Similarities between metallothionein and low molecular weight testicular cadmium-binding protein

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Summary. The incorporation of ^{35}S-cysteine and ^{3}H-glutamic acid was studied in mouse hepatic and renal metallothionein and in testicular cadmium-binding protein of similar molecular weight. Preferential incorporation of ^{35}S-cysteine over ^{3}H-glutamic acid was observed not only in hepatic and renal metallothionein, but also in testicular cadmium-binding protein. When the antigenic reactivity of these proteins was compared, all three proteins reacted with the metallothionein antibody. These similarities suggest that the low molecular weight testicular cadmium-binding protein is apparently metallothionein.
Key words. Metallothionein; cadmium; cadmium-binding protein; testis; liver; kidney.

Administered cadmium accumulates preferentially in liver and kidney and very little in the testis. Yet acute doses of cadmium, that produce no gross toxicity in liver or kidney, result in selective damage to the testis[1]. Several studies to understand the biochemical mechanism of testicular sensitivity have been carried out[2–10]. These have centered mainly on the low molecular weight testicular cadmium-binding protein (Cd-BP).

In liver and kidney, cadmium is bound to metallothionein (MT), which also binds zinc and copper, and has an abundance of cysteine residues but lacks the aromatic amino acids[11]. Exposure to cadmium induces the synthesis of MT in most tissues examined. The testicular Cd-BP from several mammalian species is similar to hepatic MT in its molecular weight[2–9,12–18], charge characteristics[5,6,14–16], and immunoreactivity with the antibody[7,19]. However, there are other reports that claim that the Cd-BP in mouse[9], rat[8,15–17] and monkey[20] testis is quite different from hepatic MT in its amino acid composition. Waalkes et al.[9] reported that the purified isoforms of the mouse testicular Cd-BP contained 8–24 mol % glutamic acid and no cysteine, as compared to the mouse hepatic MT that contained only 5–6 mol % glutamic acid but had 28–29 mol % cysteine. Because of this marked contrast in amino acid composition, we examined the comparative incorporation of radiolabeled glutamic acid and cysteine in hepatic and renal MT and testicular Cd-BP in an effort to further delineate similarities and differences between these proteins. The immunoreactivities of MT and Cd-BP with MT antibody were also compared to get additional comparison of structural homology.

Materials and methods

Male mice of DBA/2J strain were obtained from Jackson Laboratory, Bar Harbor, ME. Prolab rat chow 3000 (Agway Inc., Syracuse, NY) and tap water were provided ad libitum. The mice were injected s.c. with 10 µmol $CdCl_2$/kg b. wt. Some of these animals received, 6 h later, i.p. injections of 100 µCi each of ^{3}H-glutamic acid (21 mCi/µmol) and ^{35}S-cysteine (1.44 Ci/µmol), obtained from Amersham Corporation, IL. All animals were sacrificed 24 h following the cadmium administration. A different group of animals were injected s.c. with 10 µmol/kg of ^{109}Cd-labeled $CdCl_2$ (New England Nuclear Corp., Boston, MA) and sacrificed 24 h later. The animals were sacrificed under light ether anesthesia by exsanguination. Liver, kidneys and testes were removed, homogenized (10 %, w/v) immediately in 5 mM Tris-HCl, pH 8.6, buffer and centrifuged at 105,000 × g for 60 min at 4 °C. The supernatant (cytosol) was applied to chromatography columns (0.9 × 60 cm) packed with Sephadex G-75 superfine (Pharmacia, Uppsala, Sweden) and equilibrated with the Tris buffer. The columns were eluted at a flow rate of 2 ml/h and 1 ml fractions were collected. ^{3}H and ^{35}S were determined in column fractions by liquid scintillation spectrometry and ^{109}Cd in a gamma spectrometer. Corrections were made in ^{35}S count overlap in ^{3}H channel using appropriate standards.

To determine the immunoreactivity of Cd-BP with MT antibody, liver, kidney and testis from the animals injected with non-radioactive $CdCl_2$ were homogenized (10 %, w/v) in 125 mM borate-NaOH buffer, pH 8.6, containing 0.1 % Tween-20 and 0.04 % bovine serum albumin (BNTB). The homogenates were centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant was heated at 80 °C for 10 min, cooled and centrifuged again at 10,000 × g for 10 min. Measured volume (1.45 ml) of each supernatant was applied to Sephadex G-75 superfine columns (0.9 × 60 cm) equilibrated with the BNTB buffer at 4 °C. The columns were eluted at 2 ml/h and 1-ml fractions were collected. MT antibody reactivity was examined in the column fractions by radioimmunoassay[21]. Instead of ammonium sulfate, Pansorbin (Calbiochem, La Jolla, CA) was used for separating antibody-bound ^{125}I-MT. Purified mouse liver MT, dissolved in BNTB buffer, was heat-treated as described above and used for generating the standard curve. The results of ^{3}H, ^{35}S, ^{109}Cd and MT analyses were plotted against the elution volume (V_e) to void volume (V_o) ratios.

Results and discussion

The accumulation of cadmium in mouse tissues and basal as well as induced MT levels are summarized in the table. As reported previously[5–7], in comparison to liver and kidney which together accumulated almost half of the injected dose, cadmium accumulation in testis was rather small. The cadmium concentration in testis was 44 times lower than in liver and 13 times lower than in kidney. The highest concentration of MT-antibody-reactive protein in control animals was in the testis, which was 2.8 and 4.5 times the MT concentration in liver and kidney, respectively. Thus, while this protein is involved in sequestration of cadmium, it does not appear to affect its uptake.

In response to cadmium accumulation, the MT levels increased markedly in both liver and kidney, as expected. Eventhough the MT mRNA level in mouse testis are reported to be high[22] further induction of the protein was not expected. This was because the basal MT-antibody-reactive protein concentration in testis was much more than needed to sequester the amount of cadmium taken up (table). In fact, the level of this protein decreased somewhat after cadmium administration. This was possibly related to edema and resulting increase in testis weight[23]. Dose-related decrease in testicular Cd-BP concentration has been observed by others in cadmium-sensitive mice[23] and rats[24].

Figure 1 shows the distribution of ^{109}Cd in tissue cytosol. Whereas more than 90 % of ^{109}Cd in liver and kidney cytosol was associated with MT, in testicular cytosol it was distributed between two different Cd-BP peaks. The lower molecular weight peak had the same V_e/V_o ratio as the hepatic and renal MT and the high molecular weight peak had a molecular weight of at least 70,000 daltons. These results are in agreement with previously published reports from this laboratory[5,6] and others[2–4,8,9,14–16].

The immunoreactivities of the heat-stable proteins with the MT-antibody are superimposed over the ^{109}Cd distribution patterns in figure 1. MT is a heat-stable protein, therefore, in both liver and kidney the antibody showed clear reactivity with the fractions containing the ^{109}Cd peak. Like hepatic and renal MT, the high and low molecular weight testicular Cd-BPs were also heat-stable and reacted with the antibody. The existence of MT in testis has been documented before by both qualitative immunohistochemical[7,19] and quantitative radioimmunoassay[7] techniques. The present data further show that a large part of the total immunoreactive material, determined in the heated testicular supernatant (table), is the low molecular weight protein. The high molecular weight immunoreactive protein peak is unique to testis, as this is not observed in liver or the kidney. This peak is not an artifact of heat-treatment because it was also present in the unheated cytosol (data not shown). In all probability, it is the polymerized form of the low molecular weight protein. Polymerization of copper-MT is known to occur[25]. If the low molecular weight Cd-BP contains sufficient copper, it could similarly polymerize into larger aggregates.

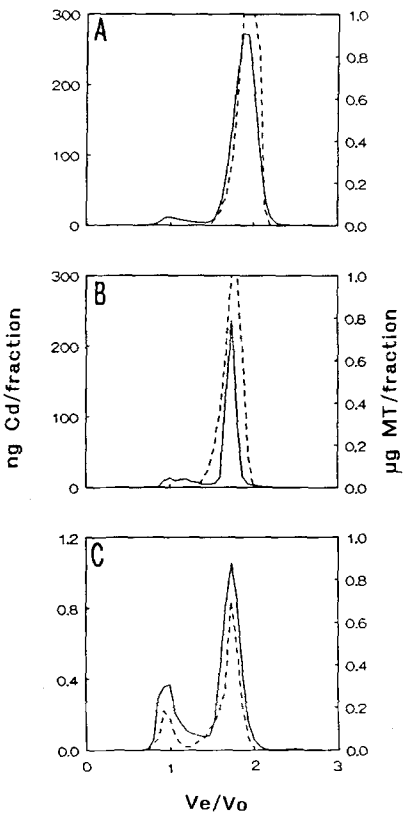

Figure 1. Distribution of ^{109}Cd and MT antibody-reactive proteins in (A) liver, (B) kidney and (C) testis cytosols. Male mice were injected s.c. with ^{109}Cd-labeled or unlabeled $CdCl_2$ (10 µmol/kg, s.c.) and sacrificed 24 h later. ^{109}Cd (—) was determined after gel filtration chromatography of 105,000 × g cytosols on 0.9 × 60 cm Sephadex G-75 superfine columns. MT (– – –) was determined by radioimmunoassay in column fractions obtained from heat-treated supernatants.

Cadmium and MT concentrations in tissues of mice injected with cadmium

Tissue	Cadmium µg/g	% Dose	MT Control	Cadmium-treated
Liver	10.10 ± 0.53	47.40 ± 1.61	6.5 ± 0.2	63.4 ± 3.5*
Kidney	3.06 ± 0.16	5.14 ± 0.39	4.0 ± 0.3	23.9 ± 1.0*
Testis	0.23 ± 0.01	0.15 ± 0.01	18.0 ± 1.7	13.2 ± 0.4 +

Mice were injected with 10 µmol $CdCl_2$/kg and sacrificed 24 h later. Each value is mean ± SE of data from 5 or 6 animals. Cadmium was determined in treated animals only. Percent dose values are for total tissue. MT is expressed as µg/g. * Significantly different from controls ($p < 0.001$). + Significantly different from controls ($p < 0.05$).

The incorporation profiles of labeled cysteine and glutamic acid into hepatic, renal and testicular cytosolic proteins of cadmium-injected mice are depicted in figure 1. Both amino acids were incorporated into high and low molecular weight proteins in all three tissues, indicating that protein synthesis was actively occurring in these tissues. Hepatic MT showed the highest activity of ^{35}S-cysteine and also of ^{3}H-glutamic acid. This was to be

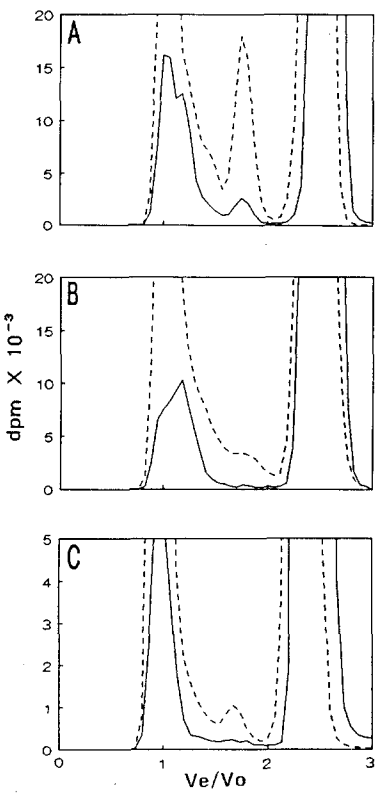

Figure 2. Distribution of ^{35}S-cysteine and ^{3}H-glutamic acid labels in (A) liver, (B) kidney and (C) testis cytosols. Male mice were injected with 10 μmol $CdCl_2$/kg s.c., 6 h later injected i.p. with 100 μCi each of ^{35}S-cysteine and ^{3}H-glutamic acid and sacrificed 24 h after the cadmium injection. ^{35}S (– – –) and ^{3}H (——) were determined after gel filtration chromatography of 105,000 × g cytosols on 0.9 × 60 cm Sephadex G-75 superfine columns.

expected, since liver had the highest level of cadmium-induced MT (table).

A distinct ^{35}S peak was evident in the low molecular weight cadmium-binding region of testicular cytosol (fig. 2). In comparison, there was little incorporation of ^{3}H in this region. Similar results were obtained in control mice and in cadmium-resistant C3H mice (data not shown). These results are supported by the study of Brady and Webb[26] who also found ^{35}S-cysteine incorporation into rat testicular Cd-BP. If this low molecular weight protein from mouse testis contains no cysteine and is indeed rich in glutamic acid[9], we should have observed the opposite results. It may be argued that the lack of ^{3}H incorporation in our study is due to lower specific activity of ^{3}H-glutamic acid and perhaps also due to larger pool size of this amino acid than that of cysteine. However, this argument does not hold because, as stated earlier, the testicular Cd-BP from mouse supposedly has no cysteine[9] and thus there should have been no incorporation of the ^{35}S label, unless the protein is similar to MT. Thus, available information on the a) molecular weight, b) charge characteristics[5,6], c) reactivity with MT antibody, and d) selective incorporation of ^{35}S-cysteine point to the low molecular weight testicular Cd-BP as being MT. The only data against this conclusion are the amino acid composition data. However, these data are not consistent, and the data published by Waalkes et al. vary considerably with respect to the cysteine content[8,9,15,16,20].

Similar controversy existed several years ago when a unique low molecular weight copper-binding protein was reported by Winge et al.[27] and Irons and Smith[28]. This protein also had low cysteine and high glutamic acid content. On further investigation, Bremner and Young[29] noted flaws in the purification procedure and established that the hepatic copper-binding protein was in fact MT. Could this also be the case with testicular Cd-BP? Further study is warranted to clarify the identity of this protein which, by most accounts, appears to be MT.

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