Sea urchin (*Anthocidaris crassispina*) egg zinc-binding protein

Cellular localization, purification and characterization

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Gel-filtration analysis of cytosol fraction obtained from unfertilized sea-urchin (*Anthocidaris crassispina*) eggs on Sephadex G-75 revealed the presence of two Zn-binding-protein fractions. The major Zn-binding protein fraction had a low molecular weight and a low absorbance at 280 nm, properties similar to those of the metallothionein found in the regenerating rat liver. These fractions were further purified by DEAE-cellulose and Sephadex G-50 chromatography. Homogeneity of the Zn-binding protein was judged by polyacrylamide-disc-gel electrophoresis and gel-permeation chromatography in the presence of 6 M-guanidinium chloride. The molecular weight determined by gel-permeation chromatography was 3900. This value is in good agreement with the minimum molecular weight calculated from the amino acid composition, which was 3655. Zn-binding protein is composed of 36 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 a complete absence of aromatic amino acids, as well as of methionine, histidine and arginine. Zn-binding protein contained 4.1 g-atoms of zinc per mol and a trace of cadmium, but no copper, iron or calcium. The molar ratio of reactive thiol groups to metal ion was calculated to be 2.73:1. Possible roles of this Zn-binding protein in the homeostasis of zinc in unfertilized sea-urchin eggs are discussed.

Metallothioneins, which are low-molecular weight cysteine-rich proteins containing unusually high amounts of zinc and cadmium, were isolated initially from equine renal cortex by Margoshes & Vallee (1957) and later characterized by Kägi & Vallee (1960, 1961). Subsequently, proteins with similar properties were also obtained from liver, kidney and other parenchymatous tissues of mammalian species [see reviews by Kojima & Kägi (1978) and Kägi & Nordberg, 1979]. Owing to its high affinity for metals and its inducible nature on administration of various metals, metallothionein has been postulated to play a role in detoxification of heavy metals. It has also been thought to function in normal zinc metabolism, i.e. the uptake and temporary storage of zinc.

We have previously reported the presence of Zn-metallothionein in the neonatal rat liver. It has also been induced in the adult rat liver when the liver

was stimulated to proliferate by administration of drug/hormone combination or by partial hepatectomy (Ohtake *et al.*, 1978; Ohtake & Koga, 1979). Zinc is known to be essential for the normal growth and development of virtually all organisms, and the necessity of zinc for nucleic acid synthesis in animal cells has been emphasized (Lieberman & Ove, 1962; Sandstead & Rinaldi, 1969; Chesters, 1972; Rubin, 1972). On the basis of these findings, a possible linkage between the appearance of metallothionein in rat liver cytosol and the regulation of DNA synthesis has been suggested (Ohtake *et al.*, 1978).

In the present paper the natural occurrence of a low-molecular-weight Zn-binding protein has been investigated in the soluble fraction of unfertilized sea-urchin eggs. From the results of amino acid composition and metal analysis, the protein was found to be a zinc- and cysteine-rich protein. To our knowledge, this is the first demonstration that Zn-binding protein with these characteristics exists in the gametes of animal species.

Abbreviation used: Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid); MT-II, metallothionein from mouse liver.

Experimental

Animals

Sea urchins (*Anthocidaris crassispina*) were induced to spawn unfertilized eggs by injecting 0.5 M-KCl into the coelomic cavity. Shed eggs were collected by centrifugation at 500 g for 5 min and washed once with 1 M-glucose.

Preparation of nucleate and anucleate merogons

Nucleate and anucleate merogons were prepared by the method of Fansler & Loeb (1969) with slight modifications as described below. A 1 ml portion of a 15% suspension of unfertilized eggs was layered on 30 ml of a linear sucrose density gradient (0.4–0.7 M) made by mixing 1 M-sucrose (prepared in deionized distilled water) and sea water. The gradient was centrifuged at 12°C in a Sorvall HB-4 swinging-bucket rotor at 10400 g for 8 min, followed by centrifugation at 21600 g for 12 min. After centrifugation, the nucleate and anucleate merogons were collected by withdrawing each separated band with a syringe. The merogons were diluted with approx. 10 vol. of sea water and collected by centrifugation.

Isolation of Zn-binding protein

For the gel-filtration analysis of Zn-binding protein, unfertilized eggs or merogons (usually 1–2 g) were homogenized in 10 vol. of ice-cold 0.05 M-Tris/HCl buffer, pH 8.2, in a Potter–Elvehjem-type homogenizer with a tightly fitted Teflon pestle. All the procedures were carried out at 0–4°C. The homogenate was successively centrifuged at 1000 g for 10 min and 140000 g for 60 min to obtain the supernatant fraction ('140S'). A portion (1 ml) of the supernatant fraction was applied to a Sephadex G-75 column (1.6 cm × 43 cm) equilibrated with 0.05 M-Tris/HCl buffer, pH 8.2, containing 0.1 M-NaCl. Elution was done with the same buffer/salt solution and fractions (1.5 ml) were collected.

Metal determination

Contents of zinc, copper, cadmium, calcium and iron were determined with a Shimadzu atomic-absorption spectrophotometer (model AA-610S). Metals contained in the whole homogenates and '140S' fractions were extracted with 0.1 M-HNO₃ before determination. Chromatographic fractions were analysed after appropriate dilution of the samples with deionized distilled water.

Protein contents

Protein contents were determined by a micro-biuret method (Westley & Lambeth, 1960) with bovine serum albumin as the standard. Protein contents of chromatographic fractions were de-

termined by the method described by Bradford (1976), which is based on the binding of a dye to proteins.

Molecular-weight estimation

The molecular weight of the unfolded polypeptide chain was estimated by gel-permeation chromatography with a Toyo Soda high-performance liquid chromatograph (model HLC 803) equipped with a TSK-G 3000SW column (0.75 cm × 60 cm) as described previously (Ohtake & Koga, 1979). The column was equilibrated with 6 M-guanidinium chloride/0.05 M-sodium phosphate buffer, pH 7.0. Blue Dextran and tryptophan were used as references. Zn-binding protein fractions obtained from a second gel-filtration chromatography of Sephadex G-50 were pooled and concentrated on an Amicon UM 2 membrane. Zn-binding protein and proteins used as molecular-weight standards were reduced with 0.1 M-2-mercaptoethanol containing 0.05 M-EDTA, pH 7.0, and portions (0.02 ml) were applied to each column. The flow rate was maintained at about 1 ml/min and the effluent fractions were monitored at 230 nm to determine the elution volume of polypeptide chains.

Distribution coefficients, K_d , were obtained from the elution volume of the polypeptide chain, V_e , the elution volume of Blue Dextran, V_0 , and the elution volume of tryptophan, V_t , as:

$$K_d = (V_e - V_0)/(V_t - V_0)$$

The proteins used as standards were bovine serum albumin (mol.wt. 66000; Dayhoff, 1976), trypsinogen (24000; Dayhoff, 1972), myoglobin (17800; Dayhoff, 1976), lysozyme (14300; Dayhoff, 1972), aprotinin (6500; Kassell & Chow, 1966), glucagon (3500; Dayhoff, 1972), insulin A chain (2300; Dayhoff, 1972) and bacitracin (1400; Bodanzsky & Perlman, 1964).

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 6 M-HCl in vacuum-sealed tubes for 24 h. The amino acid composition was determined with a Hitachi amino acid analyser (model KLA-5).

Reactive thiol groups

Reactive thiol groups were determined by titration with Nbs₂ in 6 M-guanidinium chloride/6.5 mM-Tris/HCl buffer, pH 8.0, containing 0.05 M-EDTA, by the Ellman method as modified by Birchmeier & Christen (1971).

Polyacrylamide-disc-gel electrophoresis

'Differential' polyacrylamide-disc-gel electrophoresis (a combination of 10 and 15% gels) was

carried out by the method described by Wright & Mallmann (1966), in the presence of 5M-urea. Electrophoresis was performed for 4h at constant current (4mA/tube). After electrophoresis the gels were fixed with ice-cold 30% (w/v) trichloroacetic acid and then stained with a solution of 0.5% Coomassie Brilliant Blue in 50% (v/v) ethanol/8% (v/v) acetic acid/42% (v/v) deionized water.

Chemicals

Sephadex G-50, Sephadex G-75 and Blue Dextran were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. DEAE-cellulose (DE-52) was from Whatman Ltd., Maidstone, Kent, U.K. The gel-permeation column (type TSK-G 3000SW) was from Toyo Soda Manufacturing Co., Tokyo, Japan. Amicon UM 2 membrane was from Amicon Corp., Lexington, MA, U.S.A. Bacitracin, myoglobin and trypsinogen were from Sigma Chemical Co., St. Louis, MO, U.S.A. Bovine serum albumin was from Armour Pharmaceutical Co., Chicago, IL, U.S.A. Aprotinin, insulin A chain and lysozyme were from Boehringer Mannheim, G.m.b.H., Mannheim, Germany. Glucagon was from Calbiochem-Behring Co., San Diego, CA, U.S.A. Nbs₂ was from Wako Pure Chemical Industries, Tokyo, Japan. Guanidinium chloride was from Nakarai Chemicals, Tokyo, Japan. All other chemicals were of reagent grade.

Results

Occurrence and cellular localization of Zn-binding protein in unfertilized sea-urchin eggs

The elution profile on Sephadex G-75 of the cytosol fraction ('140S') obtained from unfertilized eggs of the sea-urchin *A. crassispina* shows that zinc was associated with two fractions (Fig. 1). Peak I was eluted at the void volume of the column. The material in Peak II was determined to be proteinaceous in nature by the protein-dye-binding method, though the Peak II had a low absorbance at 280nm. These two peaks represented 90% or more of the total zinc content applied to the column. In addition, only a small amount of free zinc was recovered on gel-filtration chromatography, indicating that the zinc is almost exclusively bound to proteins or other cellular components in unfertilized eggs.

Contents of total zinc and zinc bound to low-molecular-weight protein were examined in unfertilized eggs. The bound zinc was expressed as the zinc content in the Peak II fraction. Total zinc content in unfertilized eggs was $5.58 \pm 0.34 \mu\text{mol}/10^7$ eggs (mean $\pm$ S.E.M. from ten independent analyses) and bound zinc content was $4.57 \pm 0.29 \mu\text{mol}/10^7$ eggs (mean $\pm$ S.E.M. from ten independent analyses). Therefore the content of bound zinc is 82% of total zinc in unfertilized sea-urchin eggs.

Fig. 1. *Sephadex G-75 gel-filtration profile of the cytosol fraction (140S) from unfertilized sea-urchin eggs*

The 140S fraction (1ml) was chromatographed on a column (1.6cm $\times$ 43cm) with 0.1M-NaCl/0.05M-Tris/HCl buffer, pH 8.2, at a flow rate of 10ml/h. Fractions (1.5ml) were collected and monitored for zinc content (●) and A₂₈₀ (—). The concentration of protein in each fraction was monitored at 595 nm (—) as described in the Experimental section. The position of eluted sucrose (S) is shown.

Table 1. *Distribution of Zn-binding protein in unfertilized A. crassispina eggs*

Unfertilized eggs were divided into nucleate and anucleate merogons by an isopycnic centrifugation method as described in the Experimental section. Volumes of whole eggs and merogons were calculated from the diameters, which were determined with 100 eggs or merogons. Each diameter was as follows: whole egg, $88.4 \pm 2.6 \mu\text{m}$; nucleate merogon, $61.6 \pm 4.6 \mu\text{m}$; and anucleate merogon, $77.2 \pm 3.8 \mu\text{m}$. Zn-binding protein content in whole eggs, nucleate merogons and anucleate merogons was estimated as described in the Experimental section and the legend to Fig. 1.

	Volume of egg or merogon (%)	Distribution of Zn-binding protein	
		(μg of zinc/ 10^6 eggs or merogons)	(%)
Whole egg	100	26.6	100
Nucleate merogon	33.8	16.1	60.5
Anucleate merogon	54.5	10.2	38.3

To determine the localization of Zn-binding protein in unfertilized sea-urchin eggs, eggs were divided into nucleate and anucleate merogons. Initial centrifugation of eggs in a sucrose-density gradient at low speed followed by centrifugation at an increased speed caused an egg to stratify and to separate into an upper, lighter fragment that con-

tained a nucleus (nucleate merogon) and a lower, dense fragment that lacked a nucleus (anucleate merogon). Almost all mitochondria were localized in the nucleate merogon, whereas pigment and yolk granules were enriched in the anucleate merogon. Cytosol was distributed in both nucleate and anucleate merogons, but the larger portion was localized in the nucleate merogon. As summarized in Table 1, 60.5% of the whole-egg Zn-binding protein was recovered in the nucleate merogons and 38.3% in the anucleate merogons. Since the volume of nucleate merogon was smaller than its anucleate counterpart, Zn-binding protein was found to be concentrated in the nucleate merogons as compared with whole eggs and anucleate merogons. These results suggest that Zn-binding protein is localized in the cytosol of the unfertilized sea-urchin eggs.

Purification of Zn-binding protein

Frozen eggs (50 g wet wt.) were homogenized in 10 vol. of 0.02 M-KCl/0.02 M-Tris/HCl buffer, pH 8.2, containing 0.01 M-2-mercaptoethanol. The homogenate was stirred for 30 min and centrifuged at 1000 g for 10 min, and the supernatant was further centrifuged at 140 000 g for 60 min. The supernatant ('140S' fraction) was divided into two equal portions, and each of them was applied to two preparative columns of Sephadex G-75 (each 5 cm $\times$ 95 cm) that had been equilibrated with 0.02 M-Tris/HCl buffer, pH 8.2, containing 0.01 M-2-mercaptoethanol. The column was eluted at a flow rate of 100 ml/h and

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

The Zn-binding protein fraction from two gel-filtration columns of Sephadex G-75 was applied to a DEAE-cellulose column (2.5 cm $\times$ 18 cm) equilibrated with 0.02 M-Tris/HCl buffer, pH 8.2, containing 0.02 M-KCl and 0.01 M-2-mercaptoethanol. The column was developed with a linear gradient (----) of 0.02 M- to 0.4 M-Tris/HCl buffer, pH 8.2, containing 0.02 M-KCl and 0.01 M-2-mercaptoethanol. Arrows ($\downarrow$, $\uparrow$) indicate the start and the end of the gradient. Fractions (15 ml) were monitored for zinc content (●) and A_{230} (—). The fractions denoted by the bar were pooled and processed further.

fractions (15 ml) were collected. Major Zn-binding protein fractions eluted from two columns were pooled and applied directly to a DEAE-cellulose column (2.5 cm × 18 cm) that had been equilibrated with the starting buffer, 0.02 M-Tris/HCl buffer, pH 8.2, containing 0.02 M-KCl and 0.01 M-2-mercaptoethanol. After washing the column with 270 ml of the starting buffer, elution was carried out with a 1-litre linear gradient (limiting buffer; 0.4 M-Tris/HCl buffer, pH 8.2, containing 0.02 M-KCl and 0.01 M-2-mercaptoethanol) at a flow rate of 80 ml/h and fractions (15 ml) were collected. As shown in Fig. 2, one major and two minor peaks of Zn-binding protein were obtained. After washing the column with 1 M-Tris/HCl buffer, pH 8.2, only a small amount of Zn-containing fraction was recovered.

The major peak fractions of Zn-binding protein were pooled and passed through a Sephadex G-50 column (5 cm × 90 cm) that had been equilibrated with 0.02 M-Tris/acetate buffer, pH 7.4, containing 0.01 M-2-mercaptoethanol. The fractions with a high concentration of Zn-binding protein were pooled and directly applied to a DEAE-cellulose column (1.5 cm × 20 cm) that had been equilibrated with starting buffer, 0.02 M-Tris/acetate buffer, pH 7.4, containing 0.01 M-2-mercaptoethanol. After the column was washed with 140 ml of 0.1 M-Tris/acetate buffer, pH 7.4, containing 0.01 M-2-mercaptoethanol, the elution was carried out with a 240 ml linear gradient (limiting buffer; 0.3 M-Tris/acetate buffer, pH 7.4, containing 0.01 M-2-mercaptoethanol) at a flow rate of 8 ml/h, and fractions (4 ml) were collected. The fractions with a high concentration of Zn-binding protein were pooled, concentrated by ultrafiltration on an Amicon UM 2 membrane and applied to a Sephadex G-50 column (2.5 cm × 90 cm) that had been equilibrated with 0.05 M-Tris/HCl buffer, pH 8.2, containing

Fig. 3. Polyacrylamide-disc-gel electrophoresis of purified Zn-binding protein
Zn-binding protein: (a) 10 µg; (b) 50 µg. Samples were reduced with 0.5 M-dithiothreitol in 5 M-urea and applied on 'differential' gels, which consist of 10 (O-M) and 15% (M-D) gels containing 5 M-urea. Electrophoresis was carried out at 4 mA/tube for 4 h. Staining was with Coomassie Brilliant Blue. O, origin; D, dye front.

Table 2. Purification of Zn-binding protein from unfertilized sea-urchin eggs

Frozen unfertilized eggs (50 g) were used as starting material. The protein content was determined by the micro-biuret method as described in the Experimental section. Zinc was determined by atomic-absorption spectrophotometry. The zinc content associated with Zn-binding protein fraction in the 140000 g supernatant was estimated by using 1.0 ml of the supernatant from the Peak-II fraction of each elution profile on the Sephadex G-75 column as shown in Fig. 1. Portions (0.1–0.5 ml) of samples pooled at each purification stage other than '140000 g supernatant' were used for the determination of zinc and protein content.

Stage	Volume (ml)	Total protein (mg)	Zinc content found in Zn-binding protein	
			(µmol)	(µmol/mg of protein)
140000 g supernatant	275	11 110	60.0	0.0054
Sephadex G-75 gel filtration	486	206.9	53.9	0.26
DEAE-cellulose chromatography				
(I)	89	25.7	23.0	0.89
(II)	18.5	13.2	12.7	0.96
Sephadex G-50 gel filtration	38.4	10.6	11.2	1.06

Fig. 4. High-performance gel-permeation chromatography of purified Zn-binding protein on a Toyo Soda TSK-G 3000SW column

The Zn-binding protein fraction from the second Sephadex G-50 column was chromatographed on a TSK-G 3000SW column (0.75 cm $\times$ 60 cm) with 6 M-guanidinium chloride/0.05 M-sodium phosphate buffer, pH 7.0, at a flow rate of 0.87 ml/min. Elution of Zn-binding protein was monitored by A_{230} . The insert graph shows the molecular-weight determination of reduced Zn-binding protein (Zn-BP) obtained by using a TSK-G 3000SW column. Rat liver metallothionein (MT) was also chromatographed on the column and its molecular weight was determined. Proteins used for standards were as follows: 1, bovine serum albumin; 2, trypsinogen; 3, myoglobin; 4, lysozyme; 5, aprotinin; 6, glucagon; 7, insulin A chain; and 8, bacitracin. All other details of the chromatography and of the molecular-weight determination are given in the Experimental section.

0.005 M-2-mercaptoethanol. Elution was done with the same buffer solution at a flow rate of 20 ml/h. The purified Zn-binding protein was stored at -80°C in small polypropylene tubes. Characterization of each fraction in the purification procedure is summarized in Table 2. From 50 g of unfertilized sea-urchin eggs, 10.6 mg of Zn-binding protein was obtained in the present experiment.

Polyacrylamide-disc-gel electrophoresis of the isolated Zn-binding protein showed only one visible band (Fig. 3). Further analysis of the Zn-binding protein by high-performance gel-permeation chromatography in the presence of 6 M-guanidinium

chloride yielded one major and two minor peaks of 230 nm-absorbing material (Fig. 4). From the elution profile on the gel-permeation column, the homogeneity of the isolated Zn-binding protein is calculated to be 95% or more.

Properties of Zn-binding protein

The molecular weight of reduced Zn-binding protein was estimated by using high-performance gel-permeation chromatography on a calibrated TSK-G 3000SW column in 6 M-guanidinium chloride (Fig. 4). The molecular weight of the major

Table 3. *Amino acid compositions of Zn-binding protein from sea-urchin unfertilized eggs, metallothionein from mouse liver and copper-containing protein from Neurospora crassa*

Results of duplicate analyses of proteins oxidized with performic acid and hydrolysed with 6M-HCl at 110°C for 24 h. Values are expressed as residues per molecule. Results are expressed relative to 2.0 mol of alanine per mol of Zn-binding protein. No detectable amounts of histidine, arginine, leucine, tyrosine and phenylalanine were found. Methionine was also absent, since no appreciable peaks of methionine and methionine sulphone appeared on the ion-exchange chromatogram. The absence of tryptophan is shown by the lack of absorbance of the unoxidized protein at 280 nm. The amino acid compositions of the mouse liver metallothionein (MT-II) and the *Neurospora crassa* Cu-containing protein are taken from Tsunoo *et al.* (1978) and Lerch (1980) respectively.

Amino acid	Sea-urchin Zn-binding protein		Mouse liver MT-II (no. of residues per molecule)	<i>Neurospora</i> Cu-containing protein (no. of residues per molecule)
	Relative molar quantities	Probable no. of residues per molecule		
Ala	2.0	2	6	1
Asx	2.0	2	4	3
Asp			1	1
Asn			3	2
Half-Cys*	11.7	12	20	7
Glx	4.1	4	3	
Glu			0	
Gln			3	
Gly	4.8	5	4	6
Ile	0.6	1	1	
Leu	0.7	1		
Lys	3.8	4	8	1
Met			1	
Pro	0.6	1	2	
Ser	1.0	1	10	7
Thr	2.3	2	1	
Val	0.7	1	1	
Total residues		36	61	25
Minimum chain weight		3655	6160	2220

* Determined as cysteic acid.

peak (Peak α) was estimated to be about 3900. The molecular weights of two minor peaks were 8000 (Peak β) and 12000 (Peak γ).

Table 3 shows the amino acid compositions of the sea-urchin egg Zn-binding protein, metallothionein from mouse liver (MT-II) and *Neurospora* Cu-containing protein reported by others (Tsunoo *et al.*, 1978; Lerch, 1980). A distinctive feature of the Zn-binding protein is the abundance of cysteine, since twelve residues are present per molecule, accounting for one-third of all amino acids. Another unusual feature of this protein is the total absence of aromatic amino acids, arginine and histidine. Amino acid compositions of Zn-binding protein and mouse liver MT-II are basically similar, except that Zn-binding protein lacks methionine. Leucine was present only in Zn-binding protein. It is also noted that higher amounts of serine are present in mouse liver MT-II and *Neurospora* Cu-containing protein as compared with the low serine content in Zn-binding protein. The peptide chain weight of Zn-binding protein was 3655, which was calculated from the amino acid analysis data under the assumption

Table 4. *Characterization of Zn-binding protein from unfertilized sea-urchin eggs*

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 4 g-atoms of zinc/mol of Zn-binding protein. Metal content is expressed as g-atoms of metal per 3917 g of Zn-binding protein. Reactive thiol groups were measured by the Ellman method as modified by Birchmeier & Christen (1971) as described in the Experimental section, and are expressed as groups per 3917 g of Zn-binding protein. Abbreviation: n.d., not detectable (less than 0.01).

1. Molecular size	Chain weight	3655
	Molecular weight	3917
2. Metal content	Zinc	4.13
	Cadmium	0.04
	Copper	n.d.
	Iron	n.d.
	Calcium	n.d.
3. Reactive thiol groups		11.4
4. Reactive thiol groups/ metal content		2.73

that only one residue of amino acids with the lowest values was present.

Table 4 summarizes molecular size and contents of metals and reactive thiol groups of Zn-binding protein. On the basis of the peptide chain weight, Zn-binding protein contained 4.1 g-atoms of zinc per mol. Cadmium content was negligible, and copper, iron and calcium were not detected. The content of reactive thiol groups is in close agreement with that of half-cystine. These findings suggest the absence of disulphide bonds in Zn-binding protein. The molar ratio of reactive thiol groups to metal was 2.73:1 in the present experiment. The molecular weight of Zn-binding protein was calculated to be 3917, on the basis of the binding of 4 g-atoms of zinc per mol.

Discussion

The finding that large amounts of Zn-binding protein are present in unfertilized eggs of the sea-urchin *Anthocardis crassispina* raised the question whether or not this protein is metallothionein. To investigate this possibility, we isolated Zn-binding protein from unfertilized eggs and determined the amino acid composition, metal content and molecular weight.

The criteria commonly used to identify metallothionein are as follows: (1) high metal contents, generally 7 g-atoms of bivalent-metal ions per mol; (2) very high cysteine content of 20–21 residues per molecule, constituting about one-third of the total amino acid composition; (3) no protein absorption band near 280 nm, which indicates an absolute deficiency of aromatic amino acids; (4) absence of histidine and disulphide bonds; and (5) low molecular weight, ranging from 6000 to 7000 (Vallee, 1979).

As shown in Table 2, the amino acid composition of Zn-binding protein resembles that of metallothioneins from mouse liver and other species, but differs from *Neurospora* Cu-containing protein (Kojima & Kägi, 1978; Lerch, 1980). Zn-binding protein also contained a high amount of half-cystine, accounting for one-third of all amino acids. The titration experiment of reactive thiol groups with Nbs₂ in the presence of EDTA shows that almost all cysteine residues in the protein molecule are present in a reduced form, indicating the absence of disulphide bonds. The extremely low absorbance at 280 nm of Zn-binding protein suggests a relative deficiency of aromatic amino acids. This is well confirmed by the amino acid analysis data, which indicated that there is no tyrosine or phenylalanine. The mammalian metallothionein contains only a single methionine residue which is the *N*-terminal residue in the protein molecule. In contrast, methionine was absent in Zn-binding protein. The deficiency of methionine in the protein is also supported by the

evidence that no appreciable amount of methionine sulphone is detected in performic acid-oxidized proteins. The nature of the *N*-terminal residue in Zn-binding protein remains to be established. It is noteworthy that the serine content is extremely low in Zn-binding protein as compared with that in mammalian metallothioneins.

More than 4 g-atoms of metals were bound per mol of Zn-binding proteins, which also contained 12 cysteine residues per molecule. Therefore the molar ratio of thiol groups to metal was 3:1 in the present study. This stoichiometric relationship of three thiol groups to each metal atom bound is in good agreement with the properties of metallothioneins, although the determination of amino acid sequence and the mode of metal binding in Zn-binding protein remains to be established.

The molecular weight of Zn-binding protein measured in 6 M-guanidinium chloride was estimated to be about 3900, whereas those of two minor peaks were 8000 and 12000 (see Fig. 4). These two minor peaks might reflect the elution positions of both the dimeric and trimeric forms of Zn-binding protein formed during the course of concentration by ultrafiltration rather than those of contaminating proteins, since the preparation of Zn-binding protein used for the molecular-weight determination was purified finally by Sephadex G-50 gel filtration. The molecular weight of the zinc-free Zn-binding protein (apoprotein) was estimated to be close to 3600 when 4 g-atoms of zinc per mol were removed. This value is in good agreement with the minimum molecular weight of peptide chains calculated from amino acid-analysis data (see Table 3). The molecular weight of Zn-binding protein as estimated is about two-thirds of that of known metallothioneins. On the basis of the aforementioned criteria, it is concluded that Zn-binding protein differs from metallothionein.

Cellular localization

We have approached the question of the intracellular location of Zn-binding protein by using a procedure that avoids lysis of the unfertilized egg during the redistribution of cytoplasmic granules and nucleus in the egg. The procedure employs isopycnic centrifugation, which causes unfertilized eggs to divide into nucleate and anucleate merogons. The nucleate merogon is enclosed by an intact plasma membrane and contains a nucleus, almost all mitochondria and the larger portion of the cytosol. In the present experiment, the volume of nucleate merogons was smaller than that of the anucleate counterparts. On the other hand, about two-thirds of the Zn-binding protein present in the whole eggs was recovered in the nucleate merogons and one-third was recovered in the anucleate merogons (see Table 1). Therefore Zn-binding protein was enriched in the

nucleate merogons relative to that in the whole eggs. Apparent dilution of Zn-binding protein in the anucleate merogons as compared with whole eggs is probably explained by the large volume occupied in the anucleate merogons by yolk and pigment granules.

The peptidase of eggs of the sea-urchin *Arbacia* has been reported to be distributed evenly in the cytosol and is not bound to any cytoplasmic granules (Holter, 1936). When volumes of the nucleate and anucleate merogons were nearly the same, about 80% of the egg peptidase was found in the nucleate merogons and 20% in the anucleate merogons, because pigment and yolk granules occupied a considerable volume of the anucleate merogons (Holter, 1936; Harvey, 1956). On the basis of these findings, Zn-binding protein appears to be (a) soluble protein(s) present in the cytoplasm of unfertilized sea-urchin eggs. However, the possibility that Zn-binding protein is also located in the nucleus cannot be excluded, since the isolation of 'clean' nuclei from unfertilized sea-urchin eggs is difficult at present. Therefore the precise identification of egg Zn-binding protein in the nucleus remains to be established.

Possible roles for egg Zn-binding protein

Kojima & Kägi (1978) have postulated the probable role for metallothionein, namely that it functions homeostatically as a metal reservoir that is able to donate and/or sequester its metal intracellularly to metal-requiring metalloproteins. Udom & Brady (1980) and Li *et al.* (1980) studied the ability of Zn-metallothionein to donate its metal to apoproteins of various zinc-dependent enzymes. They showed that Zn-metallothionein was as good as, or better than, zinc salt in donating its zinc to apoproteins, and thereby restoring activity to the enzymes.

Zinc is reported to be a catalytically essential component of the highly purified DNA polymerase from the sea-urchin *Strongylocentrotus franciscanns* (Slater *et al.*, 1971). Shioda *et al.* (1980) reported that DNA polymerase- α and - β were associated with rough endoplasmic reticulum in eggs of the sea-urchin *Hemicentrotus pulcherrimus*, and that their subcellular distribution changed during early embryogenesis. On the basis of these findings it would be reasonable to assume that Zn-binding protein in the cytoplasm of the sea-urchin egg can also function in zinc homeostasis as a reservoir of zinc, donating and/or sequestering its metal to zinc-requiring metalloenzymes such as DNA polymerases.

The finding that Zn-binding protein occurs naturally in the gamete of animal species will throw light on the study of regulatory functions of metal ions in the development and normal growth of animal

cells. To discover the relationship between the primary structure and metal-binding capacity of egg Zn-binding protein, the determination of its amino acid sequence will be necessary.

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