On the reactivity of metallothioneins with 5,5'-dithiobis-(2-nitrobenzoic acid)

Ta-Yuen LI, Daniel T. MINKEL,* C. Frank SHAW III,† and David H. PETERING†

Department of Chemistry and The Laboratory for Molecular Biomedical Research, University of Wisconsin–Milwaukee, Milwaukee, WI 53201, U.S.A.

(Received 17 June 1980/Accepted 4 August 1980)

Rat liver and horse kidney metallothioneins react with 5,5'-dithiobis-(2-nitrobenzoic acid) (Nbs₂) to release 5-thio-2-nitrobenzoate and metal ions. The reactions are slow and exhibit biphasic kinetics with each process having an empirical rate law of the form: rate = $k[\text{RSM}] \cdot [\text{Nbs}_2] + k'[\text{RSM}]$, where RSM represents metal-bound thiolate groups. The pseudo-first-order rates are insensitive to pH but are modified in guanidine hydrochloride solution. Rat liver metallothioneins of variable zinc, copper and cadmium composition react similarly and give observable thiol/total metal ratios in good agreement with stoichiometries of SH/(Cd + Zn) of 3 and SH/Cu of 1. A model complex cadmium–2,3-dimercaptopropanol, resembles the protein in its reaction with Nbs₂.

The nature of the function of metallothionein has been a question of intense interest for many years since Margoshes & Vallee (1957) discovered that cadmium found in the cytosol of horse kidney is bound specifically to this protein. It is now apparent that metallothionein is a versatile metal binding protein which not only sequesters a number of toxic, heavy metals but also binds zinc and copper under a variety of normal physiological conditions (Bremner & Davies, 1974; Oh *et al.*, 1978; Sobäcinski *et al.*, 1978; Petering *et al.*, 1978; Koch *et al.*, 1980).

The structure of this protein seems ideal for reaction with these metals, for about one-third of the amino acid residues are cysteinyl groups, which are excellent ligands for such metal ions (Kägi *et al.*, 1974). Nevertheless, relatively little research has been directed toward the metal–mercaptide chemistry of metallothionein. The present study begins an investigation of the chemical properties of the metal-binding sites of this protein in order to understand the types of inorganic reactions metallothionein may undergo in cells. In this report the reaction of Nbs₂ with various metallothioneins is examined.

Abbreviation used: Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid).

* Present address: Escuela de Medicina, Universidad de Cuidad Juarez, Apartado Postal 231, Cd. Juarez, Chih., Mexico.

† To whom reprint requests should be addressed.

Experimental

Materials

Nbs₂ and Trizma base were purchased from Sigma. All other chemicals were reagent grade.

Preparation of metallothioneins

The preparation of rat liver metallothionein is detailed elsewhere (Minkel *et al.*, 1980). The horse kidney protein was isolated by a similar procedure, which resembles that of Winge & Rajagopalan (1972).

Preparation of horse kidney metallothionein

The cortex from a fresh or frozen–thawed horse kidney was homogenized by using a blender in 2 vol. of sucrose/Tris buffer (0.25 M-sucrose/0.02 M-Tris/HCl, pH 8.6) for 60 s. The homogenate was centrifuged at 23 500 *g* for 40 min in a GSA rotor on a Sorvall RC5-B Superspeed centrifuge. The supernatant was recentrifuged at 45 900 *g* in an SS-34 rotor. The resulting supernatant was used to homogenize a second kidney, and the two centrifugations were repeated. After four kidneys had been sequentially homogenized and centrifuged, a heat-treatment step was used (60°C, 1 min) before the final 45 000 *g* centrifugation. After centrifugation to remove the precipitated protein, the final supernatant was applied to a column (8 litre bed volume; 10 cm × 100 cm) of Sephadex G-50 and eluted with 0.02 M-Tris/HCl, pH 8.6, at a flow rate of 300 ml/h.

A_{280} , cadmium and zinc concentrations were determined on the fractions. Fractions containing metallothionein were pooled, lyophilized, and redissolved in a smaller volume of distilled water, then pumped onto a column (2.5 cm $\times$ 100 cm) of DEAE-Sephadex A-25. After washing with one column volume of 0.005 M-Tris/HCl, pH 8.6, the column was eluted with a linear gradient (0.05–0.50 M) of Tris/HCl, pH 8.6, at a flow rate of 25 ml/h. A_{250} , cadmium, zinc, and conductivity were determined for the fractions. Metallothionein-containing fractions were pooled, lyophilized, dissolved in water and applied to a desalting column (2.5 cm $\times$ 70 cm) of Sephadex G-25 and eluted with water. The fractions containing metallothionein were stored until used.

Preparation of metal-specific metallothioneins

The enrichment of rat liver metallothionein in either zinc or copper was accomplished by adding various amounts of ZnCl_2 or CuCl_2 to the heat-treated liver supernatant in the presence of 5 mM-2-mercaptoethanol. After incubation for 24 h at 4°C, the supernatant was chromatographed on Sephadex G-75 as previously described (Minkel *et al.*, 1980).

The horse kidney metallothionein was stripped of metals by dialysis at pH 2–2.5. The thiol groups remain reduced at low pH. CdCl_2 or ZnCl_2 was added to the apoprotein and the pH was restored to 7.5 by further dialysis against Tris/HCl buffer.

Preparation of cadmium–2,3-dimercaptopropanol

Direct titration of 2,3-dimercaptopropanol with CdCl_2 by the method of Job (Rossotti & Rossotti, 1961) indicated a complex with a ligand:metal stoichiometry of $1.7 \pm 0.2:1$. The binding of cadmium to ligand was tight. Initially, about 20% of the thiol groups in this complex reacted rapidly with Nbs_2 . This diminished to zero over several days. The thiol/metal ratio (calculated from the titration with Nbs_2 and the amount of Cd^{2+} added to make the complex) was 3.2:1. After 5 months of storage at 25°C the same ratio was obtained, indicating that the complex is stable against oxidation for long periods.

Kinetic studies

Metallothionein and Nbs_2 were mixed at time zero, placed in a Beckman Acta V spectrophotometer and maintained at 25°C. Reactions were carried out after degassing the solutions because 5-thio-2-nitrobenzoate is not indefinitely stable in aerated solutions and begins to oxidize again to Nbs_2 , thereby preventing quantification of the stoichiometry and kinetics of reaction. The change in absorbance at 412 nm, the absorbance maximum of 5-thio-2-nitrobenzoate, was measured over time against a reference sample containing buffer and an

equivalent amount of Nbs_2 . The maximum absorbance value, A_∞ , and an absorbance coefficient of $13600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ were used to measure the total reactive thiol groups. To obtain first-order plots of the reaction, $\log (A_\infty - A_t)$, which is proportional to the concentration of unreacted thiol groups, was plotted against time. Because the reactions are biphasic, the corrected first-order rates for the first step must be obtained by subtracting out the contribution of the second step to the first rate [i.e. $\{(A_\infty - A_t)_{\text{step I}} - (A_\infty - A_t)_{\text{step II}}\}$] after extrapolating the data for step II to zero time. This treatment is consistent either with consecutive or parallel reactions leading to formation of 5-thio-2-nitrobenzoate. In some cases the reaction mixtures were passed through a column (1 cm $\times$ 25 cm) of Sephadex G-50 to demonstrate that complete metal displacement occurred.

Results

Reaction of Nbs_2 with rat liver metallothionein

When the aromatic disulphide Nbs_2 is mixed with liver metallothionein 1 (as isolated after DEAE–

Fig. 1. Reaction of Nbs_2 with rat liver metallothionein 1. Reaction conditions: anaerobic 0.1 M-phosphate buffer, pH 7.85 at 25°C, containing 1 mM- Nbs_2 and the following concentrations of metallothionein-bound metals: $8.53 \mu\text{M}$ -cadmium, $8.27 \mu\text{M}$ -zinc, and $6.18 \mu\text{M}$ -copper. The observed thiol/metal ratio for the reaction is 2.11.

Sephadex A-25 chromatography), a slow reaction takes place (Fig. 1). The disulphide is cleaved and 5-thio-2-nitrobenzoate with its absorption maximum at 412nm is generated. The reaction has the properties of the standard colourimetric assay for thiol groups, except that it is slow (Ellman, 1959). In contrast, apometallothionein reacts completely with Nbs₂ within the time of mixing.

Although the metallothionein-Nbs₂ reaction is slow, it appears to go to completion. Thus, as summarized in Table 1, observed and calculated thiol/metal ratios agree closely, despite a wide range of metal ratios, if one uses an SH/Cu ratio of 1, which is expected to be approximately correct for Cu(I)-thiolate complexes, along with the usual value of 3 for SH/(Cd + Zn).

Reaction of horse kidney metallothioneins with Nbs₂

As illustrated in Fig. 1, the reaction under study displays complex kinetics; two pseudo-first-order steps which vary to some degree in observed rate constants dependent upon the metal composition of metallothionein are seen. To avoid the complications of mixed metal metallothioneins, horse kidney thionein was reconstituted with cadmium or zinc for further study on the rate process. Both the cadmium and zinc reconstituted proteins also react with Nbs₂ in the biphasic manner seen in Fig. 1. Metal is released from the protein in the process. Pseudo-first-order rate constants have been deter-

mined for steps I and II as a function of Nbs₂ concentration. One can also partition the total reaction between the two processes by comparing $A_{\infty}-A$ at time zero for step I with the extrapolated $A_{\infty}-A$ at time zero for step II. Fig. 2 summarizes the results. The numbers on the Figure are the percentages of total reaction assigned to step II. The data for step II of the reaction of liver metallothionein I with Nbs₂ are also included for comparison.

The quantitative results are similar for both cadmium- and zinc-thioneins. Both steps I and II have components of the rate equation independent of, as well as dependent upon, the concentration of Nbs₂. Table 2 lists the extracted rate equations for these reactions. For both the zinc- and cadmium-thionein reactions, step II comprises the bulk of the overall reaction.

The pH-dependence of each step of the reaction of zinc-thionein with Nbs₂ is shown in Table 3. There is no clear effect of pH on k_1 . The second step shows a small decrease in k_{II} at higher pH values. Similarly in Table 3 for metallothionein I from rat liver, there is no change in k_{II} between pH 6 and 8. The presence of 5 M-guanidine hydrochloride converts the biphasic reaction into a single exponential process (Table 3). Guanidine hydrochloride causes a 10nm red shift in the absorbance of 5-thio-2-nitrobenzoate and also decreases its intensity. Alteration of pH still has little influence on the rate of reaction. Interestingly,

Table 1. Thiol and metal contents of metallothionein

The reactions were conducted in 0.1 M-phosphate buffer, pH 6.85, containing 1 mM-Nbs₂ at 25°C. Metallothionein-I is that used in Fig. 1; samples G-75 (control, and copper-1, -2 and -3) were prepared by incubating no metal, or increasing concentrations of copper, with the 40000 g heat-treated rat liver supernatant before fractionation by Sephadex G-75 chromatography.

Sample	Thiol/total metal (mol/mol)		Observed metal ratio (mol/mol)			
	Observed	Calculated	Cd	:	Zn	: Cu
Metallothionein-I	2.2	2.3	1.0		1.0	0.7
G-75 (control)	3.1	2.6	1.0		1.3	0.5
	2.7	2.7	3.0		0.8	0.3
G-75 [copper-1 (= control)]	1.9	1.8	1.0		2.0	0.5
(copper-2)	1.3	1.5	1.0		0.5	4.5
(copper-3)	1.2	1.3	1.0		1.0	11.0

Table 2. Rate equations for reactions of metallothioneins with Nbs₂

The rate equations for each step of the reactions described by the data in Fig. 2 are given in this Table.

Sample	Rate equation (s ⁻¹) for step:	
	I	II
Zinc-thionein	0.3 [Nbs ₂] + (2.0 × 10 ⁻³)	0.048 [Nbs ₂] + (0.14 × 10 ⁻³)
Metallothionein-I		0.165 [Nbs ₂] + (0.125 × 10 ⁻³)
Cadmium-thionein	0.28[Nbs ₂] + (2.4 × 10 ⁻³)	0.028 [Nbs ₂] + (0.075 × 10 ⁻³)
Cadmium-(2,3-dimercaptopropanol) _{1,7}	0.72 [Nbs ₂] + (2.5 × 10 ⁻³)	1.0 × 10 ⁻³

Table 3. *pH-dependence of reactions of metallothioneins with Nbs₂*

The initial content of metallothionein-I, isolated after DEAE-Sephadex chromatography from rat liver, was 1.28 μM -Zn, and 0.92 μM -Cu. Its observed thiol/total metal ratio was 2.09. This reaction was carried out in 1.0 M-phosphate buffer, adjusted to the appropriate pH at 25°C, in the presence of 1 mM-Nbs₂. Only data for step II are included. The zinc in zinc-thionein, reconstituted from horse kidney thionein, was 17 μM . Its reactions were conducted in 0.1 M-KCl at 25°C. Those in the absence of guanidine hydrochloride used 1.3 mM-Nbs₂, those in the presence of 5 M-guanidine hydrochloride used 6.7 mM. In the latter set, at pH 6.3, zinc-thionein was preincubated with guanidine hydrochloride. The cadmium in the cadmium-(2,3-dimercaptopropanol)_{1,7} complex was 10.4 μM . Its reaction with 0.67 mM-Nbs₂ were carried out under anaerobic conditions at 25°C in 0.01 M-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer.

	pH	$10^3 \times k_I$ (s ⁻¹)	$10^4 \times k_{II}$ (s ⁻¹)
Metallothionein-I	6.0		1.6
	6.5		1.3
	6.9		1.6
	8.0		1.7
Zinc-thionein	5.3	3.0	3.2
	6.8	4.8	2.7
	8.0	2.6	2.2
Zinc-thionein + guanidine hydrochloride	5.6	1.7	
	6.3	2.4	
	7.1	1.0	
Cadmium-(2,3-dimercapto- propanol) _{1,7}	5.7	7.0	14.3
	6.7	4.1	7.1
	7.1	3.9	8.5
	7.8	3.1	2.4

preincubation of zinc-thionein with 0.67 mM-EDTA for 40 min, which removes much of the zinc from the protein, leads to a rapid reaction of 82% of the protein thiol groups with Nbs₂ followed by the typical two-step reaction for the remaining 18%.

Reaction of Nbs₂ with a cadmium-2,3-dimercaptopropanol complex

In an effort to find a suitable model for the reaction of metallothionein with Nbs₂, the reactions with Nbs₂ of cadmium bound to 2,3-dimercaptopropanol were studied. Fig. 2 and 3 and Tables 2 and 3 show that the complex reacts slowly with Nbs₂ in a biphasic manner and with rates that are relatively independent of pH effects. Interestingly, 6 M-guanidine hydrochloride increased the rate of the reaction between the complex and Nbs₂ by approx. 100-fold. Hence, this protein denaturant may be affecting the rate of reaction of metallothionein with

Fig. 2. *Dependence of the reactions of metallothioneins with Nbs₂ upon [Nbs₂]*

Zinc-thionein (○) reaction conditions: 9.4 μM -zinc, 0.01 M-Tris/HCl/0.1 M-KCl, pH 6.8 at 25°C under anaerobic conditions. Cadmium-thionein (■) reaction conditions: 0.062 μM -cadmium, pH 7.0 at 25°C, under anaerobic conditions. Rat liver metallothionein-I (●) reaction conditions: 0.1 M-phosphate buffer, pH 6.85 at 25°C, containing the following concentration of metallothionein-bound metals: 1.28 μM -cadmium, 1.24 μM -zinc, and 0.927 μM -copper. Cadmium-2,3-dimercaptopropanol (□) reaction conditions: 8.3 μM -cadmium, 0.01 M-Tris/HCl/0.1 M-KCl, pH 6.8 at 25°C. The percentage of the total reaction occurring in step II is given next to each data point.

Nbs₂ by a mechanism other than perturbation of the thionein structure. There are qualitative differences between this model system and the metallothionein. The first step comprises much of the total reaction in the model, whereas it is the second step which is dominant for the protein. In addition, the second step is independent of Nbs₂ concentration for cadmium-2,3-dimercaptopropanol.

Discussion

Although the reaction with Nbs₂ for the analysis of protein thiol groups has been used by others to measure the thiol content of metallothionein, it was initially surprising that such a reaction should occur,

Fig. 3. Reaction of Nbs₂ with the cadmium-2,3-dimercaptopropanol complex
Reaction conditions: 0.01 M-Tris/HCl/0.1 M-KCl, pH 6.8 at 25°C.

since the thiol groups are bound tightly to metal ions in the protein (Anderson *et al.*, 1978; Ryden & Deutsch, 1978; Tsunoo *et al.*, 1978). In the reaction of free thiol groups with the aromatic disulphide, the thiol anion acts as a nucleophile in a thiol-disulphide interchange:

Yet in the protein, where the binding constant for zinc has been estimated to be 10^{11} M^{-1} and for cadmium about 10^{14} M^{-1} , it seemed at first kinetically and thermodynamically unlikely that metal-bound thiols could dissociate and react with Nbs₂.

The rate equations for all of the reactions of metal-thiol complexes with Nbs₂ given in Table 2 suggest that there is a significant contribution from metal-thiol bond breaking (path B in eqn. 2) in the rate-determining step of the reaction as well as from direct reaction of metal-bound thiol with Nbs₂ (path A, eqn. 2). Interestingly, in the study of the reaction of EDTA with metallothioneins, EDTA-indepen-

dent contributions to the rate law were observed and were also attributed to metal-thiol bond breaking (Li *et al.*, 1980).

Zinc-thiolate complexes of $\text{Zn}_4(\text{SR})_{10}^{-2}$ stoichiometry which have been characterized crystallographically (Dance, 1980) provide a possible model for the metal binding sites in metallothionein. They involve four terminal and six doubly binding thiolates bound to a tetrahedron of zinc atoms. It is conceivable that the fast and slow phases of the reaction correspond to the reactions of the terminal and bridging cysteine thiolate ions. Ultimately, the biphasic nature of the reaction will require further study as well as a structural elucidation of the metal binding sites before a satisfactory interpretation can be given.

The examination of the reaction of Nbs₂ with cadmium-(2,3-dimercaptopropanol)_{1.7} was based on the finding of Sadler *et al.* (1978) and Otvos & Armitage (1980), using ¹¹³Cd n.m.r. spectroscopy, that the cadmium-sulphur sites in metallothionein are CdS₄ complexes, or at least involve bridging thiol ligands. Of necessity, because of the metal-thiol stoichiometry in the protein, these must contain thiol groups bridging between metal centers. Therefore, cadmium-(2,3-dimercaptopropanol)_{1.7} may serve as a useful model for CdS₄ complexes, for the stoichiometry implies that it must contain bridging ligands.

The similarity of the kinetics for the cadmium-2,3-dimercaptopropanol complex and the various metallothioneins confirms the appropriateness of the model and furthermore suggests that the cause of the biphasic reaction must be sought in the nature of the cadmium-thiolate structures, rather than in the effects of the protein on the metal binding sites.

It is also of interest in these studies that copper-loaded metallothionein can be made by displacement of zinc and cadmium from native rat liver metallothionein. The thiol/metal ratio approaches 1 as the copper content increases, in agreement with previous studies indicating a thiol: copper ratio of 1 (Rupp & Weser, 1974). What is unusual here, and was also seen in the native rat liver protein, is that the copper-thionein is stable at least for a period of hours under aerobic conditions after removal from mercaptoethanol by Sephadex G-75 chromatography (Minkel *et al.*, 1980). Other authors have found synthetic and naturally isolated copper-thionein to exhibit a great sensitivity to

oxidation and polymerization (Rupp & Weser, 1974; Bremner & Marshall, 1974; Bremner & Young, 1976).

Finally, given the indications that metallothionein plays a central role in normal zinc, and possibly copper, metabolism, mechanisms may exist for movements of metals into and out of this protein. This study shows one way in which cellular disulphides could interact with metallothionein to liberate tightly bound metal ions for redistribution into other parts of the cell.

The authors gratefully acknowledge the support of U.S.P.H.S. grant ES 01504. The skilled technical assistance of Susan Wielgus and Deborah Meng is appreciated. This is contribution no. 103 from the Laboratory for Molecular Biomedical Research.

References

- Anderson, R. D., Winter, W. P., Maher, J. J. & Bernstein, I. A. (1978) *Biochem. J.* **174**, 327–338
- Bremner, I. & Davies, N. T. (1974) in *Trace Element Metabolism in Animals* (Hoekstra, W. G., Suttie, J. W., Gauthier, H. E., & Mertz, W., eds.), vol. 2, pp. 493–496, University Park Press, Baltimore
- Bremner, I. & Marshall, R. B. (1974) *Br. J. Nutr.* **32**, 293–300
- Bremner, I. & Young, B. W. (1976) *Biochem. J.* **155**, 631–635
- Dance, I. (1980) Abstr. Meet. Am. Chem. Soc. INOR 42
- Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77
- Kägi, J. H. R., Himmelhoch, S. R., Whanger, P. D., Bethune, J. L. & Vallee, B. L. (1974) *J. Biol. Chem.* **249**, 3537–3542
- Koch, J., Wielgus, S., Shankara, B., Shaw III, C. F. & Petering, D. H. (1980) *Biochem. J.* **189**, 95–104
- Li, T. Y., Kraker, A., Shaw III, C. F. & Petering, D. H. (1980) *Proc. Natl. Acad. Sci. U.S.A.* in the press
- Margoshes, M. & Vallee, B. L. (1957) *J. Am. Chem. Soc.* **79**, 4813–4814
- Minkel, D. T., Paulsen, K., Wielgus, S., Shaw III, C. F. & Petering, D. H. (1980) *Biochem. J.* **191**, 475–485
- Oh, S. H., Deagan, J. J., Whanger, P. D. & Wesig, P. H. (1978) *Am. J. Physiol.* **234**, E282–E285
- Otvos, J. & Armitage, I. (1980) *J. Am. Chem. Soc.* **101**, 7734–7736
- Petering, D. H., Koch, J., Wielgus, S. & Shaw III, C. F. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, 1349
- Rossotti, J. C. & Rossotti, H. (1961) *The Determination of Stability Constants*, pp. 47–51, McGraw-Hill, New York
- Rupp, H. & Weser, U. (1974) *FEBS Lett.* **44**, 293–297
- Ryden, L. & Deutsch, H. F. (1978) *J. Biol. Chem.* **255**, 519–522
- Sadler, P. J., Bakka, A. & Beynon, P. J. (1978) *FEBS Lett.* **94**, 315–319
- Sobäcinski, P. A., Canterbury, W. J., Mapes, C. A. & Pinterman, R. E. (1978) *Am. J. Physiol.* **234**, E399–E406
- Tsunoo, H., Kino, K., Nakajima, H., Hata, A., Huang, I.-Y. & Yoshida, A. (1978) *J. Biol. Chem.* **253**, 4172–4174
- Winge, D. R. & Rajagopalan, K. V. (1972) *Arch. Biochem. Biophys.* **153**, 755–762