CD_2Cl_2, -85 °C) δ 85.6, 81.4, 31.1, 30.6, 23.1, 22.6, 1.1; ^{27}Al NMR (78.2 MHz, CH_2Cl_2 containing 10% CD_2Cl_2, 30 °C) δ 99 ($w_{1/2}$ = 200 Hz), 91 ($w_{1/2}$ = 800 Hz), 52. Anal. Calcd for $C_9H_{19}Al_2Cl_5O_2Si$: C, 25.83; H, 4.58. Found: C, 24.81; H, 4.99.

X-ray Crystallographic Study of the Chloroaluminum Alkoxide 3. Crystallographic data and experimental parameters are summarized in Table I. A single crystal of compound **3** was mounted quickly in air on an Enraf-Nonius CAD4 diffractometer. Unit cell parameters were determined from 25 well-centered reflections in the range $10° < \theta < 11°$. The structure was solved by using direct methods (SHELXS86) and difference Fourier calculations (SHELX76).[8] Full-matrix least-squares refinement converged at $R = 0.031$ and $R_w = 0.036$. All non-hydrogen atoms were refined anisotropically. All hydrogen atoms were found by difference-Fourier synthesis and/or were calculated at idealized positions. The final ΔF map showed three peaks of 0.28-0.58 e Å^{-3} within 0.95 Å of Cl, and the general background was below ±0.31 e Å^{-3}. Scattering curves for the non-hydrogen atoms[9] and hydrogen atoms[10] were taken from standard sources.

Disorder was introduced to account for the large anisotropic thermal parameters for C(1), C(2), C(4), and C(5), and for the short C(1)-C(2) distance of 1.37 Å found at the end of the initial refinement. Occupancy was refined as 65% for the structure shown in Figure 1 and 35% for the closely related structure shown in Figure 2 of the supplementary material. The major occupant is derived from (1*R*,2*R*)-1,2-cyclohexanediol, whereas the minor occupant is derived from the (1*S*,2*S*) isomer.

Atomic coordinates and isotropic thermal parameters for both structures are listed in Table II, and selected bond lengths and angles are compiled in Table III. Tables of complete bond lengths and angles, anisotropic thermal parameters, fixed hydrogen atom coordinates, and observed and calculated structure factors are included as supplementary material.

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Supplementary Material Available: A figure (Figure 2) showing the minor occupant of the unit cell and tables containing complete bond lengths and angles, anisotropic thermal parameters, and fixed hydrogen atom coordinates (6 pages); a table of observed and calculated structure factors (13 pages). Ordering information is given on any current masthead page.

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On the Rapid, Monophasic Reaction of the Rabbit Liver Metallothionein α-Domain with 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB)

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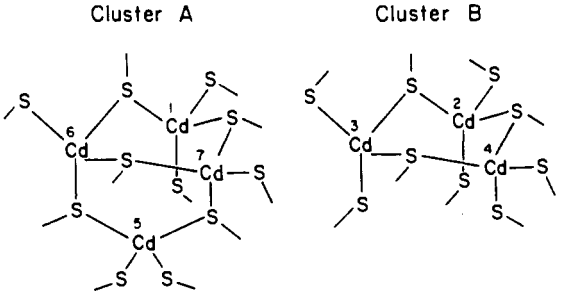
Introduction

Since its detection in 1957, metallothionein (MT) has been the subject of a great deal of research. Although the structure and composition of mammalian MTs are now well understood,[1] their chemical reactivity and the relationship of reactivity to function are just now being explored.[2] MT is a low molecular weight protein (around 6800) containing 61 amino acids, of which 20 are cysteines. These highly conserved cysteines form metal-thiolate complexes with essential metals (such as Zn and Cu), toxic metals (Cd, Hg), and therapeutic metals (Au, Pt). Thus, MT appears to have significant roles in metal storage and transfer and in the detoxification of toxic metals.

In mammalian MTs, 7 equiv of Zn(II) or Cd(II) are bound and each metal ion is tetrahedrally coordinated to four thiolate ligands. Homonuclear ^{113}Cd decoupling experiments deduced that MT contains two distinct metal-thiolate clusters, one (cluster A) containing four and the other (cluster B) containing three Cd(II) ions.[3] The models proposed from these studies are shown below. Their validity is also supported chemically from the observation that under appropriate conditions subtilisin can be used to isolate the two fragments.[4] The β-domain consists of the amino terminal fragment from residues 1-30 containing 9 cysteines and binding 3 metals (cluster B), and the α-domain consists of the carboxyl terminal fragment from residues 31-61 containing 11 cysteines and binding 4 metals (cluster A). The isolated α-domain gave an ^{113}Cd NMR spectrum similar to that of cluster A in uncleaved MT.[5]



In an effort to relate the inorganic chemistry of MT to its biological function(s) and role(s) in cells, reactions of MT with a variety of ligands were studied.[2,6,7] Among them DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) was studied extensively.[6,8] This electrophile undergoes slow thiol-disulfide interchange with the metal-coordinated thiols of MT, releasing the chromophore 5-thio-2-nitrobenzoate, TNB (λ_{max} = 412 nm). The reaction kinetics are biphasic with fast and slow steps, each displaying first- and second-order components.[6] One postulated explanation is that this behavior is due to differential reactivity of the two clusters, which predicts that isolated clusters will display cooperative, monophasic kinetics. Another possibility is that the terminal and bridging thiolates might react with distinctly different rates of reaction, which predicts that each cluster should react biphasically. In order to test these mechanistic possibilities, we isolated the α-Cd_4MT-II cluster and examined its reaction with DTNB.

Materials and Methods

Materials. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB), Trizma base, subtilisin, and Sephadex G-50 were purchased from Sigma. Ammonium bicarbonate was purchased from Aldrich. Zn_7MT-II was obtained from rabbit liver as described elsewhere.[9]

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Preparation and Characterization of the α-Cluster. α-Cd_4MT-II was prepared by using slight modifications of the methods described by Winge[4] and later by Stillman.[10] Zn_7MT-II was reacted with 4.5 mol equiv of $CdCl_2$ and a slight excess of EDTA under Ar atmosphere at room temperature. Ammonium bicarbonate buffer (10 mM, pH 7.5) was used throughout the isolation process. After 1.5 h of incubation, subtilisin was added to the solution in a 20:1 protein to enzyme ratio, and the mixture was incubated for 18 h under Ar atmosphere at room temperature. A precalibrated Sephadex G-50 column (1.5 cm × 120 cm) was used to separate the cluster from the other smaller digestion fragments and from the EDTA complexes of Zn and Cd ions. The verification of the α-cluster was accomplished by gel chromatography, polyacrylamide gel electrophoresis, and amino acid analysis. Amino acid analysis was performed at the Medical College of Wisconsin.

Kinetic Studies. The solutions of α-Cd_4MT-II and DTNB were mixed in the UV cell at time zero and immediately placed in a Beckman DU-70 spectrophotometer. Temperature was maintained at 25 °C by a circulating thermostat. The absorbance change at 412 nm (λ_{abs} of 5-thio-2-nitrobenzoate, a reaction product) was measured over time against an equivalent amount of DTNB as reference. Reactions were carried out under pseudo-first-order conditions ([DTNB] ≫ [MT Thiol]) in 5 mM Tris-HCl buffer with 0.1 M KCl at pH 7.4. DTNB concentrations ranged from 0.5 to 6 mM, and α-Cd_4MT-II concentrations measured as Cd were kept constant at 20 μM (57 μM SH). Pseudo-first-order plots were obtained by plotting ln (A_∞ - A_t) against time. Reactions were carried out at two additional temperatures, 5 and 50 °C, keeping all the other conditions the same. $\Delta H^\ddagger$ and $\Delta S^\ddagger$ were calculated for both k_1 and k_2 from a plot of ln (k/T) vs $1/T$. Reactions were also carried out at pH 6.8.

Results

The α-Cd_4MT-II cluster was prepared from Zn_7Mt-II by using Cd^{+2}, EDTA, and subtilisin as previously described. It was readily separated from traces of undigested M_7MT-II and the low molecular weight digestion products by gel-exclusion chromatography. Confirmation of the α-cluster formation was obtained by polyacrylamide gel electrophoresis. As previously reported,[4] the α-cluster is relatively immobile compared to the native protein due to the less negative charge of the cluster. Amino acid analysis also confirmed the isolation of the cluster. The analytically determined amino acid content agrees with those predicted from the published sequence[11,12] for MT-IIa and MT-IIb, which are given in parentheses (in some cases as a range due to different amino acid compositions of each subisoform): Cys, 10.0 (11); Asx, 1.3 (1); Thr, 0.6 (0–1); Ser, 4.0 (5); Glx, 1.7 (1); Pro, 2.3 (1–2); Gly, 3.6 (3); Ala, 4.5 (4–5); Val, 0.6 (0); Ile, 1.4 (1); Leu, 0.8 (0); Lys, 4.9 (5); Met, 0 (0); Arg, 0 (0); Phe, 0 (0); Tyr, 0 (0); Try, 0 (0); His, 0 (0). The absence of Met, which is present only in the β-domain, and also the absence of aromatic amino acids such as His, Tyr, Try, and Phe, which are not present in MT, provide strong evidence that the α-cluster was isolated in pure form. Recent work by Otvos and Liu[13] established that rabbit liver MT-II consists of 3 isoproteins MT-IIa, MT-IIb, and MT-IIc, which differ slightly in their amino acid composition and which change abundance with the age of the animal. Thus, the small discrepancies between our results and the published sequences are not unexpected.

The DTNB reactions were carried out under pseudo-first-order conditions (excess DTNB) at 25 °C and pH 7.4. All the reactions went to completion within 45 min and went to completion more quickly at high DTNB concentrations. Typical absorbance and first-order plots for three reactions of α-Cd_4MT (57 μM SH) with DTNB concentrations ranging from 0.5 to 5 mM are shown in Figure 1. The total absorbance change ($A_\infty - A_0$) is similar for all the runs, as demonstrated by the common intercept, ln ($A_\infty - A_0$), at $t = 0$. The value of ($A_\infty - A_0$) is determined by the total amount of TNB generated, and the concentration can be calculated by using the extinction coefficient, ϵ_{412} = 13 600 L/(mol cm). The average value of the TNB/Cd ratio obtained for seven reactions, 2.69 ± 0.23, is in good agreement with the ratio of 2.75 calculated

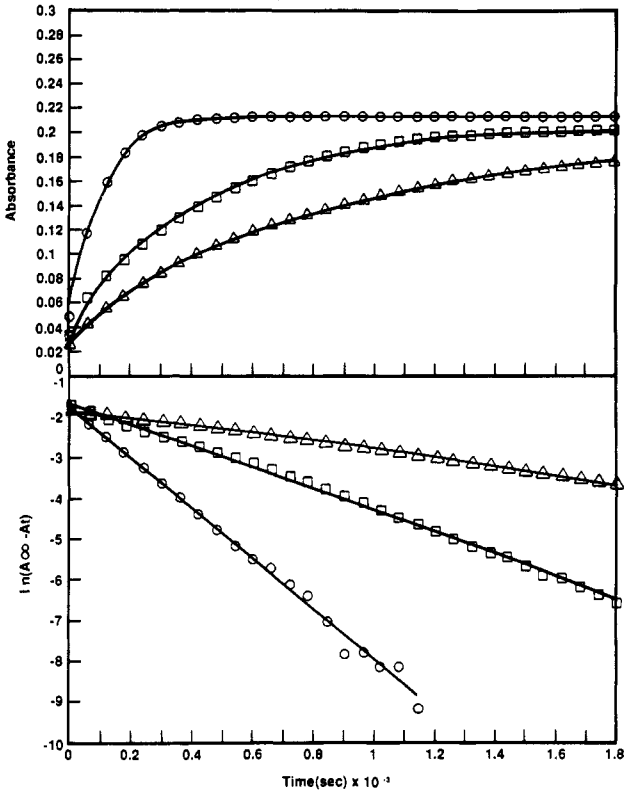

Figure 1. Absorbance at 412 nm (upper panel) and first-order plots (lower panel) vs time for the reaction of DTNB with α-Cd_4MT-II (57 μM SH) in 5 mM Tris-HCl/0.1 M KCl at pH 7.4 and 25 °C. DTNB = 0.5 mM (△), 1 mM (□), and 5 mM (○).

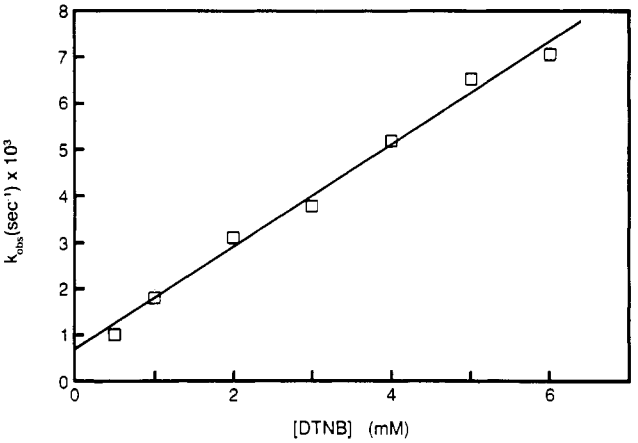

Figure 2. [DTNB] dependence of the observed rate constants, k_{obs}, for the reaction between α-Cd_4MT-II and DTNB.

Table I. Observed Rate Constants (ESDs) for Reactions of Cd_7MT and α-Cd_4MT (2.0×10^{-5} M in Cd) with DTNB in 5 mM Tris/HCl + 100 mM KCl and at 25 °C

	pH	10^3[DTNB], M	$10^3 k_{fast}$, s^{-1}	$10^4 k_{slow}$, s^{-1}
α-Cd_4MT	7.4	0.5	1.01 (0.19)	
	7.4	1.0	1.82 (0.08)	
	7.4	2.0	3.11 (0.07)	
	7.4	3.0	3.80 (0.08)	
	7.4	4.0	5.21 (0.84)	
	7.4	5.0	6.54 (0.48)	
	7.4	6.0	7.06 (0.86)	
	6.8	1.0	2.35 (0.04)	
	6.8	2.0	3.76 (0.06)	
Cd_7MT	7.4	2.0	3.11 (0.36)	6.40 (0.65)

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Table II. First- and Second-Order Rate Constants for the Reactions of Cd_7MT and α-Cd_4MT with DTNB at pH 7.4, with Reaction Conditions as in Table I

	T, °C	k_{2f}, M^{-1} s^{-1}	10^3k_{1f}, s^{-1}	k_{2s}, M^{-1} s^{-1}	10^3k_{1s}, s^{-1}
α-Cd_4MT	5	0.17	0.17		
Cd_7MT[a]	5	0.26	0.15	0.06	0.22
α-Cd_4MT	25	1.12	0.64		
Cd_7MT	25	1.75	1.26	0.12	0.42
α-Cd_4MT	50	2.53	6.91		
Cd_7MT	50	8.69	3.95	2.33	1.53

[a] The reaction of Cd_7MT with DTNB at 5 °C does not generate the net absorbance change at 412 nm predicted for a complete reaction unless anaerobic conditions are employed. The rate of autooxidation of the released TNB to regenerate DTNB is apparently comparable to the slow phase of the DTNB reaction at this temperature. The effect of aerobic reoxidation is not observed in reactions of the α-cluster, which are governed by the larger rate constant.

from the composition of the α-domain, Cd_4S_{11}. These and also other reactions studied are monophasic and first-order for at least 3–4 half-lives. The observed rate constants obtained for each DTNB concentration are presented in Table I.

The observed rate constants are clearly DTNB dependent (Figure 1) and the plot of k_{obs} vs [DTNB] shown in Figure 2 establishes a linear relationship over the range 0.5–6 mM DTNB. The intercept is finite, indicating that there are both DTNB-dependent and DTNB-independent components to the reaction. Thus, the rate law for the reaction of the α-Cd_4S_{11} cluster is

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

The value of the intercept is the first-order rate constant, $k_1 = 6.4 \times 10^{-4}$ s^{-1}, for the DTNB-independent process. The slope is the second-order rate constant for the DTNB-dependent process, $k_2 = 1.12$ s^{-1} M^{-1}.

The reactions of the intact Cd_7MT protein were repeated at pH 7.4 for comparison with the cluster reaction. The values obtained ($k_{1f} = 1.26 \times 10^{-3}$ s^{-1}, $k_{2f} = 1.55$ s^{-1} M^{-1}, $k_{1s} = 0.42 \times 10^{-3}$ s^{-1}, $k_{2s} = 0.12$ s^{-1} M^{-1}) are of the same order of magnitude and are similar to, but not identical with, those obtained at pH 7.0 in our previous study.[6]

The reactions of the α-Cd_4MT cluster were also carried out at pH 6.8 in addition to pH 7.4, to determine the effect of pH on the reactions. At pH 6.8, the rate constants for the cluster and the holo-protein were very close to the respective constants measured at pH 7.4. This establishes, as one would expect from consideration of the α-cluster amino acid side chains and DTNB pK_a values, that there are no protonatable groups which affect these results in this pH range.

Results of temperature dependence studies are collected in Table II. Plots of ln (k/T) vs $1/T$ where k is k_1 and k_2 yielded ΔH^* and ΔS^*. ΔH^* for both k_1 and k_2 were calculated from the slopes as 59 and 42 kJ/mol respectively. ΔS^* for both k_1 and k_2 were calculated from the intercepts as −105.3 and −106.7 J/(mol K), respectively. These activation parameters indicate that ΔH^* for the dissociative step (k_1) is higher than for the associative step (k_2). This result can be interpreted as additional energy required for the dissociative step.[14] However, the similarity of ΔS^* values might mean that a complicated configurational rearrangement is occuring prior to reaction since one might expect a more positive entropy for a dissociative step compared to an associative step.

Discussion

Our previous, detailed study of the reactions of DTNB with Zn and Cd thioneins revealed biphasic reactions, in which each step had DTNB-dependent and DTNB-independent processes.[6] The biphasic reactions could arise from independent reaction of each cluster with DTNB or from fast and slow reactions associated with the terminal and bridging thiolates.[6] The observation of a single reaction phase for the α-cluster is inconsistent with the possibility that the fast and slow phases are associated with the terminal and bridging thiolates, respectively. The observation of only a single reaction phase, despite the fact that 11 cysteine thiolates in the cluster react with DTNB, strongly suggests a cooperative process.

In order to compare the rates of the cluster and the whole protein reactions, the observed fast and slow step rate constants for the reaction of Cd_7MT with 2 mM DTNB are included in Table I. The reaction of the cluster ($k_{obs} = 3.11 \times 10^{-3}$ s^{-1} at 2 mM DTNB and at pH 7.4) corresponds to the fast step of the whole protein ($k_{obs,f} = 3.11 \times 10^{-3}$ s^{-1}) and is an order of magnitude greater than the slow step ($k_{obs,s} = 6.40 \times 10^{-4}$ s^{-1}). The similarity of the cluster rate constants to those of the fast-step rate constants of the holo-protein implicates the α-cluster as the more labile entity in reacting with DTNB. (While it is remotely possible that removal of the β-domain accelerates the slow reaction to exactly the rate of the fast reaction, this possibility seems unlikely.)

The X-ray crystal structure determination[15] supports an assignment of the α-cluster DTNB rate constants to correspond with the fast step of the holo-protein reaction. First, the domains are clearly independent with minimal interaction that might affect the rates of either domain.[15] Second, the two sulfur atoms with the largest solvent accessibility areas are the terminal thiolates of Cys-33 (17.1 Å^2) and Cys-36 (15.5 Å^2) in the α-domain. The remaining cysteine sulfurs have areas ranging from 13.85 to 0.[15]

In other reactions, the three-metal cluster is more labile than the four-metal cluster. For example, iodoacetamide, a neutral electrophile, appears to react preferentially at the three-metal cluster of reconstituted Cd_7MT.[8] It should be noted that only the reactions of fully metallated Cd_7MT in ref 8 can be compared to this study. The reactivity of the unsaturated protein is determined by free thiols, not the intact clusters. The three Cd^{2+} ions in the β-domain exchange sites very rapidly, which suggests a greater lability.[16] The transition states for these reactions, especially the latter, are likely to be very different from that for the DTNB reaction. The fact that the Cd^{2+} site-exchange process is several orders of magnitude faster than the DTNB reaction suggests that very different chemical processes are involved. In any event, the finding that the α-cluster reacts monophasically and rapidly with DTNB should not be extrapolated to other ligands and electrophiles that react with metallothionein, since their reactions may involve very different mechanisms and rate-determining transition states.

The two-cluster structure of metallothionein[3-5] raises the intriguing possibility that each cluster may have a unique biological function. Two-dimensional NMR studies of Cd_7MTs from various sources verify that the ligation of the β-cluster involves cysteines from the N-terminal end of the peptide and the α-cluster involves cysteins from the C-terminal end.[17] If the clusters have different functions, one might expect that each will react independently of the other. The observation here that the four-metal cluster reacts with DTNB at a single rate that is similar to one of the two phases of reaction of the holo protein provides strong evidence for independent reactions of each cluster with this reagent.

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Registry No. DTNB, 69-78-3.

(14) The ΔH^* and ΔS^* values were added at the suggestion of a reviewer and we were glad to comply. They do not, however, address the central question posed here, which is whether one phase of the biphasic reaction of MT with DTNB can be associated with a single cluster. They will be useful for addressing the mechanistic details of how DTNB reacts with specific thiolates in the cluster, which will be addressed in a future study.

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