



Interactions of growth inhibitory factor with hydroxyl and superoxide radicals

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Abstract

To show the effects of growth inhibitory factor (Cu₄Zn₃MT-III) involved in the scavenging of reactive oxygen species (ROS), a pulse radiolytic study was employed using N₂O-saturated Cu₄Zn₃MT-III aqueous solutions. It was demonstrated that the oxidizing OH[•] radical efficiently reacted with Cu₄Zn₃MT-III by forming a thiyl radical RS[•] with a second-order constant of $1.46 \times 10^{11} \text{ mol l}^{-1} \text{ s}^{-1}$, which was determined by competition kinetics against KSCN. The thiyl radical RS[•] reacted rapidly and reversibly with a thiolate in Cu₄Zn₃MT-III to form radical anion RSSR^{•-} with a constant of $1.65 \times 10^9 \text{ mol l}^{-1} \text{ s}^{-1}$ per thiolate, while the constant of the decay of this radical anion was $2.72 \times 10^5 \text{ s}^{-1}$, and the equilibrium constant of the formation for RSSR^{•-} was $6.08 \times 10^3 \text{ mol}^{-1} \text{ l}$. These values were close to those of Cd₅Zn₂MT-II. The SOD activity of Cu₄Zn₃MT-III to quench O₂^{•-} was assayed by the riboflavine-methionine-nitrobluetetrazolium (NBT) method which catalyzed the dismutation of superoxide (O₂^{•-}) at pH 7.8 with an IC₅₀ value of $1.50 \times 10^{-6} \text{ M}$ for Cu₄Zn₃MT-III and $1.62 \times 10^{-6} \text{ M}$ for Cd₅Zn₂MT-II. Additionally, the down-regulation of GIF may be a main factor in the decrease of the scavenging ability for the free OH[•] and O₂^{•-} radicals, which is possibly associated with the pathogenesis of neurodegenerative disease.

Introduction

Free radical, especially OH[•] and O₂^{•-}, are important in radiation-induced cellular damage and in many physiological and pathological reactions. The imbalance of activated oxygen species is collectively known as oxidative stress (Felix *et al.* 1993).

Metallothioneins (MTs) are cysteine-rich, low-molecular weight, metal-binding proteins found in a wide range of organisms from bacteria to humans. It is involved in the metabolism control of essential elements, detoxification of heavy metals, the pharmacology of metallodrugs and alkylating agents, and some other physiological processes. Its content of cysteine residues makes this putative protein an efficient hydroxyl radical (OH[•]) scavenger and its antioxidant properties *in vivo* have been suggested by the obser-

vation that MT protected cellular hydrogen peroxide (H₂O₂) induced DNA cleavage (Chubatsu *et al.* 1993). The radioprotective properties of yeast Cu (I)-MT and rabbit Cd, Zn-MT *in vitro* were investigated in more detail (Felix *et al.* 1993; Deters *et al.* 1994; Thornalley *et al.* 1985).

As a special member of the metallothionein family, GIF was found to inhibit the outgrowth of cortex neurons in the medium after addition of an extract from an AD patient brain (Uchida *et al.* 1991; Sewell *et al.* 1995). It is abundant in normal human central nervous system but is reduced in patient's brain of neurodegenerative diseases such as Alzheimer's disease, parkinsonism, multiple system atrophy, and progressive supranuclear palsy and amyotrophic lateral sclerosis (Hidalgo *et al.* 2001). The sharp downregulation of GIF in the brain of AD patients and the recovery

from increased neurotrophic activity by adding GIF to AD brain extract have suggested that GIF participates in the pathogenesis of AD (Sewell *et al.* 1995). On the other hand, the accumulation of β -amyloid ($A\beta$) is considered to originate from free radicals by forming senile plaques in AD pathogenesis, that could destroy proteins embedded in the membrane and thereby interrupt transport functions in neurons (Behl *et al.* 1994; Hensley *et al.* 1994.). Based on the above reports, we were intrigued by the possibility that GIF may protect neurons from oxygen or $A\beta$ toxicity through its scavenging activity of free radicals in normal aged brain. Considering the insufficient studies dealing directly with the effects of GIF on OH^\cdot and $O_2^{\cdot-}$ radicals in vitro which have been described (Ren *et al.* 2001), in this paper, the kinetic studies on the elimination of active oxygen free radicals for GIF were carried out. Thus the pulse radiolysis techniques on the N_2O -saturated aqueous GIF solution and NBT assay method were used to test the GIF scavenging ability of the free OH^\cdot and $O_2^{\cdot-}$ radicals, respectively.

Materials and methods

Materials

Porcine brain GIF (Cu_4Zn_3MT -III) and rabbit liver Cd_5Zn_2MT -II were isolated and purified according to the literature methods (Shi *et al.* in press; Comeau *et al.* 1992). The purity of each preparation was determined by amino acid analysis and HOBIN YVON HY38S inductively coupled plasma atom emission spectrometry (ICP). The protein concentration were determined spectrophotometrically by measuring the absorbance of the apoMT-II at 220 nm in 0.01 M HCl solution ($\epsilon_{220} = 47300 \text{ mol}^{-1} \text{ cm}^{-1}$) for Cd_5Zn_2MT -II (Buhler *et al.* 1979.) and the absorbance at 255 nm ($\epsilon_{255} = 48000 \text{ mol}^{-1} \text{ cm}^{-1}$) for Cu_4Zn_3MT -III (Bogumil *et al.* 1996). Sephadex G-75, G-50, G-25; DEAE-52 (Pharmacia), Nitrobluetetrazolium chloride (Fluka Chemie A.G. Switzerland), methionine from E. Merck A.G. (Germany), Tris (Sigma), KSCN and other reagents were of A.R. grade. All aqueous solutions were prepared with triple distilled water and saturated with high purity nitrous oxide (N_2O). All procedures were carried out at room temperature.

Pulse radiolysis

Pulse radiolysis experiments were conducted by using a linear accelerator providing 8-MeV electron pulse with a duration a 8 ns in the Laboratory of Radiation Chemistry, Shanghai Institute of Nuclear Research, Academia Sinica. The dosimetry of the electron pulse was determined by a thiocyanate dosimeter containing 0.01 M KSCN solution saturated with nitrous oxide and by taking $\epsilon_{(SCN_2)^{\cdot-}} = 7600 \text{ mol}^{-1} \text{ cm}^{-1}$ at 480 nm. Detailed descriptions of the set-up of pulse radiolysis equipment and the experimental conditions have been described elsewhere (Hou *et al.* 1993). The dose per electron pulse was 10 Gy.

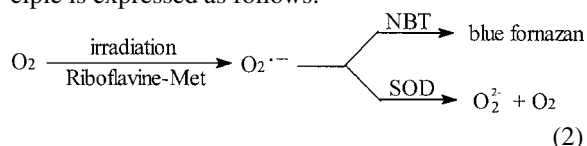
The rate of the reaction of Cu_4Zn_3MT -III with hydroxyl radical (OH^\cdot) was determined by competition kinetic studies employing KSCN as the standard solute and by monitoring the absorbance of $(SCN)_2^{\cdot-}$ species at 520 nm for various molar ratios of Cu_4Zn_3MT -III/ SCN^- . The rate constants were calculated utilizing the following equation modified from the literatures (Felix *et al.* 1993; Adams *et al.* 1965):

$$\frac{A_0}{A} - 1 = \frac{k_{MT/OH^\cdot}}{k_{SCN^-/OH^\cdot}} \cdot \frac{[MT]}{[SCN^-]} \quad (1)$$

Where A_0 and A represent the initial absorbance of $(SCN)_2^{\cdot-}$ generated by an electron pulse of 10 Gy absorbed energy in an aqueous KSCN and N_2O -saturated solution ($C_{KSCN} = 5 \times 10^{-5} \text{ mol l}^{-1}$) in the absence and presence of metallothionein. Numeric values of k_{MT/OH^\cdot} were obtained from linear regression analysis of $\frac{A_0}{A} - 1$ versus $\frac{[MT]}{[SCN^-]}$, where the slope represented $\frac{k_{MT/OH^\cdot}}{k_{SCN^-/OH^\cdot}}$, and the k_{MT/OH^\cdot} and k_{SCN^-/OH^\cdot} are the apparent rate constant for the reaction of hydroxyl radicals with MT and KSCN, respectively. $[MT]$ and $[SCN^-]$ represent the respective concentrations of MT and KSCN in the reaction mixture.

SOD activity assay

The SOD activity of Cu_4Zn_3MT -III was determined by using a riboflavine-methionine-nitrobluetetrazolium (NBT) method (Beauchamp *et al.* 1971). Riboflavine was analyzed by the method in the literature. The principle is expressed as follows:



The reduction of nitrobluetetrazolium (NBT) was followed at 560 nm in a series of solutions containing different concentration of $\text{Cu}_4\text{Zn}_3\text{MT-III}$, $3.3 \times 10^{-6}\text{M}$ riboflavin, 0.01 M methionine, and $4.6 \times 10^{-5}\text{M}$ NBT and 0.05 M phosphate buffer at pH 7.8 on a Shimadzu UV-3100 spectrophotometer. All photoinduced reactions were performed at 30 °C. For the calculation of the catalyzing activity of MT in the NBT assay, the following expression was used:

$$I = \frac{k_0 - k_c}{k_0} \times 100\%. \quad (3)$$

k_0 and k_c are the reaction rate of absence and presence of MT in the system, which deduced from the slope of the absorbance vs. time recorded at 560 nm. I is the inhibitory percent of MT catalyzing activity in the NBT assay. The chromophore concentration required to yield 50% inhibition of the reduction of NBT (IC_{50}) was determined by following the literature method (Weser *et al.* 1981).

The same experiment of native $\text{Cd}_5\text{Zn}_2\text{MT-II}$ with OH^\cdot and $\text{O}_2^{\cdot-}$ were also measured and compared with those of $\text{Cu}_4\text{Zn}_3\text{MT-III}$.

Results and discussion

In order to investigate the reactivity of GIF towards both hydroxyl and superoxide radicals, radiolytic studies and assay of SOD activity were undertaken.

In this experiment, OH^\cdot generated in the radiolysis of N_2O -saturated aqueous $\text{Cu}_4\text{Zn}_3\text{MT-III}$ solution reacted predominantly with the thiolate moieties RS^- of the protein by electron transfer to form thiyl radicals RS^\cdot (Asmus *et al.* 1983):



The second-order rate constant for the reaction of OH^\cdot with $\text{Cu}_4\text{Zn}_3\text{MT-III}$ was determined by kinetic competition studies using KSCN as the standard solute ($k_{\text{SCN}^-/\text{OH}^\cdot} = 1.1 \times 10^{10} \text{ mol}^{-1} \text{ l s}^{-1}$) (Ellison *et al.* 1972).

Figure 1a is the transient absorption spectra obtained from the pulse radiolysis of N_2O -saturated $\text{Cu}_4\text{Zn}_3\text{MT-III}$ aqueous solution ($2 \times 10^5 \text{ mol}^{-1} \text{ l}$) at room temperature of $25 \pm 1^\circ\text{C}$ and pH 7.0. It can be found that there were two dominant band with maximum near 450 nm and 330 nm, the former was assigned to the characteristic feature absorbance of the anion radicals $\text{RSSR}^{\cdot-}$, formed by the combination of RS^\cdot radical and RS^- anion in metallothionein

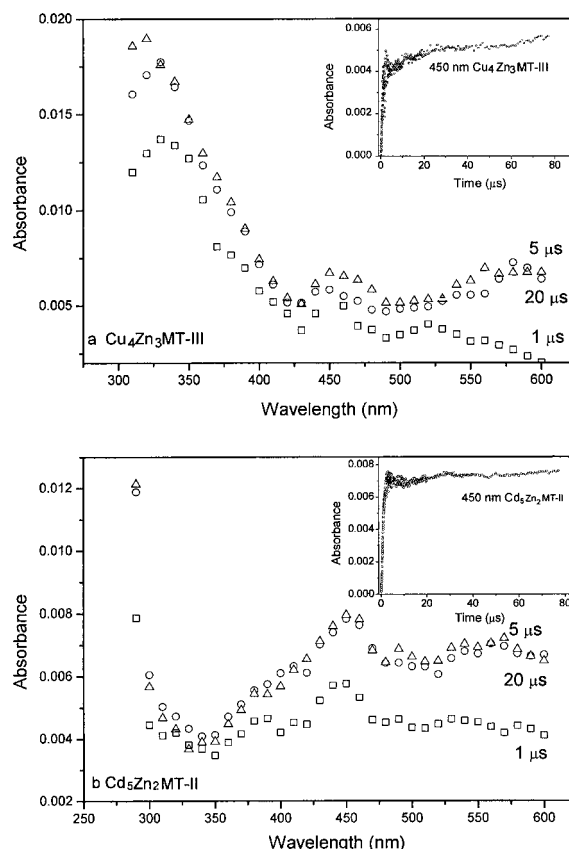
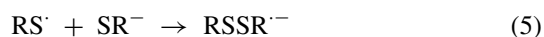


Fig. 1. Transient absorption spectra from pulsed radiolysis of N_2O -saturated $\text{Cu}_4\text{Zn}_3\text{MT-III}$ aqueous solution ($20 \mu\text{M}$) (a) and $\text{Cd}_5\text{Zn}_2\text{MT-II}$ aqueous solution ($20 \mu\text{M}$) (b) at room temperature at 1 μs , 5 μs and 20 μs after the pulse. Inset: growth trace of transient absorbance at 450 nm.



This reaction was reversible even at room temperature (Swallow *et al.* 1978; Sonntag *et al.* 1987).

In the competition kinetics study employing KSCN as the standard solute, the reaction of OH^\cdot with SCN^- produced anion radicals $(\text{SCN})_2^{\cdot-}$, with the absorption maximum at 500 nm (Adams *et al.* 1965). Considering the possible overlap from the absorbance of $\text{RSSR}^{\cdot-}$ with a maximum absorption at 450 nm ($\epsilon_{450} = 9 \times 10^3 \text{ mol}^{-1} \text{ l cm}^{-1}$), we monitored the competition kinetics at 520 nm, but not 500 nm at which recorded in the literatures. According to Equation (1), the linear regression analysis of $(A_0/A)-1$ versus $([\text{MT}]/[\text{SCN}^-])$ from the competition system of MT with SCN^- was plotted in Figure 2, where A was the absorbance of different concentration of MT in presence of KSCN. It was revealed that the rate constant for the reaction of $\text{Cu}_4\text{Zn}_3\text{MT-III}$ with OH^\cdot

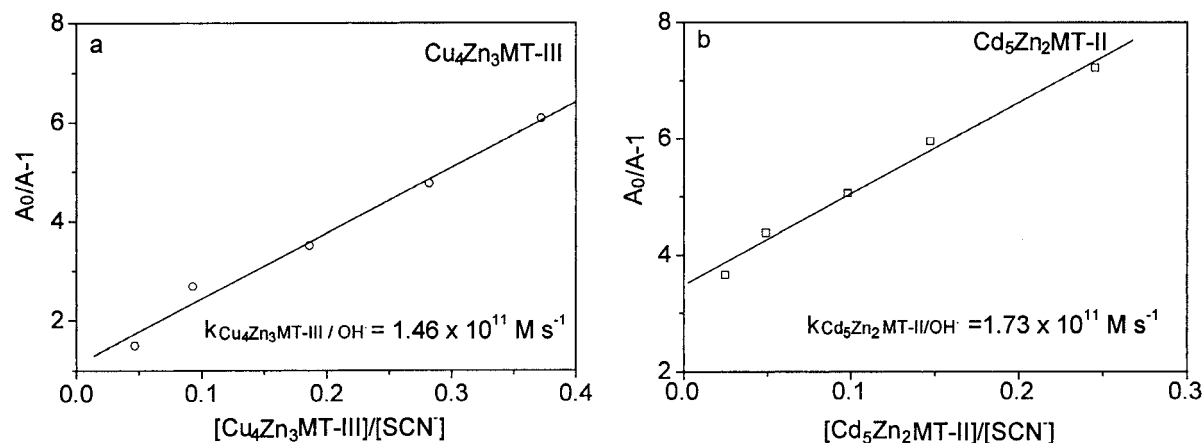


Fig. 2. Competition kinetics of porcine brain $\text{Cu}_4\text{Zn}_3\text{MT-III}/\text{OH}^\cdot - \text{SCN}^-/\text{OH}^\cdot$ (a) and rabbit liver $\text{Cd}_5\text{Zn}_2\text{MT-II}/\text{OH}^\cdot - \text{SCN}^-/\text{OH}^\cdot$ (b). The measurements were performed in N_2O -saturated KSCN solution ($50 \mu\text{M}$) with $0\text{--}20 \mu\text{M}$ of MT monitored at 520 nm . Linear regression plot. Competition kinetics values for the reaction of MT with hydroxyl radicals were calculated according to Equation (1).

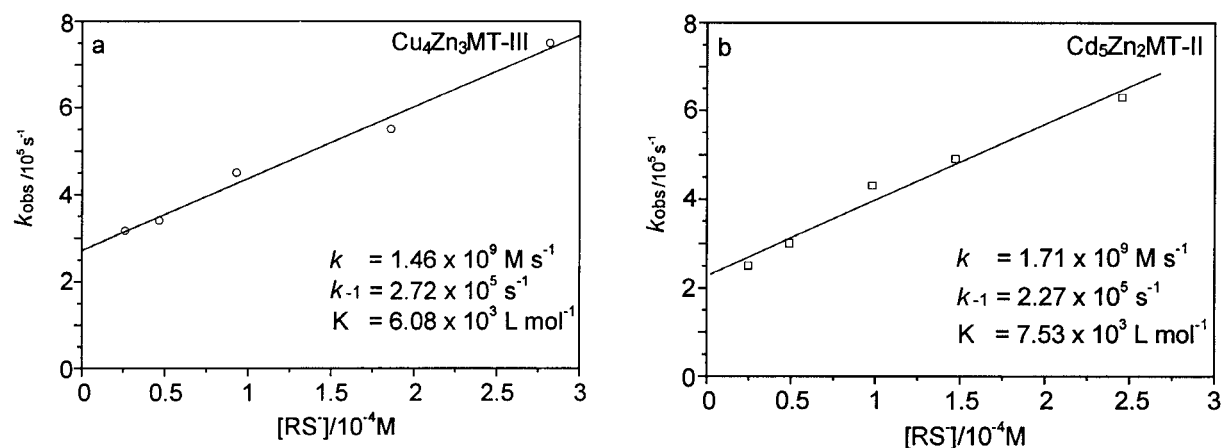


Fig. 3. Rate constants of the absorbance buildup at 450 nm as a function of thiolate concentration ($[\text{RS}^-] = 20[\text{MT}]$) from the pulse radiolysis of N_2O -saturated aqueous $\text{Cu}_4\text{Zn}_3\text{MT-III}$ (a) and $\text{Cd}_5\text{Zn}_2\text{MT-II}$ (b) solutions.

Table 1. Rate constants and equilibrium constants for reactions of hydroxyl radical with $\text{Cu}_4\text{Zn}_3\text{MT-III}$ and $\text{Cd}_5\text{Zn}_2\text{MT-II}$.

Reaction	Rate constant	$\text{Cu}_4\text{Zn}_3\text{MT-III}$	$\text{Cd}_5\text{Zn}_2\text{MT-II}$
$\text{OH}^\cdot + \text{RS}^- \rightarrow \text{RS}^\cdot + \text{OH}^-$	$K_{\text{MT}/\text{OH}^\cdot} (\text{mol l}^{-1} \text{ s}^{-1})$	1.46×10^{11}	1.73×10^{11}
$\text{RS}^\cdot + \text{RS}^- \rightarrow \text{RSSR}^{\cdot-}$	$k (\text{mol}^{-1} \text{ l s}^{-1})$	1.65×10^9 ^a	1.71×10^9 ^a
$\text{RSSR}^{\cdot-} \rightarrow \text{RS}^\cdot + \text{RS}^-$	$k_{-1} (\text{s}^{-1})$	2.72×10^5 ^a	2.27×10^5 ^a
$\text{RS}^\cdot + \text{RS}^- \rightleftharpoons \text{RSSR}^{\cdot-}$	$K (\text{l mol}^{-1})$	6.08×10^3 ^a	7.53×10^3 ^a

^a Average per thiolate function in MT molecule $[\text{RS}^-] = 20[\text{MT}]$.

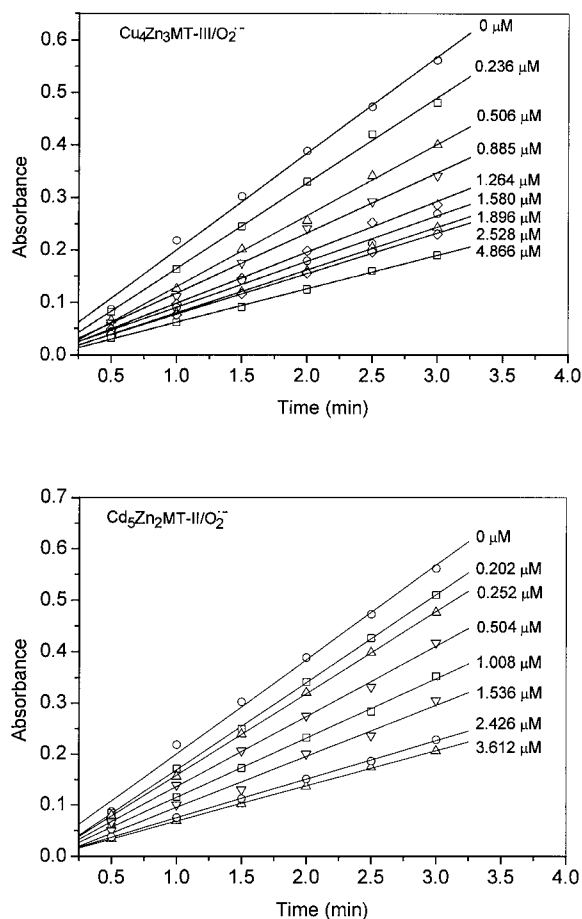


Fig. 4. The absorbance versus time recorded at 560 nm in the system of porcine brain $\text{Cu}_4\text{Zn}_3\text{MT-III}$ (a) and rabbit liver $\text{Cd}_5\text{Zn}_2\text{MT-II}$ (b) inhibiting the reduction of NBT at 30 °C.

was $1.46 \times 10^{11} \text{ mol}^{-1} \text{ l s}^{-1}$, which was just close to that of the reaction between $\text{Cd}_5\text{Zn}_2\text{MT-II}$ with OH^\cdot obtained in the experiment ($1.73 \times 10^{11} \text{ mol}^{-1} \text{ l s}^{-1}$), and a little lower to those of the reaction of Cu (I)-MT with OH^\cdot ($2.2 \times 10^{11} \text{ mol}^{-1} \text{ l s}^{-1}$) (Felix *et al.* 1993). These values of $k_{\text{MT}/\text{OH}^\cdot}$ obtained in this experiment were smaller comparing with the value reported for the reaction of Cd, Zn-MT with OH^\cdot in the competition by using ferrocyanide ($1.2 \times 10^{12} \text{ mol}^{-1} \text{ l s}^{-1}$) as standard solute and measuring the absorbance of the reaction system at 420 nm (ϵ_{420} of ferrocyanide was $1027 \text{ mol}^{-1} \text{ l cm}^{-1}$) (Schuler *et al.* 1981), but not deducting the contribution of the absorbance from $\text{RSSR}^{\cdot-}$ ($\epsilon_{450} = 9000 \text{ mol}^{-1} \text{ l s}^{-1}$) (Fang *et al.* 1995).

A pulse radiolysis of N_2O -saturated $\text{Cu}_4\text{Zn}_3\text{MT-III}$ aqueous solutions without KSCN was also employed to study the kinetics of OH^\cdot with $\text{Cu}_4\text{Zn}_3\text{MT-III}$. It is

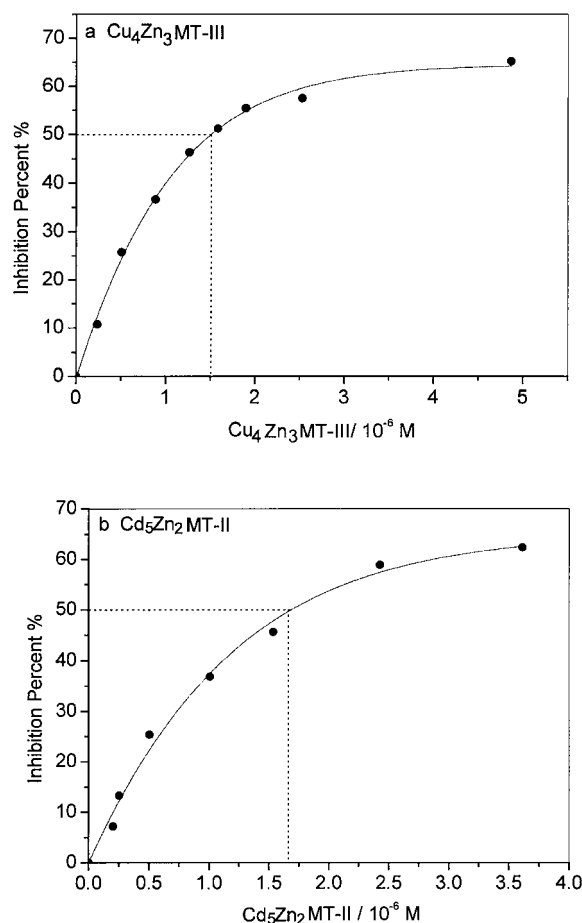


Fig. 5. SOD activity of $\text{Cu}_4\text{Zn}_3\text{MT-III}$ (a) and $\text{Cd}_5\text{Zn}_2\text{MT-II}$ (b) in the riboflavine-methionine-NBT assay ($\text{IC}_{50} = 1.50 \times 10^{-6} \text{ mol l}^{-1}$ for $\text{Cu}_4\text{Zn}_3\text{MT-III}$, $\text{IC}_{50} = 1.61 \times 10^{-6} \text{ mol l}^{-1}$ for $\text{Cd}_5\text{Zn}_2\text{MT-II}$).

well known that thiyl radicals formed from reaction of OH^\cdot with thiolate groups of MT can rapidly react with other thiolate groups and yield sulfur-centered radical species including perthiyl radicals (RSS^\cdot), radical anions ($\text{RSSR}^{\cdot-}$), radical cations ($\text{R}_2\text{S}^{\cdot+}$), $(\text{R}_2\text{S})_2^+$ and $(\text{RSSR}^{\cdot+})$ (Tamba *et al.* 1986), the radical anions $\text{RSSR}^{\cdot-}$ may be the dominant species with a maximum absorption near 450 nm, and the reaction of RS^\cdot and $\text{RS}^{\cdot-}$ was reversible (Equation 6).



The absorbance build-up at 450 nm followed first-order kinetics and the Kobs was strongly dependent on the concentration of thiolates (Figure 3):

$$k_{\text{obs}} = k[\text{RS}^-] + k_{-1} \quad (7)$$

Where the slope in Figure 3 was assigned to the forward rate constant k and the intercept to the reversed rate constant k_{-1} for there was a large excess of RS^- compared with RS^\cdot and the consume of $\text{RSSR}^{\cdot-}$ was relatively slow under the reaction conditions (Fang *et al.* 1995). The equilibrium constant for the $\text{RSSR}^{\cdot-}$ formation reaction was $K = k/k_{-1} = 6.1 \times 10^3 \text{ mol}^{-1} \text{ l}$ for the reaction of $\text{Cu}_4\text{Zn}_3\text{MT-III}$ with OH^\cdot , which was similar to that of the reaction of $\text{Cd}_5\text{Zn}_2\text{MT-II}$ with OH^\cdot , $7.5 \times 10^3 \text{ mol}^{-1} \text{ l}$ obtained in this experiment. The rate constants and the calculated equilibrium constants of the $\text{RSSR}^{\cdot-}$ formation reaction for $\text{Cu}_4\text{Zn}_3\text{MT-III}$ were close to those of $\text{Cd}_5\text{Zn}_2\text{MT-II}$ (Hoffman *et al.* 1973) (Table 1). The pulse radiolytic investigations revealed that $\text{Cu}_4\text{Zn}_3\text{MT-III}$ as well as native $\text{Cd}_5\text{Zn}_2\text{MT-II}$, was an excellent scavenger for the oxygen species radicals.

Superoxide dismutase activities

The quenching of superoxide radicals ($\text{O}_2^{\cdot-}$) by $\text{Cu}_4\text{Zn}_3\text{MT-III}$ appears to be similar when compared with that of $\text{Cd}_5\text{Zn}_2\text{MT-II}$. In present work, SOD activity of $\text{Cu}_4\text{Zn}_3\text{MT-III}$ and $\text{Cd}_5\text{Zn}_2\text{MT-II}$ to quench $\text{O}_2^{\cdot-}$ radicals was quantitated in terms of its ability to inhibit the formation of the blue formazan by indirectly using the riboflavine-methionine-nitrobluetetrazolium (NBT) assay (Beauchamp *et al.* 1971). The relationship between the inhibition percent (%) and initial concentration of MT is shown in Figure 4. Illumination of the reaction mixture containing riboflavine, methionine and NBT caused an increase in absorbance at 560 nm of 0.1839/min in aerobic solutions. When $\text{Cu}_4\text{Zn}_3\text{MT-III}$ was added to this system, the increasing of absorbance per minute was reduced with a concentration-dependent manner (Figure 5). The IC_{50} which represented the concentration of MT causing 50% inhibition of the rate of reduction of NBT exerted the SOD activity equivalent one unit of native SOD, and was $1.50 \times 10^{-6} \text{ mol l}^{-1}$ for porcine brain $\text{Cu}_4\text{Zn}_3\text{MT-III}$, which was similar to that of rabbit liver $\text{Cd}_5\text{Zn}_2\text{MT-II}$ ($1.61 \times 10^{-6} \text{ mol l}^{-1}$) but was about 400 times that of Cu, Zn-SOD ($4.0 \times 10^{-8} \text{ mol l}^{-1}$) (Buxton *et al.* 1988). These results indicated that the SOD activity of MT was weaker than that of native Cu, Zn-SOD.

Conclusion

Results obtained in our study demonstrate that GIF ($\text{Cu}_4\text{Zn}_3\text{MT-III}$) can scavenge OH^\cdot radicals formed during radiolysis and $\text{O}_2^{\cdot-}$ radicals in a BNT radical producing system. It suggests that GIF could act as an efficient scavenger against free radicals in vivo. In addition, the down-regulation of GIF in Alzheimer's disease (AD) patient's brain may be a main factor for the decrease of its scavenging ability of the free OH^\cdot and $\text{O}_2^{\cdot-}$ radicals, which is possibly related to the pathological process of Alzheimer's disease.

Acknowledgements

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