The reaction of metallothionein with mercuribenzoate

A dialysis and ^{113}Cd -n.m.r. study*

Douglas M. TEMPLETON,†|| Philip A. W. DEAN‡ and M. George CHERIAN§

†Department of Clinical Biochemistry, The Banting Institute, University of Toronto, 100 College Street, Toronto, Canada M5G 1L5, and Department of Biochemistry, The Hospital for Sick Children, Toronto, Canada M5G 1X8, ‡Department of Chemistry, University of Western Ontario, London, Canada N6A 5B7, and §Department of Pathology, University of Western Ontario, London, Canada N6A 5C1

Reaction of rat liver cadmium-metlothionein-II (Cd-MT-II) with *p*-hydroxymercuribenzoate ($p\text{HOHgBzO}^-$) causes displacement of bound Cd. When $p\text{HOHgBzO}^-$ -induced displacement of ^{109}Cd is observed after dialysis of the reaction mixture, the stoichiometry is consistent with stepwise displacement of tetra-co-ordinate Cd atoms by non-random entry of reagent into the polynuclear clusters. ^{113}Cd n.m.r. allows direct observation of the effects on bound Cd of stepwise titration of ^{113}Cd -MT-II with $p\text{HOHgBzO}^-$. The first equivalent reduces all resonances approximately equally. Subsequently differential reactivity of the protein thiolates towards the reagent gives rise to differential decreases in the ^{113}Cd signal intensities. Resonances previously attributed to a three-metal cluster are lost before those arising from the four-metal cluster. These results are interpreted in terms of current models of the MT structure. They are distinct from the results of reaction of MT with 5,5'-dithiobis-(2-nitrobenzoic acid), which distinguishes between only two classes of thiolates, terminal and bridging. Such different patterns of reactivity of the protein thiolates may underlie a biological activity of this protein.

INTRODUCTION

Metallothionein (MT) is a widely studied low- M_r metal-binding protein important to the biology of the essential metals Zn and Cu, as well as toxic metals such as Cd (Kojima & Kägi, 1978; Nordberg & Kojima, 1979). The mammalian protein provides binding sites for these and other metals with 20 cysteine thiolate ligands. Evidence indicates the arrangement of these into two domains, such that two distinct metal clusters of tetrahedrally co-ordinated Zn and Cd are formed (Boulanger *et al.*, 1983). These consist of four- and three-metal clusters, termed A and B respectively, which account for the 7 g-atoms of Zn and Cd that can be accommodated in tetrahedral co-ordination.

In addition to providing metal-binding sites, the thiol groups of MT may play a role in the detoxification of electrophilic xenobiotics (Rugstad, 1984) and scavenging of oxygen radicals (Endresen *et al.*, 1981; Thornalley & Vašák, 1985). Resistance to the alkylating agent chlorambucil is conferred by binding of the drug to intracellular MT (Endresen *et al.*, 1981), presumably through thioether linkages. On the basis of their reactivity towards Nbs_2 , two classes of thiols can be distinguished in adult (Li *et al.*, 1981), foetal (Templeton *et al.*, 1985) and chemically polymerized (Templeton & Chierian, 1985) MTs. These two classes are thought to represent bridging and terminal thiolates. In order to probe further the thiolate reactivity in MT, we have studied the reactivity of the protein with the thiol-group-modifying mercurial reagent $p\text{HOHgBzO}^-$.

MATERIALS AND METHODS

Protein purification

Rat liver MT-II was isolated as previously described (Templeton & Chierian, 1984) after injection of $^{109}\text{CdCl}_2$, at 18 mg/kg body wt. in six divided doses. For the n.m.r. experiments, animals were injected with $^{113}\text{CdCl}_2$ prepared from Cd metal enriched in ^{113}Cd (Technabs-export, Moscow, U.S.S.R.) as described previously (Dean *et al.*, 1983). Samples of ^{109}Cd -MT-II or ^{113}Cd -MT-II were then dialysed against solutions containing a 2-fold excess of the appropriately labelled metal in order to achieve further Cd enrichment, and excess metal was removed by dialysis against 10 mM-Tris/HCl buffer, pH 8.6. Protein concentrations were determined by absorption, based on an absorption coefficient ($A_{254}^{1\%}$) of 13.1 for the Cd-enriched material, and confirmed by the detection of 18 thiol residues by $p\text{HOHgBzO}^-$ titration (Templeton & Chierian, 1984).

Reaction with $p\text{HOHgBzO}^-$

Portions, typically of 50 μg , of ^{109}Cd -MT-II in 2.0 ml of Tris/HCl (pH 7.5, 0.1 M) were degassed, equilibrated with N_2 , and allowed to react with stoichiometric amounts of a solution of $p\text{HOHgBzO}^-$ (Sigma, St. Louis, MO, U.S.A.) in the same buffer. Concentrations of reagent were adjusted so that 10 μl contained one equivalent (based on $\epsilon_{232} = 1.69 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$). Thiol groups were determined in some such experiments as described by Boyer (1954). When higher concentrations

Abbreviations used: MT, metallothionein; $p\text{HOHgBzO}^-$, *p*-hydroxymercuribenzoate; Nbs_2 , 5,5'-dithiobis-(2-nitrobenzoic acid); n.o.e., nuclear Overhauser effect; equiv., equivalent(s).

* This is the third of a series of papers on the chemical modification of metallothionein; paper I is Templeton & Chierian (1984) and paper II is Templeton & Chierian (1985).

|| To whom correspondence and reprint requests should be addressed.

of $p\text{HOHgBzO}^-$ were needed for the n.m.r. experiments (see below), conc. NaOH was added to $p\text{HOHgBzO}^-$ in water until all material was dissolved.

To measure ^{109}Cd displacement after reaction with $p\text{HOHgBzO}^-$, solutions were dialysed extensively against Tris/HCl (0.1 M, pH 8.6) at 5°C , using Spectrapor dialysis tubing with a nominal M_r cut-off of 3 kDa, and retained Cd was determined by counting ^{109}Cd radioactivity. To account for possible dissociation of metal, or a decrease in the radioactivity due to binding of ^{109}Cd -MT to the dialysis tubing, controls of untreated ^{109}Cd -MT were always dialysed. All experiments were performed at least in triplicate.

N.m.r. experiments

Samples of 5.3 or 8.5 μmol of ^{113}Cd -MT-II were dissolved in 1.8 ml of Tris/HCl (10 mM, pH 8.6) containing 0.1 M-NaCl and 10% $^2\text{H}_2\text{O}$ as the field lock. Samples in tubes of outer diameter 10 mm were purged with Ar gas at the outset and immediately before and after each addition of $p\text{HOHgBzO}^-$. After a baseline spectrum of starting material, spectra were recorded after additions of reagent in 10 μl portions, each corresponding to one equivalent based on protein thiolate. Additions of reagent in basic solution resulted, over all experiments, in a maximum pH of 9.68.

Spectra were recorded with a Varian XL-200 n.m.r. spectrometer system operating at 44.37 MHz. Chemical shifts are reported as p.p.m. downfield from the ^{113}Cd resonance of 0.1 M- $\text{Cd}(\text{ClO}_4)_2$; referencing was by sample interchange. For the baseline spectrum, 25000 transients were accumulated by using a 10 μs (45°) pulse, with a pulse-repetition interval of 2.5 s that included a 2.0 s relaxation delay, sufficient to give fully relaxed spectra (Vařák *et al.*, 1985). Broad-band ^1H decoupling was gated-off during the pulse delay to avoid possible unfavourable n.O.e.s. Later spectra were obtained analogously, with correction for the dilution upon addition of reagent made by appropriately incrementing the number of transients from the value of the baseline spectrum. An exponential line-broadening of 10 Hz has been applied to the spectra shown in Fig. 2.

Miscellaneous methods

Metal content was determined by atomic absorption using a Jarrell Ash 810 spectrophotometer in flame (Cd, Zn) or flameless (Cu) mode. An LKB 1270 Rackgamma γ -radiation counter was used to determine ^{109}Cd , with 70% counting efficiency. Stokes radii (R_0) were determined by gel filtration on a column (0.8 cm $\times$ 50 cm) of Sephadex G-75 eluted with Tris/HCl (10 mM, pH 8.5) using the relationship:

$$R_0 \propto (-\log K_{av.})^{\frac{1}{2}}$$

where the partition coefficient, $K_{av.}$, is given by:

$$K_{av.} = (V_e - V_0)/(V_t - V_0) \quad (\text{McGuinness, 1973})$$

V_e is the elution volume of the protein, V_0 the void volume as determined with Blue Dextran, and V_t the total column volume. The column was calibrated with proteins of known Stokes radii, namely: ribonuclease A, 1.64 nm; cytochrome *c*, 1.56 nm; α -chymotrypsinogen A, 2.09 nm; ovalbumin, 3.05 nm; and bovine serum albumin, 3.55 nm.

RESULTS

Rat liver MT-II enriched in ^{109}Cd contained 5.3 ± 0.1 , 0.8 and 0.2 g-atoms of Cd, Zn and Cu respectively. Reaction of this material with $p\text{HOHgBzO}^-$ results in displacement of ^{109}Cd (Fig. 1). The first equivalent of mercurial displaces 0.4 equiv. of Cd, and little further displacement is seen up to 5 equiv. Atomic absorption shows loss of all Zn at 4 equiv. of $p\text{HOHgBzO}^-$, with a total displacement of 1.3 g-atoms of Cd plus Zn (Table 1). Between 4 and 10 equiv., total displacement increases to 2.9 g-atoms, and above 18 equiv., less than one Cd atom remains bound.

The above displacement experiments were carried out on separate samples of ^{109}Cd -MT-II, with single anaerobic additions of appropriate amounts of mercurial. In contrast, because of the large amount of material needed to obtain the ^{113}Cd n.m.r. spectra, increasing amounts of $p\text{HOHgBzO}^-$ were added to a single sample

Table 1. Metal contents and Stokes radii (R_0) of $p\text{HOHgBzO}^-$ -modified metallothioneins

Metal contents are determined by atomic absorption unless otherwise indicated, and expressed as mol/mol of protein, based on an M_r of 6200. —, value not determined.

Sample	Metal content (mol/mol)			R_0 (nm)
	Cd	Zn	Cu	
(A) Native ^{109}Cd -MT-II	5.4*, 5.2	0.8	0.2	1.64 ± 0.08
+ 1 equiv. of $p\text{HOHgBzO}^-$	5.0*	—	—	—
+ 4 equiv. of $p\text{HOHgBzO}^-$	4.7*	0	—	—
+ 10 equiv. of $p\text{HOHgBzO}^-$	3.1*	—	—	> 3.5
+ 18 equiv. of $p\text{HOHgBzO}^-$	1.0*	—	—	—
+ 36 equiv. of $p\text{HOHgBzO}^-$	0.55*	—	—	2.11 ± 0.02
(B) Native ^{113}Cd -MT-II, first preparation	5.3	0.6	0	—
(C) Native ^{113}Cd -MT-II, second preparation	4.3	1.7	0	—
+ 9 equiv. of $p\text{HOHgBzO}^-$	3.7	0.7	—	> 3.5

* Determined from specific radioactivity of bound ^{109}Cd .

Fig. 1. Decrease in bound ^{109}Cd upon reaction of ^{109}Cd -MT-II with different amounts of $p\text{HOHgBzO}^-$

Values are means $\pm$ s.d. determined by dialysis of the reaction mixture. Details are given in the Materials and methods section.

Fig. 2. ^{113}Cd -n.m.r. spectra of ^{113}Cd -MT-II after anaerobic stepwise addition of increasing amounts of $p\text{HOHgBzO}^-$

Spectra in order from the bottom represent starting material, then 0.9, 1.8, 2.7, 3.6, 4.5, 5.4, 6.3 and 8.1 equiv. of reagent. Numbered arrows indicate peak assignments used in the text.

of ^{113}Cd -MT-II in a stepwise manner, with accumulation of spectra between additions. Two samples of ^{113}Cd -MT gave similar spectra and comparable results in displacement experiments, despite somewhat different metal compositions (Table 1). The increase of 0.8 pH unit over the course of the experiment, due to addition of $p\text{HOHgBzO}^-$ solution, does not affect the interpretation of the spectra, which were found to be pH-independent over this range.

Stepwise addition of $p\text{HOHgBzO}^-$ results in stepwise displacement of ^{113}Cd , as determined from the decreasing integrated intensity of the n.m.r. signals (Figs. 2 and 3).

Fig. 3. Decrease in integrated intensity of assigned ^{113}Cd -n.m.r. peaks upon addition of $p\text{HOHgBzO}^-$ to ^{113}Cd -MT-II

Assignments are as in Fig. 2, and are indicated on each curve. The corresponding points are: $\circ$, peak 1; $\bullet$, peak 2; $\square$, peak 3; $\blacksquare$, peak 4; $\triangle$, peaks 5+6; $\blacktriangle$, peaks 7+7'. Peaks 5 and 6 are not well separated and so are integrated together.

Addition of the first equivalent causes an approximately equal decrease (of about 15%) in all ^{113}Cd signals. Subsequent addition of up to 4 equiv. causes a more rapid decrease in the intensities of signals 2, 3 and 4 than in all other resonances. Peaks 2 and 3 are obliterated with 4 equiv. of $p\text{HOHgBzO}^-$, whereas peaks 1 and 5+6 are still at 55% and 70% of their original intensities respectively. Whereas peak 4 disappears after 6 equiv., peaks 1, 5, 6 and 7 remain up to more than 8 equiv. Peaks 7 and 7' disappear more quickly than others of the four-metal cluster; 9 equiv. obliterate the entire ^{113}Cd spectrum.

After completion of the above stepwise additions, half the added Hg was dialysable, and 4.4 g-atoms of Cd and Zn remained bound to the MT (Table 1). A Stokes radius of more than 3.5 nm was found for this modified MT, compared with a value of 1.6 nm for native MT-II. Polymerization of MT could accompany exposure of thiol groups by stepwise displacement of Cd and Zn. To test for this, the Stokes radii were measured after one-step addition of either 10 or 36 equiv. of $p\text{HOHgBzO}^-$ to portions of the same sample of ^{109}Cd -MT used for the dialysis experiment. When 36 equiv. were added, a Stokes radius of 2.1 nm was found, but with only 10 equiv. the value was above 3.5 nm (Table 1).

DISCUSSION

These results are consistent with current proposals of the structure of MT, with independent three- and four-metal tetrahedrally co-ordinated clusters. In this structural context, differential reactivity of classes of thiolates can be discussed. Treatment of MT with Nbs_2 distinguishes between two classes of thiols based on reaction kinetics (Li *et al.*, 1981; Templeton & Cherian, 1984; Templeton *et al.*, 1985). One-third of the thiols react within minutes, whereas reaction of the remaining two-thirds goes to completion over a period of hours. The former represent the bridging thiolates of both clusters, and the latter the terminal thiolates. Upon treatment of MT with $p\text{HOHgBzO}^-$, however, reaction is complete

Scheme 1. One possible mode of reaction of the three-metal cluster of MT with $p\text{HOHgBzO}^-$

The first equivalent of reagent opens the cluster, releases one Cd atom, and exposes three thiols. These subsequently react before further displacement of Cd. See the text for details. Φ represents *p*-benzoate

within the time of mixing. The order of thiol reactivity with $p\text{HOHgBzO}^-$ can be deduced from the stoichiometry of Cd displacement, and from direct observation of bound Cd by ^{113}Cd n.m.r.

We have chosen to perform these experiments on Cd-rich MTs containing 0.6–1.7 g-atoms of Zn. Such material is more comparable in composition with naturally occurring protein than is fully Cd-replaced Cd₇-MT. Its use also circumvents exposure of the protein to very low pH, a procedure that is necessary for production of Cd₇-MT, but which may give rise to material spectrally distinct from freshly isolated MT (Dean *et al.*, 1983). The observed displacement of all Zn before most Cd (Table 1) is consistent with non-random entry of mercurial into the cluster structure (see below). Zn is well known to occur in the three-metal cluster of Cd,Zn-MTs, where it thus gives rise to some heterogeneity with respect to site occupation (Boulanger *et al.*, 1983). Its presence does not jeopardize the utility of ^{113}Cd n.m.r. for monitoring the displacement sequence, since ^{113}Cd signals attributable to all seven metal-binding sites remain after loss of all Zn.

In the dialysis experiments, 4 equiv. of $p\text{HOHgBzO}^-$ can be added with displacement of only 1.3 g-atoms of Cd and Zn. This suggests that the four thiols (two terminal and two bridging) liberated by displacement of the first metal atom all react before displacement of a second metal (Scheme 1); 10 equiv. of $p\text{HOHgBzO}^-$ displace 2.9 metal atoms, as would be expected if the nine thiols of the three-metal cluster were all modified before those of the four-metal cluster. After stepwise addition of 9 equiv., the ^{113}Cd n.m.r. spectrum is completely obliterated, despite more than half the Cd remaining protein bound. This could be due to the creation of multiple chemical environments for Cd upon disruption of the three-metal cluster and/or polymerization.

Polymerization is of course indicated by the large Stokes radius seen on partial modification, and by the slight line-broadening observed at this point in the n.m.r. spectrum. A longer rotational correlation time for the

polymer could lead to a longer spin-lattice relaxation time, T_1 , and saturation of the spectrum (Armitage & Boulanger, 1983) rendering bound Cd n.m.r.-silent. This would mean that integrated intensities are unreliable for quantitative description of Cd displacements. More significant are the initial rates of decrease in signal intensity and the order of the loss of signal.

Resonances 2, 3 and 4 are conventionally attributed to the three-metal cluster, based on homonuclear decoupling experiments (Boulanger *et al.*, 1983). With addition of the second and subsequent equivalents of $p\text{HOHgBzO}^-$, these signals decrease more quickly than the others, and disappear while other spectral features remain. These observations provide direct evidence for the preferential reactivity towards electrophiles of the thiols of the three-metal cluster. If such activity contributes to a general role of MT in detoxification *in vivo*, as has been suggested (Rugstad, 1984), these observations may point to a functional significance of the three-metal cluster. Not unexpectedly, stepwise disruption of the metal co-ordination of MT leads to oxidative polymerization. Such oxidative damage may be reversible by glutathione if it occurs *in vivo* (Thornalley & Vařák, 1985). When a 2-fold excess of $p\text{HOHgBzO}^-$ over thiol is added anaerobically, reagent competes successfully with trace O₂ and/or autooxidation, and displacement of all but 0.5 g-atom of Cd is accompanied by an increase in Stokes radius to 2.1 nm. This agrees closely with the value of 2.08 nm found for the monomeric apoprotein (Phillips, 1983).

Of interest is the behaviour of the ^{113}Cd spectrum upon addition of the first equivalent of $p\text{HOHgBzO}^-$. As expected, about 1 g-atom of metal (Cd and Zn) is displaced in the dialysis experiment, and this is accompanied by a decrease of about 15% in the intensities of all seven Cd resonances. This permits several possible explanations. (i) It could indicate random entry of the first $p\text{HOHgBzO}^-$ molecule into either metal cluster, with at least one thiol group of each metal-binding site of native MT equally reactive towards this reagent. (ii) A subset of 15% of the MT molecules could react

extensively with $p\text{HOHgBzO}^-$, and be rendered n.m.r.-silent before the remainder are modified. (iii) The first thiolate modified could participate directly or indirectly in all Cd-binding sites. This would be difficult to reconcile with the existence of two distinct clusters. With regard to (iii), the insignificant changes observed in the ^{113}Cd chemical shifts could be reconciled by noting that such shifts are quite insensitive to changes in the terminal/bridging nature of attached thiolates (P. A. W. Dean & Vittal, unpublished work). Whatever the explanation, reaction of MT with one equivalent of $p\text{HOHgBzO}^-$ decreases by one the number of metals that can be accommodated in tetrahedral co-ordination. This modified MT then shows preferential reactivity of the thiols thought to belong to the three-metal cluster. More subtle distinctions are also possible. Within the three-metal cluster, one site (resonance 4) is less reactive than the other two, whereas within the four-metal cluster at least one site (resonances 7 and 7') is more reactive than the rest.

The appearance of such specificity only after addition of the first $p\text{HOHgBzO}^-$ molecule suggests that the supposition of distinct co-ordination sites provided by specific thiols in native MT may be an oversimplification. Recently two-dimensional J-resolved ^{113}Cd -n.m.r. spectra have been interpreted to imply the existence of several Cd resonances under each peak of the one-dimensional spectrum (Vašák *et al.*, 1985). This could account for the non-integral signal intensities and multiple resonances seen in the present, as well as previous (Vašák *et al.*, 1985; Otvos & Armitage, 1982), studies. This evidence in part has led Vašák *et al.* (1985) to conclude that MT has a 'flexible structure', with stabilization of several interchangeable Cd-thiolate clusters, possibly resulting from the rapid breaking and reforming of thiolate bridges (Neuhaus *et al.*, 1984; Vašák *et al.*, 1985). Such a model would be consistent with explanation (iii) above. However, whether the thiolate ligands are associated with static metal centres or are involved in intramolecular-exchange reactions, we conclude here that they are to some extent distinguishable in

the extent of their reaction with $p\text{HOHgBzO}^-$. This may relate to the function and reactivity of MT.

This work was supported by the Natural Sciences and Engineering Research Council of Canada and the Medical Research Council of Canada.

REFERENCES

- Armitage, I. M. & Boulanger, Y. (1983) in *NMR of Newly Accessible Nuclei*, vol. 2 (Laszlo, P., ed.), pp. 337–365, Academic Press, New York
- Boulanger, Y., Goodman, C. M., Forte, C. P., Fesik, S. W. & Armitage, I. M. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1501–1505
- Boyer, P. D. (1954) *J. Am. Chem. Soc.* **76**, 4331–4337
- Dean, P. A. W., Law, A. Y. C., Szymanska, J. A. & Stillman, M. J. (1983) *Inorg. Chim. Acta* **78**, 275–279
- Endresen, L., Bakka, A. & Rugstad, H. E. (1981) *Cancer Res.* **43**, 2918–2926
- Kojima, Y. & Kägi, J. H. R. (1978) *Trends Biochem. Sci.* **3**, 90–94
- Li, T. Y., Minkel, D. T., Shaw, C. F. & Petering, D. H. (1981) *Biochem. J.* **193**, 441–446
- McGuinness, E. T. (1973) *J. Chem. Educ.* **50**, 826–830
- Neuhaus, D., Wagner, G., Vašák, M., Kägi, J. H. R. & Wüthrich, K. (1984) *Eur. J. Biochem.* **143**, 659–667
- Nordberg, M. & Kojima, Y. (1979) in *Metallothionein* (Kägi, J. H. R. & Nordberg, M., eds.), pp. 41–116, Birkhäuser, Basel
- Otvos, J. D. & Armitage, I. M. (1982) in *Biochemical Structure Determination by NMR* (Bothner-By, A. A., Glickson, J. D. & Sykes, B. D., eds.), chapter 4, Marcel Dekker, New York
- Phillips, J. L. (1983) *Biochem. Biophys. Res. Commun.* **114**, 998–1000
- Rugstad, A. E. (1984) *Eur. Surg. Res.* **16**, (Supple. 2), 102–112
- Templeton, D. M. & Cherian, M. G. (1984) *Biochem. J.* **221**, 569–575
- Templeton, D. M. & Cherian, M. G. (1985) *Toxicol. Lett.* **25**, 279–286
- Templeton, D. M., Banerjee, D. & Cherian, M. G. (1985) *Can. J. Biochem. Cell Biol.* **63**, 16–22
- Thornalley, P. J. & Vašák, M. (1985) *Biochim. Biophys. Acta* **827**, 36–44
- Vašák, M., Hawkes, G. E., Nicholson, J. K. & Sadler, P. J. (1985) *Biochemistry* **24**, 740–747

Received 28 August 1985/1 November 1985; accepted 14 November 1985