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ISOLATION OF A (COPPER, ZINC)-THIONEIN FROM THE SMALL INTESTINE OF NEONATAL RATS

W. Thomas Johnson and Gary W. Evans

United States Department of Agriculture
Science and Education Administration
Grand Forks Human Nutrition Research Center
Grand Forks, North Dakota 58202

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Summary

A Cu- and Zn-binding protein was isolated from the small intestine of 5-day-old rats whose only source of these metals was maternal milk. Elution behavior on Sephadex G-75 indicated that the molecular weight of this protein was about 9,000 daltons. Furthermore, the protein exhibited low absorbance at 280 nm, was separated into two subfractions by DEAE Sephadex chromatography and had low aromatic amino acid and high cysteine content. The protein, therefore, meets the criteria for classification as a metallothionein. Evidence suggested that the metallothionein is located primarily in the distal portion of the small intestine.

Introduction

The importance of Cu for normal development and health maintenance in animals is well established, but the mechanism by which this essential trace element is absorbed from the gastrointestinal tract is not well understood. Since Starcher (1) reported the presence of a low molecular weight (10,000 daltons) Cu binding protein in chick intestinal cytosol, similar low molecular weight Cu binding proteins have been isolated from bovine duodenum (2) and rat intestinal cytosol (3). Functional roles in the Cu absorptive mechanism have been suggested for these proteins. A low molecular weight Zn-binding protein also was isolated from rat intestinal cytosol following Zn injections (4,5) and from the intestinal mucosa of chicks given high dietary levels of Zn (6). While the identity of the Cu-binding proteins has not been unequivocally established, the Zn binding protein was classified as a metallothionein (6,7).

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Levels of Cu in the neonatal rat small intestine have been found to increase significantly during the week following birth (8). Furthermore, the Cu in the intestinal cytosol was sequestered in part by a low molecular weight protein of unknown identity. Since metallothionein has been identified in adult rat intestinal cytosol (7), and has also been found to be highly concentrated in neonatal rat liver (9), we have investigated the possibility of metallothionein being the low molecular weight Cu binding protein in the neonatal rat small intestine.

Materials and Methods

Litters from Long-Evans dams were culled to ten pups immediately following birth. The dams were maintained on Purina[1] rat chow throughout pregnancy and lactation. Five days after birth, the ten pups were decapitated and bled. The entire small intestine, unless otherwise indicated, was immediately excised, rinsed and flushed thoroughly with ice-cold 0.01 M Tris-acetate, pH 7.4, and then homogenized in 2-3 volumes of this same buffer using a motor-driven Potter-Elvehjem homogenizer fitted with a Teflon pestle. High-speed supernatant (cytosol) was prepared by centrifuging the homogenate at 130,000 x g for 40 min at 4°C.

The intestinal cytosol was fractionated immediately using a combination of gel filtration and ion exchange chromatography (10,11,12). Four ml of cytosol were applied to a 1.5 x 90 cm column of Sephadex G-75 equilibrated with 0.01 M Tris-acetate, pH 7.4. The column had been previously calibrated using Blue Dextran 2000 (Pharmacia Fine Chemicals, Piscataway, NJ) and the following proteins of known molecular weights (Sigma Chemical Company, St Louis, MO): cytochrome C (12,400), α-chymotrypsinogen A (25,000) and ovalbumin (45,000). Fractions collected from the Sephadex G-75 column were monitored for absorbance at 280 nm and Cu and Zn content by atomic absorption spectrophotometry with a Varian Techtron 1250 spectrophotometer. Tubes containing Cu and Zn and corresponding to a protein fraction with a molecular weight of 9,000-10,000 daltons were pooled immediately following elution and applied to a 0.9 x 13 cm column of DEAE-Sephadex A-25 which had also been equilibrated with 0.01 M Tris-acetate, pH 7.4. The DEAE-Sephadex column was eluted with a 100 ml linear gradient of 0.01-0.2 M Tris-acetate, pH 7.4, and the collected fractions were assayed for metal content as described above. All buffers used in the fractionation procedures were N_2 saturated, which was imperative for the successful isolation of this protein.

Subfractions from the DEAE-Sephadex chromatography were concentrated under N_2 pressure using an Amicon UM-2 ultrafiltration membrane. The concentrated samples were then oxidized with performic acid (13), hydrolyzed with 6N HCl at 110° for 24h, and analyzed with a Beckman model 119 amino acid analyzer.

[1] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

Results and Discussion

Figure 1 shows the pattern for the elution from Sephadex G-75 of cytosol prepared from the small intestines of 5-day-old rats. Cu and Zn eluted in two peaks corresponding to molecular weights of approximately 60,000, or greater, and 9,000 daltons. Absorbance at 280 nm was associated primarily with the high molecular weight proteins and the smaller peptides and amino acids that eluted last from the column. Relatively little absorbance at 280 nm was associated with the 9,000 dalton Cu and Zn binding fraction.

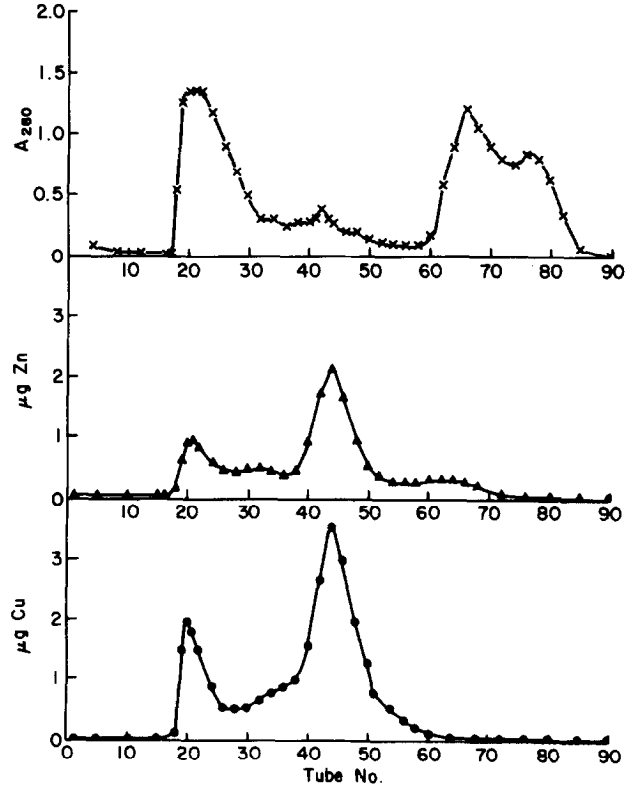

Figure 1. Sephadex G-75 chromatography of cytosol prepared from the small intestines of 5-day-old rats. Cytosol (4 ml) representing 1.5 g of tissue was applied to the column (1.5 x 90 cm, V_o = 39 ml) and eluted at 4°C with N_2 saturated 0.01 M Tris-acetate, pH 7.4. Fractions (2 ml) were collected and absorbance at 280 nm (X), Zn (▲) and Cu (●) were monitored.

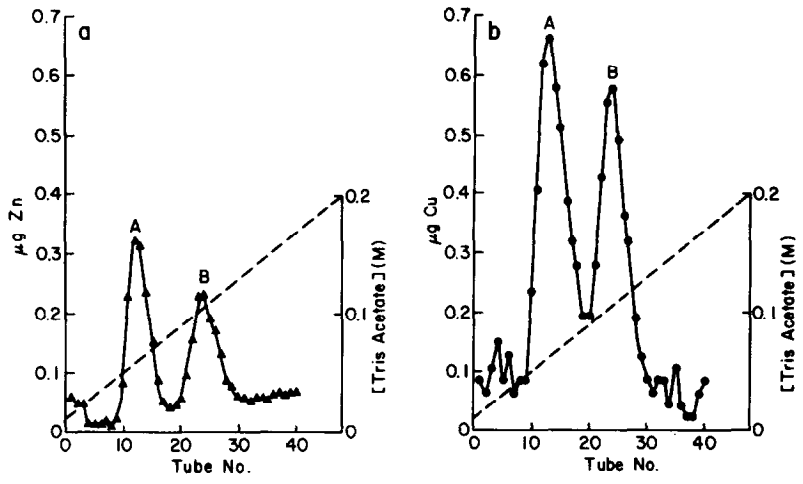

Figure 2. Chromatography of the low molecular weight, Cu-, Zn-binding protein from Sephadex G-75 gel filtration on DEAE Sephadex A-25. Fractions (2.1 ml) were monitored for the presence of Zn (a) and Cu (b). Buffers used to form the gradient were N_2 saturated and elution was performed at 4° with a 15 ml/hr flow rate.

The low molecular weight metalloprotein fraction obtained from the Sephadex G-75 column (tubes 39-50) was applied to a DEAE-Sephadex A-25 column. As shown in Figure 2, elution of the ion-exchange column with a 0.01-0.2 M Tris-acetate gradient yielded two subfractions containing both Cu and Zn. Subfraction A eluted at a Tris-acetate concentration of about 0.06 M and subfraction B eluted at about 0.11 M. Of the amount applied to the DEAE Sephadex column, A and B each contained 20% of the total Zn, while A contained 26% and B 22% of the total Cu. Since only about 2% of the total Cu and Zn did not bind to the column, and 40-50% of the total Cu and Zn was eluted in subfractions A and B, 50-60% of the total Cu and Zn was not recovered from the DEAE column with the Tris-acetate gradient.

The separation of the low molecular weight Cu, Zn-binding fraction into two major subfractions by DEAE chromatography is characteristic of the elution behavior observed for hepatic and intestinal metallothioneins (7,11,14,15). Amino acid analysis helped to substantiate the identity

Table I

Amino acid analysis of subfractions A and B from DEAE-Sephadex chromatography

	% of total residues	
Amino acid	A	B
cysteine[a]	28.6	27.7
aspartic acid	7.9	7.5
threonine	6.0	5.8
serine[b]	7.0	8.3
glutamic acid	6.4	7.3
proline	5.9	8.4
glycine	7.5	6.5
alanine	5.1	6.9
valine	5.1	4.8
isoleucine	3.0	3.2
leucine	4.4	4.3
phenylalanine	2.2	<0.5%
lysine	7.6	6.6
arginine	2.5	2.1

[a]Determined as cysteic acid.

[b]Corrected for standard losses.

of subfractions A and B as metallothioneins. As shown in Table I, the cysteine content was 28.6% and 27.7% for A and B, respectively. This high cysteine content is consistent with data obtained from the analysis of hepatic, renal and intestinal metallothioneins isolated from a variety of mammalian species (7,9,11,14,15,16). The absence or low content of aromatic amino acids and histidine is also characteristic of metallothionein.

The elution behavior on Sephadex G-75 and DEAE Sephadex, the low absorbance at 280 nm and the high cysteine content, are lines of evidence

establishing the identity of this Cu- and Zn-binding protein from the small intestines of neonatal rats as metallothionein. While intestinal Zn-thionein has been identified in adult rats following parenteral Zn administration (7), our evidence suggests that an intestinal (Cu, Zn)-thionein exists under normal physiological conditions in neonatal rats whose primary source of Cu and Zn is maternal milk.

If metallothionein is involved in the absorption of Cu, it is reasonable to suspect that this protein would be present in the upper regions of the small intestine, since this is the reported area for absorption of this metal (17,18). However, Sephadex G-75 chromatography of cytosol prepared from the first 10 cm of small intestine from 5-day-old rats did not yield the usual elution pattern observed for cytosol prepared from the entire small intestine. In the cytosol from the proximal small intestine, the low molecular weight, metal-binding protein contained, as an average, 1.8 μg Zn/g wet tissue and only 0.3 μg Cu/g wet tissue, compared to 3.8 μg Zn/g wet tissue and 9.1 μg Cu/g wet tissue found in this fraction from cytosol prepared from the entire small intestine. Furthermore, the low molecular weight, metal-binding protein from the proximal small intestine could not be resolved into two subfractions by DEAE Sephadex chromatography, and, in fact, did not elute from the column under the conditions described in this paper.

The evidence suggests that the metallothionein identified in the present study is concentrated primarily in the distal region of the neonatal rat small intestine, but does not controvert the suggested involvement of this protein in the absorption of metals from the gastrointestinal tract. Pinocytosis is very active in the ileum and jejunum of neonatal rats (19) and has been implicated as a mechanism for Cu absorption in the ileum of neonates (20). Furthermore, the limiting factor in the absorption of Cu from the ileum may be the transfer of Cu to the circulation rather than mucosal uptake (20). The presence of

metallothionein in the distal small intestine, then, suggests that this protein may function in regulating the transport of Cu, and possibly Zn, from the mucosa of this region by serving as an intermediary in maintaining the balance between the uptake of these metals by pinocytosis and their eventual transfer to the portal circulation or excretion following exfoliation of the absorptive cells.

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