitored by cadmium atomic absorption. Panel a: Metallothionein and its dimer fraction (200 μl solution containing 4.5 μg Cd) as shown in Fig. 2a was applied to a gel permeation column (Toyo Soda TSK GEL SW 3000, 21.5 × 600 mm) and eluted with 50 mM Tris–HCl buffer solution (pH 8.6 at 25°) containing 0.1% sodium azide. The eluate was directly introduced to a flame atomic absorption spectrophotometer (Hitachi 508) and cadmium atomic absorption was continuously monitored. Chart speed, 0.25 cm/min. Panel b: The separated metallothionein dimer solution (same solution as used for Fig. 3) was applied to a gel permeation column as indicated above. Chart speed, 0.5 cm/min. Panel c: One of the dimers (I–II, 200 μl solution containing 4 μg Cd) isolated on an anion exchange column as illustrated in Fig. 3 was reduced with mercaptoethanol (30 μl) and applied to a gel permeation column after adding $CdCl_2$ (22.4 μg Cd) to fill all binding sites in the reduced monomers with cadmium. Chart speed, 0.25 cm/min.

onein monomers (Cd-thioneins) did not produce the dimers, probably due to intramolecular rather than intermolecular sulfide bond formation *in vitro*.

Acknowledgement—We thank Dr. K. Kubota for encouragement.

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