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Effect of metal ions on the stability of metallothionein in the degradation by cellular fractions *in vitro*

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Abbreviations: MT, Metallothionein

Abstract

Metallothioneins (MT), small molecular weight metal binding proteins are known to play an important protective role against heavy metal toxicity, either as antioxidants or pre-oxidants. However, the mode of metabolic fate of MTs in various metal complexes is not clearly understood. This study was carried out to better understand the mode of selective turnover rate of various form of MT in complexes with different metals. The degradation of *in vitro* translated mouse ^{35}S-cysteine-MT was examined in lysosomal or cytosolic fractions from mouse liver by gel electrophoresis and autoradiography. Overnight incubations of MT showed extensive proteolysis in the lysosomal fraction but not in cytosolic fractions. However, Cu^{2+}-MT was found to be stable under the same experimental condition. In contrast, Zn did not interfere with MT degradation. These results suggest that lysosomes are chiefly responsible for MT removal and appears to be selective on the metals involved in the MT complex. *In vitro*, translated, radiolabeled MT provides a suitable substrate for investigating the characteristics of MT degradation.

Keywords: metallothionein, lysosome, degradation, copper, zinc

Introduction

Metallothioneins (MT) are a group of cysteine-rich low molecular weight (approximately 6000 to 7000 D) intracellular proteins that avidly bind heavy metals. Isoforms include MT-I, MT-II and MT-III, with similar molecular weights but slightly different amino acid compositions. Since MTs are inducible by cadmium (Cd), zinc (Zn), copper (Cu), and dexamethasone, they are considered to play an important protective role against heavy metal toxicity (Hamer, 1985). Zn-MT appears to serve as an antioxidant, and Cu-MT has been shown to enhance lipid peroxidation *in vitro* (Stephenson *et al*., 1994). Depending upon its associated heavy metals, MT acts either as an antioxidant or pre-oxidant, although the mechanism remains unclear. Recent studies using MT-I and MT-II knockout mice have also demonstrated that MT-I and MT-II help protect against Cd and Zn toxicities (Michalska *et al*., 1993; Liu *et al*., 1996a; Liu *et al*., 1996b; Kondo *et al*., 1997; Klaassen *et al*., 1998), while their protective roles against copper are still debated (Danks, 1995).

Recently, the potential toxicity of Cu-MT was evaluated in the *toxic milk* (*tx*) mouse, a model for Wilson disease. In this murine model, MT was present in the nuclei of liver cells along with increased numbers of apoptotic cells (Xi Deng *et al*., 1998). Abnormally high levels of MT and Cu accompanied striking morphologic abnormalities in the livers of *tx* mice. The presence of high amounts of copper-MT, and of copper in the nucleus, could be genotoxic and lead to an enhanced apoptosis. However, the exact mechanism of liver toxicity in the *tx* mouse is not well understood.

Despite of considerable advances in understanding the physiochemical properties of MT, the mechanism of the MT cellular degradation still remained unresolved. Most of the studies on metabolism of MT have used *in vivo* pulse-labeling method either using labeled-divalent metals or sulfur labeled amino acids either cysteine and/or methionine. The half-lives of MT-I and MT-II in the liver cytosol of Cu^{2+} injected rats were only 15.4 ± 1.5 and 18.2 ± 1.1 h, respectively, but in *in vitro* studies of copper-metallothionein in the rat liver, no significant degradation of the Cu-MT complex was observed (Mehra *et al*., 1985).

In order to better understand a possible mode of cellular turnover rate of MT in various state of metal complexes, we have prepared radiolableled mouse MT by translation in *in vitro* reticulocyte lysate system and used it in the proteolysis experiments containing various mouse liver cellular fractions. Using this method, we demonstrated that degradation of MT occurs in the lysosome and that copper interferes with the degradation of MT while Zn showed no effect.

Materials and Methods

Preparation of labeled MT

Radiolabeled mouse MT was prepared using the TnT coupled reticulocyte lysate system, as described by the manufacturer (Promega, WI, USA), and ^{35}S-cysteine (specific activity, 980 Ci/mmol; Amersham, Buckinghamshire, England). Mouse MT cDNA was kindly provided by Dr. Dean Hamer at the National Institutes of Health in Bethesda, MD, USA 1.5 µl of synthesized ^{35}S-cysteine labeled MT was diluted in 30 µl of 0.05 M sodium acetate pH 5.0 containing 2.5 mM 2-mercaptoethanol or 100 mM Tris pH 7.4, containing 100 mM $CaCl_2$. 5 µl of mixture was used to calculate the radioactivity after TCA precipitation.

Isolation of mouse liver lysosomal and cytosolic fractions

Mouse liver lysosomal and cytosolic fractions were prepared by the method of Regab *et al*. The final pellet, obtained from 40-50 g of liver, was suspended in 1 ml of 0.02 M citrate buffer, pH 4.8, containing 0.2%(w/v) Triton X-100 and 0.02 M 2-mercaptoethanol, freeze-thawed five times, and then centrifuged at 100,000 *g* for 1 h. The supernatant fraction was stored in aliquots at -20°C. The cytosolic fraction was suspended in 100 mM Tris pH 7.4, containing 100 mM $CaCl_2$ and stored at -20°C.

In vitro proteolysis of ^{35}S-labeled MT by lysosomal extracts and cytosolic extracts

^{35}S-labeled MT (5 µl) in sodium acetate buffer was incubated with 7 µl (10 µg) of mouse liver lysosomal extract as described with minor modifications (Mehra *et al*., 1985). After incubation for 1-12 h at 37°C, the reaction was stopped by freezing, treated with iodoacetic acid at neutral pH, and followed by electrophoresis on 20% SDS polyacrylamide gels as described (Hahn *et al*., 1994; Hahn *et el*., 1995). The gel was dried, exposed to X-ray film at -70°C and autoradiographed. 5 µl of ^{35}S-labeled MT was incubated with 7 µl (20 µg) of mouse liver cytosol containing Tris buffer, pH 7.4. Proteolysis of MT was stopped by freezing, followed by gel electrophoresis. The gel was dried, exposed to X-ray film at -70°C and autoradiographed. ^{35}S-labeled MT was also incubated with trypsin in Tris buffer, followed by electrophoresis and autoradiography. The control contained ^{35}S-labeled MT and buffer without lysosomal or cytosolic fractions.

In vitro proteolysis of ^{35}S-labeled MT by lysosomal extracts in the presence of copper or zinc

Copper sulfate and zinc sulfate (1 and 10 µM) were added to the ^{35}S-labeled MT prior to incubating with lysosomal extracts for 12 h. The reaction was stopped as above, electrophoresed, and autoradiographed.

Results and Discussion

In vitro proteolysis of ^{35}S-labeled MT by lysosomal and cytosolic fractions

^{35}S-labeled MT was degraded completely by lysosomal fractions of mouse liver, but not by cytosolic fractions when incubated overnight (Figure 1). Control buffer without either lysosomal or cytosolic fractions did not show any effect on the stability of the ^{35}S-labeled MT. When ^{35}S-labeled MT was incubated with cytosolic fractions, its molecular weight was slightly increased, with smearing of the band. When ^{35}S-labeled MT was adjusted to pH 7.4 in the buffer without cytosolic extracts, the same smearing pattern appeared with increased molecular weight band suggesting a possible conformational change due to pH. ^{35}S-labeled MT was shown to be sensitive to proteases in the neutral pH as demonstrated by the complete digestion with trypsin. This study demonstrates that MT is readily degraded by mouse liver lysosome at slight acidic environment, but not by neutral cytosolic fractions.

Although we have used equivalent levels of cellular fractions to simulate the *in vivo* cell state, it may still created the instability of neutral proteases during overnight incubation period in comparison with compartmentalized lysosomal enzymes. Since lysosomes are known to function as cellular clearing house, it is not surprising to find that MT degradation occurs mainly in this fraction. Whether there is a specific MT-degrading enzyme in the lysosome is not known yet. However, the *in vitro* technique we describe for assaying MT proteolysis can be employed to purify this putative enzyme(s).

In vitro proteolysis of ^{35}S-labeled MT by lysosomal extracts in the presence of copper or zinc

When copper was added to the reaction, ^{35}S-labeled MT was not digested by the lysosomal extracts, while the

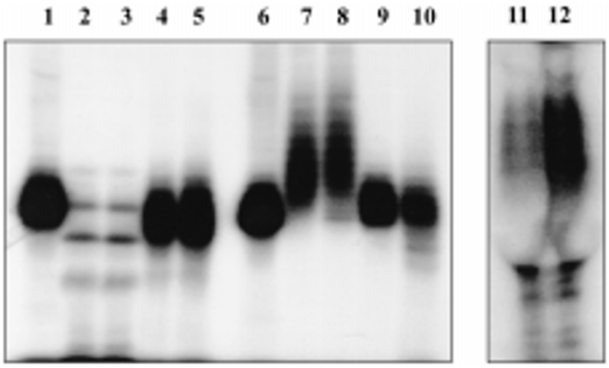

Figure 1. *In vitro* digestion of ^{35}S-labeled mouse-MT by lysosomal and cytosolic fractions. Lane 1: MT control, Lane 2, 3: MT incubated with lysosomal extracts, Lane 4, 5: MT in citrate buffer, Lane 6: MT control, Lane 7, 8: MT incubated with cytosolic extracts, Lane 9, 10: MT in Tris buffer, Lane 11, 12; MT incubated with trypsin. The proteins were incubated overnight, loaded on a 20% SDS-PAGE gel, dried and autoradiographed.

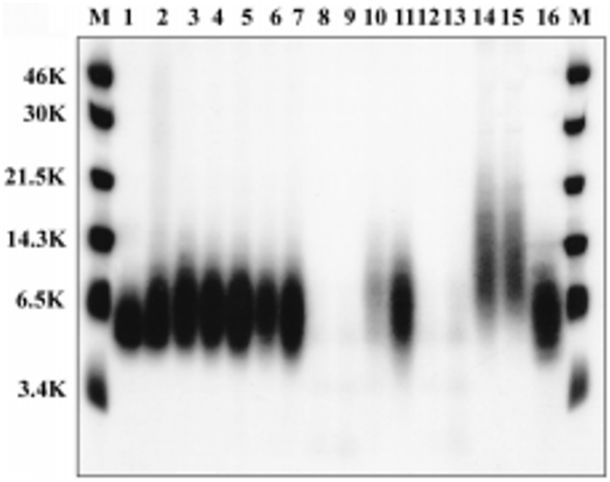

Figure 2. *In vitro* digestion of ^{35}S-labeled mouse-MT by lysosomal extracts in the presence of copper or zinc. M: Marker, Lane 1: MT control, Lane 2, 3: MT incubated with lysosomal extracts for 1h; Lane 4, 5: MT incubated with lysosomal extracts for 2 h; Lane 6, 7: MT incubated with lysosomal extracts for 3 h; Lane 8, 9: MT incubated with lysosomal extracts overnight; Lane 10: MT incubated with lysosomal extracts and 1 μM copper overnight; Lane 11: MT incubated with lysosomal extracts and 10 μM copper overnight; Lane 12: MT incubated with lysosomal extracts and 1 μM Zn overnight; Lane 13: MT incubated with lysosomal extracts and 10 μM Zn overnight; Lanes 14, 15: MT in Tris buffer pH 7.4, overnight incubation; Lane 16: MT control overnight incubation without buffer.

zinc did not interfere with the complete digestion of ^{35}S-labeled MT (Figure 2). The difference may not be contributed by the level of metal ion complexed with MT (binding capacity of MT toward copper or zinc) but more likely due to the oxidative and reductive transition state of each metals. Excess copper is very toxic and damages lipids, proteins and nucleic acids. Exposure of DNA to H_2O_2 in the presence of Cu salts can result in DNA strand breaks and base modification *in vitro* (Kawanishi *et al*., 1991; Marshall *et al*., 1981; Que *et al*., 1980). Cu(I) in the presence of H_2O_2 preferentially generates Cu-peroxide complexes leading to production of singlet $\cdot O_2$ and/or hydroxyl radicals (·OH), whereas Cu(II) in the presence of H_2O_2 generally forms ·OH by a Fenton reaction. These reactive oxygen species are capable of damaging critical cellular molecules.

The major hepatic copper binding protein both in Wilson's disease and its animal model, the *toxic milk* mouse, has been identified as metallothionein (MT). MTs are a group of low molecular weight (approximately 6000 D), cysteine rich proteins with high affinity for heavy metals such as Zn, Cd and Cu. MT combines with 11 or 12 copper ions, 5 or 6 bound to the amino terminal portion and another 5 or 6 to the carboxyl terminal. In the *toxic milk* mouse liver, accumulation of high levels of MT and Cu along with striking morphologic abnormalities have been observed. MT was also present in the nucleus along with increased numbers of apoptotic cells (Deng *et al*., 1999). MT appears to be involved in protection against apoptotic changes (Deng *et al*., 1999; Cabre *et al*., 1999; Tsangaris *et al*., 1998). MT associated with Cd has been shown to promote apoptosis in human kidney 293 cells (Hamada *et al*., 1996). In previous studies, addition of Zn-MT was found to protect against Cu (II)-induced DNA damage (Cai *et al*., 1999), whereas Cd/Zn-MT induced plasmid DNA strand breaks (Muller *et al*., 1991). It is likely that MT is involved not only in metal detoxification and homeostasis, but also in scavenging free radicals during oxidative damage (Cai *et al*., 1999). Transcriptional induction of MT-I, and MT-II genes in the livers of Cu, Zn-superoxide dismutase knockout mice demonstrated that MTs are potent scavengers of reactive oxygen species and protect cells from oxidative stress (Ghoshal *et al*., 1999). Zn-MT appears to be involved in antioxidant systems, while Cu-MT isolated from the livers of *tx* mice enhances lipid peroxidation *in vitro* (Stephenson *et al*., 1994). Depending on the heavy metals associated with it, MT appears to act as either an antioxidant or pre-oxidant.

It has been postulated that MTs are mainly involved in Zn and not Cu homeostasis. MT serves as means of sequestering excess zinc and providing a zinc reservoir that can be utilized when zinc is deficient (Kelly *et al*., 1996). MT-III, a brain specific member of the MT family (Palmiter *et al*., 1995), may be an important regulator of zinc in the central nervous system; its absence has been implicated in the development of Alzheimer disease (Erickson *et al*., 1997).

Although considerable advances have been made in studies of the physicochemical properties of MT and induction of its synthesis, comparatively little attention has been paid to its degradation. In rat pulse labeling studies *in vivo*, the degradation rates of MT depended upon which metals were bound to MT (Bremner *et al*., 1978). The half-lives of MT induced by Cd, Zn, and Cu were estimated to be 80, 20, and 17 h, respectively. Several experiments showed that removal of metals from MT is the rate-limiting step in MT degradation (Feldman *et al*., 1978), but it is hard to estimate the accuracy of this process, because protein aggregation makes chromatographic analysis difficult. Furthermore, there has been little progress on the study of the exact location of MT degradation in cells, although it was presumed that degradation occurs mainly in the lysosome. Aggregate forms of the protein appear to be located within lysosomes (Porter *et al*., 1974), suggesting that Cu-MT is not readily degraded in that organelle. When MT is bound to copper, its degradation in the lysosome may not be taken place effectively due to a conformational change of MT. In our study, Cu inhibited the digestion of MT *in vitro* while Zn did not affect MT digestion. Improperly degraded Cu-MT could play an important role in the liver toxicity of Wilson's disease *via* lipid peroxidation or apoptosis.

Copper may inhibit all lysosomal enzymes, including

the proteases that degrade MT, or copper may bind to MT and stabilize it specifically. In our experiments, we added copper to MT first in order to minimize the effect of copper on lysosomal enzymes. Our findings also imply that Cu cannot be effectively removed from cells by binding to MT under normal physiological conditions. We have not eliminated the possibility that there is a non-lysosomal system for Cu-MT degradation or a protein required for removal of Cu from MT, which was absent from our *in vitro* experiments.

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