Isolation of (Copper, Zinc)-Thioneins from Pig Liver

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The copper-binding proteins with mol.wt. of approx. 12000 in the cytosol of pig liver have been identified as copper-thioneins. They contain variable amounts of zinc, with approx. 10% total metal in the protein. The molar cysteine/metal ratio is approx. 2:1.

Injection of copper salts into rats induces synthesis of a major hepatic copper-protein with mol.wt. in the range 8000–12000 (Bloomer & Sourkes, 1973; Bremner & Davies, 1974), which has now been characterized as copper-chelatin (Winge *et al.*, 1975*b*). Although zinc may also be bound to this protein, the composition and properties of chelatin are quite different from those of zinc-thionein, the analogous protein induced in rat liver after injection of zinc salts (Bremner & Davies, 1976; Winge *et al.*, 1975*a*).

However, there is some evidence that in more physiological situations, not involving acute administration of copper, at least some of the hepatic copper is present as copper-thionein (Bremner & Marshall, 1974*b*; Bremner & Davies, 1976), which may occur in a particulate polymeric form (Porter, 1974). The tendency of copper-thionein to form aggregates has previously prevented the isolation of the pure protein. Nevertheless it is significant that the amount of copper present in proteins of this low molecular weight is markedly influenced by the zinc status of the animal. Up to 40% of the hepatic copper usually occurs in this form in zinc-supplemented pigs, whereas virtually none is present in zinc-deficient pigs (Bremner, 1976) or ruminants (Bremner & Marshall, 1974*a*). It was suggested (Bremner, 1976) that zinc might be essential for stabilization of the copper-protein, perhaps by maintaining some particular conformation. This explanation, however, would only be tenable if both metals were bound to the same protein and this has never been unequivocally demonstrated. The current study deals with the separation and characterization of the copper- and zinc-containing low-molecular-weight proteins in pig liver.

Methods

Details of the pigs, whose diet was adequately supplemented with zinc, of the concentration and distribution of hepatic copper and zinc, and of the general experimental procedures have been published (Bremner, 1976; Bremner & Davies, 1975). CM-

Sephadex C-50 was from Pharmacia, Uppsala, Sweden. Spectral analyses were carried out on a Unicam SP.800 spectrophotometer (Pye Unicam, Cambridge, U.K.). All fractionation procedures were carried out under N₂. Polyacrylamide-gel electrophoresis was by the method of Davis (1964) with 105 g of photopolymerized gel/litre. The electrolyte contained 4.9 mM-Tris and 38.5 mM-glycine (pH 8.3). Gels were stained with Amido Black. Thiol groups were measured with 5,5'-dithiobis-(2-nitrobenzoic acid) (Jocelyn, 1962).

The liver was homogenized in 10 mM-Tris/acetate (pH 7.4) (1:1.5, w/v) and centrifuged at 100000*g* (*r*_{av} 7.38 cm) for 1 h at 1°C (Bremner & Davies, 1975). A preliminary crude fractionation of the supernatant (80 ml) was carried out on a column (60 cm × 5 cm) of Sephadex G-75, with the same buffer as eluent (Scheme 1). The third copper- and zinc-containing fraction to emerge (III), eluted at between 720 and 860 ml of eluent, contained all the low-molecular-weight protein. This was collected and applied over several hours to a column (30 cm × 1.6 cm) of DEAE-Sephadex A-25 pre-equilibrated in 10 mM-Tris/acetate (pH 7.4) and the column was washed with the same buffer until the concentration of protein, copper and zinc in the eluent was almost zero. The initial eluent and washings were combined to give fraction (III-1).

The column was then eluted with a linear gradient (800 ml) of 10 mM–1 M-Tris/acetate (pH 7.4) at a flow rate of approx. 30 ml/h, 13.5 ml fractions being collected. One major copper- and zinc-containing fraction (III-2) was eluted at a Tris concentration of about 40 mM and a minor one (III-3) at about 80 mM (Fig. 1*a*). Fraction (III-2) was concentrated by ultrafiltration and purified by chromatography on a column (90 cm × 1.6 cm) of Bio-Gel P-10, with 10 mM-Tris/acetate (pH 7.4) as eluent, at a flow rate of approx. 10 ml/h, to give one main metal-containing fraction (Fig. 1*b*). As the copper and zinc distribution over the peak was non-uniform two separate 'head' and 'tail' fractions were collected, concentrated and desalted by repeated ultrafiltration to give fractions (III-2*a*) and (III-2*b*) respectively.

Scheme 1. Flow diagram for isolation of (copper, zinc)-thioneins

Experimental details are given in the text. Quantitative results are given for one typical liver and are expressed as recoveries/g of fresh liver.

Fig. 1. Separation of copper-thionein from pig liver

(a) The crude copper-thionein fraction obtained by gel filtration on Sephadex G-75 was separated on DEAE-Sephadex as described in the text. The fraction (III-1), eluted with the equilibrating buffer, is not shown. The positions of the main fraction (III-2) and of fraction (III-3) are shown. (b) The copper-thionein fraction (III-2), obtained after chromatography on DEAE-Sephadex, was purified by gel filtration on Bio-Gel P-10, as described in the text. Fractions (III-2a) and (III-2b) were collected in tubes 44–48 and 49–53 respectively. In both cases fractions were analysed for zinc (○), copper (●) and E_{280} (Δ).

Fraction (III-1) was applied to a column (30cm×1.6cm) of CM-Sephadex C-50, pre-equilibrated with 10mM Tris/acetate (pH 7.4). The column was washed with the same buffer until the concentrations of copper and zinc in the eluate were almost zero. The combined washings were concentrated by ultrafiltration and fractionated on Bio-Gel P-10 as above to give one main fraction (III-1a). Further elution of the CM-Sephadex column with a linear gradient of 1 litre of 10–500mM Tris/acetate (pH 7.4) gave further, separate copper- and zinc-containing fractions, but only in small amounts.

Because the purified proteins in fraction (III) showed little absorbance at 280nm it was possible to monitor their purity by estimation of the ratio of copper concentration (μg/ml) to E_{280} ([copper]/ E_{280}).

Results and Discussion

In the pig livers examined, 30–40% of the total copper was generally present in the low-molecular-weight form (III) separated on Sephadex G-75 (Bremner, 1976). Subsequent separation of this on DEAE-Sephadex gave a major fraction (III-1) (Scheme 1), which appeared to have little anionic charge at pH 7.4 and showed considerable heterogeneity on polyacrylamide gel electrophoresis. On application of fraction (III-1) to CM-Sephadex C-50 most of the copper and zinc was eluted directly with 10mM-Tris/acetate (pH 7.4), whereas most of the contaminant proteins were adsorbed and removed by this procedure. After a final purification on Bio-Gel P-10 fraction (III-1a) was found to be homogeneous on polyacrylamide-gel electrophoresis with a mobility relative to Bromophenol Blue of 0.49, and to have a [copper]/ E_{280} ratio of 15. Both copper and zinc were present in fraction (III-1a), but it was enriched in copper compared with fraction (III), with a copper/zinc ratio of 2.5 compared with 1.7. The molar ratio of thiol groups to metal atoms was 1.3 in fraction (III-1a). It is to be stressed that these ratios were not constant and varied in different livers. The amino acid composition of fraction (III-1a) (Table 1) shows clearly that this protein is of the metallothionein type, with about 30% cysteine residues, a high serine content, no aromatic residues and relatively little glutamic acid, leucine and isoleucine. The proportion of metal in the protein was about 11% by weight, when the protein concentration was estimated from the amino acid composition. The molar ratio of cysteine residues to metal atoms, 1.9, was greater than the thiol/metal ratio, suggesting that some disulphide bonds may exist in the protein. However, these may have been introduced during the isolation procedure, despite the use of N_2 -saturated buffers and collection of fractions under N_2 . Alternatively the thiol assay procedure may not be suitable for copper proteins.

The other main fraction (III-2), obtained by gradient elution of the DEAE-Sephadex, had a high [copper]/ E_{280} ratio, but some heterogeneity was evident on electrophoretic examination. Further, the copper/zinc ratio increased progressively from 0.8 to 2.2 during elution of this fraction from the column. At no time, however, were separate copper- and zinc-containing fractions obtained, even when much shallower gradients were used for elution.

Contaminant protein was successfully removed by gel filtration on Bio-Gel P-10. Only one copper- and zinc-containing peak was obtained, but there was again a tendency for the metals to partially separate. The 'head' and 'tail' fractions were therefore combined separately to give fractions (III-2a) and (III-2b), with ratios of copper/zinc of 1.1 and 1.8 respectively and of [copper]/ E_{280} of over 18. Amino acid

Table 1. *Amino acid composition of copper-thionein from pig liver*

Copper-thioneins were isolated from pig liver by a combination of ion-exchange chromatography and gel filtration. Amino acid analysis was carried out on samples hydrolysed in 6M-HCl for 24h at 110°C after performic acid oxidation. The compositions of rat liver copper-chelatin and of metallothionein are taken from the papers of Winge *et al.* (1975b) and Bremner & Davies (1975).

Amino acid composition (% of total residues)						
Fraction	...	Pig liver copper-thioneins			Rat liver copper-chelatin	Rat liver zinc-thionein A
		(III-1a)	(III-2a)	(III-2b)		
Lysine		11.06	11.41	10.70	13.1	11.86
Arginine		1.36	1.38	1.95	2.7	0.15
Aspartic acid		7.94	7.46	8.42	9.8	7.11
Threonine		4.40	4.61	4.55	5.1	6.22
Serine		12.54	13.12	12.88	4.2	16.38
Glutamic acid		1.63	1.97	1.94	11.1	2.79
Proline		4.44	—	—	3.6	3.99
Glycine		9.49	11.20	10.94	8.0	9.71
Alanine		10.32	11.70	11.80	6.0	5.56
Cysteine*		32.0	33.1	32.3	14.6	29.30
Valine		1.61	0.90	0.54	5.2	3.17
Methionine†		1.50	1.69	1.45	1.6	1.99
Leucine		—	—	—	6.3	0.25
Isoleucine		1.06	1.46	1.56	4.3	0.34
Phenylalanine		—	—	—	2.1	0.15
Copper/zinc ratio		2.51	1.07	1.79		
Metal/protein (w/w, %)		10.7	8.0	9.6		
Cysteine/metal (mol)		1.9	2.6	2.1		

*Measured as cysteic acid.

†Measured as methionine sulphone.

analysis of these two sub-fractions did not reveal any significant differences in their composition (Table 1). They were both clearly of the metallothionein type and similar to fraction (III-1a), with less than 80% of their cysteine residues being measured with 5,5'-dithiobis-(2-nitrobenzoic acid). The electrophoretic mobilities of fractions (III-2a) and (III-1a) were identical, but no protein band was detectable on gels after electrophoresis of fraction (III-2b). A similar phenomenon has been reported for some fractions of copper-chelatin (Winge *et al.*, 1975b).

The copper-thioneins isolated were similar to copper-chelatin in their elution volume from Sephadex G-75 and in that they gave no e.p.r. (electron-paramagnetic-resonance) signal. However, they differed markedly in amino acid composition especially in cysteine, serine and glutamic acid contents (Table 1). Moreover, the copper-thioneins had at most only a limited affinity for DEAE-Sephadex at pH 7.4, whereas chelatin binds irreversibly to DEAE-cellulose (Winge *et al.*, 1975b). As 85% of the copper in fraction (III) could be accounted for by the sub-fractions eluted from DEAE-Sephadex, it is clear that no significant amounts of chelatin occurred in these pig livers.

One notable property of the copper-thioneins is their tendency to form aggregates, probably involving disulphide bond formation, during their isolation and subsequent storage. For example, the electrophoretic behaviour of fraction (III-2a) changed during storage with the development of very diffuse bands, though the original pattern could be partly restored by treatment of the sample with dithiothreitol. Refractionation of fraction (III-1a) on Bio-Gel P-10 revealed the presence of copper proteins of higher molecular weight. In one sample of fraction (III-1a) with low zinc content, only 3% of the cysteine residues were estimated with 5,5'-dithiobis-(2-nitrobenzoic acid). Similar aggregation behaviour has been reported in copper-chelatin (Winge *et al.*, 1975b) and in cupredoxin (Prinz & Weser, 1975). This is of interest as the particulate copper-protein in neonatal bovine liver may be a polymeric species of metallothionein (Porter, 1974). The copper content of this protein is only 4%, however, and the cysteine residues occur mainly in oxidized form. Nevertheless they are probably still involved in metal binding (Porter, 1971), as has been found in both native metallothionein (Kagi & Vallee, 1961) and the derivative produced on

replacement of the 9g-atoms of cadmium and zinc/mol of protein with copper (Rupp & Weser, 1974). Up to 15g of copper could be introduced, which is similar to the amount found in the natural copper-thioneins. These generally contained 2 cysteine residues/metal atom, in contrast with the usual ratio of 3/1 in (cadmium, zinc)-thioneins (Kagi & Vallee, 1961). It is perhaps significant that the cysteine/metal ratio was 2.6 in fraction (III-2a), which contained almost as much zinc as copper. The proportion of cysteine residues detectable by 5,5'-dithiobis-(2-nitrobenzoic acid) was also greater in this fraction.

The absorption spectra of the three pig copper-thioneins were similar, with no maximum occurring at 280nm, but with a shoulder at about 265–270nm. This is close to the spectrum reported for the copper-substituted metallothionein (Rupp & Weser, 1974), suggesting similar copper-binding in these proteins.

In conclusion, it appears that in a normal physiological situation copper and zinc occur in pig liver not as copper-chelatin, but as a range of mixed (copper, zinc)-thioneins of constant amino acid composition. The relative proportion of the metals influences the charge and molar volume of the metallo-proteins, with a high copper/zinc ratio increasing their tendency to form aggregates during isolation.

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