Biochem. J. (1979) **183**, 683–690
Printed in Great Britain

Purification and Characterization of Zinc-Binding Protein from the Liver of the Partially Hepatectomized Rat

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(*Received* 26 *April* 1979)

Zn-binding protein in liver of the partially hepatectomized rat was purified by column chromatography on Sephadex G-75 and DEAE-cellulose. Homogeneity was judged by polyacrylamide-disc-gel electrophoresis. The molecular weight determined by gel-permeation chromatography in 6M-guanidine hydrochloride was 6700. This value is in good agreement with the molecular weight calculated from the amino acid composition, which was 6073. Zn-binding protein was composed of 61 amino acid residues, and the distinctive features include an extremely high content of cysteine, which accounted for one-third of the total amino acid residues, and an absolute absence of aromatic amino acids as well as of histidine, leucine and arginine. The amino acid composition was similar to that of the metallothioneins previously isolated from rat liver and mouse liver. These observations suggest that the Zn-binding protein can be classified as a type of metallothionein. Zn-binding protein contained 8.2g-atoms of zinc per mol and traces of copper, but no cadmium. The molar ratio of thiol groups to zinc was calculated to be 2.5:1. Possible roles of this Zn-binding protein in the transport and storage of zinc in the liver are discussed.

Zinc is essential for the normal growth and development of virtually all species. The necessity of zinc for DNA and RNA synthesis in animal cells has been emphasized (Lieberman & Ove, 1962; Fujioka & Lieberman, 1964; Sandstead & Rinaldi, 1969; Chesters, 1972; Rubin, 1972).

We have previously reported that Zn-binding protein appeared during the G_1 phase in the liver of rats after partial hepatectomy. Zn-binding protein was also found in the adult rat liver stimulated to proliferate after the administration of isoprenaline followed by glucagon. In addition, a high amount of Zn-binding protein was present in the liver of the neonatal rat, although it became undetectable 40 days after birth (Ohtake *et al.*, 1978). These findings suggest a close linkage between the appearance of Zn-binding protein in the liver cytosol and the regulation of DNA synthesis. However, how Zn-binding protein participates in sequential events of hepatic DNA synthesis is not known at present.

Several studies have been shown that metallothioneins, low-molecular-weight cytoplasmic proteins which bind zinc and other group-IIB metals, are present in liver (Bühler & Kägi, 1974; Kägi *et al.*, 1974), kidney (Kägi & Vallee, 1960; Pulido *et al.*, 1966) and intestinal mucosa (Richards & Cousins, 1975). The available evidence strongly suggests that metallothionein functions in the regulation of zinc metabolism, i.e. the uptake and temporary storage of zinc in liver (Richards & Cousins, 1975).

To clarify the role of the Zn-binding protein, we initiated its purification and characterization from the regenerating rat liver. The results reported herein show that the Zn-binding protein isolated by us can indeed be classified as a metallothionein type.

Materials and Methods

Chemicals

5,5′-Dithiobis-(2-nitrobenzoic acid) was obtained from Wako Pure Chemicals Industries, Tokyo, Japan. Bovine serum albumin was from Armour Pharmaceutical Co., Chicago, IL, U.S.A. Ovalbumin, myoglobin and cytochrome *c* were from Schwarz/Mann, Orangeburg, NY, U.S.A. Ribonuclease A and trypsin inhibitor from lima bean were from Sigma Chemical Co., St. Louis, MO, U.S.A. Chymotrypsinogen A, aprotinin and insulin B chain were from Boehringer Mannheim G.m.b.H., Mannheim, Germany. Sephadex G-15, G-75 and Blue Dextran 2000 were from Pharmacia Fine Chemicals, Uppsala, Sweden. DEAE-cellulose (DE-52) was from Whatman, Maidstone, Kent, U.K. The gel-permeation column (type TSK-G 3000 SW) was from Toyo Soda Manufacturing Co., Tokyo, Japan. UM2 membranes were from Amicon Corp., Lexington, MA, U.S.A.

Treatment of animals

Female rats of Wistar strain with body weights of

130–150g were used. The animals were kept in separate cages with free access to food and water and with illumination between 08:00 and 20:00h each day.

Rats were partially hepatectomized under light diethyl ether anaesthesia by removing about 70% of the liver (Higgins & Anderson, 1931). At 14h after the operation, rats were anaesthetized with diethyl ether and the liver *in situ* was perfused via the portal vein with a sufficient amount of ice-cold 0.9% NaCl. After perfusion, the liver was quickly frozen on solid CO_2 and stored at −30°C.

Isolation of Zn-binding protein

All procedures were carried out at 0–4°C. Frozen livers (about 50g) were homogenized in 2vol. of 0.85% NaCl/20mM-Tris/HCl buffer, pH8.6, in a Waring Blendor for 20s at top speed. The homogenate was stirred for 30min and then centrifuged at 18000*g* for 20min. The supernatant fraction was collected and the precipitate fraction was re-extracted with 1vol. of NaCl/Tris as above. The supernatant fractions were combined and re-centrifuged at 140000*g* for 60min. The supernatant fraction (140S) was collected.

Metal analysis

Zinc, copper and cadmium were determined with a Shimadzu atomic-absorption spectrophotometer (model AA-610S) after appropriate dilution of the samples with water.

Protein content

Protein contents of chromatographic fractions were determined by the method described by Bradford (1976), which was based on the binding of a dye to proteins. Protein-bound dye was monitored at 595nm. The fractions of Zn-binding protein were combined at each purification step, and protein content was measured by a micro biuret method (Westley & Lambeth, 1960), with bovine serum albumin as the standard. For the calculation of the metal content and thiol-group content of the Zn-binding protein, a portion of the purified Zn-binding protein was freeze-dried and dried over P_2O_5 under reduced pressure for 3 days. The dried Zn-binding protein was weighed gravimetrically.

Molecular-weight estimation

The molecular weight of the unfolded, oxidized peptide chain of Zn-binding protein was obtained by gel-permeation chromatography with a Toyo Soda High-Speed Liquid Chromatograph (model HLC 803) equipped with a TSK-G 3000 SW column (0.75cm×60cm). The pump used in this chromatograph was a reciprocating-plunger positive-displacement-type pump (the Instrument Pump model 296/2396 miniPump; Milton Roy Co., Riviera Beach, FL, U.S.A.). The column was equilibrated with 6M-guanidine hydrochloride/0.05M-sodium phosphate buffer, pH7.0, and Blue Dextran 2000 and tryptophan were used as references. The flow rate was maintained at about 1ml/min, and one operation finished within 30min. Zn-binding protein and some of the proteins used for standard were oxidized with performic acid for 4–16h by the method described by Moore (1963). Oxidized peptide chains were recovered by gel filtration on a column (2.5cm×40cm) of Sephadex G-15 with 0.2M-acetic acid. Some of the proteins used for standard were also *S*-carboxymethylated by the method of Crestfield *et al.* (1963), or reduced with 0.1M-2-mercaptoethanol. The effluent fractions were monitored at 280nm to determine the elution volume of peptide chains. For Zn-binding protein, 1ml of 10% (w/v) trichloroacetic acid was added to 0.1ml of sample. After mixing, the samples were left for 30min. Turbidity was measured as A_{450}. Distribution coefficients, K_d, were obtained from retention volume of the solute, V_e, retention volume of totally exluded Blue Dextran 2000, V_0, and the retention volume of totally permeating tryptophan, V_t, as $K_d = (V_e - V_0)/(V_t - V_0)$. The proteins used as standards were: ovalbumin (mol.wt. 43000) (Castellino & Barker, 1968), chymotrypsinogen A (mol.wt. 25000) (Hartley, 1964), myoglobin (mol.wt. 17200) (Edmundson, 1965), ribonuclease A (mol.wt. 13700) (Smyth *et al.*, 1963), cytochrome *c* (mol.wt. 12400) (Weber & Osborn, 1969), trypsin inhibitor from lima bean (mol.wt. 8400) (Jones *et al.*, 1963), aprotinin (mol.wt. 6500) (Trautschold *et al.*, 1967) and insulin B chain (mol.wt. 3500) (Gutfreund & Ogston, 1949).

Amino acid analysis

Amino acid analysis was carried out on both performic acid-oxidized and non-oxidized samples. Each sample was hydrolysed at 110°C with 6M-HCl in vacuum-sealed tubes for 24 and 72h. The amino acid composition was determined with a Hitachi amino acid analyser (model KLA-5). The content of half-cystine was determined from that of cysteic acid in performic acid-oxidized protein. The recovery of cysteic acid was calculated to be 90% by the method of Moore (1963). Methionine was determined as methionine sulphone.

Reactive thiol groups

Reactive thiol groups were determined by titration with 5,5′-dithiobis-(2-nitrobenzoic acid) in 6M-guanidine hydrochloride, pH8.0, containing 50mM-EDTA by the Ellman (1959) method modified by Birchmeier & Christen (1971).

Polyacrylamide-disc-gel electrophoresis

A differential polyacrylamide-disc-gel electrophoresis (a combination of 7% and 15% gels) was

Regenerating livers

↓ Homogenization and extraction in 0.85% (w/v) NaCl/0.02M-Tris/HCl buffer, pH 8.6. 18000*g* for 20min

Crude extract

↓ 140000*g* for 60min

140S

↓ Sephadex G-75 gel filtration, 0.02M-Tris/HCl buffer, pH 8.6

Peak-III fraction

↓ DEAE-cellulose chromatography (I), 0.02–0.2M-Tris/HCl buffer, pH 8.6

Zn-binding protein fraction

↓ DEAE-cellulose chromatography (II) 0.04–0.08M-Tris/HCl buffer, pH 8.6; concentration with UM2 membrane

Zn-binding protein fraction

↓ Sephadex G-75 gel filtration, 0.02M-NH_4HCO_3; concentration with UM2 membrane

Zn-binding protein

Scheme 1. *Purification of rat liver Zn-binding protein*

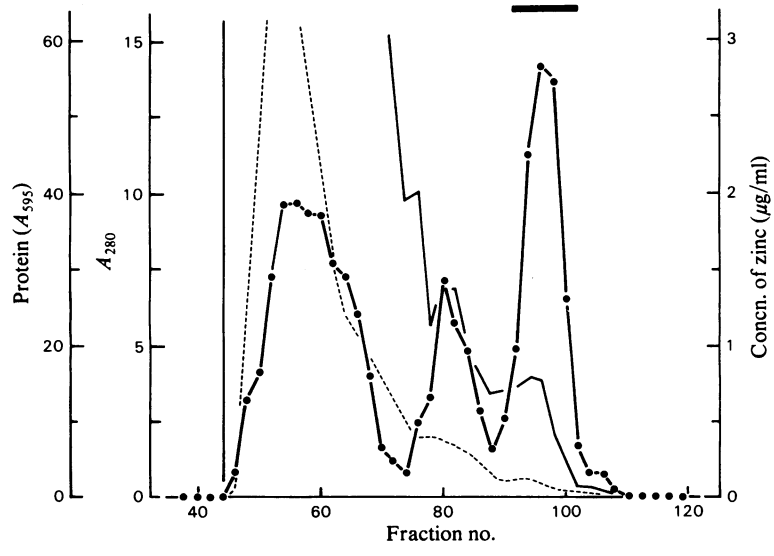

Fig. 1. *Sephadex G-75 elution profile of the cytosol fraction* (140*S*) *from the liver of partially hepatectomized rats*
The 140S fraction (130ml) was chromatographed on a column (5cm × 100cm) with 0.02M-Tris/HCl buffer, pH 8.6, at a flow rate of 100ml/h. Zinc (●) and A_{280} (----) were measured. Concentration of protein in each fraction was monitored at 595nm (——) as described in the Materials and Methods section. Fractions (15ml) were collected. The fractions denoted by the bar were pooled and frozen for further processing.

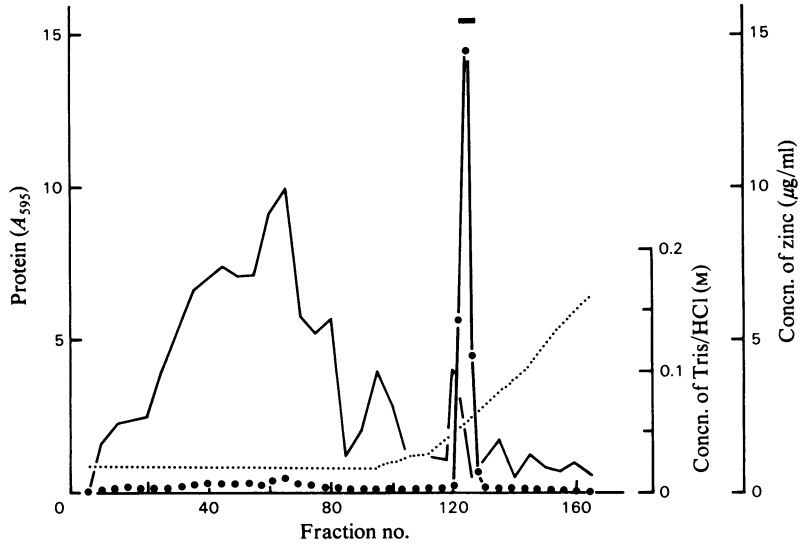

Fig. 2. *Chromatography of Zn-binding protein fraction on DEAE-cellulose column*

Zn-binding protein fraction pooled from five preparations by gel filtration was applied to a DEAE-cellulose (DE-52) column (3cm×8cm) equilibrated with 0.02M-Tris/HCl buffer, pH8.6. Elution was carried out by a 0.5litre linear gradient (limiting buffer, 0.05M-Tris/HCl buffer, pH8.6) followed by a 1.3litre linear gradient (starting buffer, 0.04M-Tris/HCl buffer, pH8.6, and limiting buffer, 0.2M-Tris/HCl buffer, pH8.6). Fractions (10ml) were collected. Concentrations of zinc (●), protein (——) and Tris (····) were measured. Concentration of Tris was measured by the micro biuret method (Itzhaki & Gill, 1964). The recovery of zinc was 90% or more of the concentration placed on the column in each experiment. The fractions indicated by the bar were processed further.

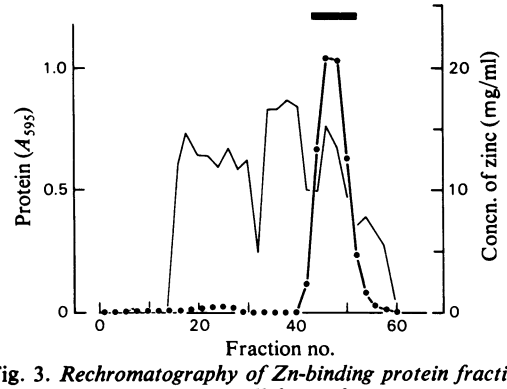

Fig. 3. *Rechromatography of Zn-binding protein fraction on DEAE-cellulose column*

Zn-binding protein fraction from the DE-52 column (Fig. 2) was applied to a DE-52 DEAE-cellulose column (1.2cm×31cm) equilibrated with 0.04M-Tris/HCl buffer, pH8.6. Elution was carried out by a 0.3 litre linear gradient (limiting buffer, 0.08M-Tris/HCl buffer, pH 8.6). Fractions (5ml) were collected. Concentrations of zinc (●) and protein (——) were measured. Fractions indicated by the bar were combined and processed further.

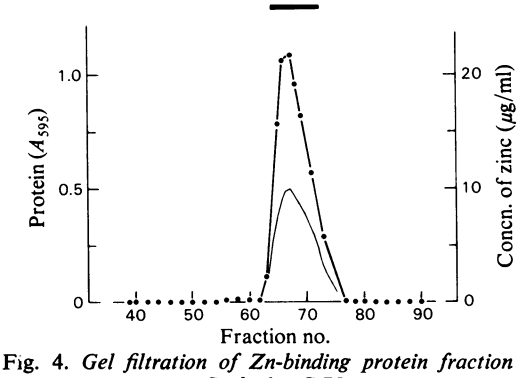

Fig. 4. *Gel filtration of Zn-binding protein fraction on Sephadex G-75*

Zn-binding protein fraction from the second DE-52 DEAE-cellulose column (Fig. 3) was applied to a Sephadex G-75 column (2.5cm×100cm) equilibrated with 0.02M-NH_4HCO_3. Elution was carried out with the same solution. Fractions (5ml) were collected. Concentrations of zinc (●) and protein (——) were measured.

carried out by the method described by Wright & Mallmann (1966), in the presence of 4M-urea, and electrophoresis was performed for 2h at constant current (4mA/tube). After electrophoresis, the gels were fixed with 10% (w/v) trichloroacetic acid and then stained with Coomassie Brilliant Blue.

Results

Purification of hepatic Zn-binding protein

The purification procedure is summarized in Scheme 1. Livers from the partially hepatectomized rats were homogenized and the cytosol fraction (140S) was prepared. About 130ml of the 140S fraction was applied to a Sephadex G-75 column. As shown in Fig. 1, three zinc-containing fractions were obtained. Peak-III fraction was found to contain Zn-binding protein free from much of the other hepatic protein. Peak-III fractions obtained from each Sephadex G-75 column were pooled and stored at −30°C until further processing.

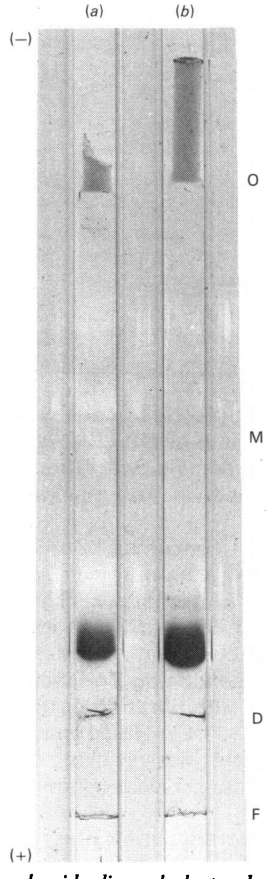

Fig. 5. *Polyacrylamide-disc-gel electrophoresis of purified Zn-binding protein*

Zn-binding protein: (*a*), 20μg; (*b*) 50μg. Samples were reduced with 0.5M-dithiothreitol in 5M-urea and applied on differential gels, which consist of 7% and 15% gels containing 4M-urea. Electrophoresis was carried out at 4mA/tube for 2h. Staining was with Coomassie Brilliant Blue. O, origin; D, dye front. Gel concentrations were 7% (O–M) and 15% (M–F) respectively.

Table 1. *Purification of Zn-binding protein from regenerating rat livers*

Frozen regenerating livers (285g) were used as starting material. Protein concentrations were determined by the micro biuret method as described in the Materials and Methods section. Zinc was determined by atomic absorption spectrophotometry. Copper content was negligible. Concentration of zinc associated with Zn-binding protein fraction in the liver cytosol (140S) was estimated from the peak-III fraction of each elution profile on the Sephadex G-75 column as shown in Fig. 1.

Stage	Volume (ml)	Total protein (mg)	Zinc concn. found in Zn-binding protein fraction (μmol)	(μmol/mg of protein)
140000*g* supernatant	639	22392	34.4	0.0014
Sephadex G-75 gel filtration	1365	734	31.7	0.043
DEAE-cellulose chromatography (I)	123	103	17.2	0.17
DEAE-cellulose chromatography (II) and concentration	5.2	11.6	14.2	1.22
Sephadex G-75 gel filtration and concentration	4.2	8.8	11.8	1.34

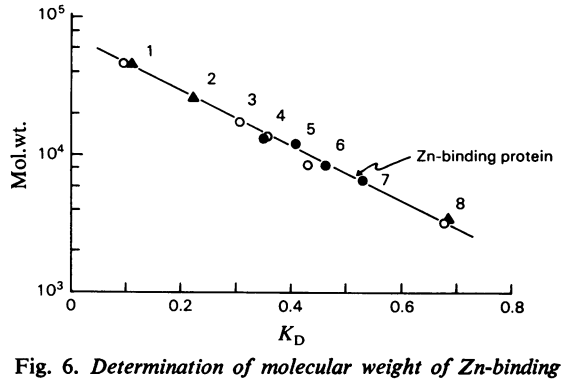

Fig. 6. *Determination of molecular weight of Zn-binding protein*

Molecular weight of performic acid-oxidized Zn-binding protein was determined by gel-permeation chromatography in 6M-guanidine hydrochloride on a TSK-G 3000SW column. Proteins used for standards were as follows: 1, ovalbumin; 2, chymotrypsinogen A; 3, myoglobin; 4, ribonuclease A; 5, cytochrome *c*; 6, trypsin inhibitor from lima bean; 7, aprotinin; 8, insulin B chain. They were performic acid-oxidized (●), *S*-carboxymethylated (○), or reduced with 2-mercaptoethanol (▲), as described in the Materials and Methods section.

Table 2. *Amino acid compositions of rat liver Zn-binding protein and metallothioneins from rat liver and from mouse liver*
Values represent the average of duplicate samples hydrolysed for 24 and 72h. Values for threonine and serine were corrected for loss by hydrolysis by extrapolating to zero time of hydrolysis. Calculations were made relative to 5.0mol of alanine/mol of Zn-binding protein. No detectable amounts of histidine, arginine, tyrosine, phenylalanine and tryptophan were found. The compositions of rat zinc-induced metallothionein and mouse cadmium-induced metallothionein are taken from the papers of Bremner & Davies (1975) and Tsunoo *et al.* (1978) respectively.

	Zn-binding protein		Metallothionein			
			Rat liver		Mouse liver	
Amino acid	(Relative molar quantities)	(Probable no. of residues)	Form A (% of total residues)	Form B (% of total residues)	Form I (Probable no. of residues/molecule)	Form II (Probable no. of residues/molecule)
Asp	4.2	4	7.11	6.74	4	4
Thr	2.1	2	6.22	3.86	5	1
Ser	7.2	7	16.38	14.07	9	10
Glu	3.6	4	2.79	6.32	1	3
Pro	1.5	2	3.99	4.87	2	2
Gly	4.5	5	9.71	7.34	5	4
Ala	5.0	5	5.56	8.14	5	6
Half-Cys*	19.6	20	29.3	27.2	20	20
Val	1.2	1	3.17	2.37	2	1
Met†	1.0	1	1.99	1.75	1	1
Leu			0.25	1.03		
Ile	0.7	1	0.34	1.74		1
Phe			0.15	0.54		
Lys	7.8	8	11.86	12.49	7	8
Arg			0.15	—		

* Determined as cysteic acid.
† Measured as methionine sulphone.

The peak-III fractions pooled from five preparations by gel filtration was then applied to a column of DEAE-cellulose. As shown in Fig. 2, a single peak of Zn-binding protein resulted. Even after washing the column with 1M-Tris/HCl buffer, pH8.6, no other zinc-containing fraction was obtained.

Table 3. *Characterization of Zn-binding protein from regenerating rat livers*
Chain weight was calculated from amino acid analysis, under the assumption that only one residue of each of the amino acids with the lowest values was present. Molecular weight is based on a content of 8g-atoms of zinc/mol of Zn-binding protein. Metal content is expressed as g-atoms of metal per 6596g of Zn-binding protein. Reactive thiol groups were measured by the Ellman (1959) method, and are expressed as groups per 6596g of Zn-binding protein. Abbreviation: n.d., not detectable (less than 0.01).

1. Molecular size	Chain weight	6073
	Molecular weight	6596
2. Metal content	Zinc	8.20
	Copper	0.029
	Cadmium	n.d.
3. Reactive thiol groups		17.9
4. Reactive thiol groups/ metal content		2.18

The Zn-binding protein fraction was re-chromatographed on a column of DEAE-cellulose. As shown in Fig. 3, considerable separation between Zn-binding protein and zinc-free material was achieved by this step.

The fractions containing Zn-binding protein were pooled and concentrated by ultrafiltration through a UM2 membrane. The concentrated material was applied to a Sephadex G-75 column equilibrated and eluted with 0.02M-NH_4HCO_3 (Fig. 4). The fraction containing Zn-binding protein was concentrated by ultrafiltration as described above and stored at −30°C. Homogeneity of purified Zn-binding protein was evident from analytical polyacrylamide-disc-gel electrophoresis as shown in Fig. 5.

Typical data for the purification procedure of the Zn-binding protein are summarized in Table 1. From 285g of livers of partially hepatectomized rats, 8.8mg of Zn-binding protein was obtained.

Properties of Zn-binding protein

The purified Zn-binding protein was analysed to ascertain whether physical similarities to metallothionein existed.

The molecular weight of performic acid-oxidized peptide chains of Zn-binding protein was estimated

to be about 6700 by gel-permeation chromatography in the presence of 6M-guanidine hydrochloride (Fig. 6).

Table 2 shows the amino acid composition of the Zn-binding protein and those of metallothioneins from rat liver and mouse liver reported by others (Bremner & Davies, 1975; Tsunoo *et al.*, 1978). The distinctive features are the abundance of cysteine and the total absence of aromatic amino acids and of histidine and leucine. This is in close agreement with the amino acid compositions of the two metallothioneins mentioned in Table 2.

Table 3 summarizes the molecular size and contents of metals and reactive thiol groups of Zn-binding protein. The peptide-chain weight of Zn-binding protein, which was calculated from the amino acid analysis data by assuming that only one residue of each of the amino acids with the lowest values was present, was 6073. Zn-binding protein contained 8.2 g-atoms of zinc per mol. Copper content was negligible and cadmium was not detected. The molecular weight of Zn-binding protein was calculated to be 6596 based on the binding of 8 g-atoms of zinc per mol. The content of reactive thiol groups is coincident with that of half-cysteine, which was measured by amino acid analysis. The molar ratio of reactive thiol groups to zinc was 2.18:1.

Discussion

In the present study, we isolated Zn-binding protein from the liver of partially hepatectomized rats and determined the molecular weight, amino acid composition and metal content. The similarities in amino acid composition and molecular weight, especially as reflected in the unusually high cysteine content, suggest strongly that the Zn-binding protein isolated in the present study can be classified as a metallothionein (e.g. Kägi *et al.*, 1974).

Since metallothionein was discovered as a cadmium-binding protein in horse kidney (Margoshes & Vallee, 1957), proteins with similar properties have been isolated from the cytosol fraction of liver, kidney and other parenchymatous tissues of a wide variety of animal species (for review, see Kojima & Kägi, 1978). Although the natural contents of metallothionein are extremely low in most experimental animals, the tissue content can be increased by administration of large doses of inducer metals such as zinc (Webb, 1972; Bremner & Davies, 1975; Richards & Cousins, 1975), or cadmium (Shaikh & Lucis, 1971; Webb, 1972; Winge & Rajagopalan, 1972). Metallothionein consists of a single peptide chain of approx. 60 amino acid residues, of which cysteine is the major amino acid, accounting for one-third of all amino acid residues. The occurrence of two molecular forms, which can be separated by anion-exchange chromatography, has also been reported for hepatic metallothionein, whether induced by the injection of zinc in the rat (Bremner & Davies, 1975; Feldman & Cousins, 1976), induced by the injection of cadmium in the mouse (Tsunoo *et al.*, 1978), or occurring naturally as the zinc-binding protein in human (Bühler & Kägi, 1974).

In the present study, it is shown that only one form of Zn-binding protein was isolated by the DEAE-cellulose chromatography (see Fig. 2). Other forms were not detected even when the experimental conditions reported by Bremner & Davies (1975) and Tsunoo *et al.* (1978) were used. The possibility that we failed to detect another form cannot be ruled out, but it may be postulated that the Zn-binding protein purified in the present study corresponds virtually to the predominant form in the regenerating rat liver. Whether other forms of Zn-binding protein are present in the regenerating rat liver remains to be established.

The molecular weight of Zn-binding protein was measured in the presence of 6M-guanidine hydrochloride with a high-speed liquid chromatograph equipped with a TSK-G 3000 SW gel-permeation column. The molecular weight of the performic acid-oxidized peptide chain of the Zn-binding protein was estimated to be about 6700, which is consistent with the molecular weight of metallothionein obtained by using 6% agarose as a gel-filtration medium (Bühler & Kägi, 1974; Kägi *et al.*, 1974; Tsunoo *et al.*, 1978). The method used in the present study permits useful molecular-weight estimates between the extreme limits of 43000 and 3500, decreases greatly the time of operation, and provides a high reproducibility. Imamura *et al.* (1979) also reported precise molecular-weight estimates of proteins in the presence of 0.1% (w/v) sodium dodecyl sulphate by using this column system.

Metallothionein was reported to contain 6 g-atoms of metals and 20 cysteine residues per mol, and the stoicheiometric relationship of 3 thiol groups to each metal atom bound was reported (Kägi & Vallee, 1961; Pulido *et al.*, 1966; Bühler & Kägi, 1974; Kägi *et al.*, 1974; Bremner & Davies, 1975). As shown in Table 3, 8 g-atoms of zinc were bound/mol of the Zn-binding protein, which also contained 20 cysteine residues per molecule, therefore the molar ratio of thiol groups to zinc atoms was 2.5:1 in the present study. However, Kojima *et al.* (1976) reported 7 g-atoms of metals per mol of horse renal metallothionein 1-B, which contained 20 cysteine residues. Webb (1972) suggested that each metal atom was bound to two thiol groups. But the findings were based on partially purified and uncharacterized proteins. Hence, a definite conclusion about the metal-binding capacity of Zn-binding protein in regenerating rat liver must be awaited until the mode of metal binding is clarified.

It was reported that metallothionein in the liver

could be induced by stimuli such as restriction of food intake (Bremner & Davies, 1975), various stresses (Oh *et al.*, 1978) or inflammation (Sobocinski *et al.*, 1978), in addition to the administration of metals to animals. These observations strongly suggest that metallothionein is involved in the regulation of normal metabolism of zinc, perhaps in transport or temporary storage in addition to detoxification of heavy metals.

Although the function of Zn-binding protein during the G_1 phase is not known at present, zinc was reported to stabilize the structures of RNA and DNA, and affect their metabolism (Fuwa *et al.*, 1960). The occurrence of zinc in several enzymes associated with nucleic acid synthesis and speculation about the possible molecular basis for a zinc requirement have been reviewed (Vallee, 1976).

In our previous paper (Ohtake *et al.*, 1978), almost all of the zinc accumulated in the liver cytosol of rats after partial hepatectomy was present as a form of Zn-binding protein. From these findings, it is possible that Zn-binding protein, identified as a metallothionein in the present study, is important in the regulation of DNA synthesis through the synthesis or activity of enzymes required for nucleic acid.

We thank Dr. J. Enami, Dr. K. Hasegawa and Dr. T. Umeda for their constant interest and valuable suggestions during the course of this work and for their kind criticism in preparing the manuscript. Thanks are also due to Dr. H. Furuta for performing the amino acid analyses.

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