Biochem. J. (1984) **220**, 811–818
Printed in Great Britain

Cadmium-binding proteins of rat testes

Characterization of a low-molecular-mass protein that lacks identity with metallothionein

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(*Received 23 January 1984/Accepted 7 March 1984*)

Cadmium-binding proteins in the cytosol of testes from untreated rats were separated by Sephadex G-75 gel filtration. Three major testicular metal-binding proteins (TMBP), or groups of proteins, with relative elution volumes of approx. 1.0 (TMBP-1), 1.7 (TMBP-2) and 2.4 (TMBP-3) were separated. Elution of Zn-binding proteins exhibited a similar pattern. TMBP-3 has previously been thought to be metallothionein (MT), and hence this protein was further characterized and compared with hepatic MT isolated from Cd-treated rats. Estimation of M_r by gel filtration indicated a slight difference between MT (M_r 10000) and TMBP-3 (M_r 8000). Two major forms of MT (MT-I and MT-II) and TMBP-3 (TMBP-3 form I and TMBP-3 form II) were obtained after DEAE-Sephadex A-25 anion-exchange chromatography, with the corresponding subfractions being eluted at similar conductances. Non-denaturing polyacrylamide-gel electrophoresis on 7% acrylamide gels indicated that the subfractions of TMBP-3 had similar mobilities to those of the corresponding subfractions of MT. However, SDS (sodium dodecyl sulphate)/12% (w/v)-polyacrylamide-gel electrophoresis resulted in marked differences in migration of the two corresponding forms of MT and TMBP-3. Co-electrophoresis of MT-II and TMBP-3 form II by SDS/polyacrylamide-gel electrophoresis revealed two distinct proteins. Amino acid analysis indicated much lower content of cysteine in the testicular than in the hepatic proteins. TMBP-3 also contained significant amounts of tyrosine, phenylalanine and histidine, whereas MT did not. U.v.-spectral analysis of TMBP-3 showed a much lower A_{250}/A_{280} ratio than for MT. Thus this major metal-binding protein in testes, which has been assumed to be MT is, in fact, a quite different protein.

Metallothioneins (MT) are metal-binding proteins containing high levels of cysteine (approx. 18 residues/molecule) with an M_r of approx. 6000 (Kagi & Vallee, 1974). These proteins are thought to be involved in detoxication of certain heavy metals, such as Cd (Leber & Miya, 1976; Probst *et al.*, 1977*a*,*b*), because of the avidity with which they bind such metals (Kagi & Vallee, 1960, 1961). In addition, MT may play an important role in the homoeostatic control of Zn metabolism, particularly during perinatal development, where high levels of Zn-containing hepatic MTs have been found in various mammalian species (Bell, 1979; Wong & Klaassen, 1979; Waalkes & Bell, 1980; Bakka *et al.*, 1981).

Several reports have indicated the presence of a MT-like protein in rodent testes. Work by Nordberg (1971), Chen *et al.* (1974), Singh *et al.* (1974) and Chen & Ganther (1975) have provided evidence for rodent testicular MT after Cd treatment. Webb (1975) has also detected a Zn-binding MT-like protein in untreated rat testes. More recently, Brady & Webb (1981) have quantified MT in developing rat testes. These studies have predominantly depended on gel-filtration profiles of testicular cytosol, detecting Cd- and/or Zn-binding proteins of the approximate M_r of MT.

Abbreviations used: MT(s), metallothionein(s); SDS, sodium dodecyl sulphate; TMPB(s), testicular metal-binding protein(s).

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Various assays for MT, including the Cd-binding method of Onosaka *et al.* (1978) and the Hg-binding method of Piotrowski *et al.* (1973), will detect relatively high levels of MT in untreated rat testes (Wong & Klaassen, 1980; Onosaka & Cherian, 1981, 1982), providing more suggestive evidence for MT in testes. However, these assays measure either acid-stable Hg-binding moieties or non-heat-denaturable Cd-binding ligands and their specificity, although confirmed for liver (Kotsonis & Klaassen, 1977; Eaton & Toal, 1982), have not been adequately tested for other organs.

The reported MT-like protein in testes has several characteristics that differ from those of MT. Chen *et al.* (1972) and Singh *et al.* (1974) have reported that the u.v.-absorption spectra of the testicular MT-like protein differs markedly from that of hepatic or renal MT. It has also been reported that, unlike hepatic MT, the testicular MT-like protein does not accumulate Zn after Cd exposure (Whanger *et al.*, 1980). Furthermore, unlike other major organs where Cd treatment results in increased MT concentrations, a decrease is observed in testes (Onosaka & Cherian, 1981). Chen & Ganther (1975) have reported that the rat testes also contains a 30 kDa protein that has a higher affinity for Cd than the testicular MT-like protein, in contrast with the Cd-binding proteins of other major organs.

Despite numerous reports of testicular MT in the literature, definitive proof of its identity, such as amino acid analysis, is lacking. Therefore, the present study was performed in order to further characterize this testicular metal-binding protein (TMBP) and to determine if it is, in fact, MT.

Part of this work was presented at the 1983 Annual Meeting of the American Society of Pharmacology and Experimental Therapeutics, Philadelphia, PA, U.S.A.

Materials and methods

Experimental

Chemicals. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless otherwise noted. Radioisotopic Cd (^{109}Cd) and Zn (^{65}Zn) in HCl were obtained from New England Nuclear Corp. (Boston, MA, U.S.A.). Sephadex G-75 and DEAE-Sephadex A-25 resins were obtained from Pharmacia (Piscataway, NJ, U.S.A.). All chemicals for electrophoresis were obtained from Bio-Rad (Richmond, CA, U.S.A.) and were of electrophoretic-grade quality.

Animals and sample preparation. Adult male Sprague–Dawley rats (200–250g) were allowed food and water *ad libitum*. Rats were decapitated and testes removed, trimmed, weighed and homogenized (1:4, w/v) in 10mM-Tris/acetate buffer, pH7.4, at 4°C. Cytosol was derived by differential centrifugation at 10000*g* for 10min, followed by 100000*g* for 60min, and used immediately for gel-filtration chromatography. Hepatic cytosol from Cd-treated (2.25mg of Cd/kg, 24 and 48h before the animals were killed) rats was prepared in the same fashion.

Gel-filtration chromatography. For gel filtration, samples were initially saturated with Cd by the addition of 1.2μmol of Cd/g wet weight equivalent (containing 0.125μCi of ^{109}Cd/μmol of Cd), an amount previously reported to saturate the low-M_r Cd-binding proteins of testes (Chen & Ganther, 1975). Such an addition *in vitro* has also been shown to duplicate the binding of Cd to testicular proteins *in vivo* (Brady & Webb, 1981). Samples (5ml) of cytosol (1g wet wt. equivalent) were applied to a column (2.6cm × 60cm) of Sephadex G-75 previously equilibrated with 10mM-Tris/acetate, pH7.4, containing 0.02% NaN_3, and proteins were eluted with a flow rate of 24ml/h at 4°C. Samples (100 × 10min) were collected (4ml each) and analysed for Cd γ-radiation spectrometry (Packard model 5130 auto-gamma scintillation spectrometer).

In a separate series of experiments, cytosols were saturated with 1.2μmol of Zn/g wet weight equivalent (containing 0.75μCi of ^{65}Zn/μmol of Zn). Zn-binding proteins of testicular cytosol were subsequently separated by gel filtration.

Columns were calibrated with the following proteins for determination of M_r by gel filtration: bovine albumin (M_r 66000), ovalbumin (45000), glyceraldehyde-3-phosphate dehydrogenase (36000), carbonic anhydrase (29000), trypsinogen (PMSF-treated) (24000), α-lactalbumin (14200) and insulin B-chain (3500). The elution volumes of standards were determined by A_{215}, and the void volume was determined with Blue Dextran.

Anion-exchange chromatography. Anion-exchange chromatography was accomplished by applying the low-M_r metal-binding proteins of either testes or liver from gel filtration to a column (2.6cm × 35cm) of DEAE-Sephadex A-25 resin previously equilibrated with 10mM-Tris/acetate. The column was then eluted (flow rate 24ml/h) with a linear gradient of Tris/acetate (10–200mM). Fractions (140 × 10min) were collected and analysed for Cd. The conductivity of the fractions was measured by using a conductivity bridge (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.; model 31). The major peaks were then either directly used for non-denaturing polyacrylamide-gel electrophoresis or dialysed for 48h against several changes of distilled water, freeze-dried and used for SDS/polyacrylamide-gel electrophoresis, amino acid analysis or u.v.-spectral analysis.

Polyacrylamide-gel electrophoresis. The Cd peaks from anion exchange were used directly for non-denaturing polyacrylamide-gel electrophoresis by the method of Davis (1964) as modified by Wong & Klaassen (1979). An acrylamide concentration of 2.5% (w/v) was used in the concentrating gel and one of 7% in the separating gel. For SDS/polyacrylamide-gel electrophoresis a modification of the method of Sobocinski *et al.* (1978) was used, 12%-acrylamide gels being used. Separations were performed at 24°C in an electrophoretic chamber (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). Samples (approx. 15–30 μg of protein) were applied and electrophoresed with a constant current of 2.5 mA/gel. Gels were fixed and stained in a solution of 0.1% Coomassie Blue in 7% (v/v) acetic acid for 4 h (7% gels) or overnight (12% gels). Gels were then de-stained in acetic acid/propan-2-ol/water (1:1:8, by vol.), by using a diffusion destainer apparatus (Bio-Rad, model 172 A) and scanned at 650 nm with a Gilford model 240 spectrophotometer.

Amino acid analysis. Amino acid analysis was performed by the method of Dean *et al.* (1983). Samples were first hydrolysed in glass-distilled constant-boiling 6 M-HCl at 100°C for 24 h. Hydrolysates were then analysed on a Dionex (Sunnyvale, CA, U.S.A.) D-300 instrument with a single micro-bore column. Half-cysteine was measured by the method of Schram *et al.* (1954) as cysteic acid and corrected for standard losses.

U.v.-spectral analysis. Freeze-dried protein samples derived from anion-exchange chromatography of testes or liver were dissolved in Tris/acetate buffer and scanned between 220 and 310 nm with a Beckman model 25 spectrophotometer.

Results

Fig. 1 shows a representative gel-filtration elution profile for Cd-saturated testicular cytosol. Results indicate the presence of three major TMBP species or groups of proteins with relative elution volumes of approx. 1.0, 1.7 and 2.4, henceforth referred to as 'TMBP-1', 'TMBP-2' and 'TMBP-3' respectively. TMBP-3 has previously been classified as MT (Brady & Webb, 1981). Similar results were obtained with Zn-saturated testicular cytosols, whereas hepatic cytosol from Cd-treated rats showed a predominant Cd-binding protein (MT) with a relative elution volume of approx. 2.25 (results not shown).

After calibration of the Sephadex G-75 column, the M_r values of TMBP-3 and hepatic MT were estimated by gel filtration. The M_r of hepatic MT was determined to be 10000, whereas that of TMBP-3 was found to be slightly less (8000).

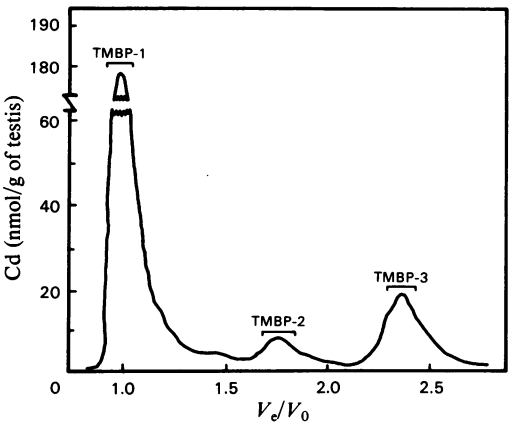

Fig. 1. *Sephadex G-75 elution profile of testicular cytosol from untreated rats after saturation with Cd in vitro*
The column was eluted with 10 mM-Tris/acetate buffer, pH 7.4, at a rate of 24 ml/h. Three distinct Cd peaks were discernible, termed TMBP-1, -2 and -3 respectively.

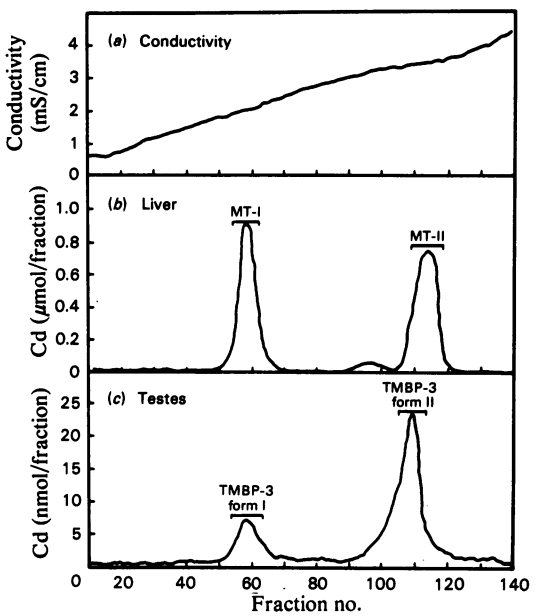

Fig. 2. *Anion-exchange chromatography on DEAE-Sephadex A-25 resin of hepatic MT and TMBP-3 after Cd-saturation*
The proteins were eluted with a linear gradient of Tris/acetate (10–200 mM) as described in the Materials and methods section. Conductivity of the effluent (mS/cm) is shown in (*a*). Hepatic MT (*b*) and TMBP-3 (*c*) both separated into two major forms (termed MT-I and MT-II and TMBP-3 form I and TMBP form II respectively) and were eluted at similar conductivities.

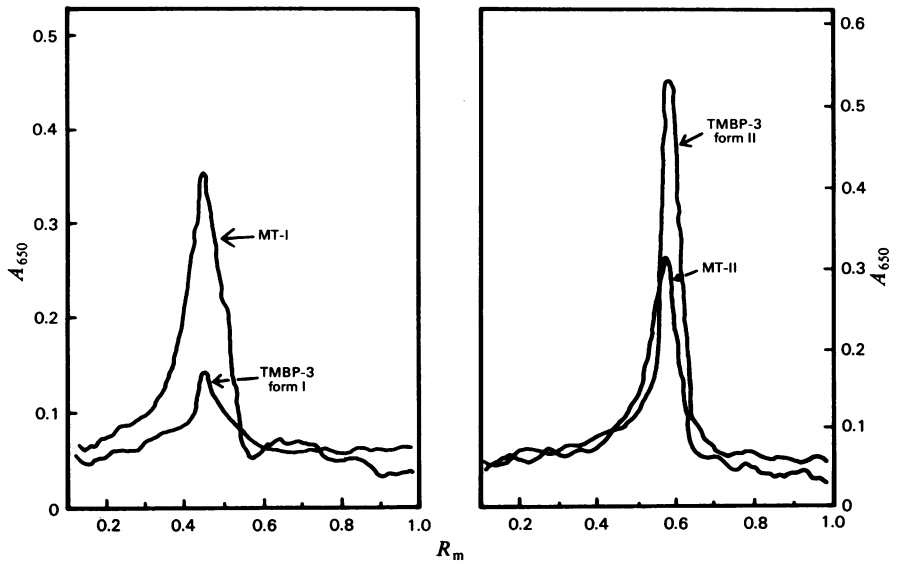

Fig. 3. *Non-denaturing polyacrylamide-gel electrophoretic profiles of MT-I, MT-II and TMBP-3 form I and II*
The gels were scanned at 650nm and relative mobility (R_m) was based on the mobility of the tracking dye (Bromophenol Blue).

TMBP-3 and MT were subjected to anion-exchange chromatography (Fig. 2). Both proteins separated into two major subfractions, being eluted at conductivities of approx. 2mS/cm (MT-I and TMBP-3 form I) and 3.4mS/cm (MT-II and TMBP-3 form II). Similar results were obtained with Zn-saturated testicular cytosols (results not shown). TMBP-3 form II appeared to predominate, whereas the two forms of hepatic MT were approximately equivalent.

Non-denaturing polyacrylamide-gel electrophoresis was performed with the two major forms of MT and TMBP-3 from anion-exchange chromatography (Fig. 3). Results indicate that the two protein forms from the liver and testes migrated similarly.

SDS/polyacrylamide-gel electrophoresis (12% gels), however, showed marked differences between the two forms of MT and TMBP-3 (Fig. 4). MT-I showed a predominant peak with a relative mobility (R_m) of 0.64, whereas TMBP-3 form I had an R_m of 0.58. MT-II had an R_m of 0.72, whereas that of TMBP-3 form II was 0.59. MT-I also showed a minor peak with an R_m of 0.34. When MT-II and TMBP-3 form II were co-electrophoresed (Fig. 5), two distinct protein peaks were apparent.

Amino acid analyses of the two forms of MT and TMBP-3 are shown in Table 1. Marked differences are evident, particularly in cysteine content, which is much lower in TMBP than in MT. MT also has higher serine and lysine contents, whereas the forms of TMBP contain more leucine and isoleucine. TMBP also contains significant amounts of tyrosine, phenylalanine and histidine, whereas, consistent with previous reports (Kagi & Vallee, 1974; Sobocinski *et al.*, 1978; Wong & Klaassen, 1979), MT does not.

U.v. spectral analysis of TMBP-3 form II and hepatic MT-II also showed a marked difference between the two proteins (Fig. 6). The absorbance spectra of TMBP indicates a much lower A_{250}/A_{280} ratio than that of MT.

Discussion

The results of the present study indicate that the Cd/Zn-binding protein of the cytosol of rat testes, previously assumed to be MT (Nordberg, 1971; Chen *et al.*, 1974; Chen & Ganther, 1975; Webb, 1975; Brady & Webb, 1981), is not MT. The TMBP does display several striking similarities to MT. In agreement with several previous studies, the present results demonstrate that the TMBP behaves similarly to MT on gel-filtration chromatography (Nordberg, 1971; Singh *et al.*, 1974; Chen & Ganther, 1975; Brady & Webb, 1981), indicating a similar M_r. However, gel filtration gives only an approximation of M_r and has been shown to be a rather poor indicator of the M_r of MT, yielding values that are approximately twice that obtained by amino acid analysis (Pulido *et al.*, 1966; Winge & Rajagopalan, 1972). The present study confirms the results of Brady &

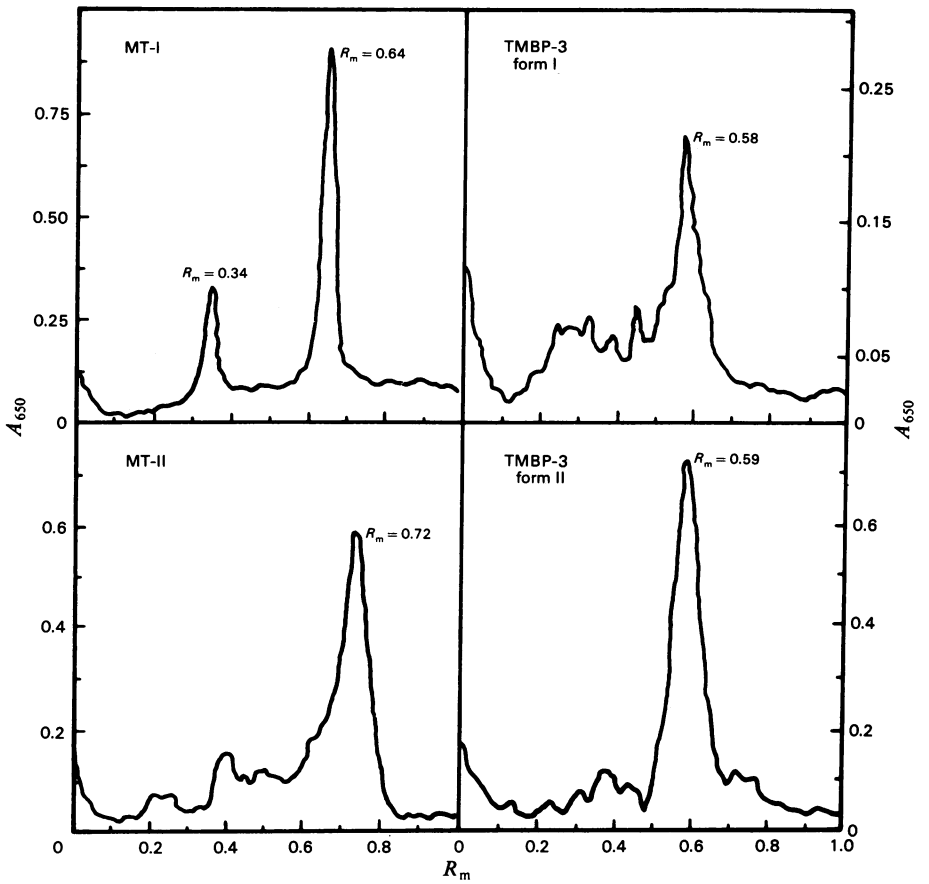

Fig. 4. *SDS/polyacrylamide-gel electrophoretic profiles of MT-I, MT-II, TMBP-3 form I and TMBP-3 form II*
The gels were scanned at 650nm and relative mobility (R_m) was based on the mobility of the tracking dye (Bromphenol Blue).

Webb (1981) that the low-M_r TMBP can be divided into two major subfractions by anion-exchange chromatography. The predominance of one form of the testicular protein has been previously noted (Brady & Webb, 1981). The subfractions of TMBP were eluted at conductivities similar to those of eluent eluting the corresponding forms of MT, indicating similar charges but not necessarily identity. Likewise, non-denaturing polyacrylamide-gel electrophoresis also showed a similarity of charge between the corresponding forms of the TMBP and MT.

The behaviour of this TMBP on gel filtration, anion-exchange chromatography and non-denaturing polyacrylamide-gel electrophoresis constitute the evidence used to indicate that this protein is MT (Brady & Webb, 1981). However, extension of these observations to include SDS/polyacrylamide-gel electrophoresis and amino acid and u.v.-spectral analysis shows the lack of identity of the TMBP with MT. Results obtained with SDS/polyacrylamide-gel electrophoresis indicate small differences in M_r between the forms of MT and TMBP. The two peaks detected after co-electrophoresis of MT-II and TMBP form II strongly suggest that they are different proteins. The minimum M_r values of TMBP and MT as determined by amino acid analysis were only slightly different, and would probably not fully account for the separation detected. Several factors other than M_r can affect protein migration in an SDS/polyacrylamide-gel electrophoresis system, including extent of SDS binding and structural features (Tung & Knight, 1972). Furthermore, several authors have shown that MT will migrate anomalously with regard to M_r in such systems (Weser *et al.*, 1973; Sobocinski *et al.*, 1978), and this may account for the separation of the TMBP-3 and MT detected in the present study.

The strongest evidence that this TMBP is not MT is amino acid analysis. In accord with numerous reports (Nordberg *et al.*, 1972; Weser *et*

al., 1973; Kagi & Vallee, 1974; Sobocinski *et al.*, 1978; Wong & Klaassen, 1979; Whanger & Deagen, 1982), purified MT was found to contain high levels of cysteine (17–18 residues/molecule),

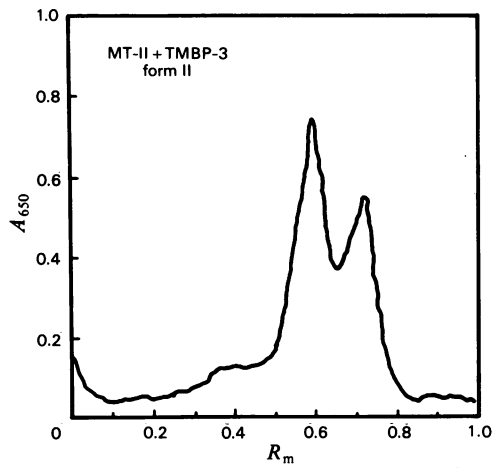

Fig. 5. *SDS/polyacrylamide-gel co-electrophoresis of TMBP-3 and MT-II*

Samples of TMBP-3 and MT-II were mixed before electrophoresis. Gels were scanned at 650nm, and relative mobility (R_m) is based on the mobility of the tracking dye (Bromophenol Blue)

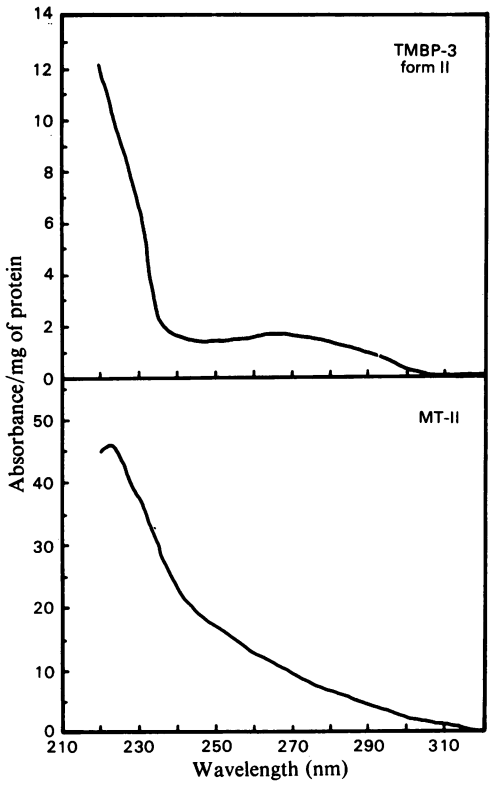

Fig. 6. *U.v.-spectral analysis of hepatic MT-II and TMBP-3 form II*

The absorbance spectra of the two proteins was determined between 310 and 220nm.

Table 1. *Amino acid compositions of hepatic MT and TMBP-3*

Results are means of two determinations. Values in parentheses are nearest whole numbers.

	Amino acid composition (residues/molecule)			
	MT		TMBP-3	
Amino acid	Form I	Form II	Form I	Form II
Asp	5.40 (5)	4.74 (5)	3.98 (4)	5.28 (5)
Thr	0.40 (0)	2.60 (3)	3.18 (3)	2.56 (3)
Ser	11.35 (11)	6.89 (7)	4.63 (5)	3.15 (4)
Glu	1.54 (2)	4.11 (4)	6.02 (6)	6.45 (6)
Pro	2.64 (3)	2.33 (2)	2.50 (3)	3.83 (4)
Gly	7.76 (8)	5.00 (5)	6.90 (7)	6.28 (6)
Ala	3.93 (4)	5.57 (6)	5.00 (5)	3.48 (3)
Val	2.74 (3)	1.59 (2)	3.45 (3)	3.99 (4)
½-Cys	16.79 (17)	17.68 (18)	4.16 (4)	3.01 (3)
Met	1.63 (2)	1.26 (1)	1.46 (1)	0.78 (1)
Ile	0.20 (0)	0.86 (1)	2.66 (3)	1.48 (1)
Leu	0.39 (0)	0.72 (1)	3.90 (4)	5.04 (5)
Tyr	0.09 (0)	0.17 (0)	0.85 (1)	1.27 (1)
Phe	0.12 (0)	0.24 (0)	1.36 (1)	1.46 (1)
His	0.20 (0)	0.15 (0)	1.46 (1)	1.49 (1)
Lys	7.68 (8)	8.25 (8)	4.36 (4)	4.30 (4)
Arg	0.15 (0)	0.23 (0)	2.27 (2)	2.09 (2)
Total no. of residues ...	63	63	57	54
Minimum M_r ...	6132	6334	6090	6010

in contrast with the forms of TMBP, which contained only three to four residues/molecule. Among several other differences, the TMBPs also contained approximately half the amount of serine and lysine found in the two forms of MT. The presence of aromatic amino acids in TMBP may account for the u.v.-spectral differences between it and MT found in present and previous studies (Chen *et al.*, 1972; Singh *et al.*, 1974).

Several authors have suspected that this low-M_r TMBP is different from MT. Earlier work by Singh *et al.* (1974) has been cited as evidence for testicular MT [see reference no. 12 in Brady & Webb (1981)], although the authors state that the testicular protein, though similar to MT in some respects, is quite different from the Cd-binding protein of liver and kidney in several characteristics. Onosaka & Cherian (1981), commenting on the observation that Cd treatment caused a decrease in MT-like protein in rat testes, contrary to results in other tissues, suggest that the metal binding of testes may involve proteins other than MT. Whanger's group has also frequently stated that the protein in testes is not MT (Whanger *et al.*, 1980, 1981; Whanger & Deagen, 1982), on the basis of various observed differences. The results of the current study confirm these suspicions.

The present study suggests that more rigorous standards of characterization should be applied before classification of a protein as MT. This would appear to be true for the low-M_r Cd-binding protein of rat testes. A further example is the Cd-binding protein isolated from metal-exposed oysters, which, although similar to MT in M_r, amino acid analysis shows not to be MT (Ridlington & Fowler, 1979). Likewise, the Cd-binding protein found in rabbit lung after inhalation of Cd, although similar to MT by gel filtration, appears to be predominantly non-MT protein(s) by behaviour on anion-exchange chromatography (Post *et al.*, 1982).

Because MT is thought to play an important role in detoxication of Cd (Leber & Miya, 1976; Probst *et al.*, 1977*a*,*b*), the apparent absence of MT from rat testicular cytosol may possibly account for the unique sensitivity of this organ to Cd (Parizek & Zahor, 1956). Despite the observation that only a small portion (approx. 0.2%) of a dose of Cd localizes in testes (Wong & Klaassen, 1980), this organ readily develops oedema, haemorrhage and necrosis at doses which are non-toxic to other organs (Parizek & Zahor, 1956).

In conclusion, the present data strongly suggest that the low-M_r metal-binding protein of rat testes is quite different from MT. The apparent lack of MT in the rat testes may, in part, account for the unique sensitivity of this organ to the toxic effects of Cd.

Funding for this work was provided by U.S. Public Health Service grant ES-01142. M. P. W. was supported by funds from National Institutes of Environmental Health Sciences training grant ES-07079. C. D. K. is the recipient of a Burroughs–Wellcome Toxicology Scholar Award.

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