# ISOLATION OF AN INTESTINAL METALLOTHIONEIN INDUCED BY PARENTERAL ZINC

Mark P. Richards and Robert J. Cousins+

Department of Nutrition
Rutgers University
New Brunswick, New Jersey 08903

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SUMMARY

An intestinal zinc-binding protein, induced by parenteral zinc administration, has been isolated and characterized. Based upon its elution behavior in two chromatographic systems, $Zn^{2+}$/protein ratio of 5.0-5.6 gram atoms/mole, a $Zn^{2+}$/SH ratio of about 3.0, paucity of both aromatic amino acids and absorbance at 280 nm, abundance of cysteic acid residues (28-31%), and low molecular weight (6,000-7,000 daltons), the protein meets the criteria for classification as a metallothionein and is more properly named zinc-thionein. Orally administered $^{65}Zn$ was found to bind to intestinal zinc-thionein and thus this intracellular protein may function as a component of the mechanism responsible for mammalian zinc homeostasis at the level of intestinal absorption.

## INTRODUCTION

The regulation of intestinal zinc absorption is an important aspect of zinc homeostasis. The nature of this mechanism is still not clearly defined. Starcher (1) reported the presence of a low mw (10,000 daltons) protein, in chick intestinal cytosol, which was capable of binding radionuclides of Cu and Zn. It was proposed that this protein functioned directly in the transport of these metals from the intestinal lumen to the plasma. A similar protein has been identified in bovine duodenum (2) and rat intestinal cytosol (3). These reports, however, failed to clearly establish the properties of this protein(s).

Recent investigations have shown that a low mw copper-binding protein, isolated from both rat liver (4-6) and intestinal cytosol (7), is induced by copper injections. Similarly a protein of about the same size, isolated from both rat liver (8-11) and intestinal cytosol (11,12), is induced by zinc injections. The copper-induced proteins have a cysteine content of between 3% and 15%. In contrast, the zinc-induced protein in rat liver (8,10) has been

+To whom correspondence should be addressed.





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shown to be a metallothionein which characteristically is composed of at least 25% cysteine (13) and is called zinc-thionein. However, the nature of the zinc-binding protein in intestinal cytosol has not been defined.

The induction of the synthesis of both zinc-thionein and the intestinal zinc thionein-like protein is inhibitable by administration of actinomycin D, cordycepin and cycloheximide (14,15). Moreover, these liver and intestinal zinc-binding proteins seem remarkably similar with respect to zinc-binding affinity, incorporation of labeled amino acids and chromatographic properties. Based upon these similarities, intestinal metallothionein has been proposed to function in an inducible mechanism that regulates zinc absorption by acting as an intracellular sequestering agent (11).

The data presented here establish the identity of the intestinal zinc-binding protein as a metallothionein and show that it is induced by parenteral zinc and is capable of binding orally administered zinc. Thus zinc-thionein could have a function in the regulation of intestinal zinc absorption.

## MATERIALS AND METHODS

Male Sprague Dawley rats were housed individually in stainless steel cages and were fed a commercial diet (50 ppm $Zn^{2+}$) until they reached 150-200 g. Rats were injected, intraperitoneally, with 25 umoles of $Zn^{2+}$ (as $ZnSO_4$). Twenty-four hrs later the rats were killed by decapitation. The entire small intestine was immediately removed, placed on an ice-cold plate, rinsed thoroughly with 30 ml of ice-cold 0.9% NaCl and then split lengthwise. Mucosal cells were harvested by scraping the intestine with a microscope slide. The mucosal cells was homogenized in 3 volumes of ice-cold 0.25 M sucrose in 10 mM Tris-acetate (pH 8.6) using a glass-Teflon, motor driven Potter-Elvejhem type homogenizer. The homogenate was centrifuged at 42,000 x g for 30 min. at 4°C. The supernatant was recentrifuged at 166,000 x g (av) for 1 hr at 4°C.

The resulting supernatant (cytosol) fraction was applied, immediately, to a 2.5 x 60 cm column of Sephadex G-75 equilibrated with 10 mM Tris-acetate (pH 8.6) at 4°C. Fractions were monitored for absorbance at 280 nm and for $Zn^{2+}$ content by atomic absorption spectrophotometry. Those $Zn^{2+}$-containing fractions, corresponding to the elution characteristics of known hepatic zinc-thioneins, were pooled and applied to a 2.5 x 30 cm column of DEAE Sephadex A-25 which had been equilibrated with 50 mM Tris acetate (pH 7.4). The column was eluted at 4°C with a linear gradient of 50-200 mM Tris acetate (pH 7.4). Fractions were analyzed as above. Those fractions corresponding to the first zinc containing component eluted (peak A) and the third zinc containing component eluted (peak B) were pooled and concentrated under $N_2$ pressure using an Amicon UM-2 ultrafiltration membrane. The concentrated samples were dialyzed against 3 changes of deionized $H_2O$ for 24 hr at 4°C using a dialysis membrane (Spectropore III). These samples were hydrolyzed at 110°C for 22 hr with 6N HCl in sealed evacuated tubes. A second sample of each component was oxidized with performic acid (16), before the hydrolysis, in

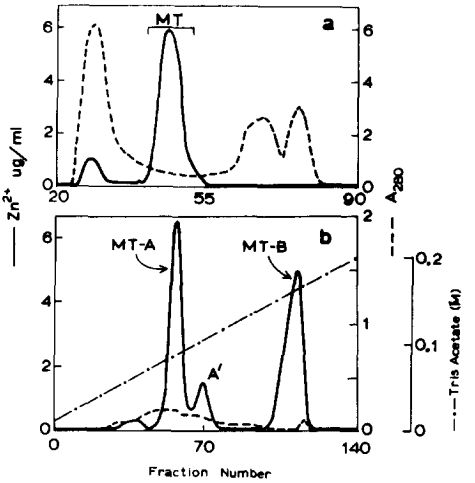


Figure 1. (a) Chromatography on Sephadex G-75 of intestinal cytosol from rats injected with 25 umoles $Zn^{2+}$ (ip) 24 hrs prior to sacrifice. Five ml fractions were collected. MT designates the metallothionein-containing fractions. (b) Chromatography on DEAE Sephadex A-25 of pooled MT fractions from Sephadex G-75 chromatography. Four ml fractions were collected. MT-A and MT-B designate the A and B components of metallothionein. The $Zn^{2+}$ content of the fractions was measured by atomic absorption spectrophotometry.

order to estimate the cysteine content as cysteic acid. Amino acid analysis was performed with a modified Beckman Amino Acid analyzer, Model 120. In order to satisfactorily estimate the number of gram atoms of $Zn^{2+}$ bound per mole of protein, a purified and concentrated sample was mixed with $Zn^{2+}$(as $ZnSO_4$)in a molar ratio of 3 to 1, respectively for 4 hr at 4°C. The mixture was then passed through a column of Sephadex G-25 to remove residual $Zn^{2+}$ and the $Zn^{2+}$ content of the protein was determined. The protein content (17) was measured based on a known calibration curve for standard metallothionein. Reactive sulfhydryl groups were estimated by a spectrophotometric method utilizing 2,2'-dithiodipyridine (18) in a 100 mM Na acetate buffer (pH 4.0). A standard curve was derived using bovine serum albumin. The molecular weights of MT-A & B were estimated from both the amino acid analysis and according to the gel filtration method of Andrews (19). For the latter, a column (1.6x90 cm) was equilibrated and eluted with 5 mM Tris acetate, 100 mM Na acetate (pH 8.6). Chymotrypsinogen (bovine pancreas), cytochrome c (equine heart), myoglobin (equine skeletal muscle) and tryptophan (all from Sigma Chem. Co.) as well as insulin B chain (bovine pancreas; from Schwartz Mann) were used as standards. The column was monitored for absorbance at 215, 280, 410 nm or for $Zn^{2+}$ in the case of MT-A&B. Disc gel electrophoresis was performed with 7% acrylamide gels using the procedure of Davis (20), except that the Tris-glycine buffer was pH 9.0 and a constant current of 2mA per tube was used. The gels were stained with Coomassie Blue (21) and scanned at 563 nm.

In a second experiment rats were injected with 25 umoles of $Zn^{2+}$, intraperitoneally, as $ZnSO_4$. After 24 hr each rat received 10 uCi of $^{65}ZnCl_2$ (109 mCi/mmole) orally, via gastric intubation. One hr later the rats were killed by decapitation and the intestinal mucosal tissue processed as described above. The cytosol was fractionated first on Sephadex G-75 and then on DEAE Sephadex A-25 as described above. All chromatographic steps were performed at 4°C. The $^{65}Zn$ content of the fractions was measured by liquid scintillation counting (22).

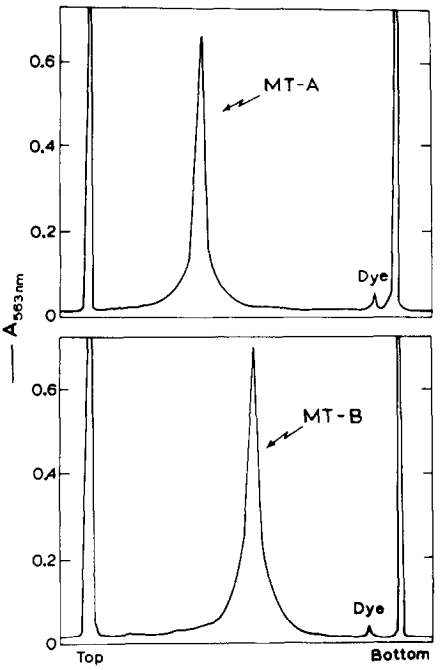


Figure 2. Disc gel electrophoresis of intestinal MT-A (top) and MT-B (bottom) after purification by DEAE-Sephadex chromatography. Gels were 7% acrylamide. The current was 2 mA per tube. After staining with coomassie blue the gels were scanned for A563nm.

## RESULTS AND DISCUSSION

The Sephadex G-75 elution profile for intestinal mucosal cell cytosol is shown in Figure 1a. The cytosol separated into two $Zn^{2+}$-containing peaks, one peak eluted at the void volume of the column which comprised the bulk of the zinc metalloproteins, i.e. those with a mw of greater than 75,000 daltons, and a second peak in the 6,000-10,000 dalton range, designated as metallothionein (MT). The majority of absorbance at 280 nm was associated with the high mw proteins and with fractions eluted last from the column, i.e., small peptides and free amino acids. Relatively little absorbance at 280 nm was associated with the MT-containing fraction.

When the MT fractions were pooled and the combined MT fraction was re-chromatographed on DEAE Sephadex A-25, it was resolved into 2 major and 1 minor $Zn^{2+}$-containing components, designated A, B, A', respectively (Figure

TABLE 1

AMINO ACID COMPOSITION OF INTESTINAL ZINC-THIONEIN

| AMINO ACID | (% TOTAL RESIDUES)* A | B |
|---|---|---|
| ASPARTIC ACID | 6.9 | 8.1 |
| THREONINE | 5.4 | 4.0 |
| SERINE | 12.3 | 10.6 |
| GLUTAMIC ACID | 4.8 | 7.5 |
| PROLINE | 5.0 | 6.8 |
| GLYCINE | 10.4 | 8.1 |
| ALANINE | 5.5 | 9.5 |
| CYSTEINE** | 30.5 | 28.4 |
| VALINE | 5.4 | 3.6 |
| METHIONINE | 2.3 | 0.9 |
| ISOLEUCINE | 2.7 | 2.4 |
| LEUCINE | 1.5 | 1.2 |
| LYSINE | 6.3 | 8.1 |

* ARGININE, PHENYLALANINE AND TYROSINE WERE DETECTED AT LESS THAN 0.5% OF THE TOTAL RESIDUES.

** DETERMINED AS CYSTEIC ACID.

1b). Peak A (MT-A) contained 44% of the recovered zinc, peak A', 11% and peak B (MT-B), 45%. Approximately 94% of the $Zn^{2+}$ applied to this column was accounted for in these components. Resolution into two major binding components via DEAE chromatography is in agreement with studies of hepatic and renal MT (8,9,13). MT-A&B were found to be homogeneous, based upon polyacrylamide disc gel electrophoresis (Figure 2), when the proteins were previously saturated with $Zn^{2+}$ to preclude oxidative polymerization products (23).

Table I shows the mole percentage composition of the amino acids in MT-A &B. The cysteine residue content (as cysteic acid) of 30.5% and 28.4% for MT-A&B, respectively, helps to establish these components as metallothioneins. Such large percentages of cysteine are consistent with data derived for hepatic or renal MT isolated from rat, equine, human or rabbit sources (8,10,13, 24-26). Also in agreement with known characteristics of MT is the almost complete lack of aromatic amino acids and the relative abundance of glycine and serine. There are distinct differences in the amino acid composition of MT-A&B; however, it is not known if these differences constitute some functional significance.

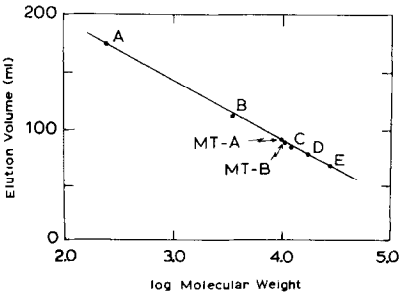


Figure 3. Elution volume of standard proteins and MT-A&B from a 1.6 x 90 cm column of Sephadex G-50 vs. the log10 of the molecular weight (daltons). Standards were, in daltons, A = tryptophan (204), B = insulin B chain (3,500), C = cytochrome c (12,400), D = myoglobin (17,000), and E = chymotrypsinogen (25,800).

Such differences have been detected previously for MT-A&B of both liver and renal MT's (8,10,13,24-26). The minimum mw based on the amino acid composition was calculated to be 6512 daltons for MT-A and 6311 daltons for MT-B. The relationship of elution volume from the Sephadex G-50 column vs. the log10 mw is shown in Figure 3. The estimated mw was 10,000 for MT-A and 10,200 for MT-B. These latter estimates are consistant with values obtained for non-denatured liver and kidney MT using gel filtration methods (8,10,13,23). In contrast Kagi *et al.* (13) have shown that denatured equine kidney MT has a mw of about 6600 which agrees with the minimum mw we calculated for rat intestinal MT. An explanation for the disparity between the calculated and estimated values is that native MT assumes an ellipsoidal configuration, accounting for its large Stokes radius (13,27), which would lead to an overestimation of size based upon gel filtration elution data. It cannot be ruled out, however, that the intestinal protein is slightly larger than either liver or kidney MT.

The metal content of MT-A&B was 5.0 and 5.6 g atoms $Zn^{2+}$ per mole of protein, respectively. This high binding capacity is consistent with that reported for MT (13,27). There were 18.9 and 18.8 reactive sulfhydryl groups per mole of protein for MT-A&B, respectively. Therefore the stoichiometry of metal to cysteinyl metal binding sites for intestinal MT is about 3, which is

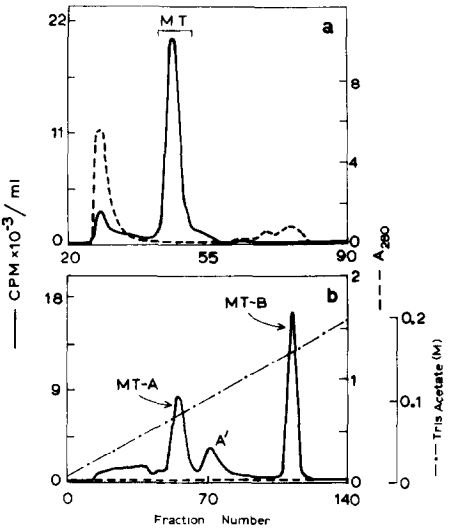


Figure 4. (a) Chromatography on Sephadex G-75 of intestinal cytosol from rats injected with 25 umoles $Zn^{2+}$ (ip) 24 hrs prior to sacrifice and administered 10 uCi $^{65}Zn$ orally 1 hr prior to sacrifice. Five ml fractions were collected. MT designates the metallothionein-containing fractions. (b) Chromatography on DEAE Sephadex A-25 of pooled MT fractions labeled with $^{65}Zn$. Four ml fractions were collected. The $^{65}Zn$ content was measured by liquid scintillation counting. MT-A and MT-B designate the A and B components of metallothionein.

consistant with liver and kidney MT (13, 27). The term zinc-thionein (Zn-MT) more properly defines the nature of this new intestinal protein.

Figure 4 shows both the Sephadex G-75 and DEAE Sephadex A-25 elution profiles of intestinal cytosol of rats injected with 25 umoles of $Zn^{2+}$, intraperitoneally, prior to oral administration of 10 uCi of $^{65}Zn$. The majority of newly absorbed $^{65}Zn$ was bound to Zn-MT fractions. Further, the $^{65}Zn$-MT eluted identically with the purified Zn-MT described above. This finding supports the proposal that Zn-MT binds newly acquired mucosal cell zinc and in this way could prevent transport of the metal to the circulation at the baso-lateral membrane (11).

This report is the first to conclusively demonstrate, by chromatography, amino acid analysis and other properties, that the intestinal protein induced by parenteral zinc, is a metallothionein. Furthermore, pre-induced intestinal

MT is capable of sequestering orally administered zinc. These findings, in addition to earlier observations concerning the regulation of the synthesis of intestinal Zn-MT (11), help to establish the regulatory function of this protein in the cellular mechanisms that regulate zinc absorption.

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