



## Kinetic studies of the reactions of some metal reconstituted metallothioneins with the electrophilic disulfide 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)

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### Abstract

Rabbit liver Pt<sub>7</sub>MT, Zn<sub>7</sub>MT, Bi<sub>7</sub>MT were reconstituted and the kinetic studies of the reactions with electrophilic disulphide 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were investigated to explore the possible mechanism of metals release from metallothioneins. It is revealed that the Pt<sub>7</sub>MT, Zn<sub>7</sub>MT react with DTNB biphasically, yielding a four-term rate law:  $\text{Rate} = k_{1f} [\text{MT}] + k_{2f} [\text{DTNB}][\text{MT}] + k_{1s} [\text{MT}] + k_{2s} [\text{DTNB}][\text{MT}]$ . Zn<sub>7</sub>MT reacts with disulphide DTNB more rapidly and has significantly greater observed rate constants  $k_s$ ,  $k_f$  than Pt<sub>7</sub>MT. The kinetic data for Bi<sub>7</sub>MT indicate that the reaction is monophasic and the rate law is proved to be:  $\text{Rate} = k_1 [\text{MT}] + k_2 [\text{DTNB}][\text{MT}]$ . The observed pseudo-first order rate constants for above MTs are insensitive to pH value. Based on the available experimental data, the different kinetic behaviors of MTs reactions with electrophilic disulphide DTNB and a possible mechanism to release the coordinated metal ions are discussed.

### Introduction

Metallothionein (MT) is a low molecular weight cytosolic protein with unusually high cysteine residues (20 cys in mammalian proteins), all of which are in reduced forms, conferring the ability to bind seven divalent metal ions, such as Cd<sup>2+</sup> and/or Zn<sup>2+</sup> per mole protein (Margoshes *et al.* 1957; Kagi *et al.* 1979; Vasak *et al.* 1984; Nordberg *et al.* 1987). It has been well established that MT may play an important role in heavy metals detoxification, essential metals metabolism, radical scavenging and the pharmacology of metal drugs and alkylating agents (Endresen *et al.* 1983; Karin *et al.* 1985; Thornalley *et al.* 1985). These unique behaviors are found to be based on the character of its metal-thiolates structure. Mammalian MTs contain two metal clusters located in the distinct domains: M<sub>4</sub>S<sub>11</sub> and M<sub>3</sub>S<sub>9</sub> (M = Cd(II) or Zn(II), S = cysteinyl thiolate) in the  $\alpha$  (C terminal) and  $\beta$  (N terminal) domains, respectively. Each metal ion is tetrahedrally coordinated by terminal and bridging

thiolates (Otvos *et al.* 1980; Arseniev *et al.* 1988; Robbins *et al.* 1991).

The cysteine residues, in addition to chelating various heavy metal ions, provide a source of nucleophilic sulfhydryl groups (Li *et al.* 1981; Bernhard *et al.* 1986; Otvos *et al.* 1990; Maret *et al.* 1994; Munoz *et al.* 1999). They have been highly conserved through the long history of mammalian evolution. The conservation of the MT cysteines suggests their functional importance exceeds the more ability to coordinate promiscuously to heavy metal ions. Thus, exploring the kinetics of reactions between MT and various ligands and electrophiles is an essential approach to understanding the putative functions of MT (Otvos *et al.* 1990; Savas *et al.* 1991; Zhu *et al.* 1995). The well-studied reactions of mammalian Cd-MT with the electrophile, 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB or ESSE) suggested that DTNB attacked the metal-thiolates and showed the biphasic kinetics, which corresponded to the reactions of  $\alpha$  and  $\beta$  clusters, respectively (Savas *et al.* 1991; Munoz *et al.* 1999).

However, the reactions of other MT with DTNB, such as Pt<sub>7</sub> MT, Bi<sub>7</sub> MT, and Zn<sub>7</sub> MT were not or only little studied previously. Available evidence strongly suggests that MT is one of the major binding sites for metabolites of *cis*-diamminedichloroplatinium(II) and other platinum anti-cancer drugs (Sharma *et al.* 1983; Zelazowski *et al.* 1984; Zhang *et al.* 1995; Zhong *et al.* 1997). In addition, the administration of bismuth complexes can induce the synthesis of MT in kidney but not in tumor tissue. This reduces the renal toxicity of CDDP without compromising its chemotherapeutic activity (Naganuma *et al.* 1987; Satoh *et al.* 1997). In the present work, we investigated the detailed reactions of DTNB with Pt<sub>7</sub>MT, Bi<sub>7</sub>MT and Zn<sub>7</sub>MT under different pH conditions and concentrations of DTNB. Based on the experimental data, the different kinetic behaviors of Pt<sub>7</sub>MT, Bi<sub>7</sub>MT, and Zn<sub>7</sub>MT toward DTNB and possible mechanism to release the coordinated metal ions were discussed.

## Experimental section

### Materials

Sephadex G-50, G-25, and DEAE Cellulose DE-52 were purchased from Pharmacia. Ultrafilter and YM-1 membrane (cut off 1000 Da.) from Amicon. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB or ESSE) was from Sigma. All the chemicals were reagent grade or better. Deionized water was used in the whole experiment.

### Preparation and characterization of MTs

Rabbit liver Cd<sub>5</sub>Zn<sub>2</sub>MT was isolated and purified as the reference reported (Comeau *et al.* 1992). The purified MT was characterized by HPLC and amino acid analysis (Otvos *et al.* 1985). Apo-MT was prepared from native MT by passing the protein down a Sephadex G-25 gel column (1.6 × 40 cm), which had previously been equilibrated with 0.01 M HCl (Buhler *et al.* 1979). All the solutions were degassed on a vacuum line and saturated with nitrogen gas. Reconstitution of Zn<sub>7</sub>MT was carried out by addition seven folds molar of zinc equivalents to apoMT. The pH value was then adjusted to 7.4 by using 0.5 M tris base solution. The reaction mixture was left standing for 24 h at 25 °C. Thus Zn<sub>7</sub>MT was reconstituted. Pt<sub>7</sub>MT and Bi<sub>7</sub>MT were prepared by the similar procedures. The excessive metals and salts were removed in ul-

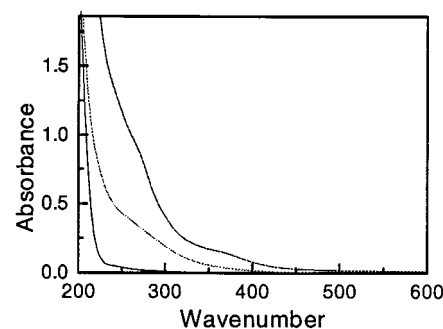


Figure 1. UV-visible spectra of metallothioneins (from top to bottom, Bi<sub>7</sub>MT, Pt<sub>7</sub>MT and apoMT). The broad band centered at 260 nm, 350 nm for Bi<sub>7</sub>MT indicated the formation of Bi-S bonds. The absorbance at 280 nm for Pt<sub>7</sub>MT was due to Pt-S charge transfer band.

trafiltration with a YM-1 membrane. All the solutions were stored below -20 °C (Vasak 1991).

Metal and sulfur concentrations of MT solutions were determined by inductively coupled plasma atomic spectrometer (ICP) performed on JOBIN JV 38S ICP spectrometer (Bongers *et al.* 1988). All the results showed that the constitution of the MT was in accordance with the equation of M<sub>7</sub>MT (M = Zn, Bi and Pt). UV spectra of Pt<sub>7</sub>MT (Figure 1) showed a broad shoulder at 280 nm due to a ligand to metal charge transfer (LMCT). Two weak, broad bands at 260 nm and 350 nm in Bi<sub>7</sub>MT due to ligand to metal charge transfer were also present. These results were consistent with the reports by Sadler (1999), Kagi (1983) and Bongers (1988).

### Kinetic study

The solutions of metallothioneins and DTNB were mixed at time zero in UV cuvettes. The absorbance change at 412 nm ( $\lambda_{obs}$  of 5-thio-2-nitrobenzoate, the reaction product) was measured over time against an equivalent amount of DTNB as reference in Shimadzu UV-3100 spectrometer (Zhu *et al.* 1995). Temperature was maintained at 25 °C. The reaction was set up under pseudo-first order conditions ( $[DTNB] \gg [MT \text{ thiol}]$ ) in 0.01 M Tris-HCl buffer with 0.1 M KCl at pH 7.4. DTNB concentrations ranged from 0.4 to 2 mM. Metallothionein concentration was measured as metal and kept constant at 6.0  $\mu$ M. Pseudo-first-order plots were obtained by plotting  $\ln(A_{\infty} - A_t)$  vs time. Reactions were carried out at different pHs, 6.0, 7.4 and 8.1, keeping all other conditions the same.

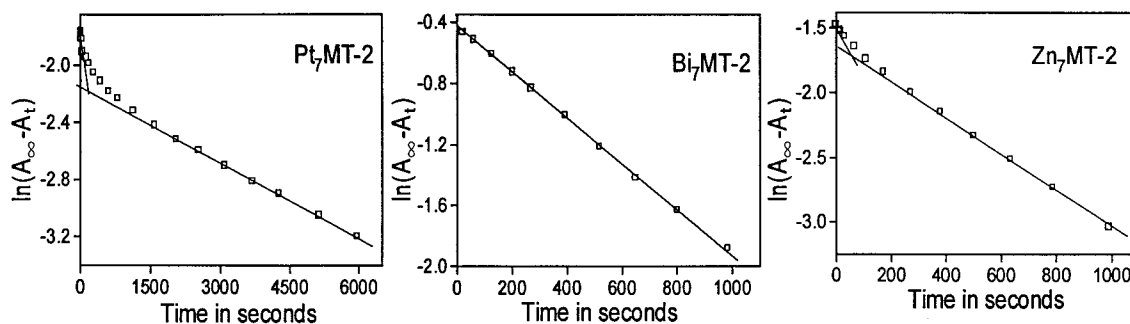


Figure 2. Kinetic reaction of Metallothioneins with DTNB, Conditions: [DTNB] = 1.1 mmol/L, [MT] = 6.0  $\mu$ mol/L, in 0.01 mmol/L Tris-HCl buffer with 0.1 mol/L KCl, pH 7.4.

## Results

The DTNB reactions were carried out under pseudo-first-order conditions (excess DTNB) at 25 °C, pH 7.4. The reaction of Zn<sub>7</sub>MT, Pt<sub>7</sub>MT and Bi<sub>7</sub>MT with DTNB went to completion within 40 min, 420 min and 70 min, respectively. All were complete more quickly at higher DTNB concentrations. The first-order plots for the reactions of three MTs with DTNB are shown in Figure 2. Both the reactions of Zn<sub>7</sub>MT and Pt<sub>7</sub>MT showed biphasic kinetics with fast and slow steps. The two observed rate constants ( $k_f$  and  $k_s$ ) could be calculated according to the standard kinetic treatments for parallel reactions (Espenson 1981). These results were similar to the previous reactions of Cd-MT with DTNB (Savas *et al.* 1991; Munoz *et al.* 1999). While the reaction of Bi<sub>7</sub>MT exhibited a monophasic kinetic process. At pH 7.4, the order of the observed rate constant  $k_f$  and  $k_s$  is  $k_{Zn7MT} > k_{Pt7MT}$ . The observed rate constant of Bi<sub>7</sub>MT was one order of magnitude larger than the value of the slow reaction of Pt<sub>7</sub>MT and similar to those obtained in the fast step of Pt<sub>7</sub>MT reaction (Tables 1 and 2).

Figure 3 exhibits the linear relationship between the observed rate constant and concentration of DTNB. The plots for the fast and slow steps each extrapolated to non-zero intercepts, indicating that there were both DTNB-dependent and DTNB-independent components to the reactions. Thus, the four-term rate laws for the reactions of Zn<sub>7</sub>MT and Pt<sub>7</sub>MT were:

$$\text{rate} = k_{1s} [\text{MT}] + k_{2s} [\text{DTNB}][\text{MT}] + k_{1f}[\text{MT}] + k_{2f} [\text{DTNB}][\text{MT}].$$

Where 'f' and 's' denoted fast and slow, respectively, and, '1' and '2' designated first-order and second-order, respectively.

Table 1. Observed rate constants for the reaction of metallothioneins with DTNB

[DTNB] mM	pH	Zn <sub>7</sub> MT		Pt <sub>7</sub> MT	
		$10^2 k_f/s^{-1}$	$10^3 k_s/s^{-1}$	$10^3 k_f/s^{-1}$	$10^4 k_s/s^{-1}$
0.4	7.4	0.282	0.531	0.473	0.410
0.8	7.4	0.530	0.893	0.941	0.643
1.1	7.4	0.725	1.190	1.400	1.043
1.6	7.4	0.980	1.581	2.110	1.345
2.0	7.4	1.270	2.101	2.321	1.759
0.8	6.0	0.491	0.824	0.943	0.647
0.8	8.1	0.526	0.884	0.952	0.650

\* [MT] = 6.0  $\mu$ mol/l, in 0.01 mmol/l Tris-HCl buffer with 0.1 mol/l KCl, at 25 °C.

Table 2. [DTNB] and pH dependence of the observed rate constants for the reaction of Bi<sub>7</sub>MT and DTNB\*

[DTNB] (mM)	pH	$10^3 k/s^{-1}$
0.4	7.4	0.291
0.8	7.4	0.471
1.1	7.4	0.682
1.6	7.4	0.790
2.0	7.4	0.973
1.1	6.0	0.707
1.1	8.1	0.716

\*[MT] = 6.0  $\mu$ mol/L, in 0.01 mmol/L Tris-HCl buffer with 0.1 mol/L KCl, at 25 °C.

The observed rate constants of Bi<sub>7</sub>MT to DTNB also established a linear relationship over the concentration of DTNB (plot not shown). The value of intercept was the first-order rate constant for DTNB-independent process. The slope was the second-order

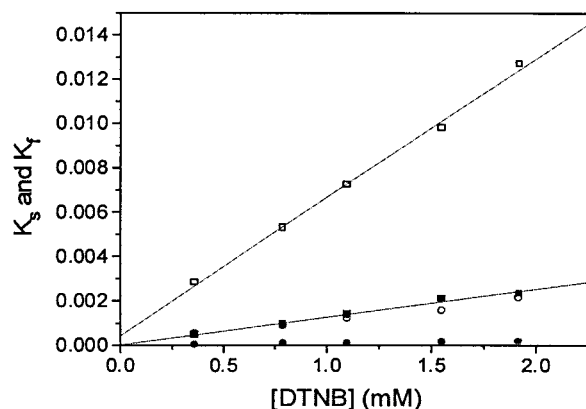


Figure 3. Plots of the observed rate constants vs. [DTNB]. The symbols represent  $k_f$  (□) and  $k_s$  (■) for Zn<sub>7</sub>MT and the symbols  $k_f$  (○) and  $k_s$  (●) for Pt<sub>7</sub>MT.

rate constant for DTNB-dependent process. Its two-term rate law was listed as follows:

$$\text{rate} = k_1 [\text{MT}] + k_2 [\text{DTNB}][\text{MT}]$$

The values of rate constants are given in Table 3.

The reactions of MTs were also carried out at pH 6.0, 7.4 and 8.5 with DTNB fixed. The variation in pH did not influence the rate constant (Tables 1 and 2). These results agreed with expectations based on the  $pK_a$  values of DTNB and the protein amino-acid side chains: There were no groups that were protonatable in this pH range that might affect the kinetic results.

## Discussion

Previous studies of DTNB with mammalian Cd-thioneins (Savas *et al.* 1991; Munoz *et al.* 1999) exhibited the biphasic reactions, in which both the fast and slow steps contained the DTNB-dependent and DTNB-independent components. Its biphasic kinetic behavior was due to different reactivities of the two metal-thiolates clusters of Cd-MT. Savas (1991) and Munoz (1999) examined the reactivities of the isolated  $\alpha$  and  $\beta$ -domain of Cd-MT for comparison with that of the holoprotein. Their results established unequivocally that the slow and fast phases of the holoprotein reactions were the sum of the independent reactions of the two domains. The fast step was exclusively in the  $\alpha$ -domain and the slow step in the  $\beta$ -domain.

However, the structural details of Pt<sub>7</sub>MT and Bi<sub>7</sub>MT have not revealed by NMR or X-ray crystallographic studies. The previous studies (Pattanaik

*et al.* 1992; Xing *et al.* 1999) by EXAFS revealed that each platinum in Pt-MT was coordinated by four sulfurs at the distance of 2.31 Å. The Pt...Pt contact was also detectable at the distance of 4.29 Å. These data demonstrated the similar metal-thiolate and metal-metal distance to the values obtained from native Zn-MT and Cd-MT. In addition, our previous detailed study (Huang *et al.* 1998) by FT-IR also confirmed that the secondary structure of Pt<sub>7</sub>MT was very similar to that of Zn<sub>7</sub>MT and Cd<sub>5</sub>Zn<sub>2</sub>MT. Therefore, it was reasonable to hypothesis that Pt<sub>7</sub>MT may contain the two clusters structure (M<sub>4</sub>S<sub>11</sub> and M<sub>3</sub>S<sub>9</sub>) as in native Zn-MT and Cd-MT. In this work, both the reactions of Pt<sub>7</sub>MT and Zn<sub>7</sub>MT with DTNB exhibited the biphasic kinetic behavior with fast and slow steps, each displaying the first-order and second-order components. Their biphasic kinetic behaviors were similar to those of the reaction of, Cd-MT to DTNB (Li *et al.* 1981; Savas *et al.* 1991; Munoz *et al.* 1999). So the kinetics of reaction should be explained by the similar mechanism. The biphasic reactions could also arise from independent reaction of each cluster with DTNB too. Due to the kinetic stability of Pt-S bond, Pt<sub>7</sub>MT reacted with DTNB more slowly and had lower observed rate constants  $k_f$ ,  $k_s$  than those of Zn<sub>7</sub>MT. However, whether the  $\alpha$ -domain and  $\beta$ -domain half-reactions corresponded to the faster and slower phases or vice versa in Pt<sub>7</sub>MT remained unclear here and needed further study.

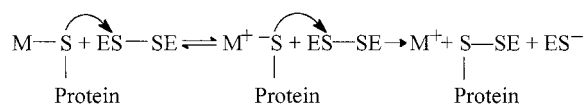
The extended X-ray absorption fine structure spectrum of Bi<sub>7</sub>MT suggested that Bi(III) was coordinated strongly to only three cysteine sulfurs with Bi-S bond length of 2.55 Å. Some sites also appeared to contain two longer Bi-S bonds at 3.1 Å and one short Bi-O bond at 2.2 Å (Sadler *et al.* 1999). This result strongly suggested that the coordination environment of Bi in Bi<sub>7</sub>MT should be significantly different from those in native Zn-MT and Cd-MT. Moreover, NMR data also confirmed the different overall three-dimensional structure of Bi<sub>7</sub>MT in comparison with those of native Zn-MT and Cd-MT (Sadler *et al.* 1999). Therefore, it is reasonably hypothesized that the structural changes of Bi<sub>7</sub>MT may cause the altered reactivity toward DTNB. On the one hand, it is possible that the  $\alpha$ ,  $\beta$  domains in Bi<sub>7</sub>MT may not contain the M<sub>4</sub>S<sub>11</sub>, M<sub>3</sub>S<sub>9</sub> clusters as those in native MT, but may still contain other clusters consisted of BiS<sub>5</sub>O structural units. The two domains do not exhibit the different kinetic behaviors in the reaction with DTNB. Therefore, the kinetics is monophasic. On the other hand, we can assume that the  $\alpha$ ,  $\beta$  domain structure of Bi<sub>7</sub>MT may not have

Table 3. Rate constants for the reactions of Metallothioneins with DTNB

Reactants	Slow step components		Fast step components	
	$k_{1s} \times 10^4 / \text{s}^{-1}$	$k_{2s} / \text{M}^{-1} \text{s}^{-1}$	$k_{1f} \times 10^4 / \text{s}^{-1}$	$k_{2f} / \text{M}^{-1} \text{s}^{-1}$
Zn <sub>7</sub> MT	1.30	0.983	6.10	6.014
Pt <sub>7</sub> MT	0.0423	0.085	0.20	1.250
Bi <sub>7</sub> MT	1.50	0.428	—	—

existed any more due to the different coordination environment of Bi atom. So the reaction exhibited the monophasic kinetics too.

The X-ray crystal structure of both domains in mammalian MTs shows that solvent accessibility to the interior of the clusters and their sulfhydryl components in the two domains is severely structurally hindered by amino acid side chains except in a crevice located in each domain (Savas *et al.* 1991; Munoz *et al.* 1999). DTNB acts as an electrophile to attack initially on one of the more accessible thiolates. This process is DTNB-dependent. The reorganization of protein is hypothesized to happen in these crevice regions (Munoz *et al.* 1999). Then, the mixed disulfides of MT-S-SE and the free chromophore thionitrobenzoate ( $\text{ES}^-$ ,  $\lambda_{\text{max}} = 412 \text{ nm}$ ) are formed. As a result, the M-S bond would be dissociated and the coordinated metal ions are released. This step is independent of DTNB. The course of the reaction can be described as below:



## Conclusion

The reactions of Pt<sub>7</sub>MT and Zn<sub>7</sub>MT with DTNB exhibit the biphasic kinetics. The biphasic character of the reactions arises from differential reactivities with the two domains structure. The observed rate constants  $k_f$  and  $k_s$  of Zn<sub>7</sub>MT are larger than those of Pt<sub>7</sub>MT. The reaction of Bi<sub>7</sub>MT with DTNB is different, and shows a monophasic behavior, and does not exhibit the different reactivities of  $\alpha$ ,  $\beta$  domains as those in Zn-MT, Cd-MT and Pt-MT. These results imply that the structure of the protein may have important effects on the reactivity and presumably on the biological functions of metallothioneins.

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