The Effects of Metallothionein on the Activity of Enzymes Involved in Removal of Reactive Oxygen Species

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To show the effects of metallothionein (MT) on the activity of enzymes involved in the removal of reactive oxygen species, MT has been added to the assay systems of superoxide dismutase (SOD), catalase and peroxidase. We have used assay systems of SOD based on NADPH oxidation and nitrite formation from hydroxylammonium chloride as an assay of superoxide breakdown rate. The two assay systems showed different results at the high concentration of MT. MT showed the scavenging of superoxide in the SOD assay system in the presence and absence of SOD. MT added to the SOD assay system behaved as an activator of SOD, but apo-MT behaved as an inhibitor. When MT was added to the assay system in the presence of a fixed amount of SOD, the breakdown rate of superoxide increased. The effects of MT on the decomposition of hydrogen peroxide and the activity of catalase and peroxidase decomposing hydrogen peroxide were evaluated. MT decreased the activities of catalase and peroxidase. We have concluded that the function of MT as an antioxidant might effect the level of superoxide scavenging and not the level of hydrogen peroxide.

Keywords : Metallothionein, SOD, Catalase, Reactive oxygen.

Introduction

MTs are cystein-rich, low molecular weight, metal-binding proteins found in a wide range of organisms from bacteria to humans.[1] Although MTs are thought to have an important role in the defense against metal toxicity, the biological role of MTs has not been completely elucidated.[2] Recently, it was suggested that MTs may play a direct role in cellular defense against oxidative stress by functioning as antioxidants.[3-5] The expression of yeast or mammalian MT in the presence of copper suppresses a number of the growth defects of yeast strains lacking Cu,Zn-SOD.[5] Reports show that MTs are indeed induced as a compensatory mechanism to detoxify ROS when Sod1 is absent in the livers of Cu,Zn-SOD knockout mice.[6]

In both prokaryotes and eukaryotes, it has been well established that many genes serve to protect cells during oxidative stress, either in the removal of reactive oxygen species or by the repair of damage from oxygen radicals. The one-electron reduction of O_2 yields $O_2^{\cdot-}$, the superoxide anion, a highly reactive and destructive radical. Superoxide anions can be scavenged by superoxide dismutase, an enzyme that catalyzes the conversion of two of these radicals into hydrogen peroxide and molecular oxygen. The hydrogen peroxide formed by superoxide dismutase can be scavenged by catalase, which catalyzes the dismutation of hydrogen peroxide into water and molecular oxygen, and peroxidase, which catalyzes an analogous reaction in which hydrogen peroxide is reduced to water and an alcohol by a reductant. Both enzymes are heme proteins.

SOD, the enzyme that catalyzes the dismutation of superoxide ($O_2^{\cdot-}$) into O_2 and H_2O_2, and catalase, the enzyme that catalyzes the removal of H_2O_2, are considered as natural defense systems against the deleterious effect of reactive oxygen species in cells. In the case of Cu,Zn-SOD, Cu constitutes the active center and is essential for the enzyme activity. On the other hand, Zn maintains the tertiary structure and facilitates the enzyme activity.[7] The reactivity in the transfer of Cu and Zn to their binding sites of SOD was examined *in vitro* by HPLC/atomic absorption spectrophotometry.[8]

In the present study, to evaluate effect of MT on the activity of SOD, metal-free apo-MT and native MT were added to an assay system of SOD. The effects of MT on the decomposition of hydrogen peroxide and the activity of catalase and peroxidase in the decomposition of hydrogen peroxide were determined.

Experimental Section

Materials. Metallothionein (from rabbit muscle), SOD (from bovine erythrocytes), xanthine, xanthine oxidase (from butter milk), peroxidase (from horseradish), catalase (from bovine liver), EDTA, NADPH, sodium carbonate, sodium bicarbonate and 2,2'-azinobis-(2-ethylbenzthiazoline-6-sulfonate) (ABTS), EDTA, sodium phosphate, sodium acetate and dialysis membranes were purchased from Sigma Chemical Co. Tris(hydroxymethyl)aminomethane, hydroxylammonium chloride, sulfanilic acid, α-naphthylamine and potassium cyanate were obtained from Aldrich Co. Nano pure water was used to prepare all the solutions used.

Preparation of apo-MT. The preparation of apo-MT was done by a modified Bernard[9] method. To release the bound metal ions quantitatively from the protein an equal volume of 1 M HCl was added. After 3 min of incubation at room temperature the metal ions were removed by gel filtration on Suprose-12 equilibrated with 0.01 M HCl. All fractions absorbing at 210 nm are pooled and stored as aliquots at -70

°C.

Assay of SOD activity. Superoxide dismutase activity was measured by the inhibition of NADPH oxidation and inhibition of nitrite formation from hydroxylammonium chloride.[10,11]

To measure the inhibition of NADPH oxidation, different concentrations of MT (0.1 mL) were incubated with 0.8 mL of TDB (triethanolamine-diethanolamine (100 mM each)-HCl buffer, pH 7.4), 40 μL of 7.5 mM NADPH, 25 μL of EDTA-$MnCl_2$ (100 mM/50 mM). The mixture was stirred thoroughly and read at 340 nm against air for a stable baseline recorded over a 5-min period. 0.1 mL of mercaptoethanol was added to the mixture and the decrease in absorbance was monitored for about 20 min to allow full expression of chain length leading to NADPH oxidation.

To measure the inhibition of nitrite formation, different concentrations of MT were incubated with 1.49 mL of 65 mM potassium phosphate buffer, 0.1 mL of 1.5 mM xanthine and 0.1 mL of 100 mM hydroxylammonium chloride. The reaction was initiated by the addition of 0.25 mL of xanthine oxidase (30 μg) and kept at 25 °C for 20 min (total volume 2 mL). After incubation time 0.5 mL of sulfanilic acid was added to 0.5 mL of the incubation mixture and incubated at room temperature for 5 min followed by 0.5 mL of a-naphthylamine. The absorbance was recorded at 530 nm.

Measurement of peroxidase activity. The peroxidative activity was measured by using a chromogen, ABTS. ABTS is water-soluble and has a strong absorption at 340 nm with a molar extinction coefficient ε_{340} of 3.66×10^4 M^{-1} cm^{-1}.[12] On oxidation, ABTS forms a stable blue-green product presumed to be the cation radical, $ABTS^{+\cdot}$ is conveniently followed at λ_{max} at 415 nm ($\varepsilon_{415}=3.6 \times 10^4$ $M^{-1}cm^{-1}$).[13] The assay mixture contained 23.5 mM $NaHCO_3/CO_2$ buffer (pH 7.6), 0.1 mM EDTA, 100 μM ABTS and 11.0 units enzyme in a total volume of 0.9 mL. The reaction was initiated by addition of 0.1 mL hydrogen peroxide, and the following increase in absorbance at 415 nm was measured by using a UV/VIS spectrophotometer. The assay solution was prepared by bubbling a 5% CO_2, 95% N_2 gas mixture.[14]

Measurement of catalase activity. The catalase activity of catalase was determined at 20 °C by following the decomposition of H_2O_2 at 240 nm according to the Aebi[15] method. Employing a molar extinction coefficient of 43.6 M^{-1} cm^{-1}, we used the rate for the first 30 s to calculate the units of activity. One unit is equal to the decomposition of 1 μmol H_2O_2/min/mL.

Results and Discussion

Preparation of apo-MT. 2.4 mg of MT was incubated in 0.5 M HCl for 3 min to release metals from MT. The metal-released MT was purified on the Suprose-12 gel filtration column equipped in LC10-AD HPLC system (Shimadzu). The main peak of apo-MT appeared at 28 mL from sample loading. The content of protein in the main peak was determined as 1.2 mg from the absorption at 220 nm and molar absorption coefficient.[9]

Scavenging superoxide by MT. Two assay methods were used to determine the superoxide scavenging. The first assay system was based on NADPH oxidation as an assay of superoxide breakdown rate. The method consisted of a purely chemical reaction sequence that generated superoxide form molecular oxygen in the presence of EDTA, manganese (II) chloride, and mercaptoethanol. The inhibition of NADPH oxidation was monitored at 340 nm. The second assay system was based on nitrite formation from hydroxylammonium chloride. Superoxide was produced by xanthine 0.02 mg/xanthine oxidase 0.01 U. Inhibition of nitrite formation from hydroxylammonium chloride was monitored at 530 nm. Different concentrations of MT were incubated in the superoxide generating system. The results showed dose-dependent inhibition of NADPH oxidation (Figure 1a) and nitrite formation (Figure 1b). MT scavenged the superoxide radical at nearly an 80% rate in higher MT concentrations. This indicated that MT scavenged the superoxide radical effectively without the help of other enzymes.

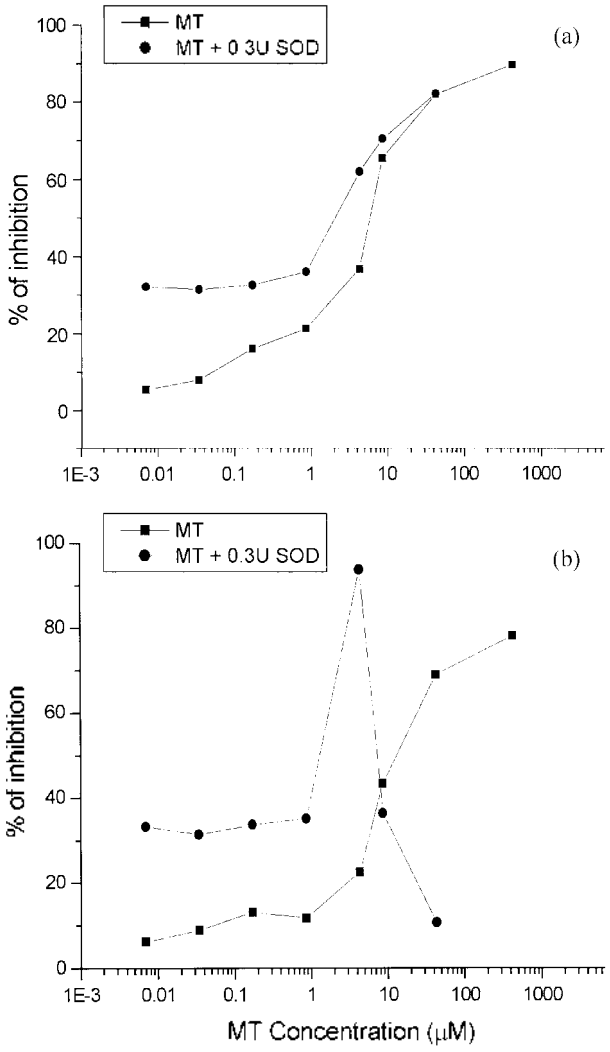

Figure 1. MT inhibits NADPH oxidation (a) and nitrite formation (b) by scavenging superoxide radical *in vitro*. The percentage of inhibition was calculated and expressed as a percentage of inhibition versus mM concentration of MT.

Effects of MT on the activity of SOD. To investigate the effect of MT on the activity of SOD. the amount of SOD was fixed at 0.3 U and the amount of MT was varied from 0 to 429 μM. The extent of inhibition of NADPH oxidation was augmented with the increasing concentration of MT in the reaction mixture (Figure 1a). The scavenging effect increased proportionally with the amount of MT. The extent of inhibition of nitrite formation increased to the 4.29 μM concentration of MT, but beyond that concentration it decreased abruptly (Figure 1b). Since this decrease appeared only in the nitrite formation assay methods. we concluded that some abnormal reaction occurred during of the assay method. The assay system did not show the abrupt decrease in the assay of superoxide removing activity by MT, suggesting that some abnormal reaction might have occurred between SOD and MT. The difference between the two assay methods was that the NADPH oxidation method was based on a purely chemical system, whereas the nitrite formation method used an enzyme system.

Effect of superoxide concentration on the scavenging activity of MT. SOD activity was assayed in the presence of 0.12 μg of MT by the NADPH oxidation method. SOD showed increased activity in the presence of MT (Figure 2). To investigate the effect of apo-MT on the activity of SOD, the same assay condition as above was set except that apo-MT was used instead of MT. Apo-MT did not have the scavenging activity and decreased the activity of SOD. It followed that apo-MT had a reciprocal effect on the activity of SOD compared with MT.

As superoxide production increased. the superoxide dismutation by SOD decreased above the standard assay condition (data not shown). An excess amount of superoxide may oxidize SOD and MT and deprive the scavenging activity of SOD and MT.

Effects of MT on the activity of catalase and peroxidase. MT or divalent metal ion was added to the assay system of catalase and the concentration of hydrogen peroxide was determined. Our results show that MT decreased the breakdown rate of hydrogen peroxide by catalase. MT showed a 10-32% decrease of catalase activity in the investigated range, 0 to 429 μM (Figure 3). Zn^{2+} and Cd^{2+} ions also showed a 50-70% decrease of catalase activity in the same concentration range.

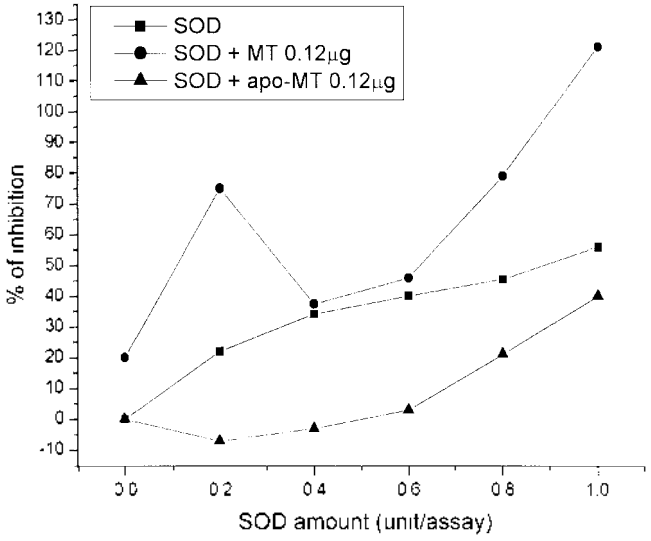

Figure 2. SOD activity was assayed by NADPH oxidation method in the presence of MT or apo-MT. MT or apo-MT amount was fixed as 0.12 μg and SOD concentration was varied from 0 to 1.0 U/assay.

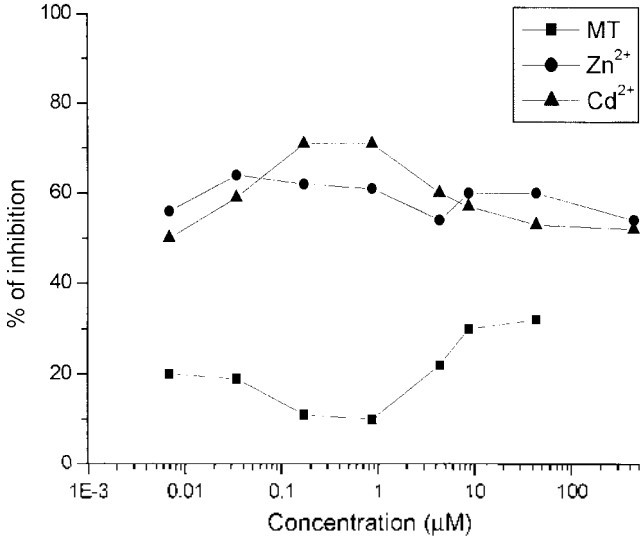

Figure 3. MT inhibits the activity of catalase *in vitro*. Different concentrations of MT (Sigma, from rabbit liver) were incubated with 2.8 mL of substrate solution, and 0.1 mL of MT solution. The reaction was initiated by the addition of 0.1 mL enzyme solution (total volume 3 mL) and measured the time required for A240 to decrease from 0.450 to 0.400. The percentage of activity inhibition was calculated and expressed as a percentage of inhibition verses concentration of MT (μg/assay).

MT or divalent metal ion was added to the assay system of peroxidase and the effect was determined by ABTS oxidation. MT inhibited the activity of peroxidase effectively. It decreased 63-99% of the activity in the range of 0.0069 to 429 μM (Figure 4). Zn^{2+} and Cd^{2+} ions showed a 9-55% decrease of peroxidase activity in the same ion concentration range. MT was a more efficient inhibitor of peroxidase than the metal ions Zn^{2+} and Cd^{2+}.

Metallothionein (MT) is a low molecular weight (6-7 kDa) protein, unique in properties such as high cystein content, the absence of aromatic amino acids, heat stability and inducibility on exposure to metals.[16] The major physiological role of MTs is to detoxify and sequester toxic heavy metals by their ability to bind firmly with sulfhydryl groups.[17,18] Although most of the biological functions ascribed to MTs are related to its metal-binding properties. MT has recently received increasing attention as a result of its ability to act as a free radical scavenger.[19] Early work by Thornalley and Vasak shows that Zn-MT can react very rapidly with hydroxyl radicals (HO·).[3] This has led to the proposal that MT can play both antioxidant and cytoprotective roles against the toxicity induced by HO· and possibly other free radicals.[20]

The method by which MT scavenges the superoxide radi-

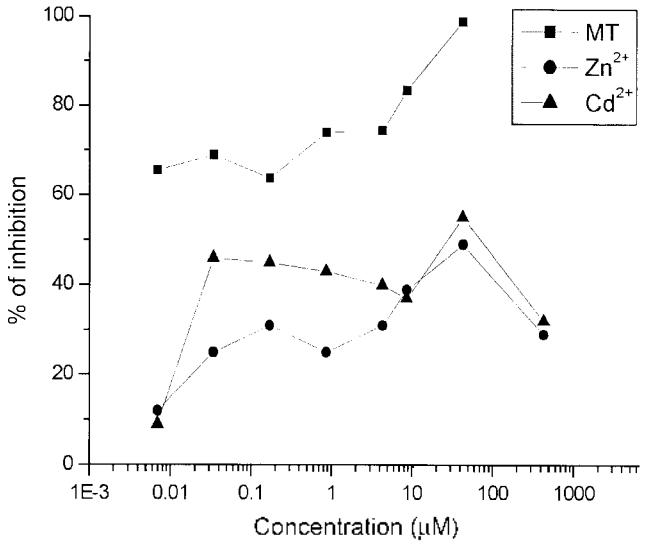

Figure 4. MT inhibits the activity of peroxidase *in vitro*. The peroxidative activity was measured by using a chromogen, 2,2'-azinobis-(2-ethylbenzthiazoline-6-sulfonate) (ABTS). The assay mixture contained 23.5 mM $NaHCO_3/CO_2$ buffer (pH 7.6), 0.1 mM EDTA, 100 M ABTS and 11.0 units enzyme in a total volume of 0.9 mL. The reaction was initiated by addition of 0.1 mL hydrogen peroxide, and the following increase in absorbance at 415 nm was measured by using a UV/VIS spectrophotometer. The percentage of activity inhibition was calculated and expressed as a percentage of inhibition verses concentration of MT (μg/assay).

cal is not clear. The first possibility is MT scavenges the superoxide radical independent of SOD and other enzymes. This possibility is supported by the result that the level of MT mRNA was elevated in the livers of mice with homozygous deletion of the gene for Cu, Zn-SOD, and the induction of MT mRNA occurred primarily at the level of transcription.[3] Previous studies support the observation that the expression of yeast or mammalian MT in the presence of copper suppresses a number of growth defects of yeast strains lacking Cu,Zn SOD.[5] Recently MTs and MT-like proteins isolated from mouse brain showed properties as neuroprotective agents by scavenging superoxide radicals.[21]

The second possibility is MT acted as an activator of SOD or metal ion supplier. We can assume that intact MT actively participates in the catalytic mechanism of SOD. MT may supply the metal to the SOD, which requires divalent metal ions for its activity. We observed the mechanism by which Cu and Zn are supplied to the primary translation product of SOD (apo-SOD) *in vitro* by HPLC/atomic absorption spectrophotometry.[8] Ionic Cu can be transferred to both Cu- and Zn-binding sites of SOD more efficiently than MT-bound Cu, whereas both ionic and MT-bound Zn is transferred only to Zn-binding site at a comparable efficiency. MT in a cell may help the SOD by supplying the metal ions. So the Cu, Zn SOD gene is co-regulated with the MT gene in yeast at the transcriptional level.[22] They propose that this effect is related to the presence in the yeast Cu, Zn SOD promoter of regions similar in nucleotide sequence and positioning to the regions of MT promoter interacting with ACE1.

Our results support both possibilities. Our result that MT shows the superoxide scavenging activity supports the first possibility. But the result that the superoxide radical scavenging activity of SOD increases in the presence of MT and the activity decreases in the presence of apo-MT suggests the idea that MT acts as an activator of SOD and apo-MT acts as an inhibitor. We could not prove that MT or apo-MT really bound to SOD or MT supplied the metal ion to SOD. Further study is needed to address this issue.

The physiological role of the cooperation of SOD and MT in cells exposed to superoxides remains to be elucidated, but the results reported in the present study point to MT having clear scavenging activity and an activator role for SOD as a part of its antioxidant activity.

The contention that MT plays a biological role as an antioxidant against H_2O_2 is still a debatable matter.[23] The few experiments that have proved the role of MT in the protection of cells from damage caused by H_2O_2 have produced conflicting conclusions about the relative importance of MT and GSH as intra cellular protective agents.[24,25]

Most of researches on the antioxidative function of MT were concentrated on the superoxide radical scavenging effects. The reaction of the sulfhydryl groups in MT with H_2O_2 has been examined.[26] When MTs were added to the catalase or peroxidase assay system, they inhibited the activity of the enzymes in proportion to the amount added (Figure 3 and 4). Especially in the case of peroxidase, MT was a more effective inhibitor than metal ion. Zn^{2+} or Cd^{2+}. This result was supported by the chemical studies that MT is a highly effective reactant with superoxide anion, hydroxyl radical, and hypochlorous acid, but is less reactive with hydrogen peroxide.[3,27] Not only MT but metal ions Zn^{2+} and Cd^{2+} served as an inhibitor of catalase and peroxidase. MTs did not affect the degradation of hydrogen peroxide alone. So it can be assumed that the inhibition by MT might be made by the metal ions derived from MT. But we could not show definitely that MT supplies metals to enzymes. Further study is required to verify the inhibition mechanism.

Conclusion

The present study was designed to evaluate the effects of MT on the activities of the ROS removal system. MT shows the scavenging of superoxide in the SOD assay system in the presence or absence of SOD. MT added to the SOD assay system behaved as an activator of SOD, but apo-MT behaved as an inhibitor. When MT is added to the assay system in the presence of a fixed amount of SOD, the breakdown rate of superoxide is increased. The results show that MTs does not affect the degradation of hydrogen peroxide. When MTs were added to the catalase or peroxidase assay system, they inhibited the activity of the enzymes in proportion to the amount added. We conclude that the function of MT as an antioxidant might have an effect on the level of superoxide scavenging and not on the level of hydrogen peroxide removal.

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