The role of Cu(I)-thiolate clusters during the proteolysis of Cu-thionein

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Rat liver Cu,Zn-[^{35}S]thionein and yeast Cu-thionein were subjected to proteolysis in vitro using equilibrium dialysis. The partially copper-loaded vertebrate thionein (2–7 Cu/mol) was affected by different proteases including thermolysin, proteinase K, protease from *Streptomyces griseus* and lysosomal enzymes. Unlike the 2Cu-thionein the respective 7Cu-thiolate-centred metallothionein was hardly proteolytically digested. In contrast to fully copper-loaded native yeast Cu-thionein both the H_2O_2-oxidized and the metal-free protein were effectively cleaved in the presence of proteinase K. It is important to realize that the native Cu(I)-thiolate chromophore survives the proteolytic attack. When the copper-sulphur bonding is broken and the same amount of copper is unspecifically bound to the thionein portion, proteolysis proceeds identically with respect to the rate observed in the presence of the apoprotein. The unsuccessful proteolysis of native Cu-thionein is not attributable to a simple copper-dependent inhibition of the proteinases. It is suggested that prior to proteolysis the copper-sulphur clusters must be destroyed.

Metallothionein *Cu-thionein* *Cu(I)-thiolate* *Proteolysis*

1. INTRODUCTION

Our knowledge on the molecular basis of copper transport is very limited. Unlike iron metabolism, in which a specific enzyme, the so-called chelatase, has been found to control iron(II) incorporation into haem proteins [1], no enzymic mechanism is known to explain how copper is inserted into the active centres of copper proteins. Furthermore, no convincing evidence exists regarding the storage of copper in the organism.

It was proposed that the ubiquitously present metallothioneins might act as storage proteins for copper and/or zinc. Quite often the copper concentration of pre- and neonatal tissues can be 10–20-times higher compared to that of adult liver [2]. A similar high hepatic copper level is observed when copper salts are administered. In Wilson's disease both liver and brain are enormously enriched with copper. However, this phenomenon was attributed to an autosomal-recessive inherited disorder of copper metabolism [2,3]. In all cases there is a common concept in that the elevated copper values paralleled the metallothionein concentration [4].

During systemic inflammation a marked rise of blood plasma copper content up to 400% is observed concomitant with the stimulated hepatic biosynthesis of ceruloplasmin [5]. It is attractive to propose that the required copper originates from catabolized Cu-thionein [6].

This observation would favour the assignment of a copper-storage function for metallothionein. In the case of freely diffusing low-M_r copper complexes, many uncontrolled and undesired reactions of the chelated copper would be the consequence. By way of contrast, sequestering copper in the form of Cu(I)-thiolate clusters as found in the metallothioneins would be very encouraging to avoid the former uncontrolled metabolic situation. The important question remains open as to how the safely sequestered copper of metallothionein will be released. Several possibilities leading to cleavage of the copper-thiolate clusters are dis-

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cussed: (i) direct transfer of Cu(I) into apo-copper-proteins; (ii) oxidative cleavage of Cu(I)-thiolate centres; (iii) proteolysis of the polypeptide chain and controlled release of low-M_r metal chelates; (iv) mixed type of mechanisms including one or more of the former possibilities.

Reconstitution experiments using Cu-thionein and the apoprotein of some copper enzymes proved to be successful. Up to 85% of the required copper was directly and specifically transferred as Cu(I) into the respective coordination centres [7–9]. Model reactions for the oxidative cleavage showed that H_2O_2-generating enzymes were well able to oxidize irreversibly the copper-thiolate chromophores of both yeast and vertebrate copper-thionein [10].

Proteolysis of metallothionein was of special interest. In general, holoproteins are much less sensitive to proteolytic attack compared to the respective apoprotein in vitro [11]. As metallothioneins contain unusually high concentrations of metals it was not surprising to observe substantially diminished proteolysis of the holoprotein. Apometallothionein is degraded very rapidly by lysosomal enzymes [12] and the removal of only Zn^{2+} from Cd,Zn-thionein renders the protein more susceptible to the action of subtilisin and other proteases [13]. Due to the high stability of the Cu-thiolate chromophore [14] the copper species of metallothionein exhibits a different behaviour with respect to proteolysis [15,16]. Thus, Cu-thionein appeared to be extremely resistant to degradation by lysosomal extracts [15–17] as measured by trichloroacetic acid precipitation of native ^{35}S-labelled metallothionein, the fragments remaining in the supernatant. Under conditions where appreciable digestion of Zn- and Cd-thionein occurred no significant degradation of Cu,Zn-[^{35}S]thionein was detected [15].

One important question remains unanswered, namely whether the metals must be specifically bound to resist proteolysis. In other words, what is the contribution of the intact metal-thiolate cluster in making metallothionein able to survive proteolysis? It was promising to examine this question in more detail.

Both ^{35}S-labelled vertebrate and yeast Cu-thionein were subjected to equilibrium dialysis in the presence of different proteases including thermolysin, proteinase K, protease from *Streptomyces griseus* and lysosomal enzymes. The course of degradation was monitored by measuring the ^{35}S radioactivity of both the remaining intact metallothionein and the dialysed fragments as well as the electronic absorption at 220 nm of the dialysate in the case of yeast Cu-thionein.

2. EXPERIMENTAL

2.1. *Reagents*

L-[^{35}S]Cysteine was from Amersham International. Thermolysin from *Bacillus thermoproteolyticus*, protease from *S. griseus* and proteinase K from *Tritirachium album* were from Serva, Heidelberg. The dialysis tubing was from Spectrum Medical Industria (Los Angeles) and had an M_r cut-off of 3500.

2.2. *Spectrometry*

Copper and zinc were measured on a Perkin-Elmer atomic absorption spectrometer equipped with an HGA 76B unit. Electronic absorption was monitored using a Beckman model 25 spectrophotometer. ^{35}S radioactivity was detected on an LKB β-Compucount liquid scintillation counter. EPR measurements were performed at 77 K on an E-109 spectrometer (Varian, Palo Alto).

2.3. *Equilibrium dialysis*

The equilibrium dialysis unit consisted of two tightly fitting polyacrylic blocks (24 × 6 × 1.5 cm) each containing 5 cylindrical holes (15 × 5.7 mm) of 1000 μl volume. The two sets of holes were separated by the membrane obtained from flat segments of the dialysis tubing. Solutions were injected perpendicularly into each cylindrical cell through channels 1.5 mm wide. A second channel ascertained the displacement of air. A remaining air bubble served as an internal stirrer. The apparatus was rotated at approx. 20 rpm.

2.4. *Preparative procedures*

^{35}S-labelled Cu,Zn-metallothionein was isolated by injecting male Wistar rats intraperitoneally with $CuSO_4$ (1.5 mg Cu^{2+}/kg body wt) and after 3 h with L-[^{35}S]cysteine (150 μCi/kg body wt). The animals were killed 18 h after injection of Cu^{2+} and the livers removed. The supernatant of the centrifuged liver homogenate was heated for 3 min

to 60°C in the presence of 1 mM 2-mercaptoethanol. The soluble fraction was subjected to a combination of gel filtration on Sephadex G-75 and ion-exchange chromatography on DEAE-Sephadex A-25. The metallothionein fractions (MT 1, MT 2) were desalted on Sephadex G-25 and freeze-dried. The isolated proteins which were essentially zinc-free had a copper content of 2–3 Cu/mol. Additional copper was introduced into the thionein by incubation of the sample with $NaBH_4$ and a desired amount of $CuSO_4$ in the presence of 20 mM Tris-HCl, pH 8.0. After desalting on Sephadex G-25 the correct incorporation of Cu(I) was ascertained, being attributable to the absence of EPR signals. Yeast Cu-thionein was isolated as described [18]. The metal content was 5.3% (w/w). The metal-free protein was prepared by dialysis against two changes of 600 mM HCl for 2 and 1 h against 100 mM HCl at 20°C. For the proteolysis experiment the pH was adjusted to pH 7 with 1 M Tris-HCl. Lysosomal enzymes were obtained from isolated rat liver lysosomes as in [19].

2.5. *Assays*

Degradation of Cu-[^{35}S]thionein was controlled by measuring the radioactivity of proteolytically digested metallothionein during equilibrium dialysis. The same dialysis method was used when native yeast Cu-thionein, the oxidized and metal-free proteins were degraded in the presence of proteinase K. The cleavage products appeared in the dialysate and were monitored at A_{220}.

3. RESULTS AND DISCUSSION

Samples of partially copper-loaded rat liver Cu,Zn-[^{35}S]thionein were subjected to equilibrium dialysis in the presence of different proteases including thermolysin, proteinase K, protease from *S. griseus* and lysosomal enzymes. Considerable proteolysis was observed when metallothionein containing 2 Cu/mol was used (fig.1). After 24 h of enzymic action at 30°C essentially all metallothionein was decomposed by either protease. Successive introduction of copper into the above 2Cu-thionein resulted in markedly diminished degradation. At 7 Cu/mol metallothionein about 25–40% of the protein was still present at the end of the experiment depending on the proteolytic serum used (fig.2). Prior to the experiment no Cu(II) EPR signal was observed in the different desalted and lyophilized Cu-thionein samples indicating a fully reduced Cu(I)-thiolate bonding. The enzymic digestion is progressively diminished depending on the number of Cu(I)-thiolate centres in metallothionein.

In contrast to in vitro experiments it was shown that Cu-thionein was degraded faster in vivo than the Zn- and/or Cd-proteins. The half-lives of the ^{35}S-labelled Cu-thioneins in Cu-injected rats were determined to be about 15 and 18 h for Cu-MT-1 and 2, respectively, and were even faster than those of the Zn- and Cd-thionein (19 h and 3–4 days) [15,16]. This led to the suggestion that the stable Cu(I)-thiolate chromophores could have been oxidized prior to proteolysis, probably by a specific Cu-thionein oxidase [10].

To support this assumption yeast Cu-thionein, which is known to bind exclusively copper, was used as a substrate for the proteolytic digestion, although this Cu-thionein is different in its

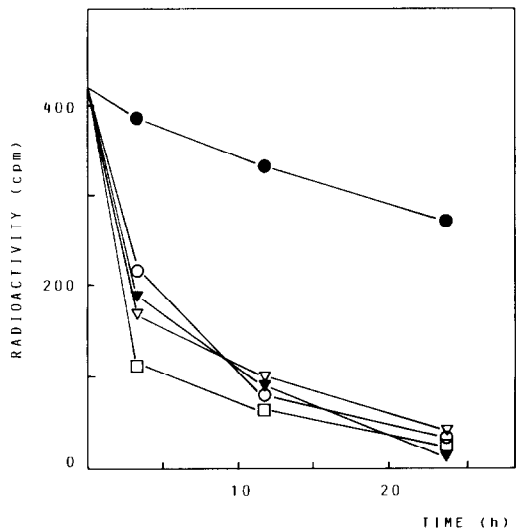

Fig.1. Degradation of rat liver Cu-[^{35}S]thionein (2 Cu/mol) in the presence of different proteases at 30°C. (●) 1 ml Cu-[^{35}S]thionein (0.5 mg/ml); (□) + proteinase K (0.2 mg, 3 U); (○) + protease from *Streptomyces griseus* (0.5 mg, 2 U); (▼) + thermolysin (0.2 mg, 10 U); (▽) + lysosomal enzymes (1.5 mg). Digestion was measured by equilibrium dialysis in H_2O. After 3, 12 and 24 h aliquots of 250 μl were used for detection of ^{35}S radioactivity. Values on the ordinate were obtained from the cpm difference between the protein-containing chamber and the dialysate.

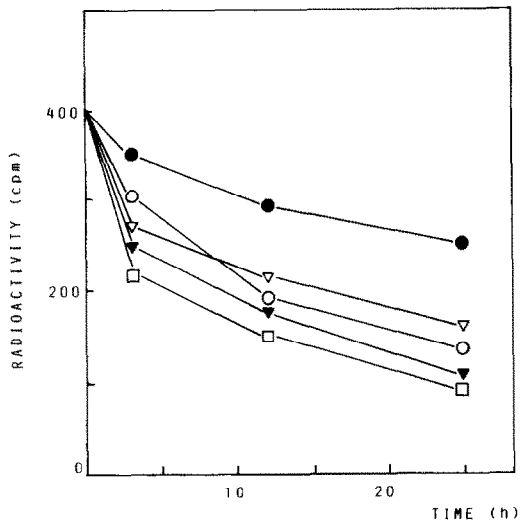

Fig.2. Degradation of rat liver Cu-[^{35}S]thionein (7 Cu/mol). Conditions were the same as in fig.1.

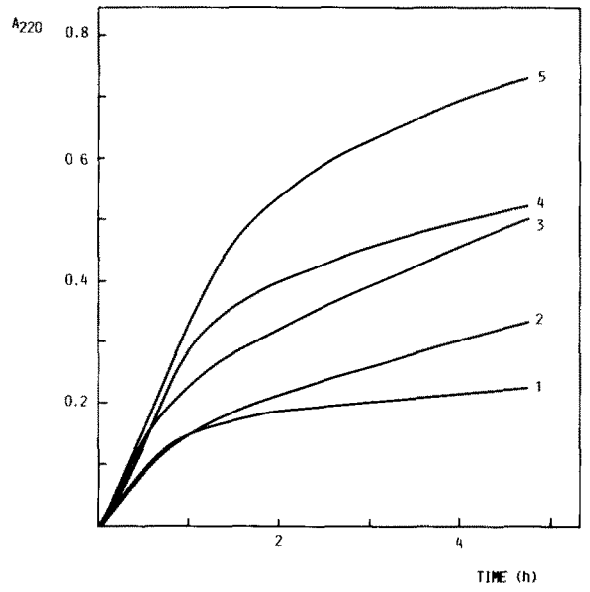

Fig.3. Degradation of differently treated yeast Cu-thionein in the presence of proteinase K. 1, control [0.1 mg (1.5 U) protease in 1 ml H_2O]; 2, + 0.25 mg native Cu-thionein; 3, + 0.25 mg apo-thionein; 4, + 0.25 mg H_2O_2-oxidized Cu-thionein; 5, + 0.25 mg apo-thionein and 4×10^{-4} M $CuSO_4$. Digestion was measured using equilibrium dialysis. A_{220} of the dialysate was monitored after 0.5, 1, 1.5, 2, 3 and 5 h.

primary structure. The metal is tetrahedrally coordinated in essentially the same manner as in the vertebrate metallothioneins [20].

Differently treated thionein samples were incubated with the unspecific cleaving enzyme proteinase K. Equilibrium dialysis was performed as described above (fig.3).

Native Cu-thionein remained virtually unaffected after 5 h at 22°C as measured by the electronic absorption of protein fragments in the dialysate at 220 nm. The small increase in A_{220} is attributable to the osmotic pressure of the protein. Approximately the same value was obtained using the protease alone. However, H_2O_2-oxidized Cu-thionein and apo-thionein were substantially degraded. The possible inhibiting effect of Cu(II) on the protease activity was additionally controlled by adding stoichiometric amounts of Cu(II) to apo-thionein. There was no detectable difference in the proteolytic rates observed in the former two experiments using the apo-thionein alone and the H_2O_2-treated holoprotein. The slightly higher absorption is possibly due to the formation of Cu-biuret complexes.

From these results we can conclude that the intact Cu(I)-thiolate cluster resists the proteolytic attack. It is assumed that copper specifically bound to metallothionein can only be released after the prior oxidation unless there is no competitive copper-removing ligand available. The demand for a specific Cu-thionein oxidase is consistent with the observed short half-life of hepatic Cu-thionein in vivo [15,16].

Unfortunately, there was no indication of the presence of such an oxidase when yeast Cu-thionein was incubated with the 100000 × *g* supernatant of rat liver homogenate. No oxidation of Cu-thionein could be seen attributable to the unchanged baseline during the course of the EPR measurements. It was concluded that ubiquitous reducing agents including glutathione might have interfered. Thus, after the complete separation of all low-M_r compounds from the homogenate by dialysis, approx. 15% of the thionein Cu was EPR-detectable within 12 h at 22°C. This result was not very encouraging for the further isolation of a possible Cu-thionein oxidase. No significant oxidation of Cu-thionein was seen in the presence of the cellular fractions containing predominantly microsomes or mitochondria. Nevertheless, being

aware of the many undesired reactions of unspecifically chelated copper, the release of copper ions ought to be enzymically controlled.

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REFERENCES

[1] Bugany, H., Flohé, L. and Weser, U. (1971) FEBS Lett. 13, 92–94.
[2] Ettinger, M.J. (1984) in: Copper Proteins and Copper Enzymes (Lontie, R. ed.) pp.175–229, CRC Press, Boca Raton, FL.
[3] Sass-Kortsak, A. and Bearn, A.G. (1978) in: The Metabolic Basis of Inherited Disease (Stanbury, J.B. et al. eds) pp.1098, McGraw-Hill, New York.
[4] Weser, U. and Hartmann, H.J. (1984) in: Copper Proteins and Copper Enzymes (Lontie, R. ed.) pp.151–173, CRC Press, Boca Raton, FL.
[5] Deuschle, U. and Weser, U. (1985) Prog. Clin. Biochem. 2, 99–130.
[6] Hartmann, H.-J., Gärtner, A. and Weser, U. (1985) Clin. Chim. Acta 152, 95–103.
[7] Hartmann, H.-J., Morpurgo, L., Desideri, A., Rotilio, G. and Weser, U. (1983) FEBS Lett. 152, 94–96.
[8] Morpurgo, L., Rotilio, G., Hartmann, H.-J. and Weser, U. (1984) Biochem. J. 221, 923–925.
[9] Brütsch, S., Hartmann, H.-J. and Weser, U. (1984) Inorg. Chim. Acta 92, 147–149.
[10] Hartmann, H.-J., Gärtner, A. and Weser, U. (1984) Z. Physiol. Chem. 365, 1355–1359.
[11] Eichhorn, G.L. (1973) Inorganic Biochemistry, vols 1,2.
[12] Feldman, S.L., Failla, M.L. and Cousins, R.J. (1978) Biochim. Biophys. Acta 544, 638–646.
[13] Winge, D.R. and Miklossy, K.-A. (1982) J. Biol. Chem. 257, 3471–3476.
[14] Rupp, H. and Weser, U. (1978) Biochim. Biophys. Acta 533, 209–226.
[15] Bremner, I. and Mehra, R.K. (1983) Chem. Scr. 21, 117–121.
[16] Mehra, R.K. and Bremner, I. (1985) Biochem. J. 227, 903–908.
[17] Held, D.D. and Hoekstra, W.G. (1979) Fed. Proc. 38, 2674.
[18] Weser, U., Hartmann, H.-J., Fretzdorff, A. and Strobel, G. (1977) Biochim. Biophys. Acta 493, 465–477.
[19] Bohley, P., Kirschke, H., Langner, J. and Ansorge, S. (1969) FEBS Lett. 5, 233–236.
[20] Hartmann, H.-J. and Weser, U. (1985) Biochem. Biophys. Res. Commun., in press.