Biliary excretion of metallothionein and a possible degradation product in rats injected with copper and zinc

Masao SATO and Ian BREMNER*

Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, Scotland, U.K.

(Received 9 May 1984/Accepted 6 July 1984)

The concentrations of metallothionein-I (MT-I) and related immunoreactive products in bile from adult female rats were measured by radioimmunoassay. Concentrations in normal animals were 20–30 ng/ml, but increased to 600 and 75 ng/ml after injection of Cu^{2+} and Zn^{2+} respectively (3 mg of metal/kg body wt.). However, only 1–2% of the biliary Cu was bound to MT, and less than 1% of the total liver MT in control or Cu^{2+} -injected rats appeared to be secreted in intact form into bile. Other major immunoreactive components in bile from Cu^{2+} -injected rats included an aggregated form of MT-I and a possible degradation product of the isoprotein.

Although the induction of synthesis of metallothionein (MT) has attracted considerable attention, comparatively little is known of the mechanisms involved in the degradation of the protein. However, there is evidence that the rates of degradation *in vitro* of different metalloforms and isoproteins of MT by rat liver lysosomal extracts are inversely related to the strength of the metal–protein bonds (Feldman *et al.*, 1978; Bremner & Mehra, 1983). Moreover Cd-MT has a longer half-life than has Zn-MT, and Zn-MT-II has a longer half-life than Zn-MT-I (Feldman & Cousins, 1976; Cain & Holt, 1979; Cain & Griffiths, 1984). It has been suggested, therefore, that degradation of the protein *in vivo* occurs within the lysosomes and that the ease of removal of bound metals is an important rate-controlling step in the degradative process (Feldman *et al.*, 1978; Bremner & Mehra, 1983).

Findings on Cu-rich MT are consistent with this concept, insofar as Cu is bound very strongly to MT (Rupp & Weser, 1974) and the Cu-protein is resistant to hydrolysis by lysosomal extracts (Bremner & Mehra, 1983). However, the turnover rate of Cu-MT in the liver cytosol of rats injected with Cu is very rapid, the half-life being only 12–17 h (Bremner *et al.*, 1978; Mehra & Bremner, 1983a), and therefore less than that of the Zn- or Cd-induced proteins (Feldman & Cousins, 1976; Cain & Holt, 1979). This implies that factors other

than the strength of the metal–thiolate bond must also influence the turnover of this form of the protein.

It has been pointed out that the pulse-labelling techniques used to measure degradation rates of MT *in vivo* have only measured rates of disappearance of the protein from a certain chromatographic fraction in the liver cytosol and have not necessarily monitored its hydrolysis (Bremner & Mehra, 1983). In rats injected with Cu, appreciable amounts of aggregated Cu-MT accumulate also in particulate fractions of the liver and may represent cytosolic protein taken up by lysosomes or other organelles (Mehra & Bremner, 1983a, 1984a). The half-life of the ‘particulate’ Cu-MT is 25 h, indicating that this pool of Cu-MT also turns over relatively quickly. If Cu-MT is indeed resistant to hydrolysis by lysosomal enzymes (Bremner & Mehra, 1983), loss of MT from lysosomes or other organelles in the liver could perhaps occur by direct excretion of intact protein, either in monomeric or aggregated form. It has already been demonstrated that MT is secreted in small amounts into the plasma of normal rats (Garvey & Chang, 1981; Mehra & Bremner, 1983b) and that this secretion is considerably increased in neonatal rats (Mehra & Bremner, 1984b) and in adult rats injected with Cd, Cu, carbon tetrachloride or endotoxin (Garvey & Chang, 1981; Mehra & Bremner, 1983b; Sato *et al.*, 1984).

In view of the importance of bile as a secretory route for Cu (Owen, 1964) and the postulated origin of certain biliary secretions in hepatic lysosomes (de Duve & Wattiaux, 1966), a study has

Abbreviation used: MT, metallothionein.

* To whom correspondence and reprint requests should be sent.

been made of the excretion of MT-I and of its degradation products in the bile of rats injected with Cu and Zn.

Experimental

Materials

Bolton and Hunter reagent [*N*-succinimidyl-3-(4-hydroxy-5-[^{125} I]iodophenyl propionate)] (5mCi/ml) was obtained from Amersham International. Donkey anti-(sheep immunoglobulin G) antiserum was kindly supplied by the Scottish Antibody Production Unit, Carlisle, Lanarkshire, Scotland, U.K.

Animals

Female Hooded Lister rats (Rowett Institute strain), weighing about 250g, were used in all experiments. They were given a stock colony diet *ad libitum* (Oxoid; H. D. Styles, Bewdley, Worcs., U.K.). Rats were injected intraperitoneally with Cu^{2+} or Zn^{2+} (as CuSO_4 or ZnSO_4) in 0.9% NaCl solution (3.0mg of metal/kg body wt.). Bile was collected under sodium pentobarbital anaesthesia over a 2h period by cannulation of the bile duct proximal to the junction of the hepatic ducts, using polyethylene tubing (0.96mm outer diameter) (Portex Limited, Hythe, Kent, U.K.). Livers, and in some instances heparinized plasma, were removed at the end of bile collection and stored with bile at -20° or $+4^\circ\text{C}$ before analysis.

Analysis

Concentrations of MT-I in bile, plasma and liver were measured using the radioimmunoassay procedure described by Mehra & Bremner (1983b). Liver was homogenized with 10vol. (w/v) of 10mM-Tris/acetate buffer, pH7.4, and centrifuged at 1500g for 15min. The supernatant fractions and bile samples were diluted as appropriate with 50mM-Tris/HCl buffer, pH8.0, containing gelatin (1g/l) and NaN_3 (1g/l) to bring the metallothionein concentrations within the range of the standard curve (1–250ng/ml).

Fractionation of bile was carried out on Sephadex G-75 (superfine grade), using 10mM-Tris/acetate, pH7.4, as eluant. In some instances, the sample was incubated for several hours at room temperature in the presence of 1% (v/v) 2-mercaptoethanol before fractionation.

Depending on the sensitivity required, Cu and Zn analyses were carried out by flame- or electrothermal-atomic-absorption spectrometry using a Perkin-Elmer model 460 instrument with an HGA-76 furnace attachment for the latter.

Results

Preliminary experiments showed that the addition of up to 50 μl of bile to the control assay system (1.6–6.35ng of MT-I) did not interfere with the radioimmunoassay and, even at the upper limit (50 μl of bile), recoveries of MT-I were complete (95–110%). Moreover, the dilution curve obtained from bile was parallel with the standard curve for authentic rat liver MT-I (result not shown).

Concentrations of MT-I and other immunoreactive components in the bile of control rats were about 20 ng/ml, but increased on injection of Cu^{2+} to 75 ng/ml after 13h and to 600 ng/ml after 24h, whereupon they declined steadily over the next 24h (Fig. 1). A similar pattern of change was observed in biliary Cu levels, except that the increase at 13h was more pronounced. Maximum concentrations of Cu (6 $\mu\text{g}/\text{ml}$) were attained at 24h after injection, and there was a good correlation between biliary MT-I and Cu concentrations. Concentrations of MT-I in the plasma of control rats and of animals injected 24h previously with Cu^{2+} were 3.9 ± 1.3 and 85 ± 15 ng/ml respectively, and were therefore considerably less than the concentrations in bile.

The increases in MT-I concentrations in bile were slightly delayed compared with the increases that occurred in liver, since the hepatic concentrations increased from 10 $\mu\text{g}/\text{g}$ wet wt. in control rats to a maximum of 210 $\mu\text{g}/\text{g}$ 18h after injection of Cu^{2+} (Fig. 1). They remained at about this level for several hours, before declining to 113 $\mu\text{g}/\text{g}$ at 48h after injection.

Injection of rats with Zn^{2+} also increased biliary concentrations of MT-I and related immunoreactive products from 30 ng/ml in control animals to a maximum of 75 ng/ml at 13h after injection (Fig. 2). Concentrations decreased gradually thereafter to 40 ng/ml at 38–48h. A similar pattern of change occurred in both liver and plasma, with increases to maxima of 135 $\mu\text{g}/\text{g}$ and 17.5 ng/ml respectively at 13–18h after injection, and with a gradual reduction thereafter (Fig. 2). In the case of plasma, control values were re-attained after 48h, but in the liver, concentrations were still twice normal at this time.

Gel filtration on Sephadex G-75 of bile collected from rats injected 18–24h previously with Cu^{2+} showed that the immunoreactive material was associated with three main fractions with V_e/V_0 ratios of 1.0, 2.0 and 2.35 respectively (Fig. 3). The second fraction corresponded in elution volume to rat liver MT-I, whose elution characteristics were shown to be unaffected by the presence of bile. The first fraction, which contained 20–30% of the total immunoreactive material, appeared to consist of an aggregated form of MT-I, since it yielded,

Fig. 1. Concentrations of MT-I and copper in liver and bile of Cu^{2+} -injected rats

Concentrations of MT-I in the bile (○) and liver (□) and of Cu in bile (●) of rats injected with Cu^{2+} (3 mg/kg body wt.) were collected over 2 h periods from 12 to 48 h after the injection. MT-I concentrations were measured by radioimmunoassay. Values quoted are means for groups of at least five rats.

Fig. 2. MT-I in the liver, bile and plasma of Zn^{2+} -injected rats

Concentrations of metallothionein-I in the liver (□), bile (○) and plasma (●) of rats injected with Zn^{2+} (3 mg/kg body wt.) were collected over 2 h periods from 12 to 48 h after the injection. MT-I concentrations were measured by radioimmunoassay. Values quoted are means for groups of at least five rats.

among other things, the component with $V_e/V_0 = 2$ on incubation with 1% 2-mercaptoethanol and re-fractionation on Sephadex G-75 (Fig. 3b). The third fraction ($V_e/V_0 = 2.35$), which was generally

Fig. 3. Fractionation on Sephadex G-75 of bile from Cu^{2+} -injected rats

(a) Bile was collected from rats injected 24 h previously with Cu^{2+} (3 mg/kg body wt.) and fractionated on a column of Sephadex G-75, with 10 mM-Tris/acetate, pH 7.4, as eluant. Concentrations of MT-I (○) and of copper (●) are shown. Authentic MT-I eluted at tube no. 40 and the apparent degradation product at tube no. 47. (b) The material eluted at the void volume (tubes 18–24) was reduced with 2-mercaptoethanol and re-fractionated on Sephadex G-75 (see the text). The concentrations of immunoreactive products (□) and of Cu (■) are shown.

the predominant one in bile from Cu^{2+} -injected animals, was not further characterized, but would appear to be a degradation product of MT-I (see below).

The copper in the bile was distributed over two main peaks of material that were eluted at the void volume and near the total volume of the column respectively (Fig. 3). However, a small proportion of the Cu in the second fraction, which was clearly heterogeneous, was associated with the second and third immunoreactive components.

Discussion

Although Nomiyama & Nomiyama (1982) obtained chromatographic evidence for the presence of MT in bile from rabbits injected with massive amounts of Cd^{2+} , the present findings provide the first quantitative information on its occurrence in bile from normal and treated rats. Concentrations of MT-I in bile increased by up to 30-fold

when hepatic metallothionein synthesis was induced by injection of Cu^{2+} and, in both Cu^{2+} -injected and normal rats, biliary concentrations were an order of magnitude greater than those in plasma. Maximum biliary concentrations of the protein occurred 24 h after Cu^{2+} injection, i.e. 6 h after maximum hepatic concentrations were attained.

Injection of Zn^{2+} also stimulated excretion of MT-I in bile and plasma, but the concentrations were considerably less than those after Cu^{2+} injection, despite the fact that hepatic concentrations of the protein were similar in the two cases. This could suggest that the rapid disappearance of metallothionein from the livers of Cu^{2+} -injected rats involves its uptake by subcellular organelles and excretion in intact form in bile. However, comparison of the rate of biliary excretion and the rate of disappearance of MT-I from the liver indicates that only 1–2% of the hepatic protein is excreted in the bile of either control or copper-injected rats. These calculations, which are based on a measured flow rate of bile of 1.3 ml/h and a half-life of MT-I of 15 h (Mehra & Bremner, 1983a), do not take into account the influence of anaesthesia and surgery (Caple & Heath, 1978) on biliary excretion of Cu and perhaps of MT, but this is unlikely to affect the basic conclusions.

The chromatographic studies demonstrate that much of the immunoreactive material in bile was present in a form other than native monomeric MT. The fraction that was eluted at the void volume was probably an aggregated form of the protein, since it dissociated on treatment with mercaptoethanol. This component may have originated in the particulate fractions of the liver, since these are known to contain aggregated Cu-MT in Cu-loaded rats and pigs (Mehra & Bremner, 1984a).

However the most interesting observation is the detection of the third and major immunoreactive component with an apparent M_r less than that of native metallothionein. This is probably a degradation product of the protein and may be related to the 'α-fragment' or 4-metal cluster that Winge & Miklossy (1982) obtained on neutral proteolysis of partially-demethylated Cd-MT. This polypeptide is also eluted from Sephadex G-75 with a V_e/V_0 ratio of approx. 2.3. Moreover the peptide from (Cd,Zn)-MT-I cross-reacts completely with Zn-MT-I in our radioimmunoassay (R. K. Mehra & I. Bremner, unpublished work). The α-fragment encompasses the C-terminal domain of MT (residues 30–61) (Winge & Miklossy, 1982), and presumably the antigenic determinants of MT-I are located principally in this region. In contrast, Winge & Garvey (1983), using a different antibody, reported that the determinants consist of two

immunologically dominant regions in the N-terminal domain (residues 1–29).

Further studies will be required to establish more precisely the antigenic sites involved in the binding of MT-I to our antibody. However, the information obtained will facilitate the characterization of the putative degradation product in bile and so provide valuable insight into the metabolism of metallothionein. The current findings have provided the first evidence for the natural occurrence of degradation products of this protein in biological tissues, since previous reports on metabolites of metallothionein in rat kidneys or urine have been based on experiments involving the parenteral administration of metallothioneins (Cherian & Shaikh, 1975; Bremner *et al.*, 1978b).

It is apparent from Fig. 3 and also from calculations of the likely metal content of the three immunoreactive components in bile that only a small proportion of the biliary Cu, probably about 1%, is associated with these fractions. Most of the copper was present in a form of lower apparent M_r , as was found previously in rats dosed with tracer amounts of ^{64}Cu (Terao & Owen, 1973). This form has never been fully characterized and there have been suggestions that it consists of Cu–amino acid, –peptide, –bile-acid or –glutathione complexes (Evans & Cornatzer, 1971; Lewis, 1973; Alexander & Aaseth, 1980). It is tempting to speculate that it could contain more extensively degraded forms of MT. However, Samuels *et al.* (1983) have recently claimed that it consists of a dye-binding protein with M_r 50000, which behaves anomalously on Sephadex G-75 and contains 5 atoms of Cu per molecule.

In conclusion, these results show that MT is excreted into bile in rats against a high bile/plasma concentration gradient. However, only a small proportion of hepatic MT appears to be excreted in intact form into bile and the discrepancy in the apparent rates of degradation of CuMT *in vivo* and *in vitro* cannot therefore be explained by biliary excretion of the protein. There is evidence for the biliary excretion of a degradation product of MT.

References

- Alexander, J. & Aaseth, J. (1980) *Biochem. Pharmacol.* **29**, 2129–2133
- Bremner, I. & Mehra, R. K. (1983) *Chem. Scr.* **21**, 117–121
- Bremner, I., Hoekstra, W. G., Davies, N. T. & Young, B. W. (1978) *Biochem. J.* **174**, 883–892
- Bremner, I., Hoekstra, W. G., Davies, N. T. & Young, B. W. (1978b) *Chem.-Biol. Interact.* **23**, 355–367
- Cain, K. & Griffiths, B. L. (1984) *Biochem. J.* **217**, 85–92

- Cain, K. & Holt, D. E. (1979) *Chem.-Biol. Interact.* **28**, 91–106
- Caple, I. W. & Heath, T. J. (1978) *Comp. Biochem. Physiol.* **61A**, 503–507
- Cherian, M. G. & Shaikh, Z. A. (1975) *Biochem. Biophys. Res. Commun.* **65**, 863–866
- de Duve, C. & Wattiaux, R. (1966) *Annu. Rev. Physiol.* **28**, 435–492
- Evans, G. W. & Cornatzer, W. E. (1971) *Proc. Soc. Exp. Biol. Med.* **136**, 719–721
- Feldman, S. L. & Cousins, R. J. (1976) *Biochem. J.* **160**, 583–588
- Feldman, S. L., Failla, M. & Cousins, R. J. (1978) *Biochim. Biophys. Acta* **544**, 638–646
- Garvey, J. S. & Chang, C. C. (1981) *Science* **214**, 805–807
- Lewis, K. O. (1973) *Gut* **14**, 221–232
- Mehra, R. K. & Bremner, I. (1983a) *Biochem. Soc. Trans.* **11**, 774–775
- Mehra, R. K. & Bremner, I. (1983b) *Biochem. J.* **213**, 459–465
- Mehra, R. K. & Bremner, I. (1984a) *Biochem. J.* **219**, 539–546
- Mehra, R. K. & Bremner, I. (1984b) *Biochem. J.* **217**, 859–862
- Nomiyama, K. & Nomiyama, H. (1982) in *Biological Roles of Metallothionein* (Foulkes, E. C., ed.), pp. 47–67, Elsevier/North-Holland, New York
- Owen, C. A. (1964) *Am. J. Physiol.* **207**, 1203–1206
- Rupp, H. & Weser, U. (1974) *FEBS Lett.* **44**, 293–297
- Samuels, A. R., Freedman, J. H. & Bhargava, M. M. (1983) *Biochim. Biophys. Acta* **759**, 23–31
- Sato, M., Mehra, R. K. & Bremner, I. (1984) *J. Nutr.* **114** in the press
- Terao, T. & Owen, C. A. (1973) *Am. J. Physiol.* **224**, 682–686
- Winge, D. R. & Garvey, J. S. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2472–2476
- Winge, D. R. & Miklossy, K.-A. (1982) *J. Biol. Chem.* **257**, 3471–3476