Sequential Proton Resonance Assignments and Metal Cluster Topology of Lobster Metallothionein-1†

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ABSTRACT: NMR studies of $^{111}Cd_6$-MT 1 from lobster have been conducted to determine coordination structure of Cd–thiolate binding in the protein. Sequential proton resonance assignments were made using standard two-dimensional ^1H NMR methods. Two-dimensional ^1H–^{111}Cd HMQC experiments were then carried out to determine the cadmium–cysteine connectivities in the protein. With this information, it was established that the six Cd ions exist in two different Cd_3S_9 clusters, each involving three bridging and six terminal thiolate ligands. Sequential cysteines in the sequence provide the sulfhydral ligands for each cluster and do not overlap, as has been found in mammalian metallothionein. Comparison of the N-terminal, Cd_3S_9 B-type cluster of lobster MT 1 with the Cd_3S_9 cluster from rabbit MT 2 shows that while eight of the nine cysteine residues occupy homologous positions in their sequences, three of the 12 Cd–thiolate connectivities are different. Similarly, the C-terminal B-cluster of lobster MT 1 was compared with the Cd_4S_{11} cluster of mammalian MT 2, excluding the two terminal cysteine sulfhydryl groups that convert this cluster from A- to B-type. As above, eight of nine cysteine positions are identical, yet five of 12 Cd–sulfhydryl connections are different. These differences are expanded when the role of each cysteine as bridging or terminal ligands in the clusters is considered.

Mammalian metallothionein is a small, sulfhydryl rich protein that binds multiple Zn and Cu ions under various physiological conditions and a variety of other nonessential metals such as Cd, Hg, Pt, and Au under other conditions (Kägi & Kojima, 1987; Stillman *et al.*, 1992). Studies of Cd_7-MT[1] and mixed metal, Cd_n,Zn_{7-n}-MT, species revealed that metal ions (M) are gathered into two thiolate ligands (S) clusters of stoichiometry, M_4S_{11} and M_3S_9 (Otvos & Armitage, 1980). In turn, these clusters are segregated into two protein domains, α and β, respectively (Winge & Miklossy, 1992; Robbins *et al.*, 1991). It was also shown in Cd_n,Zn_{7-n}-MT isolated from tissue or prepared by interprotein metal exchange between Cd_7-MT and Zn_7-MT that Cd tends to locate in the α-domain and Zn in the β-domain (Nettesheim *et al.*, 1985). This finding supported the hypothesis that the four-metal cluster sequesters toxic metals and the three-metal cluster participates in essential metal metabolism. Other studies have also concluded that there are significant differences in chemical properties between the two clusters (Otvos *et al.*, 1989). For example, there are apparent differences in affinity of the domains for metal ions (Nielson & Winge, 1983; Stillman & Zalazowski, 1989). In addition, each cluster reacts with distinctly different kinetics with the sulfhydryl reagent, DTNB[2] (Li *et al.*, 1981; Savas *et al.*, 1991).

Differential reactivity and affinity for metal ions by the two clusters might result from basic differences in the two cluster structures, from differences in protein structure around the two clusters, or some combination of these factors. One approach to examine these factors has been to study the properties of crustacean metallothionein, which binds Cd or Zn in an overall metal–thiolate stoichiometry of M_6S_{18}, consistent with the presence of two three-metal clusters in the protein structure (Brouwer *et al.*, 1989; Brouwer & Brouwer, 1993; Lerch *et al.*, 1982; Otvos *et al.*, 1982; Zhu *et al.*, 1992). Reaction of lobster Cd_6-MT 1 with DTNB reveals qualitatively similar biphasic kinetics as seen with the mammalian protein, demonstrating that differential cluster reactivity remains in a structure that may contain two Cd_3S_9 clusters (Zhu *et al.*, 1992).

To explore further the underlying basis for differential reactivity of metallothionein clusters, the determination of the 3D metal cluster topology of $^{111}Cd_6$-MT 1 from lobster has been undertaken using two-dimensional NMR methodology. Such methods first revealed the detailed structure of the α- and β-domains of a mammalian metallothionein (Frey *et al.*, 1985; Wagner *et al.*, 1986). Subsequent X-ray crystallographic studies have confirmed the basic folding and metal–thiolate binding in the molecule (Robbins *et al.*, 1991). In the present report, sequential proton resonance assignments are made, the domain nature of the binding sites of the two clusters established, and the detailed Cd–cysteinyl thiolate interactions defined.

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[1] Abbreviations: A and B refer to cluster stoichiometries and structures Cd_4S_{11} and Cd_3S_9, respectively; α and β designate the protein domains containing Cd_4S_{11} and Cd_3S_9 clusters, respectively, and subscripts n and c denote N-terminal and C-terminal; DQF-COSY, double-quantum filtered homonuclear correlation spectroscopy; DTNB, 5,5′-dithio-*bis*(2-nitrobenzoate); 1D, one dimensional; 2D, two dimensional; HMQC, heteronuclear multiple-quantum correlated spectroscopy; MT 1, metallothionein-1; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy.

[2] Rate constants for the reaction of DTNB with rabbit and lobster Cd-MT proteins at 25 °C at pH 7.4 (Savas *et al.*, 1991; Zhu *et al.*, 1992):

	$k_{1,fast}$ (α_c)	$k_{2,fast}$ (β_n)	$k_{1,slow}$ (β_n)	$k_{2,slow}$ (β_n)
rabbit Cd_7–MT 2	0.00135^{-1}	1.8 m^{-1} s^{-1}	0.00042	0.12
lobster Cd_6–MT 1	0.0048	5.8	0.0010	0.70
Cd_6–MT 2	0.0023	8.1	0.0012	0.66

The results for Cd_6-MT 1 are unpublished. Each domain of lobster MT 1 contains 5+ charged and 3– charged side chains.

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EXPERIMENTAL PROCEDURES

Sample Preparation. Lobster $^{111}Cd_6$-MT 1 protein was prepared from Cd_6-MT 1 as follows. Fresh lobster Cd_6-MT 1 was isolated as described previously (Zhu *et al.*, 1992). Then the protein was acidified to pH 0 with prechilled 6 N HCl and incubated for 15–30 min at 4 °C to dissociate Cd^{2+} and traces of Cu^{1+}. ApoMT and metal ions were separated by Sephadex G-25 gel filtration chromatography in 0.05 N HCl. Fractions containing protein were determined by UV measurements at 220 nm; Cd^{2+} was detected by atomic absorption spectrophotometry. The apoprotein fractions were pooled and the pH adjusted to 7.4 using solid Tris base. After addition of β-mercaptoethanol, 6.5 equiv of $^{111}CdCl_2$ were added to the reduced apoprotein and the sample incubated for 30–45 min. The reconstituted protein was concentrated using an Amicon YM-2 membrane concentrator and chromatographed over Sephadex G-50 (2.5 × 110.5 cm) with 20 mM Tris-HCl buffer at pH 7.4. Protein fractions were pooled and concentrated again using a YM-2 membrane system. The sulfur to ^{111}Cd ratio of the final product was assayed by reaction of the protein with DTNB and atomic absorption spectrophotometry as described previously (Zhu *et al.*, 1992). The ratio was 3.06 ± 0.06 and closely agreed with the theoretical value of 3.00. 1D ^{111}Cd NMR spectra were obtained using a 1.5-mL sample of $^{111}Cd_6$-MT 1 at a concentration of 4.3 mM ^{111}Cd in 20 mM Tris-HCl, pH 7.4, in 90% H_2O/10% D_2O. For the 2D NMR experiments, the Tris-HCl buffer was replaced with Tris-d_{11} by dilution, followed by concentration, using an Amicon Centricon-3 filtration device. In these experiments, the protein concentration was approximately 5 mM and the buffer was 5 mM Tris-d_{11}, pH 7.4, in 99.96% D_2O or 90% H_2O/10% D_2O. Additional 2D NMR experiments were carried out at pH 7.0 and 6.0 to facilitate the observation of exchangeable amide protons for sequential assignments.

NMR Experiments. NMR spectra were acquired on a General Electric (GE) GN-500 NMR spectrometer operating at ^1H and ^{111}Cd frequencies of 500.1 and 106.1 MHz, respectively. All proton pulses were generated with the low-power output of the observe transmitter and amplified with the proton decoupler amplifier. ^{111}Cd decoupler pulses were produced using the spectrometer's X-nucleus decoupler channel modified to implement GARP decoupling, with a class A amplifier (Kalmus Engineering, Inc., Woodinville, WA) and a hard-wired GARP accessory (Tschudin Associates, Kensington, MD). Data were processed on a Silicon Graphics 4D/70G computer using Felix version 2.05 software (Hare Research, Inc.). With the few exceptions noted below, water suppression was accomplished in the 2D experiments by applying a DANTE pulse sequence during the 1-s recovery delay between scans. TPPI–States phase cycling was used for quadrature detection in the F_1 dimension (Marion *et al.*, 1989).

A 1D ^{111}Cd proton decoupled NMR spectrum of MT 1 was obtained at 25 °C in 90% H_2O/10% D_2O. Proton decoupling was accomplished using WALTZ composite pulse decoupling during acquisition. The sweep width was 8475 Hz, centered at 640 ppm, and 2048 complex data points were accumulated. 10 Hz exponential line broadening was applied to the data sets before Fourier transformation. The ^{111}Cd chemical shifts are reported with respect to external 0.1 M $^{111}Cd(ClO_4)_2$ at 0.0 ppm (74 152 scans were collected).

NOESY spectra were acquired in 90% H_2O/10% D_2O with mixing times of 100 and 200 ms at 35 and 27 °C, respectively (Jeener *et al.*, 1979; Macura *et al.*, 1981). A 100-ms mixing time NOESY spectrum was also acquired in D_2O at 35 °C. To minimize saturation transfer from the water resonance to exchangeable amide protons, a 200-ms NOESY spectrum was acquired in 90% H_2O/10% D_2O at pH 7.0 and 27 °C using "short" DANTE presaturation of the water resonance, in which the water resonance was irradiated for only one-fifth of the 1-s recovery delay between scans. In addition, a NOESY spectrum using a "jump and return" water suppression sequence was acquired with a 200-ms mixing time at 25 °C, pH 6.0 (Driscoll *et al.*, 1989). In the NOESY experiments, a composite 180° pulse was inserted in the middle of the mixing period. A 5-ms z-homospoil pulse was applied at the beginning of the mixing period and after the 180° pulse to minimize the recovery of the water signal during the mixing period as described previously (Blake *et al.*, 1991). TOCSY (Braunschweiler & Ernst, 1983) spectra were acquired at 35 °C in D_2O and 27 °C in 90% H_2O/10% D_2O, with mixing times of 44 ms, using an MLEV-17$_y$ mixing scheme (Bax & Davis, 1985). In addition, a TOCSY spectrum was acquired at 25 °C, pH 6.0 in 90% H_2O/10% D_2O, with a mixing time of 50 ms, using a WALTZ-17$_y$ mixing scheme and short DANTE presaturation of the water resonance (Bax, 1989). In the NOESY and TOCSY spectra, ^{111}Cd decoupling was carried out in t_1 and t_2, using GARP composite pulse decoupling (Shaka *et al.*, 1985). Two DQF-COSY spectra were acquired in D_2O at 35 °C, with and without ^{111}Cd GARP decoupling in t_1 and t_2 (Rance *et al.*, 1983). In the NOESY, TOCSY, and DQF-COSY experiments, the sweep width was set to 6024 Hz in both dimensions with the water resonance in the center of the spectra; 1024 complex data points were acquired in t_2. Typically, 64 scans were collected for each t_1 increment and 200 t_1 increments were accumulated. The NOESY and TOCSY spectra were processed using 90° sine-bell squared window functions followed by 2 Hz exponential line broadening in t_1 and t_2, followed by zero filling to 2048 points in both dimensions, prior to Fourier transformation. The DQF-COSY spectra were processed with 60° sine-bell squared window functions, followed by zero filling to 2048 points in both dimensions prior to Fourier transformation. Polynomial baseline corrections were applied in F_1 and F_2, as required, to obtain flat baselines.

^1H–^{111}Cd HMQC spectra with mixing times ($1/2J_{HCd}$) of 15, 30, and 50 ms were acquired in D_2O at 35 °C (Bax *et al.*, 1983). To favor relay peaks from cysteine Hβ to Hα protons, a 90 °$_y$ pulse was inserted before the detection period (Frey *et al.*, 1985). ^{111}Cd GARP decoupling was carried out during the data acquisition period to remove ^{111}Cd coupling in the F_2 dimension . The F_2 sweep width was set to 6024 Hz, with the residual HOD peak in the center of the spectrum, and 1024 complex data points were acquired in t_2. The F_1 sweep width was 8475 Hz centered at 640.0 ppm. One hundred and twenty-eight scans were collected for each t_1 increment, and 128 t_1 increments were accumulated. The HMQC spectra were processed with 30° and 45° sine-bell squared window functions in t_2 and t_1, respectively, followed by zero filling to 2048 points in t_2 and 512 points in t_1 prior to Fourier transformation. Linear baseline corrections were applied in F_2 before Fourier transformation in F_1.

RESULTS

Five peaks appear in the proton decoupled 1D ^{111}Cd spectrum of lobster MT 1 shown in Figure 1. The second peak at 649.1 ppm has a shoulder at 651.1 ppm. Integration of this spectrum under fully relaxed conditions shows that this peak corresponds to one $^{111}Cd^{2+}$ ion. The shoulder may

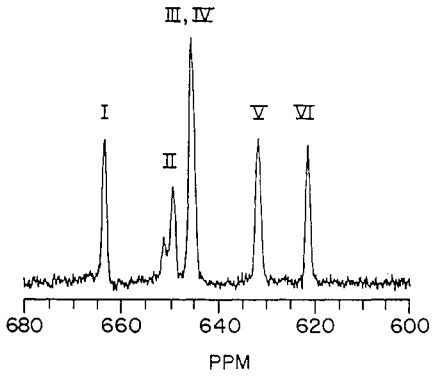

FIGURE 1: ^{1}H-decoupled 106-MHz 1D ^{111}Cd spectrum of lobster MT 1. The signals are numbered I–VI according to decreasing chemical shift. The chemical shifts were reported in parts per million (ppm) downfield from the ^{111}Cd resonance of 0.1 M $^{111}Cd(ClO_4)_2$. Conditions: [^{111}Cd] = 4.28 mM, [^{111}Cd]/[Cu] = 272; 74 152 scans collected.

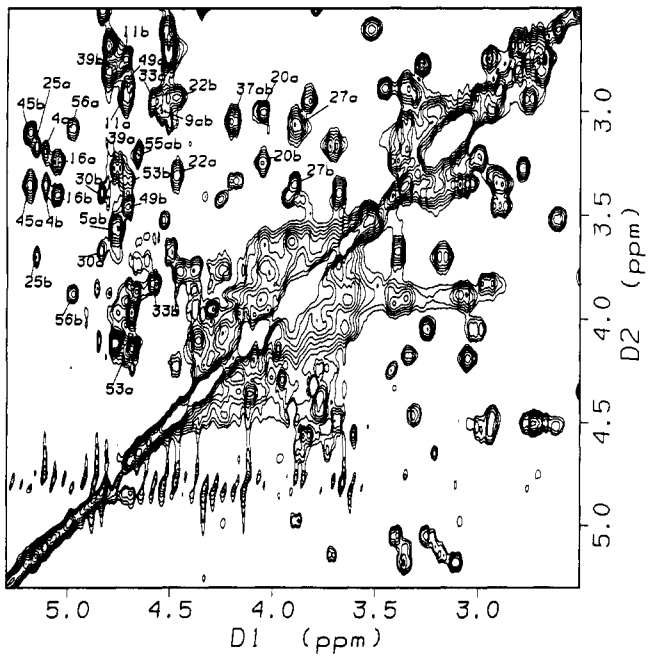

FIGURE 2: Region of the TOCSY spectrum of lobster $^{111}Cd_6$-MT 1 acquired with a 40-ms mixing time in D_2O, containing all the cysteine Hα and Hβ proton resonances. The cysteine Hβ resonances are labeled by their sequence positions, and the a and b designations indicate which resonance has the larger and smaller $^3J_{\alpha\beta}$ coupling, respectively.

arise from the presence of a second, minor isoform of MT 1 as has been observed in mammalian metallothioneins (Kägi & Kojima, 1987). The third peak at 645.2 ppm integrates to two $^{111}Cd^{2+}$ ions. The remaining peaks integrate to one $^{111}Cd^{2+}$ ion. These results show that each lobster MT 1 molecule binds six $^{111}Cd^{2+}$ ions.

Sequential proton resonance assignments were made by simultaneously assigning proton spin systems from the DQF COSY and TOCSY spectra and via the observation of sequential connectivities in the NOESY spectra, using the methodology described by Wagner (1986) and Englander and Wand (1987). The assignment of the 18 cysteine side-chain proton resonances was facilitated by the observation of ^{1}H–^{111}Cd coupling constants in proton DQF-COSY spectra acquired in D_2O by comparing a fully coupled spectrum with a ^{111}Cd-decoupled spectrum. The cysteine Hβ resonances are labeled in the portion of the D_2O TOCSY spectrum shown in Figure 2. Cysteines 25, 16, 49, and 33 were determined to be bridging cysteines, on the basis of on the observation of

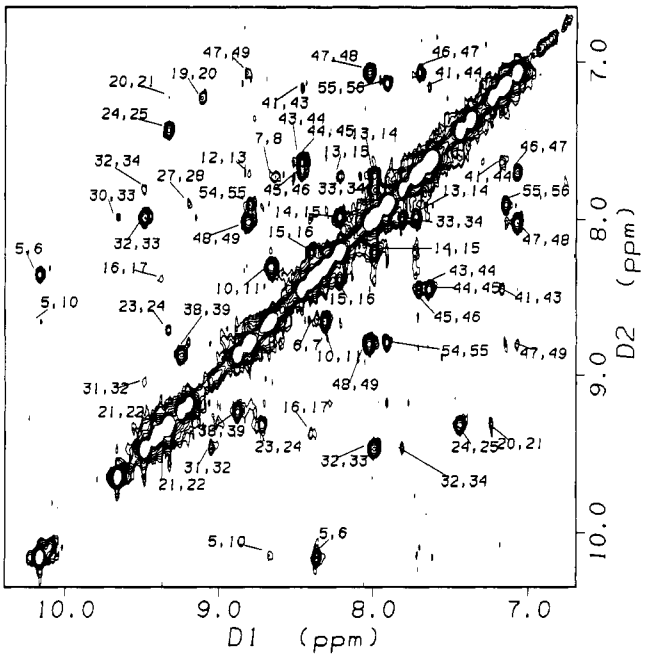

FIGURE 3: NH–NH region of the NOESY spectrum of lobster $^{111}Cd_6$-MT 1 acquired with a 200-ms mixing time under short presaturation conditions, showing the sequential $d_{NN(i,i+1)}$ connectivities and a few medium-range NOEs.

two ^{111}Cd couplings in their corresponding β,β' cross-peaks in the Cd^{111}-coupled COSY spectrum.

Sequential assignments were performed from the observation of sequential connectivities in NOESY spectra acquired in 90% H_2O/10% D_2O. Only 16 $d_{NN(i,i+1)}$ cross-peaks appeared in the 200-ms NOESY spectra acquired at 27 °C and pH 7.4 under full presaturation conditions. All but three of these sequential correlations involve cysteine residues bound to cadmium ions in the two metal–thiolate clusters in the protein. This implies that, except for the residues involved in folding about the two metal clusters, the remainder of the protein may be in a random coil configuration, exposed to surrounding water molecules. In order to obtain more sequential correlations it was necessary to acquire NOESY spectra under the short presaturation conditions described above and to use a jump and return water suppression sequence. With these approaches, an additional 12 $d_{NN(i,i+1)}$ cross-peaks were observed. A weak $d_{NN(i,i+1)}$ cross-peak between C4 and C5 that appeared in the NOESY spectrum taken under full presaturation conditions was not seen in these two NOESY spectra. The $d_{NN(i,i+1)}$ cross-peaks are labeled in the amide region of the 200-ms NOESY spectrum acquired under the short presaturation conditions (Figure 3). The cross-peak between K21 and C22 is more clearly resolved from the diagonal, when the spectrum is processed with greater resolution enhancement. Three additional $d_{NN(i,i+1)}$ cross-peaks are observed in the "jump and return" NOESY spectrum.

Many of the sequential NH–NH connectivities did not appear in the NOESY spectra, even under conditions which minimize saturation transfer. Thus, some of the sequential assignments were made from the observation of $d_{\alpha N(i,i+1)}$ and $d_{\beta N(i,i+1)}$ cross-peaks in the fingerprint region of the NOESY spectrum (Figure 4). Also, in analogy to mammalian metallothionein, the sequential assignments were made assuming that MT 1 is comprised of N-terminal and C-terminal domains, each containing a separate metal–thiolate cluster. Sequential assignments were started at the unique sequence C4-C5-K6 in the N-terminal domain. There is a break in the

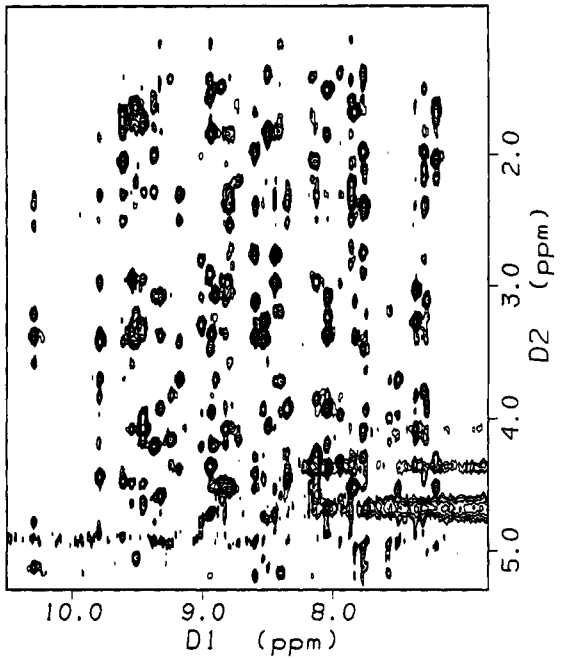

FIGURE 4: Fingerprint region of the NOESY spectrum of lobster $^{111}Cd_6$-MT 1 acquired with a 200-ms mixing time under short presaturation conditions.

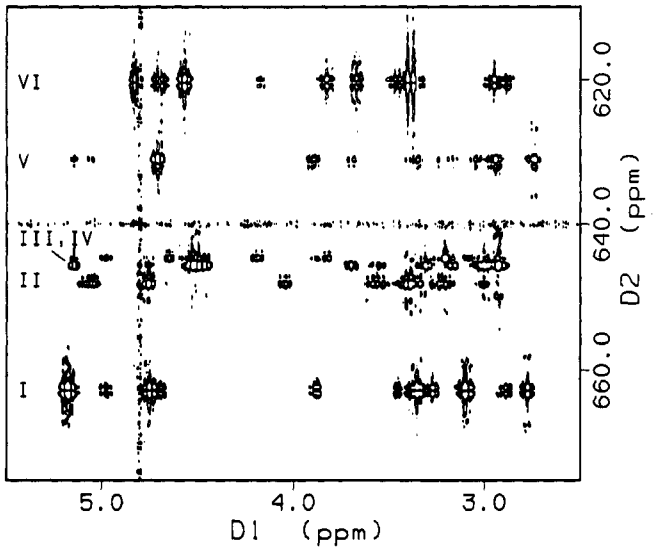

FIGURE 5: $^1H/^{111}Cd$ HMQC spectrum of lobster $^{111}Cd_6$-MT 1 acquired with a 30-ms mixing time ($1/2J_{HCd}$) in D_2O.

sequential NOEs between C9 and E10, because the amide NH chemical shifts of these residues overlap. The sequential assignments are then picked up by strong sequential $d_{NN(i,i+1)}$ and medium $d_{\beta N(i,i+1)}$ cross-peaks between E10 and C11. Nevertheless, this connectivity is somewhat ambiguous, because a $d_{\beta N(i,i-1)}$ cross-peak is also observed between C11 and E10 and because the C9 and E10 amide NH resonances overlap. This implies that the C9 and C11 assignments might be reversed, and that the $d_{\beta N(i,i-1)}$ cross-peak is actually a $d_{\beta N(i,i+1)}$ cross-peak between C9 and E10. However, we believe the cysteine amide NH resonance which overlaps the E10 resonance does indeed arise from C9, because a medium and weak $d_{\beta N(i,i+1)}$ cross-peak at the chemical shift of this proton and the two Hβ protons of K8 are also observed in the NOESY spectrum. Unfortunately, no correlation between C11 and A12 is observed in the NOESY spectrum to further confirm that the assignments are correct. Residues P57 and T58 were defined in the crab but not the lobster primary sequence

Table 1: Proton Resonance Assignments of Lobster $^{111}Cd_6$-MT 1 at pH 7.4 and 35 °C

amino acid	NH	Hα	Hβ	others
Pro1				
Gly2				
Pro3				
Cys4	7.72	5.11	3.19, 3.35	
Cys5	10.26	4.76	3.58, 3.58	
Lys6	8.47	4.05	1.82	1.41
Asp7	8.76	4.80	2.68, 2.82	
Lys8	7.83	4.47	2.06, 1.82	1.43, 1.13
Cys11	8.79	4.52	3.00, 3.00	
Glu10	8.76	4.84	2.27, 2.34	γCH_2 2.50
Cys9	8.41	4.72	2.75, 2.95	
Ala12	8.91	4.30	1.58	
Glu13	7.83	4.30	2.28, 2.48	γCH_2 2.34
Gly14	8.09	4.72, 4.25		
Glu15	8.32	4.44, 3.90		
Cys16	8.50	5.05	3.24, 3.40	
Lys17	9.48	4.88	1.64, 2.19	
Thr18	8.71	4.33	4.13	γCH_3 1.42
Gly19	9.22	4.32, 3.79		
Cys20	7.34	4.05	3.01, 3.25	
Lys21	9.43	4.63	1.71, 2.27	1.53
Cys22	9.49	4.47	2.92, 3.30	
Thr23	8.83	4.60	4.36	γCH_3 1.49
Ser24	9.43	4.65	4.14, 3.96	
Cys25	7.54	5.15	3.17, 3.70	
Arg26	8.37	4.56	1.80, 1.47	γCH_2 1.18
Cys27	9.29	3.90	3.90	3.07, 3.36
Ala28	8.02	4.35	1.48	
Pro29				
Cys30	9.75	4.83	3.39, 3.67	
Glu31	9.15	4.36	2.29, 2.29	γCH_2 2.49
Lys32	9.58	4.45	2.04, 1.77	γCH_2 1.67
Cys33	8.09	4.57	2.95, 3.83	
Thr34	7.91	4.29	3.94	γCH_3 1.36
Ser35	8.38			4.13
Gly36	7.47	4.51, 3.68		
Cys37	8.88	4.19	3.05, 3.05	
Lys38	9.35	4.75	1.98, 2.25	γCH_2 1.54; δCH_2 1.77
Cys39	8.98	4.75	2.79, 3.27	
Pro40		4.66	2.18, 2.38	γCH_2 2.08; δCH_2 3.77
Ser41	7.26	4.46	4.11	
Lys42				
Asp43	8.59	4.51	2.73, 2.73	
Glu44	7.74	4.26	1.95, 2.35	
Cys45	8.56	5.19	3.10, 3.35	
Ala46	7.80	4.50	1.67	
Lys47	7.17	4.45		1.71, 2.07, 2.20
Thr48	8.13	4.88	4.33	γCH_3 1.41
Cys49	8.90	4.70	2.88, 3.45	
Ser50	7.74		4.07	
Lys51				1.41, 1.51, 1.62
Pro52				
Cys53	8.79	4.71	3.33, 4.18	
Ser54	8.90			3.96
Cys55	8.02	4.66	3.21, 3.21	
Cys56	7.60	4.97	3.08, 3.88	
Pro57				2.18, 2.36; δCH_2 3.92
Thr82				

(Brouwer *et al.*, 1989; Lerch *et al.*, 1982). A residue T58 was not observed in these NMR experiments. The proton resonance assignments are summarized in Table 1.

Although there are some ambiguities in the sequential assignments, the sequential assignments of all the cysteines resonances are clear, except that the resonances corresponding to C9 and C11 may be interchanged. It should be possible to resolve this ambiguity in the future by carrying out 3D ^{15}N-edited NOESY and TOCSY experiments, once a clone for MT 1 becomes available.

The ^1H–^{111}Cd HMQC spectrum of MT 1 obtained with a 30-ms mixing time is shown in Figure 5. F_2 slices through each of the six cadmium resonances are displayed in Figure

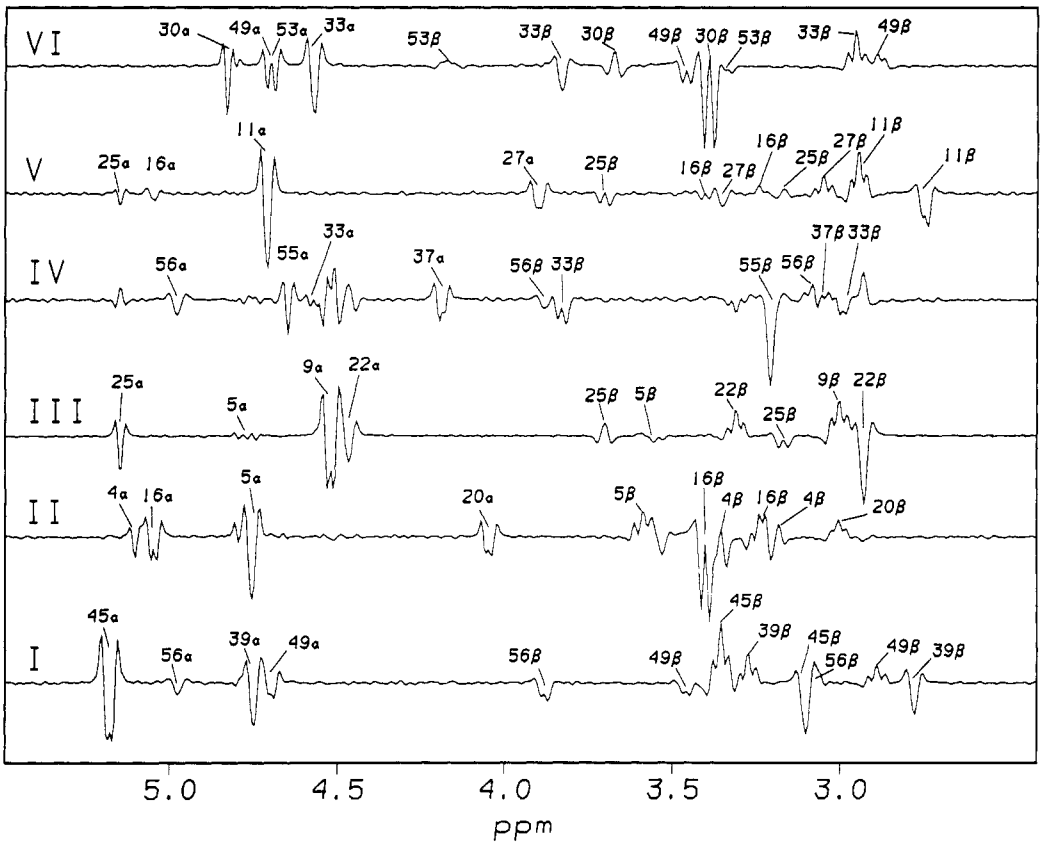

FIGURE 6: F_2 slices from the $^1H/^{111}Cd$ HMQC spectrum of lobster $^{111}Cd_6$-MT 1 taken through each of the six cadmium resonances, showing each of cysteine Hα and Hβ proton resonances.

6. The overlapping cadmium resonances III and IV shown in Figure 1 are resolved in the 2D spectrum, with chemical shifts at 644.2 and 645.2 ppm. Cross-peaks corresponding to all the Hα and Hβ protons of the 18 cysteine residues, assigned from the DQF-COSY and TOCSY spectra, are observed in the 30-ms HMQC spectrum and are labeled in Figure 6. The unlabeled cross-peaks in the F_2 slice, taken through the cadmium IV resonance, arise from overlap with the cadmium III resonance. Six bridging cysteines can be identified in Figure 6. Cysteine 25 bridges cadmium III and V, cysteine 16 links cadmium V and II, cysteine 5 spans cadmium II and III, while cysteine 56 bridges cadmium I and IV, cysteine 33 ligates both IV and VI, and cysteine 49 connects cadmium VI and I. These assignments confirm that cysteines 25, 16, 49, and 33 contribute bridging ligands, as assigned from the DQF-COSY data. The remaining 12 cysteines show cross-peaks to only one cadmium resonance. In conjunction with the cysteine Hα and Hβ sequential resonance assignments, the HMQC data determine the cadmium–cysteine connectivities, and the resulting cadmium–thiolate cluster topology for MT 1 is shown in Figure 7. These results clearly establish that cadmiums II, III, and V are contained in one metal–thiolate cluster in the protein's N-terminal domain, while cadmiums I, IV, and VI are contained in another metal–thiolate cluster in the protein's C-terminal domain.

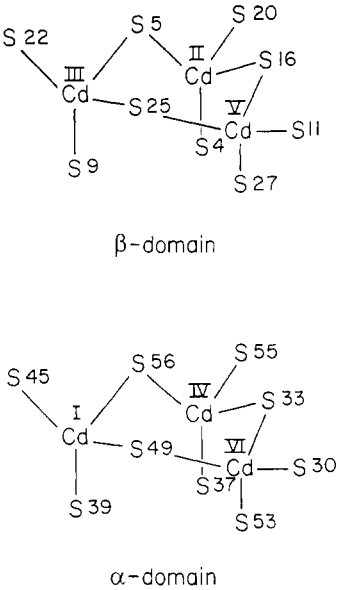

FIGURE 7: Metal–thiolate connectivities of the lobster $^{111}Cd_6$ MT 1 clusters. The 18 cysteines are labeled according to the sequence number reported by Brouwer *et al.* (1989). The six ^{111}Cd ions are labeled according to decreasing chemical shift.

DISCUSSION

Mammalian metallothionein proteins contain two metal–thiolate clusters located in separate protein domains (Winge & Miklossy, 1982). From the Cd–thiolate connectivities assigned in the present work, it is evident that the Cd_6-MT 1 from lobster is also composed of two Cd_3S_9 B-type clusters. These results are summarized in Figure 8, which shows the metal to cysteine connectivities in the two clusters. This hypothesis is supported by the fact that the first nine N-terminal cysteines (cysteines 4–27) form a metal–thiolate B–cluster located in the N-terminal half of the protein, in analogy with the β_n-domain of mammalian metallothioneins, while the second nine C-terminal cysteines (cysteines 30–56) form another B–cluster located in the C-terminal half of the protein, bearing homology with the α_c-domain of mammalian metallothioneins. The localization of all of the cysteine residues for each cluster in different parts of the sequence and the sequential NOEs strongly suggests that amino acid residues

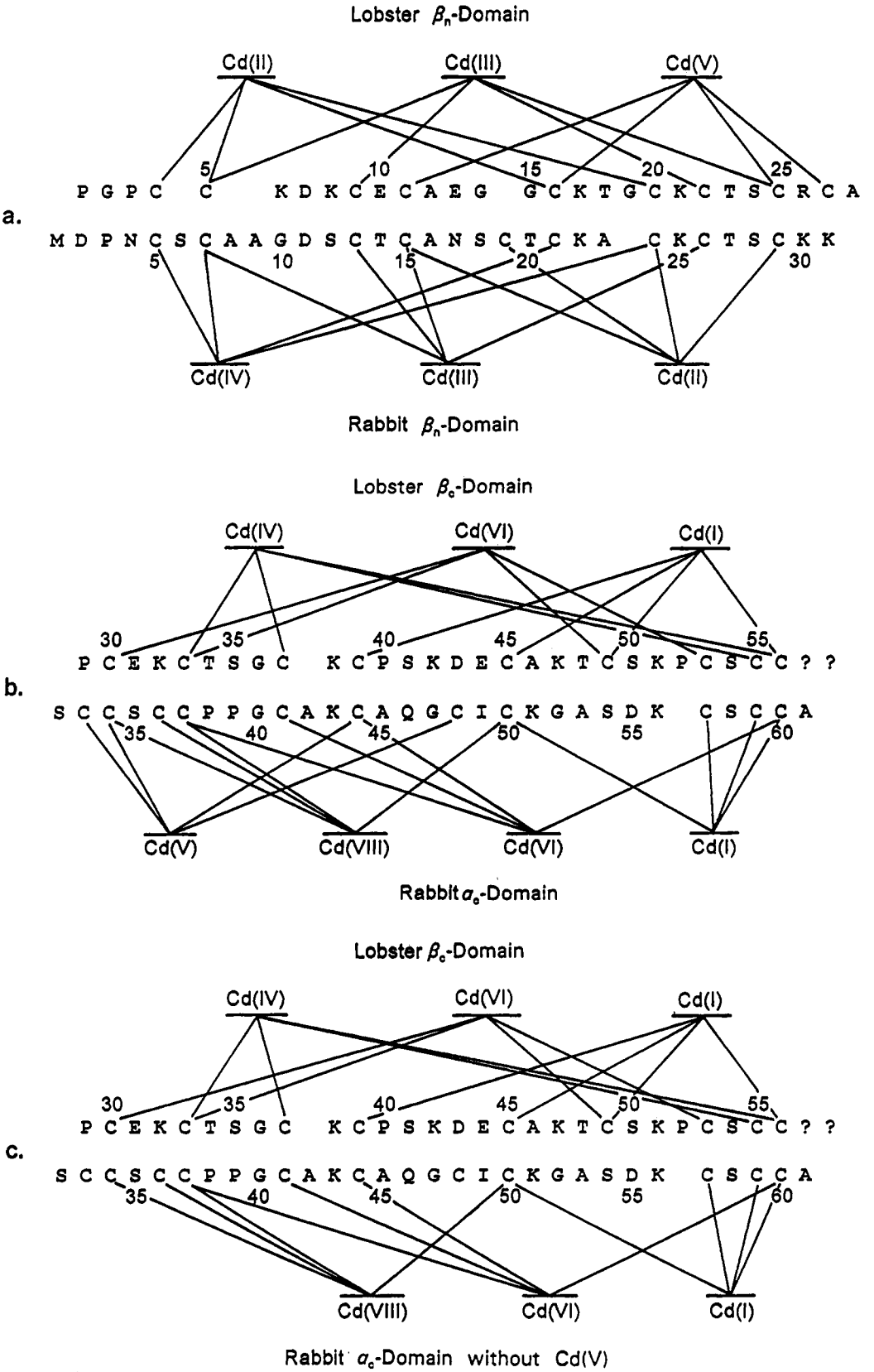

FIGURE 8: Comparison of the amino acid sequences of lobster $^{111}Cd_6$-MT 1 and rabbit MT 2a. The metal-to-cysteine coordination bonds are indicated above the sequence for lobster $^{111}Cd_6$-MT 1, below the sequence for rabbit MT 2a.

1–28 form an N-terminal domain in MT 1, while residues 29–58 constitute a second C-terminal domain in MT 1. Work is currently in progress to determine the three-dimensional structure of these domains from the NMR data.

The unexpected result of this work on the Cd–cysteine assignments was the finding that the connections between Cd ions and specific cysteinyl residues in the N-terminal cluster differ markedly from those of the mammalian MT β_n-domain, even though there are similar locations for eight of the nine cysteine residues in the two sequences (Table 2 and Figure 8) (Zhu *et al.*, 1992). In particular, although Cd II and Cd IV of lobster and rabbit, respectively, are each coordinated to four cysteinyl thiolate groups at analogous positions of the sequence, one of the two bridging sulfhydryl groups in each

Table 2: Cadmium–Cysteine Sequence Connections[a]

	sequence positions		
	lobster β_n	rabbit β_n	
Cd II	4	5	Cd IV
	[5]	[7]	
	[16]	21	
	20	[24]	
Cd III	[5]	[7]	Cd III
	9[b]		
		13	
	11[b]	[15]	
	22	26	
	[25]		
Cd V	9[b]		Cd II
	11[b]	[15]	
		(19)	
	[16]		
		24	
	[25]	29	
	(27)		

	sequence positions		
	lobster β_c	rabbit α_c[c]	
Cd VI	30	34	Cd VII
		(36)	
	[33]	[37]	
		[50]	
	([49])		
	53		
Cd IV	[33]	[37]	Cd VI
	37	41	
		44	
	55		
	[56]	[60]	
Cd I	39		Cd I
	45	[50]	
	([49])		
		57	
		59	
	[56]	[60]	

[a] Paired numbers represent the same positions in the two aligned sequences. Brackets indicate bridging sulfhydryl groups and parentheses denote cysteines that are not located at homologous positions in the two sequences. [b] Alternate Cd–thiolate connections due to possible ambiguity in the assignment of C9 and C11. C9 linkage to Cd III and C11 binding to Cd V are preferred. [c] The fourth Cd (V) of the rabbit metallothionein α_c-domain structure and its two unique cysteine connections, 34 and 48, are omitted.

structure is derived from different cysteines, residue 16 for lobster and 24 for rabbit. The structural detail around the other two Cd ions diverges even more from that of the rabbit protein such that at the Cd V (lobster), Cd II (rabbit) site, only one of the cysteines which coordinate the metal ions come from analogous positions in the sequence. The drastic difference in Cd–thiolate coordination in the β_n domains of the two proteins can be ascribed to the change in position in the sequence of only one cysteine. It is evidently not related to the fact that the lobster domain has fewer amino acids, for they are deleted near the N-terminal region where the same cysteines from both sequences coordinate to Cd II (lobster) and Cd IV (rabbit).

A similar picture emerges in the comparison of the β_c-domain of lobster with the α_c-domain of the rabbit structure. Omitting the two terminal cysteines that uniquely bind to the fourth Cd ion in the Cd_4S_{11} cluster found in mammals, one can compare the remaining Cd_3S_9 cluster structure of the rabbit MT with that of lobster. Again, eight of the nine cysteine residues in the rabbit protein occupy the same positions in the alignment of sequences shown in Figure 8. Yet, as in the comparison of Cd–cysteine coordination in the other domains of the two proteins, a shift in position of one cysteine residue leads an extensive rearrangment of metal–ligand coordination in the cluster.

From these results, it is clear that at least three different domain structures exist which form B-clusters, β_n and β_c domains from lobster and β_n from mammalian MT. Hence, it is likely that a variety of sequences of nine cysteines among about 30 residues can bind three Cd^{2+} ions in stable metal–thiolate clusters that display different Cd–thiolate binding patterns. This conclusion was unanticipated because all of the mammalian sequences of metallothioneins contain invariant locations for all 20 cysteinyl residues. That fact had suggested the need for particular spacings of cysteinyl groups to accommodate the twin requirements of the positioning of the thiolate groups about the C_3S_9 cluster and the overall tight folding of the 30-residue sequences about the core cluster. If multiple sequences are consistent with stable metal–thiolate structures, then the rigorous invariance in sequence position of all of the cysteines in mammalian MT may result from more subtle requirements of the cluster in the mammalian organism, perhaps related to its thermodynamic stability or kinetic reactivity.

Support for this view may be drawn from previous studies of the reactions of metallothioneins with DTNB. Both mammalian and lobster MT react with biphasic kinetics that each can be decomposed into first- and second-order components, $k = k_1 + k_2[\text{DTNB}]$ (Savas *et al.*, 1991; Zhu *et al.*, 1992). One of these, k_1, is thought to represent a Cd–thiolate dissociative process and the other, k_2, a direct bimolecular reaction. It has been shown with the mammalian protein that each of the biphasic kinetic steps results from the independent reaction of DTNB with one of the two clusters (Savas *et al.*, 1991). One assumes that the same pattern of reaction accounts for the biphasic kinetics with the lobster protein. Differences in cluster structure in the mammalian protein, Cd_4S_{11} vs Cd_3S_9, might account for their quantitatively different kinetic behavior. However, such differences in reactivity of each lobster cluster remain despite the fact that they are both Cd_3S_9 structures and both domains contain the same number of positive and negative side chains. Both k_1 and k_2 differ markedly for the two kinetic steps. Therefore, it is likely that cluster dynamics (k_1) and accessibility of cluster thiolates to DTNB (k_2) vary in the β_n and β_c domains because of the different folding of the domain sulfhydryl groups about the two central cluster structures. If this is so, the invariance in positioning of cysteinyl residue in mammalian sequences may, indeed, reflect common functional requirements of clusters in these organisms.

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