Biochem. J. (1976) **157**, 517–520
Printed in Great Britain

Isolation of (Copper, Zinc)-Thioneins from the Livers of Copper-Injected Rats

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(*Received* 1 *June* 1976)

The mixed copper- and zinc-binding proteins, with mol.wt. of approx. 12000, induced in rat liver after injection of copper were isolated and characterized as metallothioneins. Three separate forms were obtained, with 7–11% total metal in the protein, equivalent to 2.6–1.6 cysteine residues/metal atom.

A copper-protein with mol.wt. of about 12000 occurring in the liver of the pig has been characterized as metallothionein (Bremner & Young, 1976). Injection of copper salts into rats stimulates the production of a hepatic copper-binding protein of similar molecular weight (Bloomer & Sourkes, 1973; Bremner & Davies, 1974, 1976). The possible identity of that protein with metallothionein was considered (Bremner, 1974), but it was later claimed (Evans *et al.*, 1975; Riordan & Gower, 1975; Winge *et al.*, 1975*b*) that the copper-induced protein, termed copper-chelatin (Winge *et al.*, 1975*b*), is not metallothionein.

However, in previous studies on the induction of zinc-thioneins in rat liver (Bremner & Davies, 1975), zinc-proteins with amino acid compositions similar to that of copper-chelatin (Winge *et al.*, 1975*b*) were often obtained, but on further purification these always yielded zinc-thioneins. Further, despite the reported presence of 7% of leucine and 5–14% of cysteine residues in chelatin (Evans *et al.*, 1975; Riordan & Gower, 1975; Winge *et al.*, 1975*b*), copper injection stimulated only the incorporation of [^{35}S]-cysteine, but not of significant amounts of [^{3}H]leucine, into the copper-protein of rat liver (W. G. Hoekstra, I. Bremner & N. T. Davies, unpublished work). It is significant that rat liver metallothionein preparations contain at most only 1% of leucine residues (Bremner & Davies, 1975; Winge *et al.*, 1975*a*).

Further attempts have therefore been made to purify the low-molecular-weight copper-binding proteins occurring in rat liver after copper injection. Three forms of (copper, zinc)-thioneins were isolated and characterized.

Methods

Male Hooded Lister rats (Rowett Institute strain) weighing 170 or 250g were injected intraperitoneally with copper (as $CuSO_4$ in a solution containing 9g of NaCl/litre) at a dose rate of 1.76 or 3mg of copper/kg respectively. The rats were killed after 18h and their livers removed and stored at −20°C. The analytical and fractionation procedures used have been described previously (Bremner & Davies, 1975; Bremner & Young, 1976).

The livers from 12 rats were homogenized in 10mM-Tris/acetate (pH7.4) (1:1.5, w/v) and centrifuged at 75000*g* ($r_{max.}$ 10.8cm) for 1.5h at 1°C. A preliminary crude fractionation of the supernatant (110ml) was carried out on a column (60cm×5cm) of Sephadex G-75, with the same buffer as eluent. The third copper- and zinc-containing fraction to emerge (III) at between 720 and 860ml of eluate contained all the low-molecular-weight protein, free from most of the other hepatic proteins. This fraction was applied, over several hours, to a column (22cm×1.6cm) of DEAE-Sephadex A-25 in 10mM-Tris/acetate (pH7.4) and the column was washed with the same buffer until the concentration of protein, copper and zinc in the eluate was almost zero. The initial eluate and washings were combined to give fraction (III-1).

The column was then eluted with a linear gradient (800ml) of 10–200mM-Tris/acetate (pH7.4) at a flow rate of about 30ml/h; 13.5ml fractions were collected. Three copper- and zinc-containing fractions (III-2–III-4) were eluted at Tris concentrations of about 40, 80 and 110mM respectively (Fig. 1*a*). These were concentrated by ultrafiltration and fractionated twice on Bio-Gel P-10 (90cm×1.6cm) with 10mM-Tris/acetate (pH7.4) as eluent, at a flow rate of about 10ml/h, to give in each case one main metal-containing fraction (III-2*a*–III-4*a*).

Results and Discussion

As reported previously (Bremner & Davies, 1974, 1976), injection of copper into the rats promoted the appearance of both copper and zinc in a fraction (III), with mol.wt. of about 12000. The amount of copper and zinc in fraction (III) depended on the dose used, but in one set of livers, with mean copper and zinc

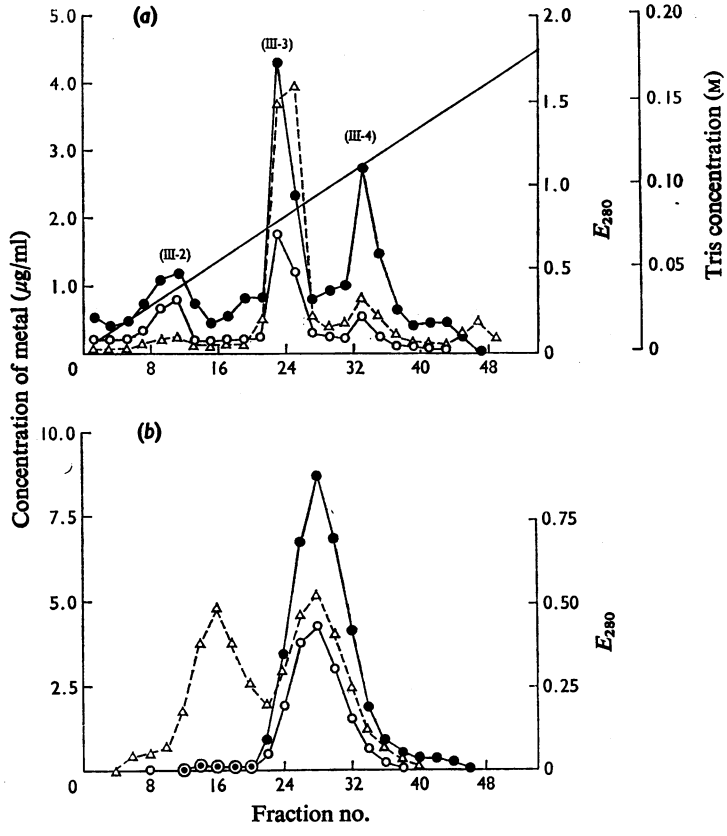

Fig. 1. *Separation of copper-thioneins from rat liver*

(*a*) The crude copper-thionein fraction obtained by gel filtration on Sephadex G-75 was separated on DEAE-Sephadex A-25 as described in the text. The fraction (III-1), eluted with the equilibrating buffer, is not shown. The position of fractions (III-2)–(III-4) and the gradient of eluting buffer are indicated. (*b*) The copper-thionein fraction (III-3) obtained from DEAE-Sephadex was purified by gel filtration on Bio-Gel P-10, as described in the text. Fraction (III-3*a*) was collected in tubes 28–33. In both cases, fractions were analysed for copper (●), zinc (○) and E_{280} (△).

contents of 42.3 and 42.5 μg/g fresh weight, 31 and 14% of the respective metals were found in this form.

Further separation of fraction (III) from these livers on DEAE-Sephadex A-25 gave four subfractions. The first (III-1) did not bind to the column and the others (III-2–III-4) were eluted at Tris concentrations of about 40, 80 and 110mM respectively (Fig. 1*a*). These four subfractions contained, in order, 31, 8, 23 and 15% of the copper and 43, 15, 24 and 7% of the zinc present in fraction (III). Fractions (III-2)–(III-4) were further purified by gel filtration on Bio-Gel P-10 (Fig. 1*b*) to give in each case only one predominant metal-binding fraction (III-2*a*–III-4*a*), with concomitant removal of other proteins.

As was found previously during the isolation of (copper, zinc)-thioneins from pig liver (Bremner & Young, 1976), the elution patterns for copper and zinc from both ion-exchange and gel-filtration columns were not quite identical. For example, copper in fraction (III-3*a*) tended to be eluted from Bio-Gel P-10 slightly before the zinc, the copper/zinc ratio increasing from 1.8 to 2.9 in tubes 28 to 33 (Fig. 1*b*). There were also differences in the overall copper/zinc ratio between the three subfractions (III-2*a*)–(III-4*a*), the values in the livers described above being 1.6, 2.1 and 6.2 respectively.

Amino acid analysis of these fractions (Table 1) showed that they were all of the metallothionein type, with a characteristic cysteine content of about 30%. Fractions (III-2*a*) and (III-3*a*) were similar to the hepatic zinc-thioneins A and B isolated from zinc-injected rats and eluted from DEAE-Sephadex with about 30 and 70mM-Tris/acetate (pH 7.4) respectively (Bremner & Davies, 1975). This suggests that the

Table 1. *Amino acid composition of copper- and zinc-thioneins from rat liver*

Copper-thioneins were isolated from the livers of copper-injected rats by a combination of ion-exchange chromatography and gel filtration. Fractions (III-2*a*), (III-3*a*) and (III-4*a*) were eluted from DEAE-Sephadex with about 40, 80 and 110mM-Tris/acetate respectively. Amino acid analysis was carried out on samples hydrolysed in 6M-HCl for 24h at 110°C after performic acid oxidation. The composition of rat liver zinc-thioneins A and B and of copper-chelatin are taken from the papers of Bremner & Davies (1975) and of Winge *et al.* (1975*b*) respectively.

	Amino acid composition (% of total residues)					
	Rat liver copper-thioneins			Rat liver zinc-thioneins		Rat liver copper-chelatin
Fraction ...	(III-2*a*)	(III-3*a*)	(III-4*a*)	A	B	
Lysine	12.0	13.6	13.1	11.9	12.5	13.1
Histidine	—	—	—	—	—	1.1
Arginine	—	—	—	0.2	—	2.7
Aspartic acid	9.2	8.5	9.0	7.1	6.7	9.8
Threonine	5.6	3.9	3.6	6.2	3.9	5.1
Serine	15.8	15.0	13.5	16.4	14.1	4.2
Glutamic acid	4.5	6.3	6.5	2.8	6.3	11.1
Proline	—	—	—	4.0	4.9	3.6
Glycine	9.6	7.4	8.1	9.7	7.3	8.0
Alanine	6.8	8.2	8.4	5.6	8.1	6.0
Cysteine*	28.9	30.1	31.0	29.3	27.2	14.6
Valine	3.2	2.3	2.3	3.2	2.4	5.2
Methionine†	1.8	1.8	1.7	2.0	1.8	1.6
Leucine	0.8	0.6	0.8	0.3	1.0	6.3
Isoleucine	1.1	1.7	1.7	0.3	1.7	4.3
Phenylalanine	0.5	0.3	0.2	0.2	0.5	2.1

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

same forms of metallothionein may be induced by copper and zinc, and by cadmium, mercury and silver (Winge *et al.*, 1975*a*). Isolation of two forms of hepatic metallothionein is quite common (Nordberg *et al.*, 1972; Buhler & Kagi, 1974), but we are unaware of any reports of the separation of additional forms.

The copper-induced proteins were also similar in their electrophoretic behaviour in 10.5% (w/v) polyacrylamide gels at pH8.3 to the zinc-thioneins (Bremner & Davies, 1975). Fraction (III-2*a*) showed a main band, with a mobility relative to Bromophenol Blue of about 0.49, similar to that reported for zinc-thionein A (Bremner & Davies, 1975). In the case of fraction (III-3*a*) and zinc-thionein B, the mobility value was about 0.67. However, unlike the zinc-induced proteins, which were electrophoretically homogeneous, the copper-proteins (III-2*a*) and (III-3*a*) each contained a minor component with mobility of about 0.31 and 0.49 respectively. Similar electrophoretic heterogeneity has been reported in the analogous copper-proteins from yeast (Prinz & Weser, 1975) and rabbit liver (Premakumar *et al.*, 1975), where the major and minor bands are apparently related and possibly arise from oxidative changes in the copper-protein (Prinz & Weser, 1975). The two bands in fraction (III-3*a*) clearly did not arise from the presence of contaminant protein or the existence of separate copper- and zinc-proteins, as analysis of acid digests of 2.5mm segments of unstained gels revealed the presence of most of the applied copper and zinc in the two protein bands. Both metals were present in each band and both were absent from the equivalent regions of control gels. Further, amino acid analysis of the 'head' and 'tail' fractions of fraction (III-3*a*) eluted from the Bio-Gel P-10 column (Fig. 1*b*), with different copper/zinc ratios, did not reveal any significant differences in amino acid composition.

The (copper, zinc)-thioneins from rat liver were similar to those isolated previously from the livers of pigs (Bremner & Young, 1976). The molar proportion of cysteine residues to metal atoms was 1.6 in proteins with a copper/zinc ratio of 2.1–6.2 [as in fractions (III-3*a*) and (III-4*a*) in the livers described above]. At lower copper/zinc ratios, however, there was a linear increase in this proportion; one fraction with a copper/zinc ratio of only 0.15 contained 2.6 cysteine residues/metal atom. There were corresponding variations in the metal contents of the proteins, which ranged from 11% to 7.5%. Only 50–80% of the thiol groups could be measured with 5,5′-dithiobis-(2-nitrobenzoic acid) (Jocelyn, 1962), the proportion decreasing with increase in the copper content of the protein. Some disulphide bridges may therefore be present,

although these could have been introduced during isolation of the proteins (Bremner & Young, 1976). The apparent increase in disulphide-bridge formation in low-zinc proteins may explain the development of polydisperse characteristics during the attempted isolation of copper-thioneins from the livers of zinc-deficient rats. These proteins contain only small amounts of zinc (Bremner & Davies, 1976) and have a shorter biological half-life than the copper-thioneins from zinc-supplemented rats (W. G. Hoekstra, I. Bremner & N. T. Davies, unpublished work).

Unfortunately it was not possible to characterize the copper-protein (III-1) which did not bind to the DEAE-Sephadex column and accounted for about 30% of the copper in fraction (III). The behaviour of this protein on subsequent chromatography on CM-Sephadex C-50 and Bio-Gel P-10 was similar to that of the equivalent copper-protein isolated previously from pig liver (Bremner & Young, 1976), but it could not be obtained in a sufficiently pure state for analysis. It is possible that this fraction also consisted of copper-thionein, as was found for the pig liver protein. Certainly the behaviour of fraction (III-1) on DEAE-Sephadex columns was completely different from that of chelatin, which was irreversibly bound (Winge *et al.*, 1975*b*).

Regardless of the identity of fraction (III-1), however, it is clear from these studies that administration of copper to rats induces the formation in liver of appreciable amounts of copper-thionein. At least one-half of the copper in fraction (III) was unequivocally shown to be in this form. It is therefore difficult to reconcile these findings with the claim that the major copper-inducible protein in rat liver is not metallothionein (Evans *et al.*, 1975; Riordan & Gower, 1975; Winge *et al.*, 1975*b*). It is noteworthy that these workers used only a combination of ultrafiltration, gel-filtration and acetone precipitation techniques to purify the proteins used for amino acid analysis. Further, Evans *et al.* (1975) assessed the purity of the protein from its electrophoretic behaviour on cellulose acetate, which has only limited resolving power. The sample analysed by Riordan & Gower (1975) was extremely polydisperse and acknowledged to be heterogeneous; no evidence was provided to support the assumption that it contained only copper-proteins. The procedure used by Winge *et al.* (1975*b*), although yielding a copper-protein which was electrophoretically homogeneous, was found by the same authors to be inadequate for the removal of all contaminant protein from rat liver metallothioneins induced by other metals (see Table 1 and Figs. 1 and 8 of Winge *et al.*, 1975*a*).

We thank Mr. W. R. Hepburn for the amino acid analyses and Dr. N. T. Davies for supplying the rat livers.

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