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Studies on the metabolism of rat liver copper-metallothionein

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The degradation of purified ^{35}S-labelled rat liver isometallothioneins (MT) by lysosomal extracts was studied. Zn-MT-I was more readily hydrolysed than Zn-MT-II, but no significant degradation of the Cu-containing metallothioneins could be detected, even after 24h incubation. The susceptibility of MT to degradation *in vitro* may be related to the strength of the metal–thiolate bonds. However, the turnover rates of cytosolic MT *in vivo*, as established by pulse-labelling techniques, are apparently subject to different controls. The half-lives of MT-I and -II in the liver cytosol of Cu^{2+}-injected rats were only 15.4 ± 1.5 and 18.2 ± 1.1h respectively. Approx. 25% of the total liver MT was present in particulate fractions (probably in lysosomes) of the liver and had a half-life of 25.1 ± 4.1h.

Although considerable advances have been made in recent years in the studies of the physicochemical properties of metallothionein (MT) and of the induction of its synthesis (see Bremner, 1981), comparatively little attention has been paid to the mechanisms involved in the degradation or turnover of the protein. Pulse-labelling experiments have shown that the turnover rate of rat liver MT is dependent on the nature of the inducing metal and tends to increase as the protein's Cu content increases (Feldman & Cousins, 1976; Shaikh & Smith, 1976; Bremner *et al.*, 1978; Cain & Holt, 1979). Moreover, the half-life for Cu-MT is less in Zn-deficient rats than in Zn-adequate animals (Bremner *et al.*, 1978) and the half-life for Zn-MT-I is less than that for Zn-MT-II (Andersen *et al.*, 1978).

It has been suggested that degradation of hepatic MT involves its uptake by lysosomes and hydrolysis by proteolytic enzymes located therein, since Cd- and Zn-MT are readily degraded *in vitro* by extracts of rat liver lysosomes (Feldman *et al.*, 1978). Degradation *in vitro* proceeds more rapidly with Zn-MT than with Cd-MT, which is consistent with the shorter biological half-life of the former metalloform (Feldman & Cousins, 1976; Shaikh & Smith, 1976; Cain & Holt, 1979). It is also consistent with the greater lability of the Zn–thiolate bond, Zn^{2+} being more readily removed from MT than is Cd^{2+} (Kagi & Vallee, 1961), and this has led to the suggestion that removal of metal, with resultant change in conformation of the protein, is an important step in the degradation of MT *in vivo* (Bremner & Mehra, 1983). The finding that selective removal of Zn^{2+} from (Cd,Zn)-MT renders the protein susceptible to proteolysis by subtilisin (Winge & Miklossy, 1982*a*) has supported this view.

However, this hypothesis cannot be readily reconciled with observations on Cu-containing MT. The half-life of Cu^{2+}-induced MT in the liver of Zn-deficient rats is only 12h (Bremner *et al.*, 1978), and therefore considerably less than that of Zn- or Cd-MT. Despite this, Cu is bound to MT much more firmly than is either of these metals (Bremner & Marshall, 1974; Weser & Rupp, 1979). Moreover, appreciable amounts of Cu-MT can accumulate in the particulate fractions of the liver of the human foetus (Riordan & Richards, 1980), of the Cu^{2+}-loaded pig and of Cu^{2+}-injected rats (Mehra & Bremner, 1984). This aggregated form of the protein appears to be located mainly within lysosomes (Porter, 1974), suggesting that Cu-MT is perhaps not readily degraded in that organelle, despite the short half-life of the cytosolic protein.

The present study was designed therefore to obtain information on the susceptibility of Cu-MT to hydrolysis *in vitro* and to compare the turnover rates of cytosolic and particulate forms of the protein in liver of Cu^{2+}-injected rats. Preliminary reports of some of the findings have been pub-

Abbreviations used: MT, metallothionein; SDS, sodium dodecyl sulphate.

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lished elsewhere (Bremner & Mehra, 1983; Mehra & Bremner, 1983*a*).

Materials and methods

Materials

Sephadex G-25 and G-75 and DEAE-Sephadex A-25 were purchased from Pharmacia Fine Chemicals and L-[^{35}S]cysteine (20–150mCi/mmol) was from Amersham International. Donkey anti-sheep IgG was kindly supplied by the Scottish Antibody Production Unit, Carluke, Lanarkshire, Scotland, U.K.

Preparation of ^{35}S-labelled MT

Male Hooded Lister rats (Rowett Institute strain) were injected i.p. (intraperitoneally) with $ZnSO_4$ (10mg of Zn^{2+}/kg body wt.) or with $CuSO_4$ (1.5mg of Cu^{2+}/kg), and then after 4h with L-[^{35}S]-cysteine (0.125mCi/kg body wt.). The rats were killed 20 and 12h after the injection of Zn^{2+} and Cu^{2+} respectively and their livers removed. The two main isoproteins of MT (MT-I and MT-II) were isolated from the livers by the procedure described by Bremner & Young (1976), using a combination of gel filtration on Sephadex G-75 and ion-exchange chromatography on DEAE-Sephadex A-25. The purified isoproteins of Zn-MT were freeze-dried and stored at −20°C, whereas those of Cu-MT were stored in distilled water at +1°C. The Cu/Zn ratios in MT-I and MT-II from the Cu^{2+}-injected rats were 2.0:1 and 1.5:1 respectively. The specific radioactivities for Zn-MT-I, Zn-MT-II, Cu-MT-I and Cu-MT-II were 57000, 69000, 111000 and 106000d.p.m./mg of protein respectively. The protein content of the Cu-MT preparations was estimated from their metal content, assuming 10g-atoms of metal/mol (Bremner & Young, 1976).

Isolation of rat liver lysosomes

Rat liver lysosomal fractions were prepared by the method of Ragab *et al.* (1967). The final pellet, obtained from 40–50g of liver, was suspended in 10.0ml of 0.02M-citrate buffer, pH4.8, containing 0.2% (w/v) Triton X-100 and 0.02M-2-mercaptoethanol, freeze–thawed five times, and then centrifuged at 100000*g* for 1h. The supernatant fraction was stored in aliquots at −20°C. The specific activity of the acid proteinase in these extracts was 10–20-fold greater than in the unfractionated liver homogenates.

Degradation of Cu- and Zn-MT in vitro

Solutions (0.3ml) of Zn- and Cu-containing isoproteins of ^{35}S-labelled MT (100μg) in 0.05M-sodium acetate, pH5.0, containing 2.5mM-2-mercaptoethanol, were treated with rat liver lysosomal extract (0.1ml) containing 51.2munits of acid proteinase activity (Barrett, 1972). After incubation at 37°C for periods of 30–120min, degradation of the Zn-MTs was stopped by the addition of 0.1ml of a solution of bovine serum albumin (20mg/ml), followed by 1.0ml of cold trichloroacetic acid solution (20%, w/v). The mixtures were centrifuged at 1500*g* for 15min and the ^{35}S content of the supernatant fractions (containing degradation products) was measured. In the case of Cu-MTs, complete precipitation of unchanged protein did not occur with trichloroacetic acid, and reaction products were therefore separated by gel filtration on Sephadex G-25 or by precipitation of unchanged protein with cold acetone (80%, v/v, final concn.). At least three determinations of the degree of hydrolysis were carried out at each time point.

Measurement of turnover rate of Cu-MT in rat liver

Groups of six Hooded Lister male rats (Rowett Institute strain), weighing 180–200g, were injected i.p. with Cu^{2+} (2mg of metal as $CuSO_4$/kg body wt.), followed after 4h with [^{35}S]cysteine (0.250mCi/kg body wt.). The rats were killed by cervical dislocation 12–96h after the first injection and their livers removed and stored at −20°C.

Livers were homogenized in 2vol. of 0.01M-Tris/acetate, pH7.4, and the supernatant fraction obtained on centrifugation at 100000*g* for 1h was separated on Sephadex G-75 equilibrated with 0.01M-Tris/acetate, pH7.4, containing 0.01M-2-mercaptoethanol. Elution was carried out with the same buffer and the Cu, Zn and ^{35}S contents of the eluate were determined.

The MT-containing fractions (eluted with $V_e/V_0 = 2.0$; see Fig. 3 below) were combined and further separated into the two isoproteins of MT by anion-exchange chromatography on DEAE-Sephadex A-25 (1.6cm × 30cm), equilibrated with 0.01M-Tris/acetate, pH7.4, containing 0.01M-2-mercaptoethanol. After elution of the column with 25ml of equilibrating buffer, the isoproteins were eluted with a linear gradient of 0.01–0.20M-Tris/acetate, pH7.4, containing 2-mercaptoethanol.

The pellets remaining after the removal of the cytosolic fraction were suspended in 5ml of 0.01M-Tris/acetate, centrifuged at 100000*g*, for 1h, and the supernatants decanted. The pellets were then extracted with 1% (w/v) SDS/1% (w/v) 2-mercaptoethanol for 3h at 37°C, whereupon they were centrifuged at 100000*g* for 1h. The extracts were then fractionated on Sephadex G-75 as described above. All chromatographic procedures were carried out at 4°C. Livers were homogenized and centrifuged individually, but pooled samples of

extracts were used for fractionation studies, which were carried out in duplicate.

Analytical methods

MT-I was measured by the radioimmunoassay procedure of Mehra &Bremner (1983*b*). Acid phosphatase and proteolytic activities were assayed by the methods of Bergmeyer (1974) and Barrett (1972) respectively. ^{35}S was measured by liquid-scintillation counting in a Packard Tri-Carb 460 CD counter.

Results

Degradation of MT in vitro

Both isoproteins of rat liver Zn-MT were readily degraded by extracts of rat liver lysosomes, but the rate of degradation of Zn-MT-I was significantly faster than that of Zn-MT-II (Fig. 1). Thus 58 and 24% of Zn-[^{35}S]MT-I and Zn-MT-II respectively were converted into trichloroacetic acid-soluble digestion fragments after 30min of incubation at 37°C. After 3h, degradation of Zn-MT-I was complete, but 34% of MT-II remained apparently unchanged. When ^{35}S-labelled isoproteins of Cu-MT were incubated with lysosomal extracts, no significant quantities of degradation products could be detected by acetone precipitation or by gel filtration on Sephadex G-25 after 3h (Fig. 1) or even after 24h (result not shown).

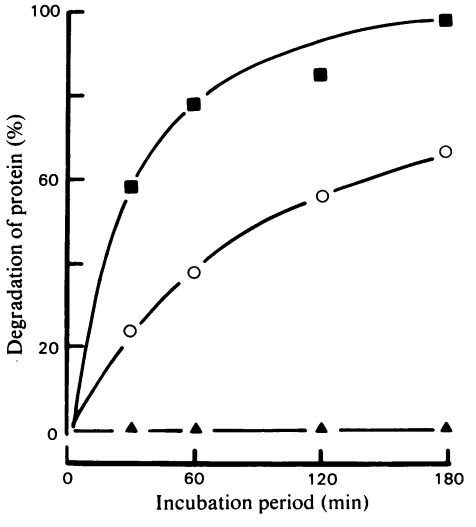

Fig. 1. *Degradation in vitro of ^{35}S-labelled zinc-MT-I* (■), *zinc-MT-II* (○) *and of copper-MT-I or -II* (▲)
The proteins were incubated at 37°C with extracts or rat liver lysosomes.

Turnover rate of Cu^{2+}-induced MT in vivo

Concentrations (±S.E.M.) ($n = 6$) of Cu in the whole liver and in the cytosolic fraction increased to 29.0±2.9 and 17.7±2.0μg/g of liver 18h after injection of Cu, but decreased gradually thereafter (Fig. 2). After 96h the concentrations were 5.9±0.6 and 2.7±0.3μg/g respectively and close to those in non-injected rats. Fractionation on Sephadex G-75 of the cytosolic fraction obtained after homogenization of the liver with Tris/acetate buffer showed that much of the Cu was associated with a fraction with $V_e/V_0 = 2$ (Figs. 2 and 3). This fraction also contained Zn and ^{35}S from the injected [^{35}S]cysteine and was known from previous studies to constitute MT (Bremner & Young, 1976; Bremner *et al.*, 1978). The Cu/Zn ratio varied with time, but the mean value was 1.22 (±0.07):1.

The crude MT obtained by gel filtration was further purified by anion-exchange chromatography on DEAE-Sephadex A-25 into two main

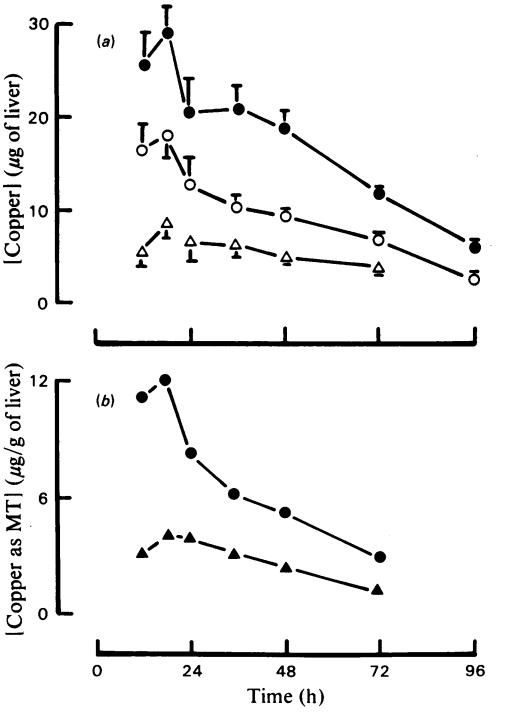

Fig. 2. *Changes in liver copper concentrations and distribution after injection of rats with copper.*
(*a*) Concentrations of copper in the whole liver (●), in the cytosolic fraction obtained after homogenizing with Tris/acetate (○), and in the SDS/mercaptoethanol extract of the non-cytosolic pellet (△). (*b*) Concentrations of copper present as MT in the Tris/acetate (●) and SDS/mercaptoethanol extracts (▲).

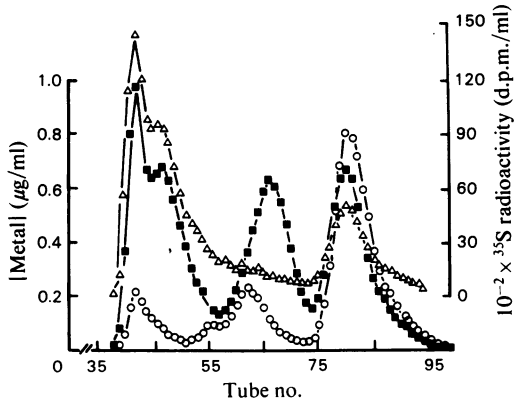

Fig. 3. *Fractionation on Sephadex G-75 of the Tris/acetate extract of the livers from rats injected 12 and 8 h previously with copper and [^{35}S]cysteine*
Concentrations of copper (○), zinc (■) and ^{35}S (△) are shown. MT was eluted at about tube 82.

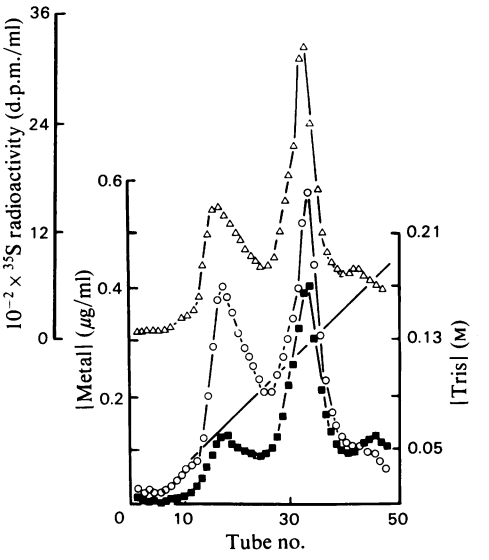

Fig. 4. *Fractionation on DEAE-Sephadex A-25 of rat liver MT*
The isoproteins MT-I and MT-II were eluted at around tubes 18 and 35 respectively. Concentrations of copper (○), zinc (■) and ^{35}S (△) are shown, along with the concentration of the eluent.

isoproteins, MT-I and MT-II, which were eluted at approximate buffer concentrations of 0.07 and 0.13 M (Fig. 4). A third minor fraction containing both Cu and ^{35}S was eluted after MT-II (around tube 46 in Fig. 4). The recovery of ^{35}S from the anion-exchange column was over 80%. The ratio of MT-II to MT-I, based on their ^{35}S contents, increased from 1.63:1 at 12 h to 2.23:1 at 72 h. When the ratio was based on the metal contents of the isoproteins, almost identical values were obtained, namely 1.51:1 and 2.13:1 at 12 and 72 h respectively. Cu was the predominant metal in both isoproteins, the average Cu/Zn ratio being 3.7 (±0.3):1 in MT-I and 1.6 (±0.2):1 in MT-II.

There was an exponential decrease with time in the ^{35}S content of the two isoproteins, and the half-lives were calculated to be 15.4±1.5 h for MT-I and 18.2±1.1 h for MT-II (Fig. 5). These estimates are slightly less than that calculated from the rate of disappearance of ^{35}S from the crude MT fraction obtained after gel filtration, namely 20.9±1.5 h. However the half-life for MT-I is in good agreement with that calculated from the direct estimation of the isoprotein by radioimmunoassay. Concentrations of this isoprotein increased to 70 μg/g of liver at 24 h post injection, whereupon they decreased with a half-life of 15.1±0.8 h (Fig. 5).

Extraction with mercaptoethanol and SDS of the pellet remaining after centrifugation of the Tris/acetate homogenate removed a high proportion of the remaining Cu (Fig. 2). On fractionation of this extract on Sephadex G-75, about half of the Cu was again eluted in a fraction with $V_e/V_0 = 2$, which also contained Zn and ^{35}S (results not shown). This fraction was shown by immunoassay

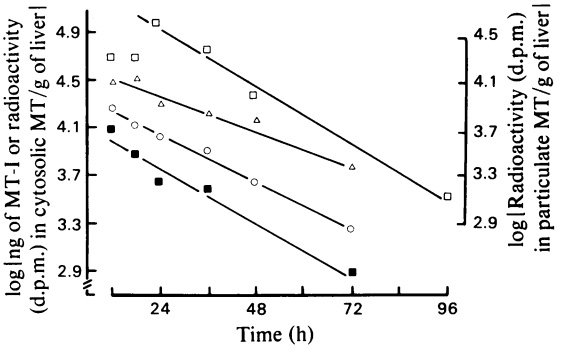

Fig. 5. *Rate of disappearance of MT from the cytosolic and particulate fractions of the livers of rats injected with copper and [^{35}S]cysteine*
Rates are calculated from the ^{35}S content of the cytosolic MT-I (■) and MT-II (○) and of the particulate copper-MT (△). The change in concentration of MT-I, as measured by radioimmunoassay, is also given (□).

to contain MT-I and can be assumed to contain MT-II also. The Cu/Zn ratio in this 'particulate' form of MT was 2.5 (±0.1):1. Maximum incorporation of ^{35}S into the protein occurred at 18 h after Cu^{2+} injection, whereupon the ^{35}S content decreased steadily with a half-life of 25.1±4.1 h

(Fig. 5). The particulate form of MT accounted for 25% of the total liver MT, the average ratio of the cytosolic to the particulate pool of MT (based on their ^{35}S content) being 3.07 ($\pm$0.28):1. Similar values were obtained when the distribution of MT was estimated from the metal content of the protein, with 22% of the protein then present in the particulate fraction.

Discussion

These studies have confirmed that Zn-MT is readily hydrolysed by rat liver lysosomal enzymes (Feldman *et al.*, 1978) and have demonstrated that MT-I is more rapidly degraded than MT-II *in vitro*. This is consistent with estimates of the relative half-lives of the isoproteins of hepatic MT in rats injected with Zn^{2+} or subjected to hepatectomy (Andersen *et al.*, 1978; Cain & Griffiths, 1984). Cain & Holt (1979) also reported differences in the half-lives of Cd^{2+}-induced MT-I and MT-II in rat liver, but attributed this to differences in the metal composition of the isoproteins. The increased susceptibility of MT-I to proteolysis may be related to the weaker binding of Zn to this isoprotein (Winge & Miklossy, 1982*b*) and reflect differences in the structures of the metal clusters in the two isoproteins (Nicholson *et al.*, 1983).

Metal removal is probably an important rate-controlling step in the degradation of Cd- and Zn-MT, as the random chain configuration of apo-MT (Vasak *et al.*, 1980) probably renders the peptide bonds more accessible to proteolytic enzymes. Even partial removal of metal atoms increases susceptibility to hydrolysis (Winge & Miklossy, 1982*a*), and it is likely that the pH within lysosomes could be sufficiently low for Zn^{2+} to be removed by competition with H^+. Weser & Rupp (1979) have reported that, at pH 4.6, 50% of the Zn atoms in Zn-MT are dissociated. This pH is not far removed from that within lysosomes (Dean & Barrett, 1976) or from the pH optimum of the enzymes involved in the degradation of Zn-MT *in vitro*, namely pH 4.2–4.4 (Mehra, 1983).

Cu-MT appeared to be extremely resistant to degradation by lysosomal extracts, which is in accord with a preliminary report on the hydrolysis of MT in which Cu was introduced by metal exchange (Held & Hoekstra, 1979). It is also consistent with the strength of the Cu–thiolate bonds in MT (Weser & Rupp, 1979) and with the concept that ease of removal of metal determines the rate of hydrolysis of the protein. However, it is at odds with the rapid turnover of Cu^{2+}-induced MT in rat liver, as indicated by the half-lives of MT-I and MT-II of 15.4 and 18.2 h respectively. These estimates are in good agreement with the value of 17 h obtained previously for the mixture of isoproteins, as separated on Sephadex G-75 (Bremner *et al.*, 1978). Direct measurement of MT-I by radioimmunoassay also confirmed the estimate of the half-life of this isoprotein. The difference in half-life of the Cu-isoproteins was not statistically significant, but the trend was nevertheless in the same direction as that for the isoproteins of Zn-MT (Andersen *et al.*, 1978; Cain & Griffiths, 1984). The fact that the half-lives of both isoproteins of Cu-MT were less than the value of 21 h obtained for the crude MT probably reflects the presence of some long-lived contaminant protein in the partially purified MT. Similar results have been described previously for Cd^{2+}-induced MT (Cain & Holt, 1979).

Pulse-labelling experiments of the type used here have generally been used to obtain information on the degradation rate of proteins, but in the case of MT, no direct evidence of hydrolysis has ever been obtained. In all cases only the rate of disappearance of MT from a particular chromatographic fraction of the liver cytosol has been measured. The results obtained here demonstrate that about 25% of the total liver MT in the Cu^{2+}-injected rats was actually present in particulate fractions. Although the Cu-binding protein in these fractions was not purified, the detection of MT-I by radioimmunoassay in the SDS/mercaptoethanol extract leaves little doubt as to its identity, especially as the equivalent 'particulate' Cu-protein in livers from the human foetus (Riordan & Richards, 1980) and Cu^{2+}-loaded pigs (Mehra & Bremner, 1984) has been characterized as MT.

The precise subcellular localization of the 'particulate' MT could not be determined, but there is immunohistochemical evidence for the intranuclear accumulation of MT in the liver of neonatal rats (Panemangalore *et al.*, 1983). However, it seems more likely that MT in the Cu^{2+}-injected rats was present mainly in lysosomes, as was reported for bovine neonatal liver (Porter, 1974), since accumulation of Cu-MT within lysosomes would be consistent with its resistance to hydrolysis by lysosomal enzymes *in vitro*. There have been no reports of the occurrence of Zn-rich MT in lysosomes or other organelles.

The mechanism involved in the removal of Cu-MT from the particulate fractions of the liver has yet to be determined. One possibility is that there is exocytosis of the particulate MT, and it is significant that MT-I has been detected in the plasma (Mehra & Bremner, 1983*b*) and bile (Sato & Bremner, 1984) of rats injected with Cu^{2+} and other metals, with some of the protein present in an aggregated form which can be dissociated with 2-mercaptoethanol. However, plasma-based MT-I concentrations are relatively low (80 ng/ml) 24 h after Cu^{2+} injection (Sato & Bremner, 1984), and

biliary MT-I only accounts for 1–2% of the isoprotein, which disappears from the liver of Cu^{2+}-injected rats (Sato & Bremner, 1984). Moreover, some of this immunoreactive material in bile appears to be present as degradation products of MT-I.

Possible explanations for the apparent anomaly of the stability of Cu-MT *in vitro* and its lability *in vivo* are that degradation of Cu-MT normally occurs by a non-lysosomal system or that the system used to study the degradation of Cu-MT *in vitro* failed to reproduce the conditions required to convert the protein into forms accessible to hydrolytic enzymes. These could include the presence of endogenous chelators capable of removing Cu from the protein or the occurrence of a redox change to convert the bound Cu(I) into Cu(II) and so reduce its affinity for the protein. Further studies are required to resolve this question.

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