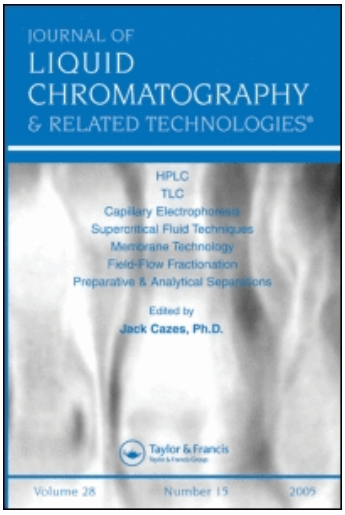

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Chromatographic Properties of Metallothioneins on a Gel Permeation Column: Changes Induced by Replacement of Cadmium with Cuprous and Cupric Ions

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CHROMATOGRAPHIC PROPERTIES OF METALLOTHIONEINS ON A GEL PERMEATION COLUMN : CHANGES INDUCED BY REPLACEMENT OF CADMIUM WITH CUPROUS AND CUPRIC IONS.

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ABSTRACT

In vitro experiments were conducted to find whether or not a similar elution profile to rat kidney metallothionein with high copper content was obtained on a gel permeation column by replacement of cadmium and/or zinc in rat liver metallothionein with copper. Stepwise replacement of cadmium in rat liver metallothionein with cuprous ion did not cause any shifts of retention times from those of the original proteins on a gel permeation column (SW 3000 column). In contrast to cuprous ion, stepwise replacement of cadmium with cupric ion induced shifts of retention times to larger values than the original ones for the two isometallothioneins on a SW column. Replacement of zinc in zinc-thionein with cupric ion but not with cuprous ion caused a retardation of elution volume on a Sephadex G-75 column. The decreases of cadmium peaks were accompanied by the increases of copper peaks in the case of replacement of cadmium in metallothionein with cuprous ion. Although stepwise decreases of cadmium peaks were observed by the replacement of cadmium in metallothionein with cupric ion, concomitant increases of copper peaks were not observed. Although the relative peak heights of isometallothionein peaks were different from those of rat kidney metallothionein, the third peak with the same retention time as that of rat kidney metallothionein was observed for the replacement of cadmium with cupric but not with cuprous ion.

INTRODUCTION

Metallothioneins induced in the livers of experimental animals by cadmium or zinc loadings are known to be a mixture of

two isometallothioneins (1 - 4) and the proteins can be separated due to differences in their isoelectric points (4 - 6). A commonly used separation procedure is a combination of gel filtration chromatography on a Sephadex G-75 column and subsequent ion exchange chromatography on a DEAE Sephadex A-25 column.

Recently, we reported a new analytical method for metallothioneins by a combination of a high speed liquid chromatograph equipped with a gel permeation column and a flame atomic absorption spectrophotometer (HLC - AAS) (7). Metallothioneins in tissue supernatants can be analyzed within an hour with consumption of a small amount of sample solution. Furthermore, a gel permeation column (SW 3000) used for the new analytical method was shown to have both gel filtration and cation exchange chromatographic properties for the elution with alkaline buffer solution. Therefore, metallothioneins in liver supernatants were separated into the two isometallothioneins on a SW column for the elution with alkaline buffer solution, metallothionein-II being eluted faster than metallothionein-I. Thus, the new analytical method (HLC - AAS) was shown to be a highly useful method for the analysis of metallothionein.

In contrast to liver metallothioneins induced by cadmium or zinc loadings, metallothionein induced in the kidneys of rats by loadings of cadmium ion is rich in copper and the kidney metallothionein shows different chromatographic properties both on a gel filtration column (Sephadex G-75) and on an anion exchange column (DEAE Sephadex A-25) (8). The kidney metallothionein with high copper content was also shown to have different chromatographic properties on a gel permeation column (SW 3000) ; namely, metallothionein-I peak is always bigger than metallothionein-II peak and a third metallothionein peak is present as a slower eluting isometallothionein peak than the metallothionein-I and -II peaks (9). Kidney metallothionein with low copper content (induced by injection of metallothionein) showed an intermediate pattern between typical liver and kidney metallothioneins (10, 11)

The present study was conducted to find whether or not *in vitro* replacement of zinc and/or cadmium in liver metallothionein with either cuprous or cupric ion induces changes of chromatographic properties both on a Sephadex G-75 column and on a SW 3000 column.

MATERIALS AND METHODS

Induction of metallothioneins Metallothionein was induced in the liver and kidneys of female rats of the Wistar strain (mean body weight 121 g) by subcutaneous injections of cadmium chloride (once with 0.3 mg Cd / Kg body weight and six times with 0.6 mg Cd / Kg body weight at intervals of 3 days) and the animals were sacrificed by exsanguination 4 weeks after the last injection. The livers and kidneys were homogenized using a polytron homogenizer in three volumes of 0.1 M Tris-HCl buffer solution (pH 7.4 at 25°) containing 0.25 M glucose under a nitrogen atmosphere. The homogenates were centrifuged at 170000 x g for 60 min.

Separation of metallothioneins Kidney metallothionein (Cd,Cu, Zn-thionein) was isolated by applying the kidney supernatant to a Sephadex G-75 column (2.6 x 90 cm). The column was eluted with 1 mM Tris-HCl buffer solution (pH 8.6) and 5 ml fractions were collected on a Diaflo UM-10 membrane. Zinc in liver metallothionein (Cd,Zn-thionein) was replaced *in vitro* by adding cadmium ion to give cadmium-thionein (Cd-thionein) and the cadmium-thionein was separated on a Sephadex G-75 column as above. Zinc-thionein (Zn-thionein) was induced by injecting zinc acetate into rats (12) and the livers were homogenized as above. Zinc-thionein was separated on a Sephadex G-75 column as mentioned above.

Gel permeation chromatography on a SW 3000 column The outlet of a high speed liquid chromatograph (Toyo Soda HLC 803, Toyo Soda

Co., Tokyo, Japan) equipped with a gel permeation column (Toyo Soda TSK GEL SW 3000, 21.5 x 600 mm with a precolumn (21.5 x 100 mm)) was directly connected to the nebuliser tube of an atomic absorption spectrophotometer (Hitachi 508) (7). Samples were applied in a 200 µl portion and the column was eluted with 50 mM Tris-HCl buffer solution (pH 8.6 at 25°) containing 0.1 % sodium azide at a flow rate of 3.75 ml / min. Cadmium or copper atomic absorbance was continuously recorded. The experiment was carried out at room temperature (20 - 22°).

Preparation of cuprous and cupric ion solutions Cuprous ion was prepared by the reported method (13) and stored in a solution of one to one mixture of acetonitrile and 50 mM Tris-HCl buffer solution (pH 8.6 at 25°). A solution of cupric ion was prepared by dissolving cupric chloride into 50 mM Tris-HCl buffer solution (pH 8.6 at 25°).

RESULTS AND DISCUSSION

Fig. 1 shows co-chromatograms on a Sephadex G-75 column of cadmium-thionein and copper-thioneins prepared by replacing zinc in zinc-thionein by cuprous (A) or cupric ion (B). Although zinc and cadmium in metallothioneins (Zn-thionein, Cd-thionein, and Cd,Zn-thionein) did not alter the chromatographic properties of metallothioneins on a Sephadex G-75 column for any metal ratios in the proteins (8), replacement of zinc in zinc-thionein with copper indicated that copper in metallothionein caused the changes of chromatographic properties depending on the valence state of copper ; namely, copper-thionein prepared by replacing zinc with cuprous ion was eluted at the same elution volume with cadmium-thionein but copper-thionein prepared by replacing zinc with cupric ion was eluted at a slower rate than cadmium-thionein. Copper-containing metallothioneins obtained from the livers and kidneys of rats after copper or cadmium loadings were shown to be

eluted at a slower rate on a Sephadex G-75 column than metallothioneins without copper as observed for the *in vitro* experiment in the present study (Fig. 1B) (8).

Figs. 2 - 5 show elution profiles of metallothioneins on a gel permeation column (TSK GEL SW 3000). The column was equipped to a high speed liquid chromatograph and the concentration of cadmium or copper in the eluate was monitored by directly connecting the outlet of the column to a nebuliser tube of a flame atomic absorption spectrophotometer (7). The column was shown to have gel filtration and cation exchange chromatographic properties for the elution with alkaline buffer solution and liver metallothionein is separated into the two isometallothioneins (metallothionein-II is eluted faster than metallothionein-I). The cation exchange chromatographic property was explained by the dissociation of hydroxyl groups in polyhydroxylated packing materials (9). Although metallothionein is apparently not separated on a Sephadex G-75 column due to shortage in the numbers of theoretical plates, the same properties were also shown for Sephadex gel materials (9).

Fig. 2 illustrates a typical elution profile of kidney metallothionein obtained by injecting cadmium chloride into rats. In contrast to the profiles of liver metallothioneins without copper (Cd,Zn-thionein and Zn-thionein), kidney metallothionein with high copper content was always accompanied by one more additional cadmium- and copper-containing peak other than the two peaks which correspond to liver metallothionein-II and -I (9, 11). Furthermore, the relative peak height of metallothionein-I was always higher than that of metallothionein-II as shown in Fig. 2. The extreme unbalance of the two isometallothionein peaks was already reported for the rat kidney metallothionein on a DEAE Sephadex A-25 column (8, 10), although the third peak observed on a SW column was not separated on a DEAE Sephadex A-25 column for the elution with exponential gradient of buffer concentration.

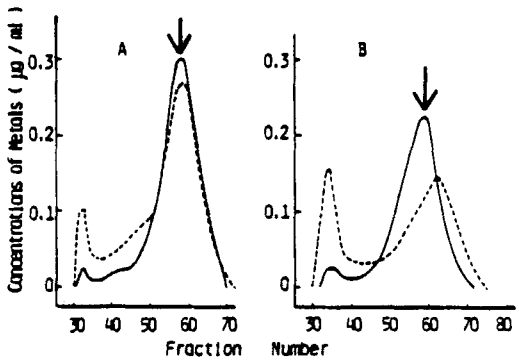

FIGURE 1. Co-chromatograms of cadmium-thionein and copper-thionein on a Sephadex G-75 column. A solution of cuprous ion (A) or cupric ion (B) was added to a solution of rat liver zinc-thionein (17 μg Zn in zinc-thionein / ml of 10 mM Tris-HCl buffer solution, pH 8.6) in the amount of 25 μg Cu (1.5 molar equivalence to zinc). Each solution was mixed after 5 min with rat liver cadmium-thionein (28 μg Cd / ml). The mixed solution (2.5 ml) was applied to a Sephadex G-75 column (2.6 x 90 cm) and eluted with 1 mM Tris-HCl buffer solution (pH 8.6). Five ml fractions were collected and the concentrations of cadmium and copper were determined in each eluate. The arrow indicates the metallothionein fraction.

Cadmium-thionein was prepared *in vitro* by replacing zinc in liver metallothionein (Cd,Zn-thionein) with cadmium ion and used for the following *in vitro* experiments to avoid possible complexities due to the presence of two metals (cadmium and zinc) and easy dissociation of zinc from the protein.

Addition of cuprous ion to cadmium-thionein induced the reduction of cadmium peaks on a SW column and the changes of relative peak heights were observed for the two isometallothionein peaks with added amounts of cuprous ion (Fig. 3). The changes may indicate that relative binding affinities for cuprous ion are changeable with the incorporated amounts of cuprous ion into the isoproteins. Although the stepwise reduction and the changes of relative peak heights were observed by the replacement of cadmium with cuprous ion, shifts of retention times of iso-

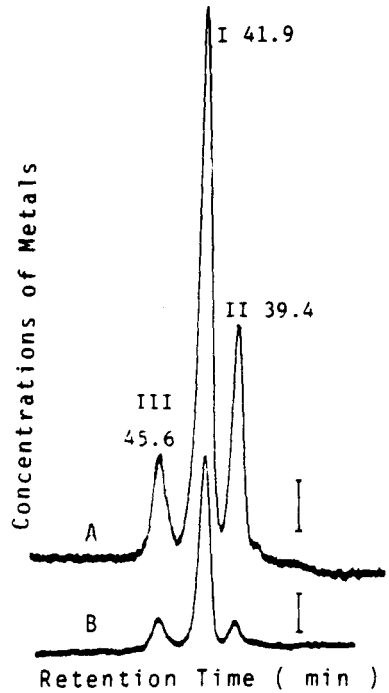

FIGURE 2. Gel permeation - atomic absorption chromatograms of rat kidney metallothionein. Rat kidney metallothionein fraction on a Sephadex G-75 column was concentrated on a Diaflo UM-10 membrane and applied to a TSK GEL SW 3000 column (21.5 x 600 mm) in a 200 µl portion and eluted with 50 mM Tris-HCl buffer solution (pH 8.6 at 25°) at a flow rate of 3.75 ml / min. Atomic absorbance of cadmium (A) or copper (B) was continuously monitored. The vertical bars show absorbances of standard solutions for cadmium and copper (0.1 µg / ml). I (41.9 min), II (39.4 min), and III (45.6 min) indicate metallothionein-I and -II, and a third metallothionein peaks, respectively.

metallothioneins were not observed by the replacement with monovalent copper.

In contrast to rather simple changes of elution profiles induced by the replacement with cuprous ion, addition of cupric ion to cadmium-thionein induced complex changes of elution profiles on a SW 3000 column ; namely, reduction of peak heights, changes of relative peak heights, and shifts of retention times

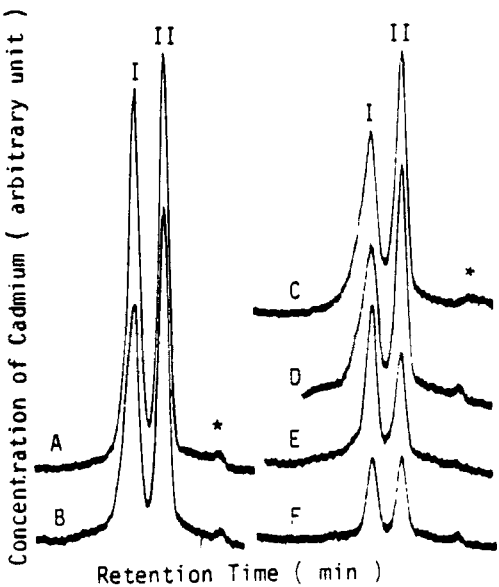

FIGURE 3. Gel permeation - cadmium atomic absorption chromatograms of cadmium-thionein with different amounts of cuprous ion. Cuprous ion (150 µl) was added to rat liver cadmium-thionein solution (11.7 µg Cd in 100 µl of 50 mM Tris-HCl buffer solution, pH 8.6 at 25°) in the amounts of 0 (A), 3.18 (B), 4.76 (C), 6.35 (D), 9.53 (E), and 12.7 µg (F), and the solutions were stood at room temperature for 5 min. A 200 µl portion of the solutions was applied to a TSK GEL SW 3000 column (21.5 x 600 mm) and the column was eluted with 50 mM Tris-HCl buffer solution (pH 8.6 at 25°) at a flow rate of 3.75 ml / min. Atomic absorbance of cadmium was continuously monitored. I (41.9 min) and II (39.4 min) indicate metallothionein-I and -II, respectively. * indicates a contamination peak, probably a metallothionein dimer peak (17).

(Fig. 4). The most dramatic change was the appearance of cadmium peak at a retention time of 45.6 min at the expense of metallothionein-I peak. The new peak at a retention time of 45.6 min was tentatively named a third peak. The retention time of a third peak was identical to that of the third peak observed for rat kidney metallothionein (Fig. 2). The selective reduction of metallothionein-I peak and appearance of the third

peak were followed by the selective reduction of metallothionein-II peak and appearance of a small fourth peak at a retention time of 50.1 min. The fourth peak was observed at first in the present *in vitro* study and has not been observed in the elution profiles of rat kidney metallothionein.

To confirm the replacement of cadmium in cadmium-thionein with copper, atomic absorption of copper was monitored for representative replacement experiments instead of cadmium. Fig. 5 illustrates the elution profiles of copper. Although addition

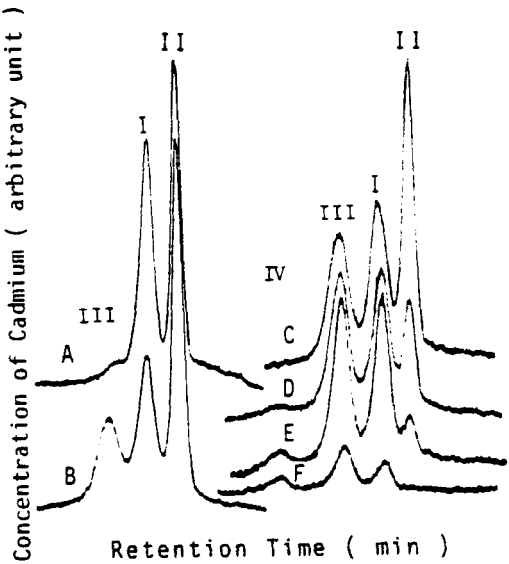

FIGURE 4. Gel permeation - cadmium atomic absorption chromatograms of cadmium-thionein with different amounts of cupric ion. Cupric ion (150 µl) was added to a rat liver cadmium-thionein solution (11.7 µg Cd in 100 µl of 50 mM Tris-HCl buffer solution, pH 8.6 at 25°) in the amounts of 0.635 (A), 1.59 (B), 3.18 (C), 4.76 (D), 6.35 (E), and 12.7 µg (F), and the solutions were stood for 5 min at room temperature. A 200 µl portion of the solutions was applied to a TSK GEL 3000 column (21.5 x 600 mm) and the column was eluted with 50 mM Tris-HCl buffer solution (pH 8.6 at 25°) at a flow rate of 3.75 ml / min. Atomic absorbance of cadmiun was continuously monitored. I (42.0 min), II (39.5 min), III (45.6 min), and IV (50.1 min) indicate metallothionein-I and -II, a third, and a fourth metallothionein peaks, respectively.

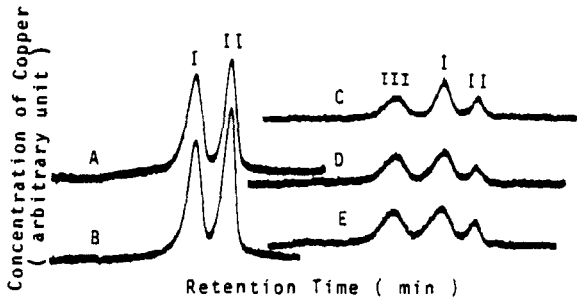

FIGURE 5. Gel permeation - copper atomic absorption chromatograms of cadmium-thioneins with different amounts of cuprous or cupric ion. Cuprous ion (A, 6.35 μg ; B, 9.53 μg in 150 μl buffer solution) and cupric ion (C, 6.35 μg ; D, 9.53 ug ; E, 12.7 μg in 150 μl buffer solution) were added to a rat liver cadmium-thionein solution (11.7 μg Cd in 100 μl of 50 mM Tris-HCl buffer solution, pH 8.6) and the solutions were stood for 5 min at room temperature. A 200 μl portion of the solutions was applied to a TSK GEL SW 3000 column (21.5 x 600 mm) and the column was eluted with 50 mM Tris-HCl buffer solution (pH 8.6 at 25°) at a flow rate of 3.75 ml / min. Atomic absorbance of copper was continuously monitored. I (42.0 min), II (39.5 min), and III (45.6 min) indicate metallothionein-I and -II, and a third metallothionein peaks, respectively.

of cuprous ion induced the increase of copper peaks and it suggested the replacement (Fig. 5A), addition of cupric ion did not induce the proportional increase of copper peaks (Fig. 5B). The addition of cuprous ion gave only two peaks which corresponded to metallothionein-I and -II. On the other hand, the addition of cupric ion gave three peaks at retention times of 39.5, 42.0, and 45.6 min which corresponded to metallothionein-II and -I, and the third peak, respectively. The fourth copper peak was not observed in Fig. 5B.

Although the redox states of copper and cysteinyl residues in metallothioneins prepared by *in vitro* replacement experiments were not studied in the present study, the elution profiles on a Sephadex G-75 and on a SW 3000 columns revealed several interesting chromatographic properties of metallothioneins. First of all, replacement of cadmium with cupric but not with cuprous ion induced the shifts of retention times of metallothionein on a SW

column and elution volume on a Sephadex column. One of the new peaks, the third peak on a SW column was eluted at the same retention time with that of the third peak in rat kidney metallothionein. The fourth peak may also be present in rat kidney metallothionein induced by cadmium loadings when relative copper content to cadmium and zinc contents is high. As far as elution profiles of only cadmium are concerned, replacement of cadmium with cuprous ion occurred proportionally with the added amounts. Although cadmium in metallothionein decreased with the increase of added cupric ion, copper in metallothionein did not increase concomitantly with the added amounts of cupric ion. Therefore, the present *in vitro* experiments did not succeed in the preparations of exactly same elution profile of rat kidney metallothionein shown in Fig. 2. However, the chromatographic properties of copper-containing metallothioneins observed in the present study have to be recognized for the identification and separation of not only tissue metallothioneins but also urinary metallothionein (14) whenever replacement of copper is possible. Multi-forms of copper-containing metallothionein may also be related to the present observation (15, 16).

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