Cadmium-binding proteins of rat testes

Apparent source of the protein of low molecular mass

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Fractionation of rat testicular cytosolic proteins by gel filtration indicates three major metal-binding proteins, or groups of proteins, termed testicular metal-binding protein (TMBP) 1, 2 and 3 by order of elution. The major heat-stable, metal-binding proteins in testes is TMBP-2, which has an M_r of approx. 25 000. In most tissues, metallothionein (MT) is the major heat-stable, metal-binding protein, but it has an M_r of 6000. This testicular protein (TMBP-2) is much larger than MT, and since polymeric forms of MT have been previously reported, further characterization of TMBP-2 was performed. TMBP-2 was separated into two forms by DEAE-Sephadex A-25 anion-exchange chromatography. Amino acid analysis of both forms of TMBP-2 revealed that they differed markedly from MT, having particularly low cysteine contents. However, amino acid analysis showed that TMBP-2 was strikingly similar to TMBP-3, with an approximate stoichiometric relationship of 4:1. Therefore, experiments were conducted to determine if TMBP-3 could be a breakdown product of TMBP-2. Heat treatment of testicular cytosol in room air before gel filtration resulted in a marked increase in TMBP-3 and loss of TMBP-2. Storing intact testes at -20°C for 2 weeks before processing for gel filtration also resulted in an increase in TMBP-3 and a loss of TMBP-2. Addition of a reducing agent (dithiothreitol) or proteinase inhibitor (*N*-ethylmaleimide) in processing of samples before gel filtration inhibited the appearance of TMBP-3. Results suggest that the low- M_r Cd-binding protein (TMBP-3) of rat testes results from either proteolytic or oxidative breakdown of a higher- M_r species, or from a combination of such factors.

Metallothionein (MT), a low- M_r (approx. 6000) metal-binding protein, is thought to be present in relatively high amounts in testes (Brady & Webb, 1981). In the absence of metal treatment, this protein contains predominantly Zn, and, because of the high Zn demand of testes, it has been proposed that MT may play an important role in homeostatic control of testicular Zn (Brady & Webb, 1981; Brady, 1982). However, the preceding paper (Waalkes *et al.*, 1984) strongly suggests that the

low- M_r Cd/Zn-binding protein of testicular cytosol with an M_r similar to that of MT, differs markedly from MT in several important characteristics. This testicular metal-binding protein (TMBP) differs from MT not only in u.v.-spectral characteristics and migratory behaviour on SDS/polyacrylamide-gel electrophoresis, but also in amino acid content, it being particularly low in cysteine.

Despite the differences between the low- M_r Cd-binding protein of testes and MT, several widely employed assay systems for MT quantification detect high basal levels in this organ. The Cd-haemoglobin assay (Onosaka *et al.*, 1978) indicates levels in control rat testes to be the highest among those in eleven tissues examined (Onosaka & Cherian, 1981, 1982). Other commonly used techniques of MT quantification, such as the Hg-

Abbreviations used: MT(s), metallothionein(s); TMBP(s), testicular metal-binding protein(s).

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saturation method (Piotrowski *et al.*, 1973), also indicate high basal testicular levels (Wong & Klaassen, 1980). Both of these assay systems are based on initial saturation of a sample with a metal of known high affinity for MT, followed by separation of MT-bound and non-MT-bound metal by taking advantage of MT's heat- or acid-stability. The Cd-haemoglobin and Hg-saturation assays have been verified for liver, having been shown to detect species of the appropriate M_r of MT on gel filtration (Kotsonis & Klaassen, 1977; Eaton & Toal, 1982). However, verification of these methods is lacking for testes, and the M_r of the protein detected by such systems is unknown.

Chen *et al.*, 1974; Chen & Ganther (1975a,b) have reported the presence of a Zn-binding protein in rat testes that has an M_r approximately three to four times that of MT and has a higher affinity for Cd than does the low- M_r protein in testes previously assumed to be MT (Brady & Webb, 1981). Furthermore, there are numerous reports in the literature concerning polymerized or aggregate forms of MT (Wolkowski, 1974; Wolkowski-Tyl & Preston, 1979; Riordan & Richards, 1980; Sharma & Verma, 1980). Wolkowski (1974) reported the presence of MT dimers in certain mouse strains, apparently dependent on endocrine status. Interestingly, the levels of MT dimers in liver have been positively correlated with prevention of testicular necrosis after Cd administration (Wolkowski-Tyl & Preston, 1979), a metal known to be highly toxic to testes (Parizek & Zahor, 1956). Riordan & Richards (1980) have suggested that MT normally exists in an aggregate form in human foetal liver that can be extracted as the monomer by using high concentrations of reducing agent. Sharma & Verma (1980) found that bovine hepatic MT consists of two monomers joined together.

The existence of polymerized forms of MT in testes could explain the apparent discrepancy between the high basal levels of MT detected in testes by metal-saturation assays *in vitro* (Onosaka & Cherian, 1981, 1982) and the apparent lack of low- M_r species of MT in testes (Waalkes *et al.*, 1984). Initial experiments separating metal-binding proteins of rat testes by gel filtration and quantification by the Cd-haemoglobin assay for MT indicated that the assay predominantly detected a protein with M_r approx. 25000. Hence the primary goal of the present study was to determine if this metal-binding protein in rat testes was a polymerized form of MT.

Materials and methods

Chemicals

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless otherwise

noted. Radioisotopic Cd (^{109}Cd) in HCl was 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.).

Animals and sample preparation

Adult male Sprague-Dawley rats (200–250g) from SASCO (Omaha, NE, U.S.A.) 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, pH 7.4, at 4°C. Cytosol was derived by differential centrifugation at 10000g for 10 min, followed by 100000g for 60 min, and used immediately for gel-filtration chromatography. Portions of testes (1g) were also placed in buffer and then in boiling water for 5 min before preparation of cytosol. In some cases intact testes were stored at -20°C for 2 weeks before processing for gel filtration. In certain instances, testes were also homogenized in buffer containing either a proteinase inhibitor (2.5mM-N-ethylmaleimide) or a reducing agent (1mM-dithiothreitol).

Gel-filtration chromatography

For gel filtration, samples were initially saturated with Cd by the addition of 1.2 μmol of Cd/g wet weight equivalent (containing 0.125 μCi of $^{109}\text{Cd}/\mu\text{mol}$ of Cd), an amount previously reported to saturate the low- M_r Cd-binding proteins of testes (Chen & Ganther, 1975a). Such saturation *in vitro* has been shown to duplicate the pattern of Cd binding in rat testes *in vivo* (Brady & Webb, 1981). Samples (5 ml) of cytosol (1g wet weight equivalent) were applied to a column (2.6cm $\times$ 60cm) of Sephadex G-75 previously equilibrated with 10mM-Tris/acetate, and proteins were separated at a flow rate of 24ml/h. Fractions (100 $\times$ 10min) were collected (4ml each) and analysed for Cd by γ -radiation spectrometry with a Packard model 5130 auto-gamma scintillation spectrometer. M_r was determined by using a calibrated column of Sephadex G-75 as reported in the preceding paper (Waalkes *et al.*, 1984).

Cd-haemoglobin assay. Metal-binding capacity of the testicular cytosol proteins was quantified in the Sephadex G-75 eluate by the method of Onosaka *et al.* (1978) as modified by Eaton & Toal (1982). Portions of the eluate (200 μl) were incubated for 10 min with 200 μl of ^{109}Cd solution (2 μg of Cd/ml; 3 μCi of ^{109}Cd /ml) in a 1.5 ml polyethylene micro-centrifuge tube before the addition of 100 μl of 2% (w/v) bovine haemoglobin (Sigma Type I). The samples were then incubated at 100°C for 1 min, cooled on ice and centrifuged at 10000g (Beckman model B micro-centrifuge) for 1 min at room temperature. Another 100 μl portion of

haemoglobin solution was added and the heat-denaturation and subsequent centrifugation repeated. A 200 μ l portion of the final supernatant was removed and Cd was determined.

Anion-exchange chromatography

Anion-exchange chromatography was performed by applying selected gel-filtration eluates containing Cd-binding proteins on to a column (2.6 cm $\times$ 35 cm) of DEAE-Sephadex A-25 resin previously equilibrated with 10 mM-Tris/acetate, pH 7.4, at 4°C. The column was then eluted with a linear gradient of buffer generated by mixing 300 ml of 10 mM-Tris/acetate and 300 ml of 200 mM-Tris/acetate. Fractions (140) were collected and analysed for Cd. Conductivity was measured by using a conductivity bridge (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.; model 31). The major Cd-binding proteins were then dialysed against several changes of distilled water for 48 h, freeze-dried and used for amino acid analysis.

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) 6M-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 microbore column. Minimum M_r was calculated from amino acid analysis by setting the methionine content to one residue/molecule.

Results

The major Cd-binding proteins of testicular cytosol after addition of Cd *in vitro* and after separation by gel-filtration chromatography are shown in Fig. 1(a). Results suggest three major metal-binding proteins, or groups of proteins, termed TMBP-1, -2 and -3 in order of elution. Fig. 1(b) illustrates the results obtained when the eluate was assayed for MT by the Cd-haemoglobin method (Onosaka *et al.*, 1978; Eaton & Toal, 1982). The assay predominantly detected proteins eluted in the range of TMBP-2. Estimation of the M_r of TMBP-2 on a calibrated column of Sephadex G-75 indicates an approximate value of 25 000. The Cd-haemoglobin method did not detect metal-binding proteins in the area of TMBP-3, the protein previously assumed to be MT (Brady & Webb, 1981).

Since polymeric forms of MT have been reported (Wolkowski, 1974; Wolkowski-Tyl & Preston, 1979; Riordan & Richards, 1980; Sharma & Verma, 1980), further characterization of TMBP-2 was performed to determine its nature. Results of anion-exchange chromatography, using DEAE-Sephadex A-25 resin, are shown in Fig. 2. The testicular protein separated into two major sub-fractions, termed TMBP-2 form I and form II res-

Fig. 1. Assay of Sephadex G-75 gel-filtration fractions of rat testicular cytosol as determined by gel filtration of Cd-saturated cytosol (a) and for metal-binding, heat-stable proteins by the Cd-haemoglobin technique (b).

Three major Cd-binding proteins, or groups of proteins, termed TMBP-1, -2 and -3, were detected by gel filtration, whereas the Cd-haemoglobin method predominantly detected TMBP-2.

Fig. 2. Anion-exchange chromatography (DEAE-Sephadex A-25) of TMBP-2 derived by gel filtration of rat testicular cytosol.

The proteins (b) were eluted with a linear gradient of Tris/acetate buffer (10–200 mM). The conductivity of the effluent is shown in (a). TMBP-2 separated into two major forms termed TMBP-2 forms I and II respectively, by order of elution.

pectively, by order of elution. These forms were eluted at conductivities consistent with those reported in the preceding paper for the two forms of MT (Waalkes *et al.*, 1984).

Amino acid analyses of the two forms of TMBP-2, TMBP-3 and hepatic MT are shown in Table 1. The analyses show that TMBP-2 and MT differ markedly in amino acid content. TMBP-2 has a much lower cysteine content than MT. The lysine content of TMBP-2 tended to be lower than MT, whereas the number of glutamate and leucine residues were generally higher. TMBP-2 also contained significant amounts of phenylalanine and histidine, amino acids not present in MT. The amino acid content of TMBP-2 was strikingly similar to that of the low- M_r Cd-binding protein of testes (i.e. TMBP-3). Comparison of the amino acid compositions of TMBP-2 and TMBP-3 indicate that they are very similar, but both forms of TMBP-3 did appear to contain more serine and glutamate than TMBP-2, whereas TMBP-2 form I contained more cysteine than TMBP-3 form I.

The minimum M_r values for TMBP-2 forms I and II, as determined by amino acid analysis, were 5800 and 6200 respectively. When these data are compared with the M_r of TMBP-2, as determined by gel filtration (25000), a stoichiometric relationship of approximately 4:1 is apparent.

Previous studies have shown that the Cd-binding protein of rat testes corresponding to TMBP-2 of the present study is rather unstable (Chen *et al.*, 1974; Chen & Ganther, 1975a,b) and that when testicular samples are not used for gel filtration

immediately after preparation, there is an apparent loss of TMBP-2 with a corresponding increase in TMBP-3. Because of the similarities between the amino acid contents of TMBP-3 and TMBP-2 and reported lability of the latter protein, experiments were performed to determine if TMBP-3 was a breakdown product of TMBP-2. Fig. 3 shows the gel-filtration elution profile for the Cd-binding proteins of testicular cytosol after heat treatment (5 min in boiling water in room air). Results show that such treatment led to an increase in Cd binding in the region of TMBP-3 and a loss in the region corresponding to TMBP-2 and high- M_r proteins (TMBP-1). Freezing and thawing whole testes to enhance the rupture of lysosomes and thus increase proteolysis before gel filtration also

Fig. 3. Gel filtration (Sephadex G-75) of Cd-saturated rat testicular cytosol after intentional oxidation of the sample by heat treatment in room air

Table 1. Amino acid analysis of TMBP-2 and -3 and hepatic MT
Results are means of two determinations.

Amino acid	Composition (% of total residues)					
	TMBP-2		MT		TMBP-3	
	Form I	Form II	Form I	Form II	Form I	Form II
Asp	8.1	11.5	8.6	7.5	7.0	9.8
Thr	5.7	5.1	0.6	4.1	5.6	4.7
Ser	12.9	10.3	18.0	10.9	8.1	6.5
Glu	14.5	13.3	2.4	6.5	10.6	11.9
Pro	3.3	7.4	4.2	3.7	4.4	7.1
Gly	13.1	11.1	12.3	7.9	12.1	11.6
Ala	6.0	8.0	6.2	8.8	8.8	6.4
Val	4.3	7.3	4.3	2.5	6.1	7.4
½-Cys	13.6	6.2	26.8	28.1	7.3	5.6
Met	1.3	1.3	2.6	2.0	2.6	1.4
Ile	1.8	2.8	0.3	1.4	4.7	2.7
Leu	3.6	8.2	0.6	1.1	6.8	9.3
Tyr	0.8	1.6	0.1	0.2	1.5	2.4
Phe	1.3	2.0	0.2	0.3	2.4	2.7
His	1.4	2.5	0.3	0.2	2.6	2.8
Lys	7.8	10.1	13.1	13.1	7.6	8.0
Arg	1.8	2.8	0.3	0.3	4.3	3.9

resulted in a loss of Cd binding in the area of TMBP-2 with a corresponding increase in TMBP-3 (Fig. 4).

The use of a reducing agent, dithiothreitol, in

Fig. 4. Gel filtration (Sephadex G-75) of Cd-saturated rat testicular cytosol prepared from whole testes that had been stored for 2 weeks at -20°C

Fig. 5. Gel filtration (Sephadex G-75) of Cd-saturated rat testicular cytosol prepared in the presence of (a) 1.0 mM-dithiothreitol (+DTT) or (b) 2.5 mM-ethylmaleimide (+NEM)

In (a) the reducing agent was also employed in the eluting buffer. In both cases there was loss of Cd binding in the region of TMBP-3.

sample preparation and chromatography (Fig. 5a) resulted in no metal binding in the region of TMBP-3. Similar results were obtained by the use of a proteinase inhibitor, *N*-ethylmaleimide, in sample preparation before gel filtration (Fig. 5). In both cases there was more binding of Cd to TMBP-1, with no apparent increase in TMBP-2. Assay of fractions for protein (results not shown) indicated its absence from the region of TMBP-3.

Discussion

Despite the various reports concerning aggregate forms of MT (Wolkowski, 1974; Wolkowski-Tyl & Preston, 1979; Riordan & Richards, 1980; Sharma & Verma, 1980), the results of the present study suggest that the intermediate- M_r (25 000) Cd-binding protein of rat testes (TMBP-2) is not a polymerized form of MT. The marked differences in amino acid composition substantiate this conclusion. It can also be ascertained from these data that the Cd-haemoglobin assay method (Onosaka *et al.*, 1978; Eaton & Toal, 1982) is not specific for MT, at least in rat testes. Furthermore, the present results, in conjunction with those of the preceding paper (Waalkes *et al.*, 1984), indicate that neither the intermediate- M_r (i.e. 24 500; TMBP-2) nor the low- M_r (i.e. 6000; TMBP-3) Cd-binding protein in rat testicular cytosol is MT.

However, the two testicular proteins, TMBP-2 and TMBP-3, did show several characteristics in common. Anion-exchange chromatography separated TMBP-2 into two major isoproteins at conductivities similar to those reported for TMBP-3 (Waalkes *et al.*, 1984). This indicates that both proteins have subfractions with similar accessible charge, as the anion-exchange resin (A-25) would not have impeded the elution of proteins in this M_r range. Amino acid analysis of TMBP-2 also showed that it is strikingly similar to TMBP-3. The minimum M_r of TMBP-2 (approx. 6000), as determined by amino acid analysis, was the same as that of TMBP-3 and, in conjunction with the M_r of TMBP-2 being 25 000 as determined by gel filtration, indicates a stoichiometric relationship of approx. 4:1.

A further relationship between TMBP-2 and TMBP-3 is evident after heat treatment or freezing and thawing of whole testes before gel filtration. These conditions resulted in a marked increase in metal binding associated with TMBP-3 and a decrease with TMBP-2. Several reports (Chen *et al.*, 1974; Chen & Ganther, 1975a,b) have previously indicated that this intermediate- M_r protein (TMBP-2) of rat testes is rather unstable and have shown this corresponding loss of Cd-binding capacity in the region of TMBP-2 and gain in TMBP-3 on storage. Furthermore, when a reducing agent, dithiothreitol, or proteinase inhibitor, *N*-ethyl-

maleimide, was used in preparing samples for gel filtration in the present study, there was no detectable Cd-binding protein in the low- M_r region, indicating that dithiothreitol and *N*-ethylmaleimide prevented the formation of TMBP-3. Chen & Ganther (1975b) also observed that dithiothreitol prevented loss of the Cd-binding protein corresponding to TMBP-2 (approx. 30000 by their estimates) when re-chromatographed, suggesting the susceptibility to oxidative loss of this protein.

Although no attempt was made to determine the function of TMBP-2 in the present study, Chen & Ganther (1975b) initially suspected that this protein might be carbonic anhydrase, because of similarity of M_r , which is known to be inhibited by Cd (Hodgen *et al.*, 1970); however, they found that carbonic anhydrase activity did not correspond with this protein (Chen & Ganther, 1975b). TMBP-2 does appear to have several characteristics in common with the non-steroidal gonadal factor, termed inhibin, which exerts a negative feedback on secretion of follicle-stimulating hormone (McCullagh, 1932). Inhibin can be detected by its absorption at 254 nm (de Jong *et al.*, 1981), perhaps indicating metal-sulphur interactions. Inhibin-like activity has been associated with a number of different- M_r proteins, including reports of 19000–25000 (Chari *et al.*, 1978a,b; Davies *et al.*, 1978) as well as 5000–10000 (Franchimont *et al.*, 1978; de Jong, 1979). These observations have suggested to de Jong (1979) that inhibin could exist in polymeric forms or in association with other proteins. Chari *et al.* (1978b) have also reported the existence of two major subunits of inhibin ('isohormones') on electrophoresis. The instability and susceptibility to proteolytic breakdown of certain inhibin preparations has also been reported (de Jong, 1979; de Jong *et al.*, 1981). Interestingly, Chen & Ganther (1975a) report that TMBP-2 is organ-specific and found only in testes. Further research is required to determine if TMBP-2 is inhibin or what the function of this Cd-binding protein is.

In summary, the results of the present study indicate that rat testes do not appear to contain polymerized forms of MT in the M_r range of 25000. Furthermore, the low- M_r (6000) Cd-binding protein appears to be an oxidative and/or proteolytic breakdown product of a larger- M_r species and not a monomeric form of MT as was previously assumed (Brady & Webb, 1981).

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