cedure compared well with those determined by ^{1}H NMR analysis.[28] Complete tables of atomic coordinates, bond distances and angles, and anisotropic displacement parameters and ORTEP drawings for all structures are given in the supplementary material.

Acknowledgment. This work was supported by the National Science Foundation (Grant CHE-9007512). G.P. is the recipient of an A. P. Sloan Research Fellowship (1991-1993) and a Camille and Henry Dreyfus Teacher-Scholar Award (1991-1996). We thank Professor R. E. Marsh and the reviewers for very helpful comments.

Registry No. $\{\eta^3$-HB(3-Bu^{t}pz)$_3\}$ZnCN, 127623-01-2.

Supplementary Material Available: Tables SI-SXXX, giving crystal and intensity collection data, atomic coordinates, bond distances and angles, and anisotropic displacement parameters, and ORTEP drawings for all structures (35 pages); listings of calculated and observed structure factors (50 pages). Ordering information is given on any current masthead page.

(28) $\{\eta^3$-HB(3-Bu^{t}pz)$_3\}$Zn(CN)$_x$Cl$_{1-x}$: x(NMR) = 0.80, x(X-ray) = 0.76. $\{\eta^3$-HB(3-Bu^{t}pz)$_3\}$Zn(CN)$_x$Br$_{1-x}$: x(NMR) = 0.95, x(X-ray) = 0.96. $\{\eta^3$-HB(3-Bu^{t}pz)$_3\}$Zn(CN)$_x$Br$_{1-x}$: x(NMR) = 0.55, x(X-ray) = 0.56. $\{\eta^3$-HB(3-Bu^{t}pz)$_3\}$Zn(CN)$_x$I$_{1-x}$: x(NMR) = 0.90, x(X-ray) = 0.91.

Contribution from the Department of Chemistry and the UWM-NIEHS Aquatic and Marine Biomedical Core Center, University of Wisconsin—Milwaukee, P.O. Box 413, Milwaukee, Wisconsin 53201

Biphasic Reactions of DTNB with Lobster Cd_6- and Cd_5Cu-Metallothionein-2 Which Have Two Type-B (M_3S_9) Clusters

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Lobster metallothionein (MT), which has two three-metal clusters instead of the four-metal and three-metal clusters found in mammalian MTs, was used to determine the origin of the biphasic reactions of MT with DTNB. Cd-rich MTs (Cd_5Cu-MT-2 and Cd_6-MT-2) were isolated from lobster hepatopancreas after Cd treatment preceded by dexamethasone injection or an applied stress. Each lobster MT-2 preparation reacts biphasically with DTNB [5,5′-dithiobis(2-nitrobenzoic acid)], as do mammalian MTs, and the reaction proceeds about 1 order of magnitude faster than that of mammalian MTs. The slow step and fast step each have first- and second-order components, resulting in a four-term rate law: rate = $k_{1s} + k_{2s}$[DTNB] + $k_{1f} + k_{2f}$[DTNB]. At 25 °C, in 5 mM Tris/HCl buffer with 100 mM KCl, at pH 7.4, the rate constants are $k_{1s} = 1.34 \times 10^{-3}$ s^{-1}, $k_{2s} = 0.706$ s^{-1} M^{-1}, $k_{1f} = 3.23 \times 10^{-3}$ s^{-1}, $k_{2f} = 2.92$ s^{-1} M^{-1} for Cd_5Cu-MT-2; $k_{1s} = 1.23 \times 10^{-3}$ s^{-1}, $k_{2s} = 0.663$ s^{-1} M^{-1}, $k_{1f} = 2.27 \times 10^{-3}$ s^{-1}, $k_{2f} = 8.13$ s^{-1} M^{-1} for Cd_6-MT-2. The biphasic nature of these reactions establish that the MT amino acid sequences, and not the structures of the individual clusters, determine the reactivity toward DTNB. Comparison of the lobster MT sequence with those for mammalian MTs leads to the prediction that the clusters of crustacean MTs are also located in separate domains. Consistent with this prediction, the presence of a single copper reduces the rate of the fast step, but does not alter the slow step for lobster MT-2.

Metallothionein (MT)[1] is a small protein which is rich in highly-conserved cysteine residues and able to bind various metal ions including Zn(II), Cd(II), Au(I), Ag(I), Cu(I), Hg(II), and Pt(II). Interest in MT has stimulated intensive research over the last several decades.[1-3] It is believed that MT plays an important role in metal ion metabolism.[4-8] A great deal of research has been done on the elucidation of the structure of this protein. Homonuclear ^{113}Cd decoupling studies[9] revealed the existence of two clusters, type A and type B, able to bind four and three metal ions, respectively. These ions are tetrahedrally coordinated by terminal and bridging thiolates from the cysteine residues of the protein. Further studies revealed that each cluster was in a separate domain of the protein (M_4S_{11} in the α-domain and M_3S_9 in the β-domain).[10] Subsequent 2-D NMR and X-ray studies confirmed this conclusion.[11-13] However, much less has been done to characterize the reactivity and putative functions of MT in terms of its unique structure. In order to fully characterize this protein, the linkage between structure and reactivity must be determined.

Examining the kinetics of bioinorganic reactions between MT and various ligands[1,14] and electrophiles[15-18] has enlarged our understanding of the reactivity of MT. The well-studied reactions of mammalian MTs with DTNB,[15,17] which attacks the metal-bound thiolates, show biphasic kinetics. Possible origins might include differential reactivity of the two domains or different rates for terminal and bridging thiolates. Any differences in reactivity of the domains might be intrinsic to the two metal-thiolate clusters or imposed by the protein chain. Kinetic studies of the reaction of rabbit liver MT α-cluster domain[17] narrowed the number of possibilities by eliminating the differences in the reactivity of bridging and terminal thiolates' MT as a possible cause. Whether the two clusters themselves or the sequences surrounding them cause the biphasic kinetics remain unanswered.

MTs from marine crustaceans[6,7,19-22] will provide important

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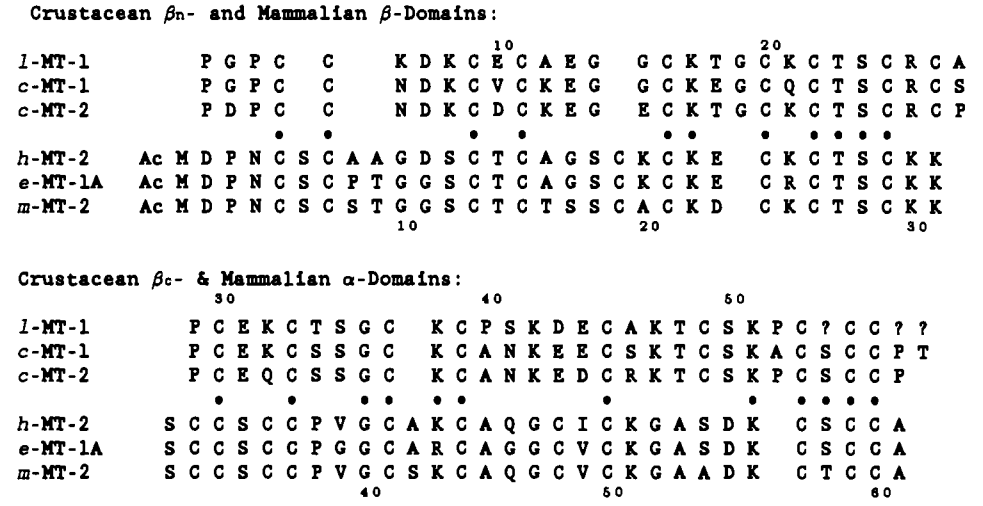

Figure 1. Amino acid sequences for lobster (l-MT-1),[22] crab (*Scylla serrata*, c-MT-1 and -2),[20] human (h-MT-2),[35] equine (e-MT-1A),[36] and mouse (m-MT-2)[37] (A = ALA; Ac = acetyl; C = CYS; D = ASP; E = GLU, G = GLY; I = ILE; K = LYS; M = MET; Q = GLN; P = PRO; R = ARG; S = SER; T = THR; V = VAL). The numbers above/below are sequence numbers for the crustacean/mammalian MTs. Note the exact conservation of the 18/20 cysteines in the crustacean/mammalian sequences and the close homology (●) of cysteines and certain lysines, threonines, and serines between the two types of MTs.

insights into the reactivity of the two MT domains and their clusters. MT-1 and MT-2 from the crab *Scylla serrata*[20,21] and MT-1 from the American lobster (*Homarus americanus*)[22] have been sequenced and found to contain 18 cysteines (Figure 1). The ^{113}Cd NMR spectra of crab MT-1 and -2 demonstrate that they contain two three-metal clusters ($\beta1$ and $\beta2$) instead of four- and three-metal (α and β) clusters of mammalian MTs.[21] The cluster structures are shown in Figure 6 of the Appendix. Lobsters are readily available, both seasonally and geographically, are an easily maintained laboratory species, and generate copious amounts of MT in their hepatopancreases.[22-24] The sequence homology,[22] including the exact conservation of the cysteine residues between the lobster MT-1 and crab MTs (Figure 1), indicates that lobster MTs (l-MTs) also have the 18-cysteine structure and, thus, will have the same metal-cluster structures as crab MTs. Since l-MTs have two distinct clusters, as do mammalian MTs (m-MTs), they are potentially important model systems for structure–function studies of metallothioniens. Because the lobster clusters are both three-metal (type B) compared to one four-metal (type A) and one three-metal (type B) in human and mammalian MTs, one can formulate specific, testable hypotheses about comparative structure–reactivity relationships for the two types of MTs.

We report in this paper isolation procedures for lobster MT-2 preparations with the metal content Cd_5Cu-MT-2 and Cd_6-MT-2, its amino acid composition, and detailed kinetic studies of their reactions with DTNB. The last are used to address the question of the origin of the biphasic kinetics. In the Appendix, we present arguments, based on the amino acid sequences of crustacean MTs, that their two type-B clusters are located in specific domains, which we designate β_n and β_c (according to the ends of the protein chain).

Experimental Section

Materials. Sephadexes G-75, G-25, and G-10, DEAE Sephadex A-25-120, Trizma base, and β-mercaptoethanol all were purchased from Sigma Biochemicals; (phenylmethyl)sulfonyl fluoride (PMSF) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), from Aldrich Chemical Co., Inc.

Lobster Husbandry. All lobsters used in these experiments were obtained from reliable commercial vendors and kept in flowing artificial sea water which was recycled after biological filtration and UV light sterilization. Water quality and temperature (15 °C) were maintained within the lobster's optimal range. To induce MT, the lobsters were injected intraperitoneally with a single dose of $CdCl_2$ (3.75 mg of Cd(II)/kg of body weight). During the course of this study it was found that surgical removal of a small walking leg preceding the Cd injection enhanced the yield of Cd-MTs and reduced the amount of copper bound to MT-1 and MT-2. Similar reductions were obtained when a dexamethasone injection (2 mg/kg of body weight) was substituted for the surgical procedure.

Isolation of l-MT-2. Fresh hepatopancreas tissue was removed from lobsters and homogenized in a Brinkman polytron homogenizer at high speed in two volumes of 20 mM Tris/HCl buffer, pH 7.9, containing 2 mM β-mercaptoethanol and 0.5 mM PMSF, and then centrifuged at 30 K for 2 h at 4 °C. The supernatant was directly loaded onto a Sephadex G-75 gel filtration column (5 × 59 cm) and eluted with 20 mM Tris/HCl buffer, pH 7.9, containing 2 mM β-mercaptoethanol. Metals were measured by flame aspiration atomic absorption spectrophotometry. The fractions containing MT were pooled. The MT was bound by DEAE Sephadex A-25 gel and applied to a DEAE Sephadex A-25 column (2.5 × 15 cm). Proteins were eluted with a gradient generated from 1000 mL of 20 mM Tris/HCl and 1000 mL of 300 mM Tris/HCl, both of pH 7.9 and containing 2 mM β-mercaptoethanol. Metals were measured again. The MT fractions were pooled and concentrated by reverse osmosis using an Amicon YM-2 filter, then applied to a Sephadex G-10 desalting column (2.5 × 39 cm), and eluted with 5 mM Tris/HCl buffer, pH 7.5. The desalted proteins were concentrated again and stored in liquid nitrogen until they were used.

Kinetics. The solutions of lobster MT were mixed with DTNB at time zero and immediately placed in a Beckman DU-70 spectrophotometer and maintained at 25 °C by a circulating thermostat. The reaction was followed by measuring absorbance at 412 nm over time against a reference of an equivalent amount of DTNB. The reactions were set up under pseudo-first-order conditions ([DTNB] ≫ [MT Thiol]) in 5 mM Tris/HCl buffer with 0.1 M KCl of pH 7.4. Cd_5Cu-MT-2 measured as Cd and Cu and kept constant at 20 μM, with DTNB concentrations varying from 0.5 to 5.0 mM. Pseudo-first-order plots were obtained by plotting $\ln (A_\infty - A_t)$ vs time. The pH dependence was determined using 2.0 mM DTNB at different pHs, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5, with all other conditions the same. The reactions were also carried out at two additional temperatures, 5 and 50 °C, at pH 7.4. ΔH^* and ΔS^* were calculated from plots of $\ln (k/T)$ vs $1/T$ for the four kinetic terms, k_{1f}, k_{2f}, k_{1s} and k_{2s}, required to determine the rate law.

Results

Lobster MT (l-MT) was induced by Cd with and without an applied stress and isolated from fresh hepatopancreases by two different procedures.[22,25] The distribution of metal ions (Table I) shows that Cd injection only, without stress, induces Cu-rich MTs in lobster hepatopancreases. Pretreating the homogenate by an ethanol/chloroform precipitation procedure did not affect

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Table I. Distribution of Cytosolic Metal Ions after Induction by $CdCl_2$ with and without Stress and Using Various Isolation Procedures[a]

isolation / induction	CH_3Cl/EtOH no stress		chromatography no stress		chromatography declawing		chromatography dexamethasone	
	Cd	Cu	Cd	Cu	Cd	Cu	Cd	Cu
homogenate	44	78	72	247	75	12	141	37
cytosol	29	44	44	173	52	10	91	20
MT fractions[b]	18	33	3.3	21	22	1.6	47	2.7
MT-2[c]					15	1.5	17	<0.2

[a] μg of metal/g of hepatopancreas. [b] Pooled fractions after chromatography over Sephadex G-75. [c] After resolution by DEAE chromatography.

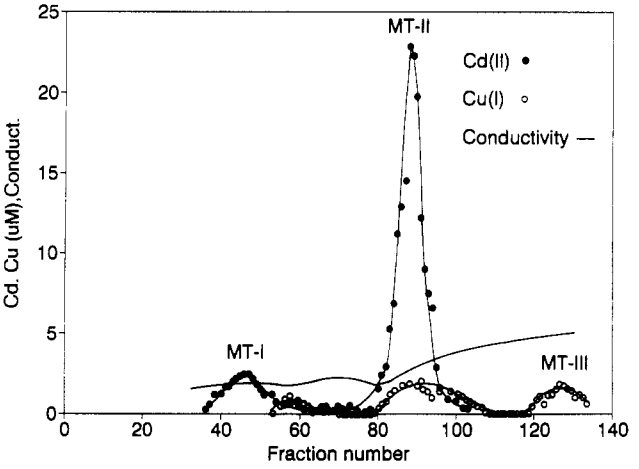

Figure 2. Sephadex DEAE-A-25 elution profile of lobster hepatopancreas cytosolic MTs. A Tris/HCl gradient (20–300 mM), pH 7.9, was used to elute the proteins. MT-1, -2, and -3 appear at fractions 40–55, 90–95, and 120–135, respectively. Key: Cd (●); Cu (○); conductivity (—).

the final copper content of the MTs. Previously, it has been found that MT isolated from lobsters fed with Cd-rich food contained predominately Cu.[23] When stress is applied by surgical procedures or when dexamethasone is injected before $CdCl_2$ administration, Cd-rich MTs are obtained. This result agrees with stress experiment studies on rat.[26] From the stressed lobsters, three MTs were isolated and characterized by standard gel-exclusion and ion-exchange chromatography methods. The predominant Cd species is MT-2 which contains some Cu(I) and no detectable Zn(II) (Figure 2). Cd(II) and Cu(I) are present in the small quantity of MT-1 detected. A species designated MT-3, which has no mammalian counterpart, is predominantly a copper species, and unlike l-MT-1 and l-MT-2, is able to reconstitute apohemocyanin,[22] was also observed here.

Conductivity measurements show that the l-MT-1 and -2 found here correspond to the lobster MT-1 and MT-2 reported by Brouwer et al.[22] The dominant Cd protein, MT-2, was analyzed by DTNB for thiolate content and by AAS for metal content. MT samples with compositions corresponding to Cd_5Cu-MT-2 after surgical stress and Cd_6-MT-2 after dexamethasone injection were selected for the kinetic studies. The amino acid composition of Cd_5Cu-MT-2 (Table II) is consistent with that for a Cu-rich MT-2 previously studied.[22] The high content of cysteine and the absence of Phe, Tyr, His, Trp, and Met residues confirm the isolation of l-MT-2.

The UV spectra of Cd_5Cu-MT-2 and Cd_6-MT-2 (Figure 3) show shoulders at about 250 nm, due to a ligand-to-metal charge-transfer transition typical of all Cd-containing MTs.[27] A broad, weak band due to ligand-to-copper charge transfer is also present at longer wavelengths for Cd_5Cu-MT-2,[27] while the Cd_6-MT-2 spectrum tails off without a new maximum.

After establishing that our metalloprotein was indeed a typical crustacean MT, we next turned to kinetic studies of its reactivity. The reagent selected was 5,5′-dithiobis(2-nitrobenzoic acid), which

Table II. Amino Acid Composition Analysis of Lobster Cd_5Cu-MT-2

AA	this work	ref 22	AA	this work	ref 22
Cys	14[a]	13[a]	Ile	1	0
Asp	5 (4.5)	3.4	Leu	1	0
Thr	4	3	Lys	6	5.8
Ser	5 (4.6)	5	Arg	1	1
Glu	6 (5.5)	4	Phe	0	0
Pro	5	4	Tyr	0	0
Gly	6 (5.6)	6.4	His	0	0
Ala	4	3	Trp	0	0
Val	1	0	Met	0	0

[a] Cys is typically underdetermined.

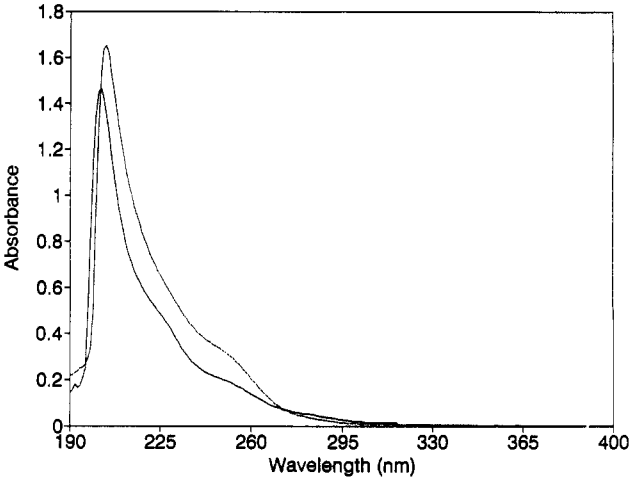

Figure 3. UV spectra of Cd_5Cu-MT-2 (—, [Cd + Cu] = 10 μM) and Cd_6-MT-2 (···, [Cd] = 20 μM). The extended absorbance tail of the Cd_5Cu-MT-2 absorbance is due to the CuS charge-transfer band at ca. 280 nm superimposed on the 250-nm Cd–S charge-transfer band.

is known to react with the thiolates of mammalian MTs in a biphasic reaction[15] and with the isolated α-domain in a monophasic reaction.[17] It also reacts with copper-rich MTs.[28-30] The reactions with lobster Cd_5Cu-MT-2 or Cd_6-MT-2 and DTNB were carried out under the same pseudo-first-order conditions used for mammalian MT[15,17] (at 25 °C and pH 7.4 with [DTNB] ≫ [MT]). DTNB (here more usefully designated ESSE) reacts with the thiolates of the metal clusters to release the chromophore ES^-, detected by its absorbance at 412 nm:

$$M_6S_{18}\text{-MT-2} + 18\text{ESSE} \rightarrow 6M^{n+} + \text{oxidized-apo-MT} + 18ES^-$$

The average values of $ES^-/(Cd + Cu)$ are 3.29 ± 0.17 for Cd_5Cu-MT-2 and 2.94 ± 0.32 for Cd_6-MT-2 and are close to the ratio of 3.00 calculated from the composition of lobster MT-2.

The slowest reactions reach completion within 56 min. The higher the concentration of DTNB used, the quicker the reactions go to completion. The observed rate constants (k_f and k_s) are obtained from plots of ln $(A_\infty - A_t)$ vs time (Figure 4) according to standard kinetic treatments.[31] The reactions of Cd_6-MT-2 and

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Table III. [DTNB] Dependence of the Observed Rate Constants (ESD) for the Reaction of Lobster MT-2 and DTNB[a]

[DTNB]/ mM	Cd_5Cu-MT-2			Cd_6-MT-2		
	$10^3k_s/s^{-1}$	$10^2k_f/s^{-1}$	% slow	$10^3k_s/s^{-1}$	$10^2k_f/s^{-1}$	% slow
0.5	1.55 (0.04)	0.38 (0.01)	43 ± 2	1.33 (0.18)	0.51 (0.09)	75 ± 7
1.0	2.03 (0.04)	0.58 (0.01)	44 ± 3	2.00 (0.23)	1.09 (0.11)	62 ± 1
2.0	2.88 (0.10)	1.03 (0.01)	49 ± 4	2.61 (0.09)	1.87 (0.12)	59 ± 2
3.0	3.69 (0.09)	1.32 (0.03)	48 ± 1	3.39 (0.18)	2.71 (0.08)	59 ± 1
4.0	4.09 (0.24)	1.44 (0.08)	43 ± 2	4.00 (0.34)	3.68 (0.19)	52 ± 3
5.0	4.77 (0.01)	1.72 (0.01)	44 ± 2	4.33 (0.40)	4.10 (0.22)	54 ± 4

[a] $[Cd + Cu]_{MT}$ = 20 μM; pH 7.4 in 5 mM Tris/HCl + 100 mM KCl at 25 °C.

Table IV. pH Dependence of the Observed Rate Constants for Reaction of Cd_5Cu-MT-2 with DTNB[a]

pH	$10^3k_s/s^{-1}$	$10^2k_f/s^{-1}$
6.0	3.33 (0.09)	1.06 (0.03)
6.5	3.51 (0.13)	1.15 (0.09)
7.0	3.39 (0.07)	1.13 (0.04)
7.5	2.88 (0.10)	1.03 (0.01)
8.0	3.27 (0.08)	1.10 (0.03)
8.5	3.34 (0.03)	1.10 (0.03)

[a] $[Cd + Cu]_{MT}$ = 20 μM; [DTNB] = 2 mM; in 5 mM Tris/HCl + 100 mM KCl at 25 °C.

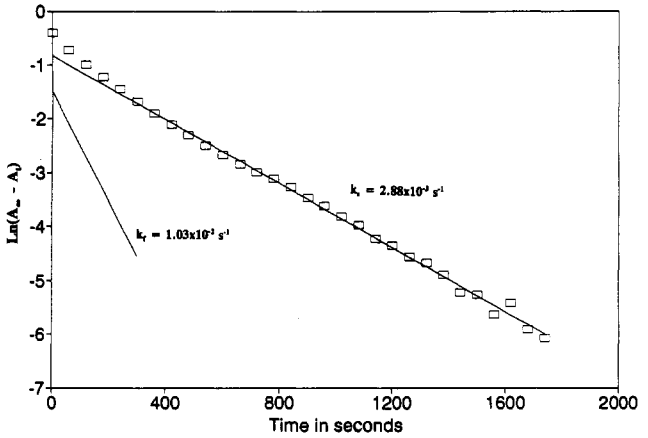

Figure 4. Typical biphasic reaction profile for the DTNB reaction with Cd_5Cu-MT-2. Conditions: [DTNB] = 2 mM; [Cd + Cu] = 20 μM; pH 7.4 in 5 mM Tris/HCl buffer with 100 mM KCl. The slope extrapolated from the latter part of the reaction provides the observed, slow-phase rate constant k_s; the absorbance changes due to the fast component, calculated from the early data points by subtraction of the extrapolated line, are then used to calculate k_f.

Cd_5Cu-MT-2 all show biphasic kinetics, similar to the reactions of mammalian MT-2 with DTNB. *Clearly, since the Cd_6-MT-2 reaction is biphasic, this aspect of the reaction is intrinsic to the protein and cannot be attributed to the presence of copper ion in the case of Cd_5Cu-MT-2.* The rate constants for the lobster protein reactions (Table III) are 1 order of magnitude larger than those for the corresponding mammalian MTs at the same concentrations.[15,17]

The observed rate constants for Cd_5Cu-MT-2 and Cd_6MT-2 are linearly dependent on [DTNB] over the range 0.5–5 mM (Figure 5). The plots for the fast and slow steps each extrapolate to non-zero intercepts, indicating that each consists of first- and second-order components. This yields for lobster MT-2, regardless of whether it contains Cu(I) or not, a four-term rate law:

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

The values of k_{1s}, k_{2s}, and k_{1f} (Table V) are unaffected by the presence of copper, but k_{2f} decreases by a factor of about 2.5 for Cd_5Cu-MT-2. Previous results with DTNB and mammalian MTs established that they react with DTNB according to the same four-term rate law and that the rate constants for Zn-MT, and

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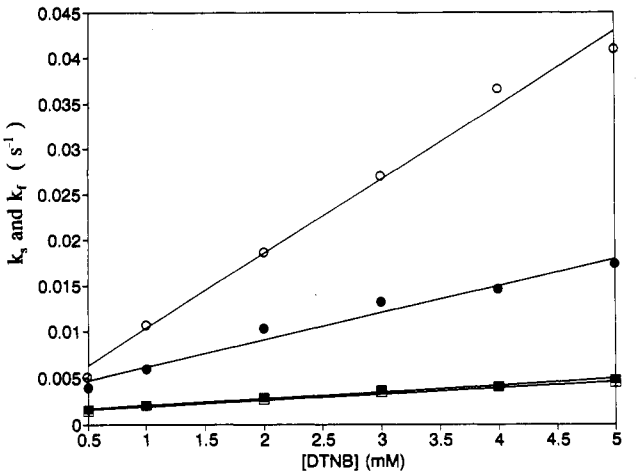

Figure 5. Plots of the observed constants k_f and k_s vs [DTNB] yielding the first- and second-order components of each reaction phase (k_{1f}, k_{2f}, k_{1s}, k_{2s}). The open symbols represent k_f (○) and k_s (□) for Cd_6-MT-2 and the closed symbols, k_f (●) and k_s (■) for Cd_5Cu-MT-2. The slow step rates are unaffected by the presence of Cu(I), but the associative component of the fast step for Cd_5Cu-MT-2 is reduced by a factor of ca. 2.5 compared to that of Cd_6-MT-2. This change is far less than the estimated differences of several orders of magnitude between the Cu(I) and Cd(II) binding constants.

Table V. Rate Constants and Activation Enthalpies and Entropies of Lobster and Rabbit Liver MT Reactions with DTNB

first-order components			second-order components		
10^3constant/ s^{-1}	$\Delta H^{\ddagger}$/ kJ/mol	$\Delta S^{\ddagger}$/ J/(mol K)	constant/ $M^{-1}\,s^{-1}$	$\Delta H^{\ddagger}$/ kJ/mol	$\Delta S^{\ddagger}$/ J/(mol K)
Lobster Cd_5Cu-MT-2					
k_{1s} = 1.34	34.2	−185	k_{2s} = 0.71	31.4	−142
k_{1f} = 3.23	29.1	−194	k_{2f} = 2.92	22.1	−162
Lobster Cd_6-MT-2					
k_{1s} = 1.23			k_{2s} = 0.66		
k_{1f} = 2.27			k_{2f} = 8.13		
Rabbit α-Domain[a]					
k_{1f} = 0.64	59	−105	k_{2f} = 1.12	42	−107
Rabbit Cd_7-MT-2[b]					
k_{1s} = 0.42			k_{2s} = 0.12		
k_{1f} = 1.26			k_{2f} = 1.75		

[a] Reference 17. [b] M. M. Savas, C. F. Shaw III, D. H. Petering, unpublished results.

Cd-MT[15] differ only slightly, so the similarity of the reaction rates found here for $CuCd_5$-MT-2 and Cd_6MT-2 from lobster is not surprising.

The reactions of Cd_5Cu-MT-2 were also carried out from pH 6.0 to 8.5 with [DTNB] fixed at 2 mM. The variation in pH did not influence the rate constants (Table IV). This result agrees with expectations based on the pK_a values of DTNB and the protein amino acid side chains: there are no groups that are protonatable in this pH range which might affect the kinetic results.

Activation enthalpies and entropies, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, were calculated from kinetic rate constants measured at 5 and 50 °C that were combined with the 25 °C data from Table V. Plots of ln

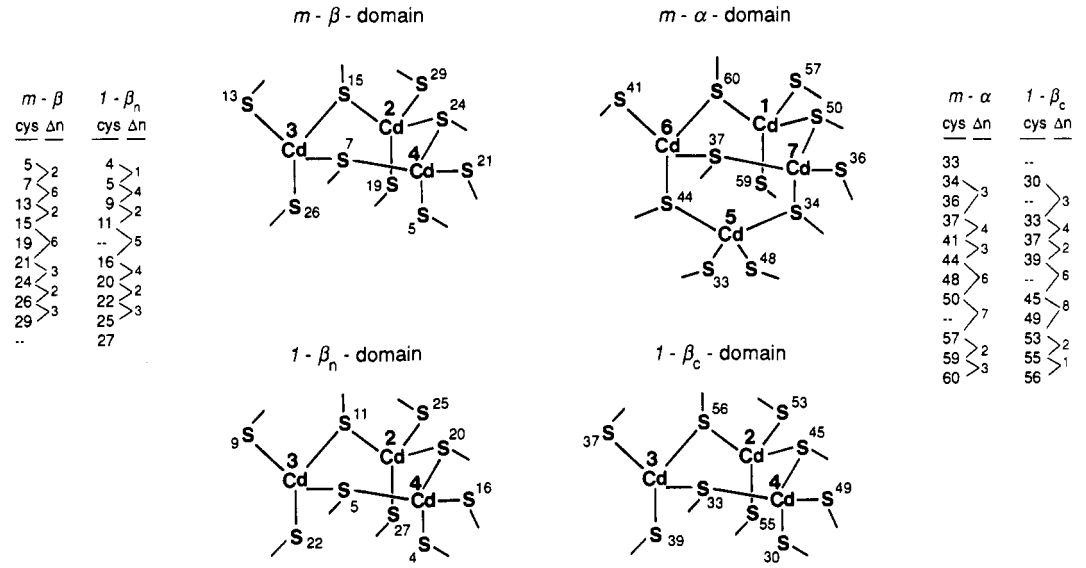

Figure 6. Predicted structures of lobster β_n- and β_c-domains, based on homologies to mammalian β- and α-domains, respectively. The loop sizes (Δn = number of peptide bonds between the cysteines) are relatively unchanged. The deletions and insertions of cysteines are consistent with the organizational motifs found in the mammalian structures.[11-13]

(k/T) vs $1/T$ were made for each of the four rate constants in the rate law, and the activation parameters obtained are listed in Table V. The activation entropies are negative for both the first- and second-order components of each step. This indicates that some entropically unfavorable rearrangement must precede the dissociative as well as the associative components of the reaction. The activation enthalpies are more positive for the first-order components, suggesting that they involve a bond-breaking contribution to the rate-determining step. The absolute and relative magnitudes of the activation parameters are similar to those obtained for the rabbit liver Cd_4-a-domain.[17]

Discussion

The observation that lobster MT reacts biphasically with DTNB, albeit more rapidly than mammalian MTs, can be used to evaluate the possible origins of the biphasic reactions: intrinsic differences in reactivity of the type A (four-metal) and type B (three-metal) clusters or differences imposed on the clusters by the protein environments that generate them (the α- and β-domains of m-MTs and the β_n- and β_c-domains of l-MTs) or perhaps differences between bridging and terminal thiolates. Previous kinetic studies of the reactions of isolated rabbit liver α-domain with DTNB revealed monophasic kinetics, inconsistent with the possibility that the terminal and bridging thiolates cause the biphasic kinetics.[17] The biphasic kinetics of lobster MT-2, which has two three-metal clusters, establishes that the amino acid sequences constituting the clusters, not the clusters themselves, determine the reactivities of MT toward DTNB. If the cluster structure alone determined the reactivities of MT toward DTNB, the reaction of lobster MT2 with DTNB would be monophasic. This result emphasizes that the sequence of the protein has important effects on reactivity and presumably therefore on the biological functions of metallothionein.

The Appendix presents detailed arguments for the existence of two discrete metal-binding domains in the crustacean MTs. The finding of two distinct reaction phases is consistent with that prediction. It suggests that the DTNB reaction with the thiolates of each cluster is cooperative and that the initial attack on one of the more accessible thiolates is rate-determining, followed by reaction of the additional thiolates at comparable or faster rates.

The finding that rate constants for Cd_6-MT-2 and Cd_5Cu-MT-2 (Tables III and V) show only a small perturbation due to the presence of Cu(I) is not surprising. The rates for mammalian MTs loaded with Zn(II) or Cd(II) are nearly independent of the metal content.[15] The only difference found here is for k_{2f}, which is smaller for Cd_5Cu-MT-2 than for Cd_6-MT-2. This difference is comparable to that between the all-copper and all-zinc mammalian MTs.[15] The fact that k_{2s} and k_{1s} are unaffected by the presence of Cu(I) is consistent with the existence of discrete metal-binding domains and the localization of the copper in one of them. This result parallels those for mammalian MTs, where copper is known to bind preferentially to the β-domain.[32-34]

This kinetic study verifies our expectation that crustacean MTs, which have two three-metal clusters,[20-22] are attractive models for MT research. Certainly, the presence of two discrete clusters[21] and the ability to bind in vivo the three metal ions, Cu(I), Cd(II), and Zn(II),[6,7,19-24] commonly found in mammalian MTs give them an advantage over yeast MTs, which have only a single cluster and bind only Cu(I) in vivo, as models for studying chemical reactivity. Once the structural details of the lobster protein are revealed by 2-D NMR or X-ray crystallographic studies, the utility of comparative studies will be greatly enhanced. The year-round, commercial availability of lobsters and their convenient husbandry in artificial sea water make them a viable laboratory species. The similarities of mammalian and lobster MTs—two domains; Cu, Zn, and Cd binding; stress induction—make the lobster a "high connectivity" model for understanding the role of metallothionein in metal metabolism and human health.

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Appendix: Proposed Structure for Lobster and Crab MTs

The recent crystal structure,[11] two-dimensional NMR studies,[12,13] and the original biochemical studies of Winge[10] demonstrated the existence of two distinct metal-binding domains in mammalian (m) MTs. The N-terminal β-domain contains the M_3SCy_9 metal cluster, and the C-terminal α-domain, the M_4SCy_{11}

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cluster. On the basis of the homology between the mammalian and crustacean proteins,[20,22] given in Figure 1, and the presence of two independent three-metal clusters,[21] we expect that each cluster in crab and lobster (l) MTs will also be located in a distinct domain (hereafter designated β_c or β_n, referring to the ends of the protein sequence). We are able to predict the structure (Cd–S connections) of l-MT (Figure 6), on the basis of these homologies and the structural patterns found for the m-MTs.[11-13] Eight of the nine cysteines in the N-terminal region of l-MT correspond to cysteines of the mammalian β-cluster. One is missing and is replaced by a new cysteine later in the sequence. Eight of the eleven cysteines of the m-α-domain are present in the C-terminal sequence of l-MT; three are absent, and one new one appears. For each lobster MT domain, the "conserved" residues easily bind to a three-metal cluster, conserving the protein loop sizes ($\Delta n \pm 0$, 1, or 2, where Δn is the number of intervening peptide bonds).

In the l-β_n-domain, there is no residue corresponding to m-CYS-19, but a new residue, l-CYS-27 appears.[20,22] m-CYS-19 is bound to Cd-2 (Figure 6, Cds numbered according to the ^{113}Cd NMR spectra for m-MTs).[11-13] The new residue l-CYS-27 is one residue removed from l-CYS-25, which is homologous to m-CYS-29 that also binds to Cd-2. Binding to the same cadmium by cysteines that are adjacent ($\Delta n = 1$) or one residue removed ($\Delta n = 2$) from one another is a common motif. Thus we propose that l-CYS-27 "replaces" m-CYS-19 as ligand to Cd-2 and that all other cysteines follow the pattern of m-MT. As shown on the left side of Figure 6, the protein loops (Δn) between metal–thiolate connections remain relatively unchanged between the mammalian and lobster clusters.

The mammalian α-domain has two types of Cd(II) ions, those with two terminal cysteines (Cd-1 and Cd-5) and those with only one (Cd-6 and Cd-7).[9,11-13] This structure can be "generated" by adding a fourth cadmium with two terminal ligands and converting two previously-terminal ligands of the three-metal cluster into bridging ligands. Thus, removing either Cd-5 or Cd-1 and their terminal thiols leaves three Cds and nine thiolate ligands arranged as three-metal clusters. Interestingly, two (of the three) cysteines omitted in the l-β_c-domain,[20,22] m-CYS-33 and -48, are ligands to Cd-5.[11-13] Thus, we propose that the l-β_c-domain is constructed with the homologous cysteines connected to Cd-1, -6, and -7, with the new l-CYS-49 replacing the missing m-CYS-36 as a ligand to Cd-7. Stout et al.[11] have also predicted that Cd-5 is likely to be omitted in crab MT.

At present, there is no evidence that the domains function in concert with one another. In the reactions of either the mammalian[17] or lobster proteins (this work) with DTNB, the domains react independently. Yet the hinge regions that connect the domains are precisely conserved in length and nature of the residues among the mammalian MTs (–K–K–S–, residues 30–32) and among the crustacean MTs (–A/S/P–P–, residues 28–29), although not between the two types. Thus, it is not clear whether there is a requirement for precise positioning of the metal clusters relative to one another. If the l-CYS-25 and -27 residues are positioned as shown in Figure 6, the hinge region begins in a stereochemical configuration that is different for lobster and mammalian MTs. If residues 25 and 27 are switched in position, then l-CYS-27 occupies the coordination site corresponding to m-CYS-29, each being the last cysteine of the corresponding domains. CYS-33, the first in the m-α-domain is not present in the lobster β_c-domain since Cd-5 is missing, and this difference may dictate that, in any event, the domains are oriented differently in mammalian and crustacean MTs.

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Magnetic Characterization of Tetranuclear Copper(II) and Cobalt(II) Exchange-Coupled Clusters Encapsulated in Heteropolyoxotungstate Complexes. Study of the Nature of the Ground States

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This paper presents a magnetic characterization of the heteropolyanions $[M_4(H_2O)_2(PW_9O_{34})_2]^{10-}$ and $[M_4(H_2O)_2(P_2W_{15}O_{56})_2]^{16-}$ (M = Cu(II), Co(II)), with emphasis on the exchange interactions. Their individual heteropolyoxometalate molecules encapsulate a rhomblike arrangement formed by four coplanar MO_6 octahedra sharing edges. The magnetic susceptibility data show that the four copper ions are antiferromagnetically coupled, while in the cobalt(II) complexes the intramolecular exchange is ferromagnetic. In all cases the ground state of the M_4O_{16} molecules has been found to be magnetic. These behaviors are discussed from isotropic (Cu_4 clusters) or anisotropic (Co_4 clusters) exchange models. In case of copper compounds, the presence of a triplet ground state is in agreement with the order of energy levels deduced from the analysis of the magnetic data and is confirmed from EPR and magnetization measurements. This intermediate-spin ground state is discussed in relation with spin frustration resulting from the presence of two competing copper–copper interactions in the rhomb. Finally, the nature of the ground state as well as the presence of intercluster interactions in the cobalt clusters are examined from low-temperature susceptibility and magnetization measurements and compared with the results obtained in the copper clusters.

Introduction

Polyoxometalate complexes resemble discrete fragments of metal oxide structures of definite sizes and shapes, which maintain their identities in solution as well as in the solid state.[1-3] In view of their topological and electronic structural versatility this class of inorganic compounds attracts current attention in analytical chemistry, catalysis, biology, medicine, geochemistry, topology and materials science.

These kinds of compounds have attracted our attention as model systems for the study of magnetic exchange interactions in clusters but also for use as components of new magnetic molecular materials. With respect to the former aspect, the magnetic properties of these compounds have been little studied up to now, due probably to the weak magnetism of the samples (the magnetic sites are encapsulated in a diamagnetic molecular metal oxide cluster). Nevertheless, they can be especially valuable in this area

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