

Metallothionein Induces Site-specific Cleavages in tRNA^{Phe}

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It is known that metallothionein (MT) plays a role in the scavenging of free radicals, which is produced under various stress conditions. MT may function as an antioxidant that protects against oxidative damage of DNA, protein, and lipid induced by superoxide anion, hydrogen peroxide, hydroxyl radical, nitric oxide, and peroxynitrite. This study was undertaken to test the hypothesis that MT also protects from RNA damage induced by peroxynitrite, an important reactive nitrogen species that causes a diversity of pathological processes. A cell-free system was used. RNA damage was detected by the mobility of tRNA^{Phe} in electrophoresis. Cleavages on tRNA were not induced by 3-morpholinosydnomine, which produces peroxynitrite directly. MT induced tRNA damage which was site specific.

Key Words : Metallothionein, MT, RNS, Peroxynitrite, tRNA^{Phe}

Introduction

Metallothioneins (MTs) belong to the class of low molecular weight proteins, unique in properties such as high cysteine content, no aromatic amino acids, heat stability and inducibility on exposure to metals, and are found in a wide range of organisms from bacteria to humans. The major physiological roles of MTs are to detoxify and sequester toxic heavy metals by their ability to bind firmly with sulfhydryl (-SH) groups¹ (Figure 1). Although MTs are thought to have an important role in the defense against metal toxicity, the biological role MTs has not been completely elucidated. Recently, it was suggested that MTs may play a direct role in cellular defense against oxidative stress by functioning as antioxidants.

Several studies have demonstrated that MTs are able to quench a wide range of reactive oxygen or nitrogen species (ROS or RNS) including superoxide (O₂^{•-}), hydrogen peroxide (H₂O₂), hydroxyl radical (HO•), and nitric oxide (NO) at a higher efficiency than other well known antioxidants such as GSH, SOD and catalase.² An important RNS is peroxynitrite (ONOO⁻), which has been shown to play a key role in many pathogeneses. The powerful oxidant, ONOO⁻ oxidizes a wide range of biomolecules, including lipids, methionine, sulfhydryls, cytochrome c, and DNA with adverse consequences.³⁻⁷ Recently it was reported that peroxynitrite nitrates tyrosine and tryptophane residues of carbonic anhydrase II.⁸ Many studies have demonstrated that

exposure of cells to 3-morpholinosydnomine (SIN-1) or directly to ONOO⁻ caused apoptotic cell death through an activation of p38 mitogen-activated protein kinase (MAPK).^{9,10} There is also increasing evidence indicating that ONOO⁻ causes mitochondrial structural changes, leading to the release of cytochrome c and thereby activating caspase-3 and causing apoptosis.^{11,12}

It is reported that ONOO⁻-induced DNA damage is protected by MT, but the protection is not directly dose-dependent on MT concentrations.¹³ The present study was undertaken to test the hypothesis that MT directly protects RNA from ONOO⁻ induced oxidative cleavage. The cleavage of tRNA^{Phe} was determined as index of ONOO⁻ induced oxidative damage. Radioactive labeled tRNA was used to show the protection effect and the damage on RNA was analyzed after running the polyacrylamide gel electrophoresis. The result showed that ONOO⁻ did not induce cleavage on tRNA and MT induced site specific cleavages independent on the presence of peroxynitrite. This result may be used to determine the structure of RNA in solution.

Experimental Section

Chemicals and Phosphate Buffer Preparation. MT (from rabbit liver, containing Cd + Zn), tRNA^{Phe} (yeast), SIN-1, RNase T1 and all other chemicals were purchased from Sigma. Phosphate buffer was prepared to contain 50 mM sodium phosphate, 10 mM NaCl, 0.1 mM diethylenetri-

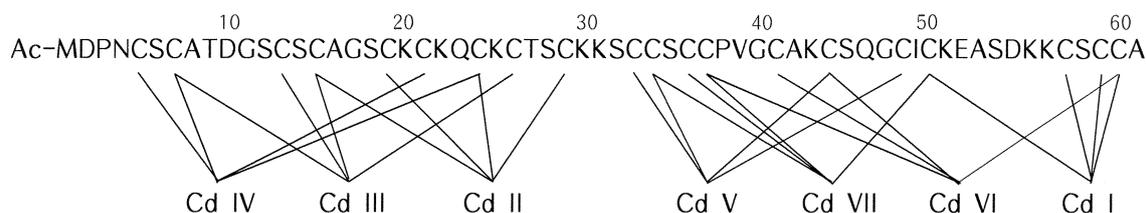


Figure 1. Cadmium-cysteine connectivities of rat liver MT2 as established by two-dimensional ¹H ¹¹³Cd NMR spectroscopy (adapted from Vasask *et al.*³²).

aminepentaacetic acid, pH 7.4.¹⁴

Radioactive End Labelling of tRNA^{Phe}. The 3' terminus of tRNA was labeled with [5'-³²P] pCp using T4 RNA ligase.¹⁵ The labeled RNA was purified on 12% polyacrylamide gel. After electrophoresis, the ³²P-RNA was located by autoradiography of the wet gel. The band was excised from the gel and RNA was eluted with high salt (0.5 M ammonium acetate) buffer.

Limited alkaline hydrolysis and guanine specific RNase T1 reaction were carried according to the method of enzymatic RNA sequencing.¹⁶

Peroxyinitrite Treatment and Analysis of RNA Strand Breaks. tRNA^{Phe} (0.5 μg) was treated with 0.1 mM SIN-1 at 37 °C in phosphate buffer with or without the presence of MT. The solution was immediately mixed after addition of SIN-1 because under these conditions ONOO⁻ spontaneously decays with a half-life of less than 2 s. RNA strand breaks in tRNA^{Phe} were analyzed after polyacrylamide gel electrophoresis. The reaction solution (30 μL) was mixed with 30 μL of electrophoresis loading buffer and loaded onto

a 12% polyacrylamide gel. After electrophoresis, the wet gel was autoradiographed.^{16,17}

Results and Discussion

After radioactively labeled tRNA^{Phe} was incubated for 10 min or 20 min at 37 °C with SIN-1 and MT, tRNA damage was detected by the polyacrylamide gel electrophoresis. When 3'-³²P-labeled tRNA^{Phe} was exposed to SIN-1, the RNA was not made breaks (Figure 2, lane 5 and 9). But MT was added to the labeled tRNA^{Phe}, the tRNA was cleaved at specific sites. This effect of MT was dose-dependent (Figure 2, lanes 6-8). This damage was also time-dependent, it showed more damages with 20 min of exposure to MT at 37 °C than 10 min of exposure (Figure 2, Lane 6 and lane 10). So tRNA cleavages assumed to be the presence of MT. The RNA cleavage did not occur randomly but occurred at specific site of tRNA^{Phe}. Strong cleavages were observed at G15, D16, C25, m²G26, U41, C48, Cm⁵49, and U59, and weak cleavages at U33, Gm34, U47, G57, C60, G65, A66 and A67. The cleavage sites were indicated on the secondary structure of tRNA^{Phe} (Figure 3).

SIN-1 generates O₂•⁻ and NO•, leading to ONOO⁻ generation. One molecular form of protonated ONOO⁻ (ONOOH) is a powerfully oxidizing cytotoxic species. The pK_a of ONOO⁻ is very difficult to determine because of the rapid loss of ONOOH. Addition of ONOO⁻ to cells, tissues or body fluids will lead to its rapid protonation, followed by ONOOH-dependent depletion of -SH groups and other antioxidants, oxidation of lipids,¹⁸ DNA strand breakage, nitration and deamination of DNA bases (especially guanine)¹⁹ and nitration of aromatic amino acid residues in proteins. ONOO⁻ has been shown to be highly responsible for pathogenesis under a diversity of disease conditions.

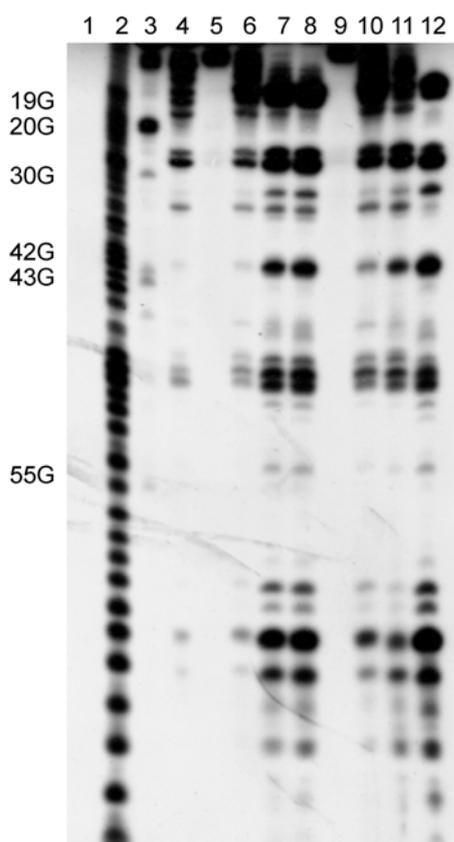


Figure 2. RNA damage induced by MT in the presence or absence of SIN-1. tRNA^{Phe} (0.5 μg/lane) was incubated with SIN-1 in the presence (lane 4-7, 10-12) or absence of MT (lane 9) for 10-20 min at 37 °C. Lane 1 represents tRNA^{Phe} alone. Lane 2 represents tRNA with alkaline hydrolysis. Lane 3 represents tRNA with RNase T1 reaction. Lane 4 represents tRNA incubated for 10 min with 100 μM SIN-1. Lane 5-8 represent tRNA incubated for 10 min with 100 μM SIN-1 and 30, 130, and 200 μM MT respectively. Lane 9 represents tRNA incubated 20 min with 100 μM SIN-1. Lane 10-12 represent tRNA incubated for 20 min with 100 μM SIN-1 and 30, 130, and 200 μM MT respectively.

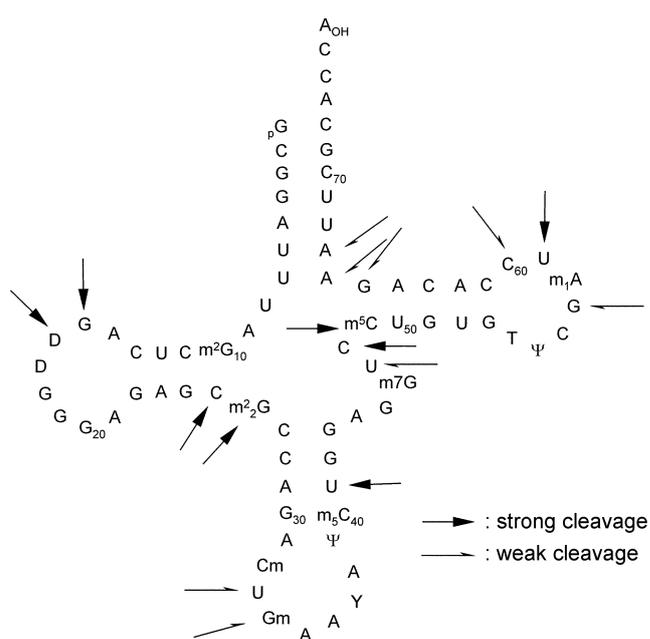


Figure 3. A schematic diagram of the secondary structure of yeast tRNA^{Phe}, summarizing positions where MT causes cleavage.

These include inflammatory and neurodegenerative disease, myocardial dysfunction, and environmental toxicity.^{20,22} The role of ONOO⁻ in pathogenesis has been demonstrated to be associated with its interaction with MAPK signaling pathway. Exposure of cultured cells to SIN-1 or directly to ONOO⁻ caused immediate activation of p38 MAPK and induction of apoptotic cell death with typical nucleosomal DNA fragmentation with activation of caspase 3-like proteases, suggesting the role of ONOO⁻ in activating signaling transduction pathways capable of inducing apoptosis.^{9,10} Peroxynitrite anion causes extensive base modification as well as single breaks in both supercoiled plasmid DNA and mammalian cellular DNA.^{23,24} Oxidation of guanine in DNA is thought to proceed through a transient formation of guanine cation-radicals. The latter are either hydrated to give 8-oxo-guanine or deprotonated to form 2,2-diamino-5(2H)-oxazolone and 2-amono-4H-imidazol-4-one.²⁵ Oxazolone and imidazolone have been reported as the major oxidation products in single-stranded DNA.²⁶ The inhibition of DNA oxidation by peroxynitrite has been investigated. Sensitive to apoptosis gene (SAG) protein, a zinc RING finger protein, significantly inhibits single strand breaks in supercoiled plasmid DNA induced by synthesized peroxynitrite and SIN-1. The formation of 8-hydroxy-2'-deoxyguanosine in calf thymus DNA by peroxynitrite and SIN-1 is also significantly inhibited by SAG.²⁷

It is known that MT plays a role in the scavenging of free radicals, which is produced under various stress conditions. So the reaction of MT with ONOO⁻ is particularly important in preventing oxidative injury. In the report of Cai *et al.*, for the first time, direct evidence that MT can react with ONOO⁻ to protect DNA and lipoprotein from oxidative damage was provided.¹³ Because SIN-1 produces NO and O₂[•] simultaneously in physiological solution at 37 °C and the reaction between NO and O₂[•] leads to the formation of ONOO⁻, DNA damage by SIN-1 is thus assumed to be the effect of ONOO⁻. ONOO⁻ is able to induce DNA damage in a dose-dependent manner, and MT conveyed significant protection from this damage. This suggests that MT is a potent scavenger of the ONOO⁻ radical. This further extends our understanding of the characteristics of MT antioxidant action.

So it was expected that MT also protect the oxidative damage on RNA from ONOO⁻. In our present study, we suggest that SIN-1 had nearly no effect on the cleavage of RNA. The effect of SIN-1 appeared different between DNA and RNA. Our result showed that SIN-1 did not induce oxidative damage on tRNA, but MT induced site specific cleavages independent of SIN-1. It can be explained by the study of the formation of 8-nitroguanosine and 8-oxo-7,8-dihydroguanosine in reactions of calf-liver RNA with various reactive nitrogen species.²⁸ 8-Nitroguanosine in RNA is found to be much stable than 8-nitro-2'-deoxyguanosine in DNA, which rapidly depurinates to release 8-nitroguanine.

It is suggested that MT might have opposite effects to DNA in nuclei, independent of metals bound to MT. At low

concentration, MT can be an excellent hydroxyl radical scavenger due to the SH groups of the various cysteine residues of MT and protect DNA from hydroxyl radical attack in nuclei. On the contrary, MT can induce DNA scission by itself at high concentration.²⁹ The in vitro DNA strand breaking activity of MT containing Cd²⁺ and Zn²⁺ in a ratio of 5 : 2 is described. Studies with radical scavengers and electron paramagnetic resonance spectroscopy indicate that DNA might be caused by a radical species formed by the native MT charged with the heavy metal ions. Further characterization showed that the DNA cleavage is more likely random than sequence- or base-specific.³⁰ There seems to be no base or site specificity in the MT-dependent DNA cleavage. The co-expression of MT and Cu, Zn-superoxide dismutase (SOD) genes has been reported in yeast where MT may act both as a donor of metals to the enzymes and as an antioxidant.³¹

In our study, site specificity was observed in the MT-dependent RNA cleavage. The cleavages were occurred at bases which did not participate in strong base pairing on the secondary structure model of tRNA^{Phe}(Figure 3). The cleavage sites by MT were located within loops or junctions between loop and stem or stem and stem except U41 which was located in the middle of the stem. So the cleavage showed structure specificity, but showed no base specificity. Further study on the conditions of cleavage induced by MT were provided, this specificity would be used as a tool determining the secondary and tertiary structure of RNA.

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