Species differences in the occurrence of copper-metallothionein in the particulate fractions of the liver of copper-loaded animals

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(Received 4 November 1983/Accepted 30 December 1983)

Large amounts of Cu-metallothionein were obtained by 2-mercaptoethanol and sodium dodecyl sulphate extractions of the particulate fractions of the liver of pigs given high-Cu²⁺ diets or rats injected with Cu²⁺. Three isoproteins were purified from pig liver and characterized on the basis of their physicochemical properties, metal content and amino acid composition. No such pool of Cu-metallothionein was present in the liver of Cu²⁺-loaded sheep or of rats given Cu²⁺-supplemented diets.

Excessive hepatic accumulation of Cu frequently results in severe changes in liver structure and function, and may even cause death, as in Wilson's disease in man and in chronic Cu toxicosis in sheep (see Bremner, 1979; Sternlieb, 1980). However, equally high liver Cu contents may also be tolerated with only minor signs of liver dysfunction, as in newborn animals and in such diverse species as the buffalo toad (Goldfischer *et al.*, 1970) and the Bedlington terrier (Ludwig *et al.*, 1980). It is possible that these differences in ability to tolerate Cu reflect differences in the intracellular distribution of the metal, as has been indicated by histochemical or electron-microscopical examination of Cu-loaded livers (see Bremner, 1979; Sternlieb, 1980).

In many cases, excess hepatic Cu has been located principally in electron-dense lipofuscin-rich granules, probably derived from lysosomes (Goldfischer *et al.*, 1970, 1980; King & Bremner, 1979). It has been suggested that this constitutes a defence mechanism against hepatotoxicity (Goldfischer & Sternlieb, 1968; Epstein *et al.*, 1981). However, this is an oversimplification of the situation, as major ultrastructural changes occur in the liver of Cu-poisoned sheep, despite the localization of the metal in lysosomes (King & Bremner, 1979; Gooneratne *et al.*, 1980).

The molecular association of the metal must determine the hepatotoxic effects of Cu. Major

differences occur in the distribution of Cu in the liver cytosol of sheep and pigs, particularly in the binding of the metal to metallothionein (Bremner & Marshall, 1974; Bremner, 1976). These species differ markedly in their tolerance of Cu, and it may be that the extreme susceptibility of sheep to Cu toxicosis is related to an impaired ability to incorporate hepatic Cu into this protein (Bremner, 1979). This view is supported by the isolation of large amounts of Cu-metallothionein from the livers of Bedlington terriers (Johnson *et al.*, 1981) and of the human foetus (Riordan & Richards, 1980). There are no major signs of Cu-induced liver dysfunction in these livers even at relatively high liver Cu contents.

Most of the Cu-metallothionein in the liver of either the human foetus or Bedlington terrier was present in the particulate (non-cytosolic) fractions of the tissue and was extracted with reducing media, whereas the earlier comparative studies on Cu-metallothionein in sheep and pig liver were concerned only with the cytosolic protein. Since the particulate fractions can account for 60–80% of the total hepatic Cu in these latter species after Cu loading, it was of interest to establish whether a non-cytosolic pool of Cu-metallothionein existed in either of these animals or in the rat. No significant amounts of the protein were found in the livers of sheep or of rats given dietary Cu²⁺ supplements. However, a large proportion of the non-cytosolic hepatic Cu in pigs and Cu²⁺-injected rats was present as Cu-metallothionein. Three isoproteins of the 'particulate' porcine protein were purified and characterized.

Abbreviations used: SDS, sodium dodecyl sulphate.

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Materials and methods

Animals

Eight Suffolk $\times$ (Finn-Dorset) lambs, aged about 12 weeks, were maintained for 12–24 weeks on a diet containing 25 mg of Cu/kg body wt. (Bremner & Young, 1978). Twelve Landrace $\times$ (Large White $\times$ Landrace) pigs, aged 6 weeks, were given a diet with 300 mg of Cu/kg for 8 weeks (Bremner, 1976). Male Hooded Lister rats (Rowett Institute strain), aged 5 weeks, were given a diet containing 500–1000 mg of Cu/kg for 6 weeks (Williams & Mills, 1970). Additional rats were injected intraperitoneally on three successive days with Cu^{2+} , as CuSO_4 (3 mg of Cu^{2+} /kg body weight), and were killed 24 h after the last injection.

Livers were removed from all animals at slaughter and stored at -20°C .

Fractionation of pig and rat liver

(i) Samples of liver were homogenized in 2.5–10 vol. of 0.01 M-Tris/acetate buffer, pH 7.4, and centrifuged at 100 000 *g* for 1 h. The supernatant was removed and fractionated on Sephadex G-75 as described previously to determine the amount of Cu and Zn bound to metallothionein (Bremner & Marshall, 1974). The pellet was suspended in 1% (v/v) 2-mercaptoethanol in water (3.5 ml/g of liver), freeze-thawed three times to aid disruption of membranes and left overnight at $+4^\circ\text{C}$. The mixture was then centrifuged and fractionated on Sephadex G-75 as described above.

(ii) In an alternative procedure, a sample of liver was homogenized with 10 vol. of 0.01 M-Tris/acetate buffer, pH 7.4, containing 1% 2-mercaptoethanol and 1% SDS. The homogenate was left for 3 h at 37°C , whereupon it was centrifuged at 100 000 *g* and the supernatant fraction separated on Sephadex G-75 as described above.

Fractionation of sheep liver

The procedures used were similar to those described in section (i) of the above paragraph, except that the extraction with 1% mercaptoethanol was carried out directly on the whole liver.

Purification of Cu-metlothionein from the particulate fractions of pig liver

Samples (about 200 g) of liver were homogenized with 1.5 vol. of 0.01 M-Tris/acetate, pH 7.4, and centrifuged at 100 000 *g* for 1 h. The supernatant was decanted and the pellet homogenized with 1% (v/v) 2-mercaptoethanol. After freeze-thawing three times the mixture was incubated at 37°C for 3 h and centrifuged again to obtain a soluble fraction that was concentrated 5-fold by ultrafiltration with an Amicon UM-2 membrane (Amicon Ltd., Stoneleigh, Coventry, West Midlands, U.K.). The

concentrated sample was centrifuged at 73 000 *g* for 1 h to remove any precipitate. The supernatant was then fractionated by a combination of gel filtration on Sephadex G-75 and G-50 and ion-exchange chromatography on DEAE-Sephadex A-25, as described for pig liver cytosol (Bremner & Young, 1976), except that all buffers contained 10 mM-2-mercaptoethanol.

Three partially purified isoproteins of Cu-metlothionein were obtained in this way. Final purification of each of these was achieved by affinity chromatography on thiopropyl-Sepharose 6B as described by Riordan & Richards (1980). Care was taken to limit the flow rate of the column, during application of the sample and during elution of the metallothioneins with 0.05 M-Tris/HCl containing 0.05 M-2-mercaptoethanol, to only 2.0 ml/h.

Analytical techniques

The procedures used for metal and amino acid analysis have been described previously (Bremner & Young, 1976).

Results

Pig liver

The pig livers contained $135 \pm 14 \mu\text{g}$ of Cu^{2+} and $81 \pm 3 \mu\text{g}$ of Zn^{2+} /g fresh weight, and about 39 and 73% of the tissue Cu and Zn respectively were recovered in the cytosol after homogenization of the livers with Tris/acetate, pH 7.4. Fractionation of the cytosol on Sephadex G-75 revealed that $88 \pm 2\%$ of the Cu and $52 \pm 3\%$ of the Zn was present in a fraction, with $V_e/V_0 = 2$, known to be metallothionein (Bremner & Young, 1976). On treatment of the 'non-cytosolic' pellet with a solution of 2-mercaptoethanol, substantial amounts of Cu and Zn, equivalent to 16 ± 1 and $9.4 \pm 0.4\%$ respectively of the liver metal, were extracted. Fractionation of this extract on Sephadex G-75 (Fig. 1) revealed that the bulk of the Cu (85%) and a small proportion (25%) of the Zn were eluted in a fraction with $V_e/V_0 = 2$, later shown to be metallothionein (see below). The average Cu/Zn ratio in the metallothionein fraction was 10:1. Sequential extraction of the liver with Tris/acetate buffer and with 2-mercaptoethanol therefore removed 54% of the tissue Cu, and 87% of this appeared to be present in the form of one protein. More efficient extraction of the hepatic Cu was achieved by treating the whole liver with a mixture of SDS and 2-mercaptoethanol. Up to 86% of the total Cu was then extracted, and 80% of this was generally present in the 'metallothionein' fraction. (The elution profile from the Sephadex G-75 column was similar to that shown in Fig. 1, except that more Zn was present in the metallothionein peak.)

Fig. 1. Fractionation on Sephadex G-75 of the 2-mercaptoethanol extract of the particulate fractions obtained from pig livers

Livers were homogenized in 0.01M-Tris/acetate, and centrifuged at 100000g for 1 h. The pellet was homogenized once more in 1% 2-mercaptoethanol, freeze-thawed three times and centrifuged as described above. The supernatant fraction was then separated on Sephadex G-75 as described in the text. The concentrations of Cu (○) and of Zn (Δ) and the position of the metallothionein peak (MT) are shown.

However, the yield of metallothionein was proportionally less when livers of high Cu content were extracted.

Isolation of Cu-metallothionein

The identity of the major Cu-binding fraction in the 2-mercaptoethanol extract was established by the isolation and characterization of the protein (it was not possible to purify the SDS/2-mercaptoethanol extracts because of the modifying effects of the detergent on the charge properties of the protein). The fractionation procedure involved an initial separation by gel filtration on Sephadex G-75 (Fig. 1), followed by anion-exchange chromatography of the main fraction on DEAE-Sephadex A-25. Half of the Cu did not bind to the ion-exchange column and was eluted with the equilibrating buffer, 0.01M-Tris/acetate. Subsequent gradient elution of the column gave two main fractions, I and II (Fig. 2). These were eluted with approx. 0.04 and 0.11M-Tris/acetate and had average Cu/Zn ratios of 4–7:1. The average recovery of Cu from the column was 85%.

Final purification of each of the three subfrac-

Fig. 2. Purification on DEAE-Sephadex A-25 of the particulate Cu-metallothionein isolated from pig livers

The crude particulate Cu-metallothionein, as obtained by gel-filtration on Sephadex G-75, was applied to a column of DEAE-Sephadex A-25. About 50% of the protein did not bind to the ion-exchanger and was eluted with the equilibrating buffer (not shown). The other main isoproteins were eluted with a linear gradient (—) of 0.01–0.2M-Tris/acetate, pH 7.4. Concentrations of Cu (□) and of Zn (■) and the positions of metallothioneins-I and II (MT-I and MT-II) are shown.

Fig. 3. Purification on thiopropyl-Sepharose 6B of Cu-metallothionein isolated from the particulate fractions of pig livers. The three isoproteins of Cu-metallothionein, obtained after DEAE-Sephadex A-25 chromatography, were each purified by affinity chromatography on thiopropyl-Sepharose 6B. After application of the sample, the column was eluted sequentially with 0.3M-ammonium acetate, pH 5.5 (Buffer A), 0.3M-ammonium acetate, pH 5.5, containing 1.0M-NaCl (Buffer B), 0.05M-Tris/HCl, pH 8.0, containing 1.0M-NaCl (Buffer C), 0.05M-Tris/HCl, pH 8.0 (Buffer D) and finally 0.05M-Tris/HCl, pH 8.0, containing 0.05M-2-mercaptoethanol (Buffer E). The concentrations of Cu (▲) and of Zn (■) are shown. Metallothionein was eluted with Buffer E.

tions (i.e. the 'unbound' fraction, I and II) was achieved by affinity chromatography on thiopropyl-Sepharose 6B (Fig. 3). Provided that very low flow rates were used, most of the Cu was eluted in a single fraction with Tris/HCl buffer containing 2-mercaptoethanol. However considerable loss of Zn occurred during the affinity chromatography, the purified proteins being virtually free of Zn.

Properties of particulate Cu-metallothionein

The amino acid composition of each of the three purified isoproteins is given in Table 1 and compared with that of cytosolic pig liver Cu-metallothionein. The 'particulate' proteins are clearly very similar to those from the cytosol, with the characteristic high cysteine and serine and low leucine and isoleucine content of metallothionein.

Because of the loss of Zn during the affinity chromatography, precise estimates of cysteine/metal ratio are not possible. However, if some

allowance is made for the Zn loss, the average ratio was 2.6:1, equivalent to a metal content of the proteins of about 6%.

The spectral properties of the Cu-proteins were also consistent with their identification as metallothioneins. Thus there was no absorption maximum in the u.v.-absorption spectrum at 280 nm, although a shoulder was present at around 270 nm. Moreover, the isoproteins showed orange luminescence when irradiated with u.v. light. Excitation of the proteins at 310 nm resulted in the appearance of broad luminescent bands at around 573 and 630 nm (Fig. 4).

Sheep liver

Concentrations of Cu and Zn in the livers of the Cu^{2+} -supplemented sheep were 247 ± 41 and $34.6 \pm 2.5 \mu\text{g/g}$ fresh weight respectively. Homogenization of the livers in 0.01M-Tris/acetate, followed by centrifugation at 100000g, resulted in the extraction of $29 \pm 1\%$ of the Cu and $61 \pm 3\%$ of

Table 1. *Amino acid composition of the isoproteins of Cu-metallothionein isolated from the particulate fractions of pig liver*
The procedure for the isolation of the isoproteins is described in the text. The 'unbound' fraction represents the material which was eluted from the DEAE-Sephadex column with the equilibrating buffer. The proteins were oxidized with performic acid and hydrolysed as described previously (Bremner & Young, 1976). Cysteine and methionine were measured as cysteic acid and methionine sulphone. The composition of the cytosolic Cu-metallothioneins is taken from Bremner & Young (1976). No histidine, tyrosine, phenylalanine or tryptophan were detected.

Cu-metallothioneins ...	Amino acid composition (residues/100 residues)				
	Particulate			Cytosolic	
	Unbound	I	II	Unbound	I
Amino acid					
Cysteine	30.8	31.7	31.5	32.0	33.1
Aspartic acid	5.8	6.9	7.4	7.9	7.5
Methionine	5.0	2.7	2.1	1.5	1.7
Threonine	6.0	5.5	6.1	4.4	4.6
Serine	13.1	13.6	13.4	12.5	13.1
Glutamic acid	3.1	2.2	2.5	1.6	2.0
Proline	—	—	—	4.4	—
Glycine	12.0	12.2	12.5	9.5	11.2
Alanine	10.7	11.6	12.0	10.3	11.7
Valine	2.3	1.5	1.7	1.6	0.9
Isoleucine	1.7	1.8	2.1	1.1	1.5
Leucine	1.5	0.8	1.1	—	—
Lysine	10.7	10.3	9.5	11.1	11.4
Arginine	—	—	—	1.4	1.4

Fig. 4. *Fluorescence spectrum of the particulate Cu-metallothionein-I from pig livers*

The spectrum was recorded by using the excitation wavelength of 310 nm and a solution of the protein in distilled water (0.12 mg/ml).

the Zn. Surprisingly, the alternative extraction procedure, involving the use of a solution of 2-mercaptoethanol, was no more efficient, and removed $27 \pm 2\%$ and $55 \pm 4\%$ of the Cu and Zn respectively.

Fractionation of the Tris/acetate extract on Sephadex G-75 revealed that most of the Cu was present in a fraction eluted at the void volume of the column (Fig. 5). This accounted for $49 \pm 9 \mu\text{g}$ of Cu/g of liver, or 20% of the total liver Cu. The resolution of the Cu-containing components was not good, but a minor fraction with an approx.

Fig. 5. *Fractionation on Sephadex G-75 of extracts of sheep liver*

The liver was homogenized in (a) 0.01 M-Tris/acetate (●) or (b) 1% mercaptoethanol (○) and then centrifuged at 100000g for 1 h. The supernatant fractions were separated on a column of Sephadex G-75. Concentrations of Cu in the eluate are shown. The liver contained 273 and 27 μg/g of Cu and Zn respectively.

V_e/V_0 of 2, typical of metallothionein, was detected. This accounted for $13.2 \pm 3.5 \mu\text{g}$ of Cu/g of liver, or 5% of the total hepatic Cu. Another Cu-containing fraction of even lower M_r was also detected (eluted at about tube 48), especially in

livers of high Cu content. No Zn was present in the metallothionein fraction (result not shown).

Similar fractionation of the 2-mercaptoethanol extracts showed only a minor increase in the amount of Cu associated with metallothionein to $17.7 \pm 4.8 \mu\text{g/g}$ of liver (Fig. 5). The main effect of the 2-mercaptoethanol was to decrease the amount of Cu eluted at the void volume to only $22.5 \pm 4.5 \mu\text{g/g}$ of liver and to increase the amount of Cu in the low- M_r fraction by a corresponding amount (to $21.3 \pm 8.1 \mu\text{g/g}$ of liver).

Rat liver

Concentrations of Cu and Zn in the livers of the rats injected with Cu^{2+} were 117 ± 11 and $46 \pm 2 \mu\text{g/g}$ fresh weight respectively. On extraction of the liver with 0.01M Tris/acetate, 52 and 63% of the Cu^{2+} and Zn^{2+} were recovered in the cytosol. Subsequent extraction of the pellet with a solution containing mercaptoethanol and SDS removed a further 25% of the total liver Cu^{2+} and Zn^{2+} , i.e. 28 ± 3 and $11.3 \pm 0.8 \mu\text{g/g}$ of liver respectively. Fractionation of the cytosolic fraction (i.e. from the Tris/acetate homogenate) revealed that 80% of the Cu was present as metallothionein, as it was eluted with a V_e/V_0 of 2 (Fig. 6). Similar fractionation of the SDS/mercaptoethanol extract (Fig. 6) indicated that 40% of the Cu was again present as metallothionein. The concentrations of Cu bound to 'cytosolic' and 'particulate' metallo-

thionein were therefore 47.5 and $10.9 \mu\text{g/g}$ of liver respectively.

In the rats given a Cu-supplemented diet for several weeks, liver Cu and Zn concentrations were 217 and $24.6 \mu\text{g/g}$ respectively. Fractionation of the Tris/acetate cytosol and of the mercaptoethanol extracts on Sephadex G-75 (results not shown) showed that only negligible amounts of Cu were eluted with $V_e/V_0 = 2$, indicating that metallothionein was absent. Most of the Cu in the cytosol was eluted at the void volume of the column, whereas a large amount of that extracted with mercaptoethanol was in a fraction of low M_r ($V_e/V_0 = 2.5$).

Discussion

Although over half of the Cu^{2+} in the liver of animals of normal Cu status is usually located in the cytosol, this proportion decreases in livers of high Cu^{2+} content. In the case of livers from the human foetus, the newborn calf and the Bedlington terrier (Riordan & Richards, 1980; Porter, 1974; Johnson *et al.*, 1981), much of the 'particulate' Cu is present as metallothionein. The present findings on the livers from Cu-supplemented pigs and Cu-injected rats extend these observations and demonstrate that a pool of 'non-cytosolic' metallothionein can occur in growing animals of those species as well as in neonatal animals and those with a specific genetic defect in Cu metabolism. They confirm the importance of metallothionein as a major Cu-binding protein in the Cu-loaded liver, as over 70% of the total hepatic Cu in the pig frequently occurred in this form, with at least half of it in the particulate fractions.

There is little doubt as to the identity of the Cu-protein extracted from the non-cytosolic pellet of pig liver with 2-mercaptoethanol, as it satisfied most of the accepted criteria for the identification of metallothionein. Thus the spectral (u.v. and luminescence) properties, the chromatographic behaviour and the amino acid composition were typical of Cu-metalllothioneins (Weser & Rupp, 1979; Beltramini & Lerch, 1981; Bremner & Young, 1976). The apparent M_r was about 12000, on the basis of the elution volume from Sephadex G-75, as has been found for most metallothioneins. However, metallothionein is known to occur as a prolate ellipsoid and to behave abnormally on gel filtration, and the true M_r of the particulate copper-metalllothionein is probably about 6500.

The metal content of the protein was very high, and the fact that it was not as great as in the cytosolic pig liver metallothionein (Bremner & Young, 1976) can be partly attributed to the loss of Zn^{2+} during affinity chromatography (Riordan & Richards, 1980). It is also possible that some loss of Zn

Fig. 6. Fractionation on Sephadex G-75 of extracts of the livers from Cu^{2+} -injected rats

Rats were injected on three successive days with Cu^{2+} (see the text) and their livers were homogenized in 0.01M-Tris/acetate buffer and centrifuged at $100000g$ for 1 h. The supernatant was separated on Sephadex G-75 (○) and the pellet was further extracted with a solution of SDS and mercaptoethanol and centrifuged as described above. The supernatant fraction from this extract was also separated on Sephadex G-75 (●). The Cu and Zn concentrations in the rat livers were 117 and $46 \mu\text{g/g}$ wet wt. respectively.

occurs on uptake of the protein by lysosomes. The apparent occurrence of three isoproteins is also typical of the cytosolic Cu-metallothioneins (Bremner & Young, 1976), although it is not absolutely clear whether the fraction that did not bind to the DEAE-Sephadex is a separate isoprotein or is derived from the other two.

Unfortunately the precise intracellular localization of the particulate metallothionein could not be established in the pig or rat livers. Panemangalore *et al.* (1983) produced immunohistochemical evidence for the intranuclear accumulation of metallothionein in the livers of neonatal rats, and subcellular fractionation of the liver of Cu-loaded animals has also indicated that substantial amounts of Cu are present in the nuclear fraction (e.g. Lal & Sourkes, 1971; Johnson *et al.*, 1981; Bremner, 1980), although it may be that some of this metal is actually present in particularly dense lysosomes (Porter, 1974; Johnson *et al.*, 1981). This would be consistent with histochemical findings on Cu localization (see Sternlieb, 1980) and also with the resistance of Cu-metallothionein to hydrolysis by lysosomal enzymes *in vitro* (Bremner & Mehra, 1983). However, studies on the turnover rate of liver particulate Cu-metallothionein in Cu-injected rats indicate that the half-life (25.1 h) is not very different from that of the cytosolic protein (20.9 h), suggesting that mechanisms must exist for the rapid removal of particulate Cu-metallothionein from the liver (Mehra & Bremner, 1983a).

As was discussed above, the concept of lysosomal accumulation of Cu acting as a protective mechanism against the hepatotoxic effects of the metal cannot be readily reconciled with the liver damage that occurs in sheep after relatively moderate increases in liver Cu content (King & Bremner, 1979; Gooneratne *et al.*, 1980). However the present investigation has revealed that the binding of the lysosomal Cu in the sheep is different from that in the pig and dog, as well as that from human foetal livers (Riordan & Richards, 1980; Johnson *et al.*, 1981). Only a small proportion of the cytosolic Cu in the sheep liver was present as metallothionein, and extraction of the tissue with mercaptoethanol did not significantly increase the yield of the protein. The main effect of the reducing agent was to increase the binding of Cu to a fraction of very low M_r .

Similar results were obtained in rats which were Cu-loaded by dietary supplementation, since no Cu was bound to metallothionein in either the cytosol or the particulate fractions. This was confirmed by direct estimation of the metallothionein content of the liver using a specific radioimmunoassay (Mehra & Bremner, 1983b). However, substantial amounts of the Cu-protein did occur in the liver of the rats injected with Cu^{2+} , indicating that

the route of administration of Cu^{2+} influences its ability to induce synthesis of metallothionein. It is noteworthy that at very high liver Cu concentrations in sheep (Bremner *et al.*, 1976), and in rats given larger dietary Cu supplements (R. K. Mehra & I. Bremner, unpublished work), proportionally more Cu is bound to metallothionein. It may be that the induction of metallothionein synthesis by Cu^{2+} is dependent on a critical concentration of labile forms of the metal, and that this is more readily attained after Cu^{2+} injection. There is also evidence that the levels of Zn in the liver are extremely important in controlling the synthesis and degradation of hepatic Cu-metallothionein (Bremner & Mehra, 1983).

The present findings therefore give support to the view that the hepatotoxicity of Cu is decreased when it is bound to metallothionein in both the cytosol and particulate fractions of the liver. However it will be necessary to establish 'dose-response' relationships more rigorously before this can be regarded as proven, since there have been large variations in experimental design and, particularly, in liver Cu content, in many of the studies on the influence of Cu on liver structure and function.

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