In Vitro and In Vivo Studies on the Degradation of Metallothionein

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Degradation of metallothionein (MT) from rat liver was examined. Degradation of apo-MT by liver homogenate was greater than that by cytosol. At pH 5.5, degradation by homogenate was more than that at pH 7.2. These findings suggest that proteases that function at acidic pH are probably involved in MT degradation. Because lysosomes are the principal subcellular organelles that contain acid proteases (cathepsins), we compared the degradation of apo-MT by lysosomes and cytosol. Apo-MT was degraded about 400 times faster by lysosomal fraction than by cytosolic fraction. To determine the relative importance of different cathepsins, we used different inhibitors. Leupeptin, which inhibits cathepsins B and L, inhibited the degradation of apo-MT by 80%, implying that cathepsins B and/or L might be very important in the intracellular turnover of MT. Cathepin D appeared to be the least significant, because apo-MT degradation was reduced by about 20% by inhibiting cathepin D. When we extended this study with purified cathepsins, we obtained the same answer, i.e., the ability of different cathepsins to degrade apo-MT was in the following order: cathepin B > cathepin C > cathepin D. While apo-MT was susceptible to degradation, ZnMT and CdMT were highly resistant to degradation. Coincubation of ZnMT or CdMT with either lysosomal extract or purified cathepsins did not result in any appreciable degradation even after 16 hr. However, longer incubations did result in some degradation, especially by purified cathepin B. Interestingly, CdMT degraded little faster than ZnMT by both lysosomal extract as well as purified cathepin B. These data suggest that metals protect MT from rapid proteolysis. By metal-titration study, we found that at least 5 moles of Zn equivalent/mole of MT dramatically reduced the degradation, by both lysosomal fraction and purified cathepin B. This indicates that metal release might be a prerequisite for the initiation of degradation. Release of metals is possible in the lysosomes because the intralysosomal pH is close to 5.0. Therefore, we determined the displacement of metals (Zn) as a function of pH. We found that at a pH of 4.5, nearly 70% Zn was removed, and at pH 4.0 nearly all Zn was displaced from MT. We propose, therefore, that lysosomes are probably important in the in vivo degradation of MT, and that metal release is a prerequisite for degradation. With the release of metals in the acidic pH of lysosomes, MT becomes more susceptible to degradation, which is probably accomplished by the cathepsins, in particular cathepin B and/or L. The in vivo study, however, suggests that there are other factors, too, in addition to metal composition and pH, that may have profound effect on determining the half-lives of various forms of MT, at various stages of development. — Environ Health Perspect 102(Suppl 3):141–146 (1994).

Key words: metallothionein, degradation, lysosomes, cathepsins, inhibitors, MT half-life, inducers

Introduction

Conditions that affect the synthesis of metallothionein (MT) provide important information concerning its importance in different tissues, and during various physiological conditions. Equally important in understanding the functions and regulation of MT, but less well understood, is the degradation of the protein and turnover of the metals bound to it.

For the last several years, research in our laboratory has focused on characterizing the degradation of hepatic MT in a rat model system. When we undertook the studies, not much information was available on the degradation of MT. Nevertheless, a few studies were done earlier on this aspect, and the information emanating from them could be distilled into the following concepts: lysosomes might be important in degrading MT; metals protect MT from rapid degradation; the order of sensitivity towards degradation is apo-MT >> ZnMT >> CdMT; and degradation of MT and metal release probably occur concomitantly.

The principal objectives of our current studies were to answer many other aspects of MT degradation that were not addressed by the previous studies, viz., to identify the subcellular site(s) in which the degradation of hepatic MT occurs; to identify the protease(s) most likely involved in the in vivo turnover of MT; to determine the relative efficiency of different proteases to degrade MT; to quantitatively compare the degradation of apo-MT, ZnMT, and CdMT; to evaluate the extent of protection provided by the metals; and finally to obtain a more thorough understanding of the biochemical processes that regulate the degradation of this important metal-binding protein.

Methods

Lysosomes were prepared by differential centrifugation followed by a phase separation technique (1). Radiolabeled apo-MT was prepared according to Sendelbach and Klaassen (2) from livers of rats that received subacute exposure to Cd or Zn, and [35S]-cysteine. To prepare apo-MT, metals were removed from the protein by dialysis against 0.1 M HCl solution. We obtained fully saturated ZnMT and CdMT by titrating MT with the respective metals (Zn or Cd) bound to it, followed by desalting to remove excess unbound metals (3,4).

Proteolytic reactions were carried out in vitro at pH 5.5 or 7.2, using 0.5 M sodium acetate or 0.1 M sodium phosphate buffer, respectively. Although the lysosomal pH is close to 5.0 (5,6), we chose pH 5.5 because preliminary experiments in our laboratory showed that, at this pH, all metals are retained by MT while the lysosomal...
acid proteases remain active. For most of our experiments, we used MT-I. The optimum ratio of apo-MT to homogenate or cytosolic protein was determined to be 1:1 (50 μg apo-MT: 50 μg homogenate or cytosolic protein). For ZnMT and CdMT the same ratio was found to be optimum. The optimum ratio of apo-MT to lysosomal protein was 80:1 (50 μg apo-MT: 0.625 μg lysosomal protein). Incubates with a final volume of 100 μL were maintained at 37°C. When apo-MT was used as a substrate, the reactions were sampled at 0, 0.5, 1.0, and 2.0 hr, after introduction of all components to the digestion mixture. When ZnMT or CdMT were used as substrates, the reactions were sampled at 0, 6, 12, and 16 hr. Samples were rapidly placed in the denaturing buffer to stop the reaction, flash-frozen in liquid nitrogen, and stored at ~80°C.

Samples of different time points were then subjected to SDS-PAGE analysis as described by Hames (7), and stained by Coomassie brilliant blue. Prior to electrophoresis, the samples were carbosymethylated according to Otsuka et al. (8), with slight modification. The degraded MT bands were excised from the gel, solubilized, mixed with scintillation cocktail, and analyzed for radioactivity using a scintillation spectrometer.

MT degradation was quantified by measuring the disappearance of the undegraded parent bands. The reduction of cpm values in the undegraded parent bands of each time point, compared to the 0 hr time point band, was used as the index of MT degradation. Based on prior determinations of the specific activity of our MT preparations (cpm/μg protein), the cpm values were converted to the amount of protein. The values thus obtained were normalized for the incubation time as well as amount of protein, because both the incubation time and the amount of lysosomal protein were different depending on the substrate: apo-MT, ZnMT, or CdMT.

In order to determine further the influence of metal composition on the proteolytic breakdown of MT, the degradation and metal contents of MT-I and MT-II in rat liver were examined in vivo during various conditions of induction: neonatal development, and acute exposure to Zn (1 mmole/kg, sc), ethanol (109 mmole/kg, po), or Cd (10 μmole/kg, sc). This was accomplished by measuring the disappearance of pulse-labeled MT-I and II in liver (9). Injections of [35S]-cysteine 24 hr after parturition or 6 hr after inducer pretreatment were employed to radiolabel each iso-

protein. High dosages of nonradioabeled cysteine (1 mmole/kg, po) were administered 4 hr after [35S]-cysteine and every 24 hr thereafter to minimize reutilization of radiolabeled amino acid. Iso-MTs were isolated by chromatography and HPLC for the assessment of the metal content and specific radiological activity, i.e., dpm of [35S]-labeled iso-MTs/g liver (9). Zn, Cd, and Cu content of MT isomers were measured by atomic absorption spectrometry (AAS). Chemical and radiological concentrations of iso-MTs were measured by HPLC coupled with AAS as described by Lehman and Klaassen (10), and liquid scintillation spectrometry, respectively.

**Results**

Lysosemes probably represent an important subcellular compartment that might be involved in the degradation of MT in vivo. Previous studies on the degradation of MT in rat liver indicated that lysosomes can degrade apo-MT (11,12). However, apo-MT was also shown to be degraded by trypsin, which is a neutral cytosolic protease (11). It was, therefore, our initial goal to identify the subcellular compartment(s) that are most likely involved in the in vivo degradation of MT. In order to do that, we investigated the relative abilities of the whole rat liver homogenate, cytosol, and lysosome fractions to catabolize apo-MT in vitro at two different pHs: pH 5.5 (lysosomal pH) and pH 7.2 (cytosolic pH). Apo-MT was chosen as a substrate because preliminary studies in our laboratory had shown that metal-bound forms of MT were highly resistant to degradation. Table 1 shows that the degradation of apo-MT reached a maximum (0.78 ± 0.16 nmole apo-MT degraded/mg protein/min) by whole rat liver homogenate at pH 5.5. This was about four times more extensive than that at pH 7.2 (0.20 ± 0.02 nmole/mg protein/min). At pH 7.2, cytosolic fraction could degrade little apo-MT (0.13 ± 0.03 nmole/mg protein/min), but at pH 5.5, it could not degrade any apo-MT. Because the homogenate contained all subcellular enzymes including lysosomal proteases, which are most active at acidic pH, these data suggest the involvement of lysosomes in degrading apo-MT.

Additional evidence that identifies lysosomes as the most likely subcellular site in MT degradation was obtained when the relative abilities of lysosomes and homogenate in degrading apo-MT were compared. Figure 1 shows that the greatest degradative activity clearly resides in the lysosomes having 65 and 390 times greater proteolytic activity over homogenate and cytosol, respectively. These observations are consistent with the previous studies that indicated lysosomes as the possible intracellular site of MT degradation in vivo (11-13).

**Table 1. Degradation of apo-MT by homogenate and cytosol at pH 5.5 and 7.2.**

| Fraction | pH  | Degradation of apo-MT (nmole/mg protein/min) |
|----------|-----|---------------------------------------------|
| Homogenate | 5.5 | 0.78 ± 0.16a |
| Homogenate | 7.2 | 0.20 ± 0.02a |
| Cytosol | 5.5 | ND |
| Cytosol | 7.2 | 0.13 ± 0.03 |

*aEach value represents the mean SE (n=3). ND, no detectable degradation.*
Lysosomal Proteases Can Efficiently Depurate MT In Vitro

Because cathepsins are the principal lysosomal proteases, it seemed probable that the lysosomal degradation of MT might be mediated by them. Lysosomes contain many different cathepsins that mediate critical catabolic pathway in liver for a wide array of exogenous and endogenous proteins (14, 15). In order to identify the most important lysosomal enzyme(s) that might be involved in MT degradation, cathepsin-specific inhibitors were used (3). Figure 2 illustrates the relative abilities of different cathepsin inhibitors to decrease the extent of apo-MT degradation in vitro. Leupeptin, which inhibits cathepsin B and L, inhibited apo-MT degradation the most (by about 80% of the control). This indicates that cathepsin B and/or L might be the most important lysosomal protease involved in the intracellular degradation of MT. By the same argument, the minimal effect of pepstatin seems to preclude the involvement of cathepsin D in MT degradation. The moderate inhibitory effect of E-64 (about 50% inhibition of degradation) suggests a role for cathepsin C also, but this should be viewed with caution in light of the fact that E-64 can also partially inhibit cathepsin B and L (16).

The relative abilities of cathepsins in degrading MT were further evaluated by extending the study to in vitro MT degradation by purified cathepsins (4). The composition of incubates was similar to that employed with crude liver extract, except for their substitution by cathepsin B (6.0 mU), C (90 mU), or D (75 mU). The concentrations of cathepsins used were also optimized by preliminary studies. The results of purified cathepsin-mediated in vitro MT degradation, corroborated nicely the results obtained from studies using the inhibitors. Cathepsin B again turned out to be the most efficient in degrading MT, followed by cathepsins C and D. The amount of apo-MT degraded by cathepsins B, C, and D were 12,500 ± 1500, 350 ± 33, and 275 ± 15 pmole apo-MT degraded/U cathepsin/min, respectively. Therefore, cathepsin B degraded apo-MT 36 and 45 times faster than cathepsins C and D, respectively. These data further support our conclusion that cathepsin B, a cysteinyl acid protease in lysosome, might be the major player in the in vivo degradation of MT.

Comparison of the Degradation of Metal-free and Metal-bound Forms of MT Reveals that Association of Metals with MT Protects it from Rapid Proteolysis

It is believed that metals bound to MT render it resistant to degradation. This was first shown by Feldman and Cousins (17) and Feldman et al. (11). Because there were no quantifications, it was not possible to compare the degradation of metal-free and metal-bound forms of MT and assess the extent of protection against degradation provided by metals. When we compared the degradation of apo-MT, ZnMT, and CdMT, by either lysosomal extract or purified cathepsins, we found that apo-MT degradation was very rapid, but ZnMT and CdMT were extremely resistant to degradation. Table 2 shows that degradation of apo-MT by lysosomal fraction was about 1500 and 2500 times faster than that of CdMT and ZnMT, respectively. Similar results were obtained from studies using purified cathepsins (Table 3). Degradation of apo-MT by purified cathepsin B was about 1000 and 6000 times faster than that of CdMT and ZnMT, respectively. These

### Table 2. Comparison of the degradation of apo-MT, ZnMT and CdMT.

| Form of MT | pH | MT degradation, pmol/mg protein/min |
|------------|----|----------------------------------|
| Apo-MT     | 5.5| 50200 ± 43000*                   |
| CdMT       | 5.5| 35.0 ± 5.2                      |
| ZnMT       | 5.5| 20.5 ± 2.80                     |

*Each value represents the mean SE (n=9).

### Table 3. Degradation of apo-MT, Cd-MT, and Zn-MT by cathepsins B, C, and D.

| Substrate | Cathepsin | B | C | D |
|-----------|-----------|---|---|---|
| Apo-MT    | 12,500 ± 1500a | 350 ± 33 | 276 ± 15 |
| Cd-MT     | 130 ± 1.6 | ND | ND |
| Zn-MT     | 2.0 ± 0.30 | 0.22 ± 0.04 | 0.63 ± 0.06 |

*Degradation based on picomoles MT degraded/U cathepsin/min. Values are means ± SEM of one to three time points from three separate incubations. ND, none detected.

Figure 3. SDS-PAGE analysis of CdMT collected at different time points following incubation with lysosomal fraction. Lane 1 on the left contains the molecular weight markers x 10^3. Lane 2 has control MT-I band containing 20 μg protein. Lanes 3 to 6 have CdMT-I samples from the experiment. In each lane 10 μg of MT-I was loaded. The figure shows that even after 16 h there was no appreciable degradation. Therefore, metals bound to MT protect it against proteolysis.

Figure 4. SDS-PAGE analysis of ZnMT-I and CdMT-I following digestion by cathepsin B (150 mU/lane). Lane 1 on the left contains the molecular weight markers x 10^3. Lane 2 has control MT-I band containing 20 μg protein. The figure shows that ZnMT was more resistant to degradation by cathepsin B than CdMT, and both metals can protect MT from rapid degradation.
observations support the existing concept that metals render MT highly resistant to degradation.

However, two findings in the present study are interesting and not reported earlier. First, either ZnMT or CdMT was found to be more resistant to degradation than previously reported. Secondly, CdMT degraded a little faster than ZnMT by both lysosomal extract and purified cathepsins. Figure 3 shows an SDS-PAGE analysis of CdMT that was incubated with lysosomal extract. After 16 hr, there was no appreciable degradation. Figure 4 shows an SDS-PAGE analysis of both ZnMT and CdMT, incubated with cathepsin B. Both ZnMT and CdMT appear more resistant to degradation than previously thought. Figure 4 and Tables 2 and 3 also clearly depict that CdMT degraded slightly faster than ZnMT. This finding was surprising considering the fact that the affinity of Cd for the protein moiety of MT is higher than that of Zn (18,19). However, compared to apo-MT, the degradation of ZnMT and CdMT were negligible, and our finding still provides strong support to the fundamental concept that metals protect MT against proteolysis.

Five moles of metals/mole MT can dramatically decrease degradation, indicating that displacement of metals from the protein may be a prerequisite for the initiation of MT degradation. In order to further assess metal-dependent stabilization of MT, Zn was added in increments into the incubation mixture containing apo-MT. Figures 5 and 6 are strikingly similar. The degradation of apo-MT by either lysosomal extract (Figure 5) or purified cathepsin B (Figure 6) decreased in a linear fashion as a function of increasing amounts of molar equivalents of Zn added to the reaction mix. The degradation reduced dramatically in the presence of the reaction mix of 5 or more molar equivalents of Zn.

The findings from the foregoing studies indicate that complete or partial displacement of metals from MT might be an important prerequisite for degradation to occur. One possible mechanism for displacement of metals from MT is the low pH, which exists in the lysosomes. Our experiment on pH-dependent displacement of Zn and Cd from MT (Figure 7) reveals that at a pH of 4.5, about 70% Zn was removed and at pH 4.0 nearly all Zn was displaced from MT. In contrast, at a pH of 3.5, only 30% Cd was removed with approximately 80% Cd displaced at pH 3.0. Therefore, it requires a higher H+ ion concentration to displace Cd because of its higher affinity toward MT (4).

**Conclusion and Future Prospects**

The findings from the present study can be broadly categorized as follows; those that corroborate the existing concepts on MT degradation and those that are different from the previous reports on certain aspects of MT degradation.

Our study provides support for the "lysosomal theory of degradation," and identifies lysosomal cathepsins, in particular cathepsin B, to be an important enzyme that may be involved in the degradation

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**Table 4. Metal contents and half-lives of MT-1 and MT-2**

| Treatment   | iso-MT | Cu | Cd | Total | Half-life, hr |
|-------------|--------|----|----|-------|---------------|
| Neonate     |        |    |    |       |               |
| MT-1        | 5.5    | 0.1| ND | 5.6   | 49            |
| MT-2        | 5.9    | ND | ND | 5.9   | 73            |
| Cd-treated  |        |    |    |       |               |
| MT-1        | 3.2    | 0.2| 3.2| 6.6   | 56            |
| MT-2        | 3.4    | 0.1| 2.8| 6.3   | 61            |
| Zn-treated  |        |    |    |       |               |
| MT-1        | 6.6    | ND | ND | 6.6   | 21            |
| MT-2        | 5.6    | 0.3| ND | 6.1   | 33            |
| Ethanol-treated |      |    |    |       |               |
| MT-1        | 6.0    | ND | ND | 6.0   | 9             |
| MT-2        | 5.6    | ND | ND | 5.9   | 9             |

ND: Not detected; the lowest detectable specific metal content was approximately 0.1 g atom metal/mole MT.

*Metal contents and half-lives of hepatic isometallothioneins were determined as described in "Materials and Methods." Metal contents represent mean values of three determinations made upon a single pooled sample prepared from six adult rats (Cd-, Zn-, or ethanol-treated) or 76 nontreated neonates.*
process. Our study also confirms that metals bound to MT protects it from proteolytic degradation, and apparently 5 mole of metal/mole MT can dramatically reduce its degradation. The experiments on metal-mediated protection strongly suggest that the release of metals from MT is a prerequisite for initiating the degradation.

However, the extent to which metals protect MT was found to be much greater in our study than previously suggested. Feldman et al. (11) reported that it takes about 1 and 3 hr to degrade about 50% ZnMT and CdMT, respectively. Our study showed that even after 16 hr, there was no appreciable degradation of MT by lysosomes. This apparent difference might be explained, at least in part, by the pH level and the nature of the MT used. In the present study, a pH of 5.5 was used. At this pH, all metals were retained by MT and therefore, its rapid degradation was inhibited (3, 4). In contrast, all previous studies, including the one by Feldman et al. (11), employed a pH of 5.0 to 5.3. At this pH, metals, especially Zn, will be partially displaced from the protein (4), which would promote rapid initiation of degradation. This effect of pH also explains why 50% degradation of CdMT was obtained in about 3 hr at pH 5.0, although Cd is not displaced from MT at this pH. The CdMT used by the previous studies was isolated following Cd treatment of the animals. The CdMT formed in vivo is actually a mixed-metal species of MT, containing both Zn and Cd (21). The Zn content of the CdMT can be displaced at pH 5.0 or lower, thereby initiating degradation of CdMT.

The slightly faster degradation of CdMT over ZnMT that was observed in the present study has not been reported earlier. It contradicts the fact that CdMT has a longer in vivo half-life than ZnMT. In our studies we used fully saturated ZnMT or CdMT. Different molecular configuration of ZnMT and CdMT might offer an explanation, but to this end no such information exists. It should be remembered that the degradation of ZnMT and CdMT was so negligible compared to that of apo-MT that this apparent difference in their degradation might be ignored.

Although recent studies on MT degradation speak strongly in favor of the "lysosomal theory of degradation," involvement of other subcellular compartments, in particular the cytosolic compartment, cannot be ruled out. In fact, in recent years, an increasing number of studies have provided strong evidence in favor of this contention (13, 22). The study by Steinebach and Wolterbeek (22) has demonstrated that MT in cell exists in two compartments; the cytosolic compartment (apo-MT) and the lysosomal compartment (metal-bound MT), each being depleted and replenished at different rates. In our laboratory, the current research interest is to identify the cytosolic enzymatic component(s) that may be involved in the degradation of MT, in particular the cytosolic pool of MT.

Apart from the different subcellular compartments, many other factors might potentially contribute to the intracellular turnover of MT. For example, it was demonstrated that MT is localized mainly in the nucleus during development (23, 24). It can be argued that, because MT is localized mainly in the nucleus during development, its limited availability to the degradation machinery (cytosol and/or lysosomes) might be a factor that can regulate its accumulation in the cell during development. This might account for the reportedly high level of hepatic MT in the livers of neonatal rats (25). Another important but unexplored area in MT degradation research is examining the possibility of involvement of the ubiquitin system in MT turnover. Finally, various post-transcriptional events, including translational control, might also play important roles. Clough et al. (26) reported that human fetal liver tissue contains a third charge-separable MT isoform. Soumillion et al. (27) termed it MT-3, and demonstrated the presence of MT-0 mRNA in adult liver, despite the complete absence of this isoform at the protein level.

Thus, the intracellular turnover of MT and its regulation appear to be much more complex than previously contemplated. The current status of knowledge suggests the involvement of lysosomal and extralysosomal (possibly cytosolic) compartments in the intracellular turnover of MT. However, it is not known yet if these compartments cooperate with each other in a concerted fashion to degrade the total cellular MT pool, or if they act independently of each other to degrade separate compartmentalized MT pools. Therefore, the existing knowledge about MT degradation is fragmentary, and more work is needed in this area in order to gain a complete insight of the steps and processes involved. For this purpose, use of special cell lines/transgenic models that either overexpress, or are deficient in specific components of the intracellular degradation machinery, might prove to be extremely helpful.

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