Mercury Binding to Metallothioneins: Formation of the Hg_{18}–MT Species

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Received July 17, 1992

We report the formation and properties of a novel mercury–protein complex formed from rabbit liver metallothionein, namely Hg_{18}–MT. Complex formation is dependent on the presence of the Cl^- ion and a pH below 6. Rabbit liver Hg_{18}–MT exhibits a strongly dichroic CD signal in the 240–360-nm region, and a unique X-ray absorption, near-edge spectrum (Lu, W.; Kasrai, M.; Bancroft, G. M.; Stillman, M. J.; Tan, K. H. *Inorg. Chem.* **1990**, *29*, 2561–2563). Hg_{18}–MT elutes with an apparent molar mass of 5500 Da from Sephadex G-50 columns. There is no indication of dimer formation. Formation of the 3-dimensional structure associated with Hg_{18}–MT is unique to rabbit liver isoform 2; unless isoform 1 is initially lyophilized and redissolved in acidic solution, the CD spectrum characteristic of Hg_{18}–MT is not observed. The CD spectrum of Hg_{18}–MT can be used to discriminate between isoforms 1 and 2 following chromatographic separation on Sephadex-DEAE columns. Isoforms of rat metallothionein do not adopt the same structure as isoforms of rabbit metallothionein. Neither isoform of metallothionein isolated from rat liver binds 18 Hg^{2+} to form a complex characterized by a similar CD signal. Each of the four MT isoforms studied binds 7 mol of Hg^{2+} to form Hg_7–MT species that exhibit similar CD spectra at pH 7. The CD bands observed for the Hg-containing metallothioneins lie under bands assigned generally as sulfur-to-mercury charge transfer, although the strongly dichroic band of Hg_{18}–MT 2 lies to the red of bands previously reported for other Hg–MT complexes. We suggest that the CD spectral intensity observed for rabbit liver Hg_{18}–MT arises from the enhanced chirality of stacked Hg–S bonds as the peptide chain self-associates into a unique 3-dimensional structure based on a single domain. This structure represents a third structural motif for metallothioneins. The essential requirement for Cl^- suggests that the coordination geometry of the Hg^{2+} may be pseudotetrahedral with bridging thiolates and outlying chlorides. This would result in a kink in the structure which could be the origin of the enhanced CD intensity. We propose that the significant differences in complexation properties observed for the different isoforms result from differences in the amino acid sequences between each of the MT isoforms in the region of a pleat required by the Hg_{18}–MT structure.

Introduction

Metallothionein, which has a unique amino acid sequence in which 20 cysteine residues make up the 61 total in the peptide chain, has been the focus of many studies that have examined its remarkable metal-binding properties.[1-16] Mammalian metallothionein binds a wide range of metals into two binding sites that involve metal–thiolate clusters. Cadmium- and zinc-containing metallothioneins have received the most detailed study by NMR,[4] X-ray diffraction,[5] and optical spectroscopy.[1,2,6,7] From these studies, the stoichiometric ratio for the sum of zinc and cadmium bound to metallothionein has been determined as 7 with a structural geometry that involves tetrahedral coordination of the metals.[4,5] Both terminal and bridging cysteinyl sulfurs form the metal-binding-site cage.[4,5] Metal:protein ratios of 12 are observed for copper[1,8,9] and silver,[10] for which trigonal coordination has been proposed. A molar ratio of 18 has also been reported by our group for Ag-MT.[10] Except for a preliminary report on the formation of Hg_{18}–MT,[11] only an Hg_7–MT species has previously been described for mercury-containing metallothioneins.[7,13-16] Mercury exhibits an inorganic chemistry in which tetrahedral, trigonal, and linear coordination geometries have been reported.[17-19] Examples of metallothionein binding metals with stoichiometric ratios of 7 and 12 suggest that more than one structure might be possible for a metal that exhibits variable coordination geometry.

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0020-1669/93/1332-0919$04.00/0

In mammals, two major isoforms of metallothionein, referred to as MT 1 and MT 2, are isolated.[1,20] These isoforms differ slightly in amino acid composition[21] and isoelectric point,[22,23] and there has been little indication that the isoforms exhibit significant spectroscopic differences in their metal-binding properties.[24,25]

In this paper, we describe the formation of Hg_{18}–MT 2, a novel mercury–protein complex, which is isolated from rabbit liver metallothionein. Hg_{18}–MT 2 exhibits new spectroscopic properties that are unlike those reported previously for mercury-containing metallothioneins.[1,13-15] In addition, differences in metal-binding properties between rabbit and rat liver metallothionein isoforms 1 and 2 are reported that provide evidence for the structural requirements of Hg_{18}–MT.

Hg_{18}–MT is characterized by strong circular dichroism (CD) intensity under the thiolate to mercury charge-transfer bands in the 240–360 nm region. The presence of this unusually intense CD spectrum suggests that Hg_{18}–MT adopts a single-domain structure not found previously for a metallothionein species, that is dependent on the coordination properties of Hg^{2+} and brings to three the structural motifs that can be adopted by metallothioneins, namely, M_7–MT, M_{12}–MT, and M_{18}–MT.

Materials and Methods

Metallothionein was prepared as described previously.[6,10,26] For preparative convenience, rabbit liver Zn–MT and rat liver Cd,Zn–MT were initially isolated. Apo-MT from both sources was prepared as described below. The preparative procedure followed was as follows: (i) Zn–MT was isolated from the livers of rabbits following a series of injections of aqueous solutions of $ZnSO_4$ (20 mg of Zn/kg of body weight, every second day, over 2 weeks). (ii) Cd,Zn–MT was isolated from rat livers following a series of injections of aqueous solutions of $CdCl_2$ (1 mg of Cd/kg of body weight, every second day, over 2 weeks). (iii) The protein was isolated following homogenization of the liver in pH 7.5 phosphate buffer. After mixing with acetone (50% v/v) for 5 min, the solution was centrifuged; the metallothionein remained in the supernatant. Increasing the acetone concentration to 80%, followed by centrifugation, formed a pellet containing the metallothionein. The sediment was redissolved in 5 mM Tris-HCl buffer, and the solution was applied to a G-75 Sephadex column. (iv) Isoforms 1 and 2 were separated using preparative gel electrophoresis. Polyacrylamide gels prepared from acrylamide and *N,N'*-methylenebis(acrylamide) (Sigma), as described by Zelazowski et al.,[27] were used. The cathode and anode buffer solutions were 0.038 M Tris-glycine, pH 8.30, and 0.06 M Tris-HCl, pH 8.10, respectively. The individual isoforms were desalted on a Sephadex G-75 column, eluted with distilled water (pH 7–8), where the pH was adjusted with NH_4OH, and, finally, lyophilized. (v) Apo-MT was prepared by passing Zn–MT or Cd,Zn–MT down a Sephadex G-25 gel column equilibrated with 0.01 M, pH 2, HCl. (vi) Rat liver Zn_7–MT was prepared by adding 7.5 mol equiv of $Zn(CH_3COO)_2$ to rat liver apo-MT at pH 2 and then raising the pH to 7.4 by adding portions of Trizma base. All spectral studies used solutions 10 nmol/mL (approximately, 10 μM) in metallothionein.

A gel filtration molecular weight marker fit (aprotinin, cytochrome *c*, carbonic anhydrase, and albumin; MW-GF-70), insulin chain B, oxidized (3496 Da), and myoglobin (16 950 Da) were obtained from Sigma. Metal concentrations are based on Pipetman volume measurements, which were confirmed using AAS (Varian 875) measurements. Mercury concentrations were determined using a mercury vapor generator as described previously.[28] The protein concentrations were calculated by estimating the concentration of SH groups using the DTNB method[29] and by measurement of the metal concentrations (AAS). In all our titrations monitored by spectroscopic methods, we assume (and have confirmed by AAS techniques[28]) that, with the large binding constants involved for mercury–thiolate complex formation, no free mercury is present prior to saturation at the 18 Hg^{2+} point. We have confirmed independently using AAS techniques for each system that the Hg:MT stoichiometric ratio in each complex can be estimated from the saturation point in the chiral intensity. Chelex-100 was used to bind free metals at each point up to 40 mol equiv of Hg^{2+}. CD spectra of Hg_n–MT 2 (n = 1–18) were recorded before and after the addition of 50 mg of Chelex-100.[30] The CD spectrum of Hg_n–MT 2 remained the same after addition of Chelex-100. The Chelex-treated solution was then digested and analyzed by cold-vapor AAS.[28] A maximum of 18 Hg^{2+} binds to the protein at low pH. Zn^{2+} is displaced stoichiometrically up to 7 mol equiv of Hg^{2+} and at both low pH and pH 7.

The molar mass of Hg_{18}–MT 2 was estimated by comparing its retention time to that of a series of standards on a Sephadex G-50 column. The gel itself influences the complex stability unless pretreated. A method introduced by Lonnerdal and Hoffman[31] to treat chromatographic gels chemically in order to eliminate metal-binding carboxyl and sulfate groups involves alkaline hydrolysis of the charged groups in Sephadex G-50 in the presence of sodium borohydride to reduce metal–gel interactions. (1) A volume of swollen G-50 Sephadex gel was taken up in 2.5 volumes of water. (2) The slurry was adjusted to a pH of 10–11 with NaOH, and $NaBH_4$ was added up to a concentration of 2.0 g/L. (3) The gel slurry was heated at 80 °C for 2 h with gentle stirring. (4) The gel was cooled and then washed with water until the pH was neutral. (5) Any globules generated during the procedure were removed. The treated Sephadex G-50 gel was packed into a column (175 cm × 2.5 cm) and washed extensively (3 column volumes) with 200 mM Hg^{2+} in a 10 mM HCl solution. A 2-mL aliquot of a 40 μM Hg_{18}–MT 2 solution in 10 mM HCl was applied to the column. Similar CD spectra were measured before and after Hg_{18}–MT 2 had been applied to the column (data not shown).

CD spectra were recorded on a Jasco J-500 spectrometer that was controlled by an IBM S9001 computer using the program CDSCAN5.[32a] The spectral data were organized and plotted on an HP 7550A plotter with the spectral database program Spectra Manager.[32b]

Results

Formation of Hg_{18}–MT 2. Hg_{18}–MT is formed most readily when 18 mol equiv of Hg^{2+} is added to rabbit liver apo-MT 2 at low pH (in our studies 10 mM HCl). Figure 1 shows the development of the characteristic, intense CD spectrum in the region of the thiolate to mercury charge-transfer bands between 240 and 360 nm. Hg_7–MT does not exhibit the same CD spectrum at low pH as at pH 7,[26] although the CD spectral intensity saturates at exactly 7:1 Hg:MT (as marked on the contour diagram). Following a stage where the CD intensity is negligible between 10 and 14 Hg^{2+}, a new band grows steeply in intensity as a function of the Hg:MT ratio. The changes in CD band intensities as a function of the Hg:MT ratio are shown in Figure 1D. This species is stable enough that this same CD spectrum is observed when lyophilized powder is redissolved under acidic (but not neutral) conditions. There is no change in the CD spectrum between 18 and 40 Hg^{2+}. The formation of Hg_{18}–MT depends on temperature.[11] CD spectra recorded during titrations carried out at pH 2 and 2 °C show formation of Hg_7–MT but only minimal development of the signature associated with Hg_{18}–MT. The full spectral envelope is observed following warming to 37 °C.[11]

pH Dependence. Figure 2 shows the steep dependence of the CD spectral intensity recorded for rabbit liver Hg_{18}–MT 2 as a

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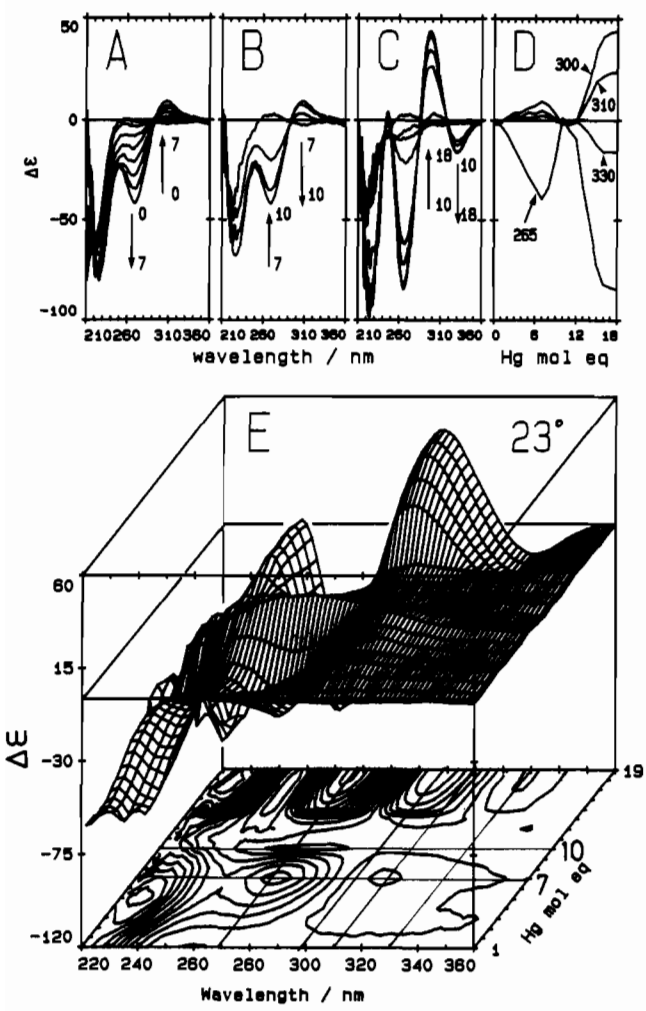

Figure 1. CD spectra recorded during a titration of rabbit liver apo-MT 2 with Hg^{2+} at pH 2 and 23 °C. Top: (A) CD spectral changes for Hg:MT = 0–7; (B) CD spectral changes for Hg:MT = 7–10; (C) CD spectral changes from Hg:MT = 10–18; (D) changes in CD spectral intensities at 265, 300, 310, and 330 nm as a function of the Hg:MT ratio from 0 to 18. (Note the steep increase in CD intensity at 265 and 300 nm at the Hg:MT ≈ 14 point.) Bottom: (E) A 3-dimensional representation of the spectral data. The *z* axis is the Hg:MT molar ratio for the Hg^{2+} added to a solution of apo-MT 2 held at pH 2. Trace 1 is for zero Hg(II) added; trace 19 is for 18 mol equiv of Hg(II) added. No further changes in the CD spectrum were observed for Hg:MT = 18–40. Grid lines are drawn across the contour diagram at 269, 298, and 330 *nm*, the band maxima for the M_7–MT and M_{18}–MT species.

function of pH. The pH of the solution was adjusted upward from pH 2 with Tris buffer. The inset shows changes in the CD intensity at 296.7 and 266.7 nm. The characteristic CD signal of rabbit liver Hg_{18}–MT 2 is unchanged between pH 2.3 and 6.9 but collapses between pH 7 and 8 and is completely quenched at pH 8.5. The formation of Hg_{18}–MT 2 can be carried out at pH 5. CD spectra recorded as from 1 to 18 Hg^{2+} were added to a solution of apo-MT made up in 10 mM HCl with the pH adjusted to 5 with Tris buffer closely resemble the CD spectra recorded in 10 mM HCl at pH 2 (the pH 5 data are not included here).

Chloride Dependence. While acidic conditions are a critical requirement for the formation of the Hg_{18}–MT 2 species, so also is the presence of chloride ion (Figure 3C). A solution of rabbit liver apo-MT 2 (10 mM) was prepared in dilute acetic acid solution (pH 2.4). Aliquots of Hg^{2+} up to 18 mol equiv, using 0.1 N standardized mercuric nitrate (Sigma), were added to the apo-MT 2 solution over a period of 2 h; the CD spectrum was recorded for each addition. At this point, although the pH was 2.4, there was no indication of the characteristic CD envelope of Hg_{18}–MT 2 (Figure 3A, line *). Twenty-milliliter aliquots of 1 M NaF, NH_4Cl, KBr, and NaI were added to separate 2-mL portions of

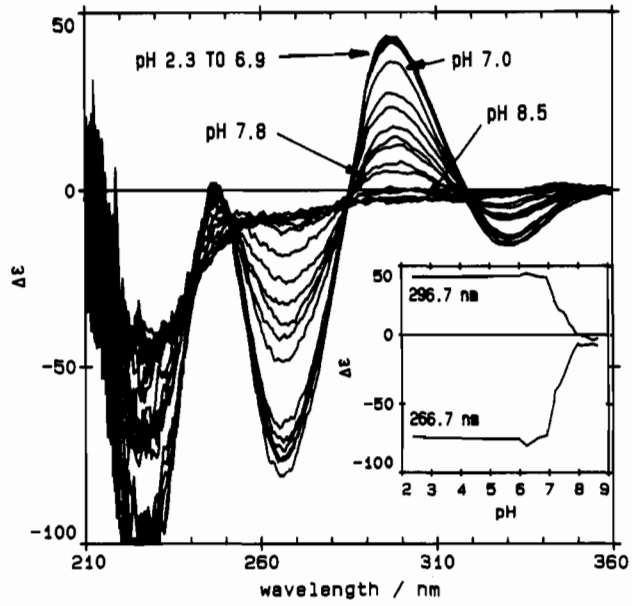

Figure 2. CD spectra recorded for rabbit liver Hg_{18}–MT 2 as a function of pH. pH value for each spectrum: 2.30, 6.00, 6.24, 6.63, 6.93, 7.00, 7.15, 7.19, 7.33, 7.49, 7.57, 7.68, 7.82, 7.89, 7.97, 8.07, 8.22, 8.50. Inset: Changes in the CD intensity at 296.7 and 266.7 nm. The spectral changes are reversible and not dependent on the direction of the change in pH. Conditions: single solution, in HCl; pH adjusted by adding Tris buffer, 10 μM, at 23 °C.

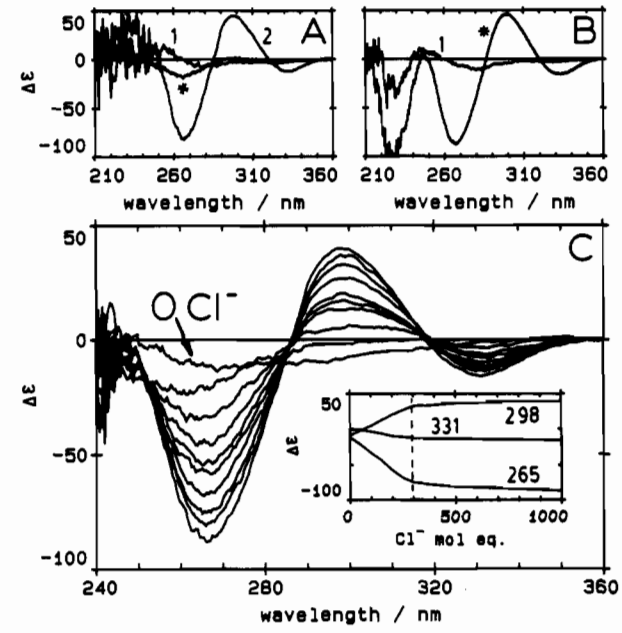

Figure 3. Haloid effects on the formation of Hg_{18}–MT 2: (A) line *, Hg_{18}–MT 2 in dilute acetic acid solution (pH 2.4); line 1, solution + 1000 mol equiv of Br^-; line 2, solution + 1000 mol equiv of Cl^-; (B) line *, Hg_{18}–MT 2 in HCl (pH 2.4); line 1, solution + 1000 mol equiv of Br^-; (C) Hg_{18}–MT 2 in dilute acetic acid (pH 2.4) with different amounts of additional Cl^-. CD spectra in (C) are with 0, 50, 100, 150, 200, 250, 300, 500, and 1000 mol equiv of Cl^-. Inset: Changes in the CD intensity at 298, 331, and 265 nm. The vertical dashed line indicates 300 mol equiv of Cl^-. Conditions: 10 μM, rabbit liver MT 2, at 23 °C.

this solution, and the CD spectra were recorded. A normal CD spectrum for Hg_{18}–MT 2 appeared (Figure 3A, line 2) when Cl^- was added. No changes were observed following addition of F^-. Addition of the Br^- resulted in a weak broad band at 250 nm (Figure 3A, line 1), which is not the same as the CD spectrum of Hg_{18}–MT. A milky precipitate of HgI_2 formed following addition of I^-, as expected with a solubility product constant (K_{sp}) for HgI_2 of 4.0×10^{-29}. In a second step, Cl^- was added to the solutions that contained excess F^- and Br^-. The CD

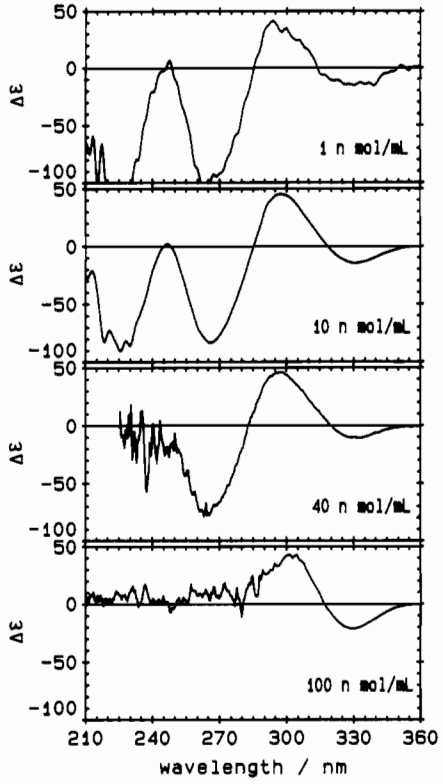

Figure 4. Concentration effects on the CD spectrum of Hg_{18}–MT 2. CD spectra were recorded for Hg_{18}–MT 2 with different protein concentrations. Conditions: rabbit liver MT 2, at 23 °C, in 10 mM HCl; cell length 1.0 cm for 1 and 10 μM solutions, 0.5 cm for 40 μM solution, and 0.1 cm for 100 μM solution.

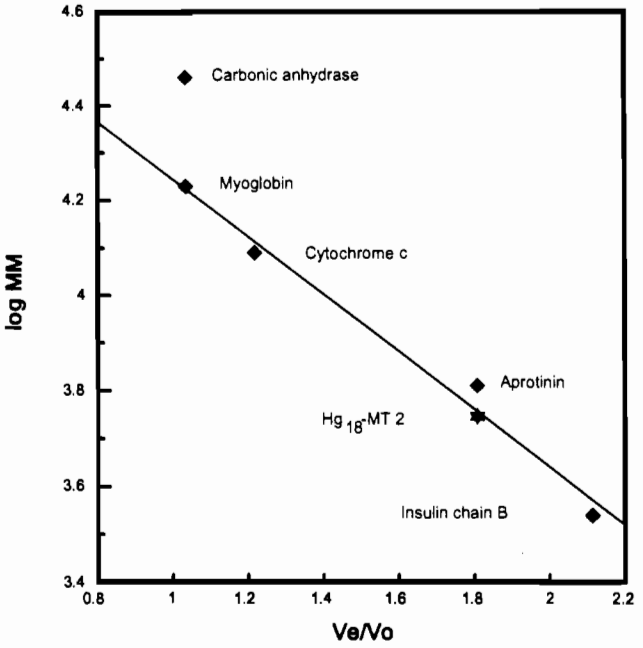

Figure 5. Calibration curve for molar mass determination of Hg_{18}–MT 2: log(molar mass) versus the ratio V_e (elution volume)/V_0 (void volume) for a range of standard molecules. The Sephadex G-50 column was equilibrated with Hg^{2+} before the protein solutions were passed down the column. The proteins (aprotinin (6500 Da), cytochrome *c* (12 400 Da), carbonic anhydrase (29 000 Da), insulin chain B, oxidized (3496 Da), and myoglobin (16 950 Da) span the molar mass range of Hg_{18}–MT 2. Blue dextran (2 000 000 Da) was used to determine the void volume (V_0). 40 mM solutions of each marker protein were applied to the treated Sephadex G-50 column, and the column was eluted with 200 mM Hg^{2+} in a 10 mM HCl solution. Three replicates were taken and average V_e volumes were calculated.

envelope of Hg_{18}–MT 2 developed only when Cl^- was added in the presence of excess F^-.

The coordination properties of each haloid for Hg_{18}–MT was determined by adding 20-mL aliquots of 1 M KBr or NaF to solutions of Hg_{18}–MT 2 in 10 mM HCl. There was no change in the CD spectrum of Hg_{18}–MT 2 when F^- was added, while addition of Br^- replaced the signal due to Hg_{18}–MT 2 with a weak broad band at 250 nm (Figure 3B, line 1). The stability constants (log *K*) for Cl^-, Br^-, I^-, and L-cysteine with Hg^{2+} are 6.62,[33] 9.40,[34] 12.87,[34] and 14.21,[35] respectively. Clearly, Cl^- only binds in the absence of the other haloids. The Cl^- dependency was determined by titrating Cl^- into a solution of apo-MT and 18 mol equiv of Hg^{2+} (from $Hg(CH_3COO^-)_2$ (Figure 3C). The CD spectrum of Hg_{18}–MT 2 reaches a maximum at a concentration of 3.0×10^{-3} M (300 mol equiv) Cl^-.

Concentration Effects. Figure 4 shows a series of CD spectra recorded following addition of 18 mol equiv of Hg^{2+} to 1, 10, 20, 40, and 100 μM solutions of rabbit liver apo-MT 2 in 10 mM HCl. Hg_{18}–MT 2 clearly forms with almost identical intensities for protein concentrations between 1 and 40 μM. The spectrum of the 100 μM solution is correct for the low-energy CD band, but high absorbance below 300 nm precludes measurement of the rest of the CD spectrum. There is no indication of a monomer–dimer equilibrium in this concentration range.

Molar Mass Determination. The molar mass of Hg_{18}–MT 2 was estimated by comparing its retention time on a Sephadex G-50 column to the retention times of the proteins insulin chain B, aprotinin, cytochrome *c*, myoglobin, carbonic anhydrase, and albumin. Blue dextran (2 000 000 Da) was used to determine the void volume (V_0). The results are summarized in Figure 5.

Hg_{18}–MT 2 elutes with an apparent molar mass of 5500 Da. The molar mass of metallothionein deduced from the sequence data is approximately 6000 Da.[36] When determined by gel filtration, the molar mass of equine and other mammalian metallothioneins is considerably higher, at about 10 000 Da. This discrepancy is attributed to the nonglobular shape of the protein.[1] The molar mass and the measured Stokes radius (1610 pm) are consistent with a prolate ellipsoid that has an axial ratio of 6.[37] The apparent molar mass of Hg_{18}–MT estimated by molecular filtration in section is approximately 5500 Da. Reported molar masses from amino acid sequence analysis for renal mercury metallothioneins with Hg:Cu:Zn ratios of 2.0:2.2:0.5 (isoform 1), 2.0:3.6:0.4 (isoform 2), and 1.8:4.4:0.2 (isoform 3) are 6580, 6880, and 7000 Da, respectively,[38] while by gel filtration the masses were 8100 Da for both isoforms 2 and 3.[38]

Spectroscopic Differences between MT Isoforms. Figure 6 shows the CD, MCD, and absorption spectra recorded during titrations of rabbit and rat liver apometallothionein (10 μM) with Hg^{2+} at a pH of about 2 (a 10 mM HCl solution). The span of the Hg:MT molar ratio shown in each panel is from *10 to 18*. Between 0 and 10 Hg^{2+}, a quite different set of CD spectra are obtained, which, although being different for the rabbit and rat MT, do not show any selectivity between each isoform. The experiments were carried out with four different MT isoforms, namely, rabbit liver Zn_7–MT 1 (Figure 6, row B, and Figure 7), rabbit liver Zn_7–MT 2 (Figure 6, row A, and Figure 1), rat liver Cd,Zn–MT 1 (Figure 6, row C), and rat liver Cd,Zn–MT 2 (Figure 6, row D, and Figure 8).

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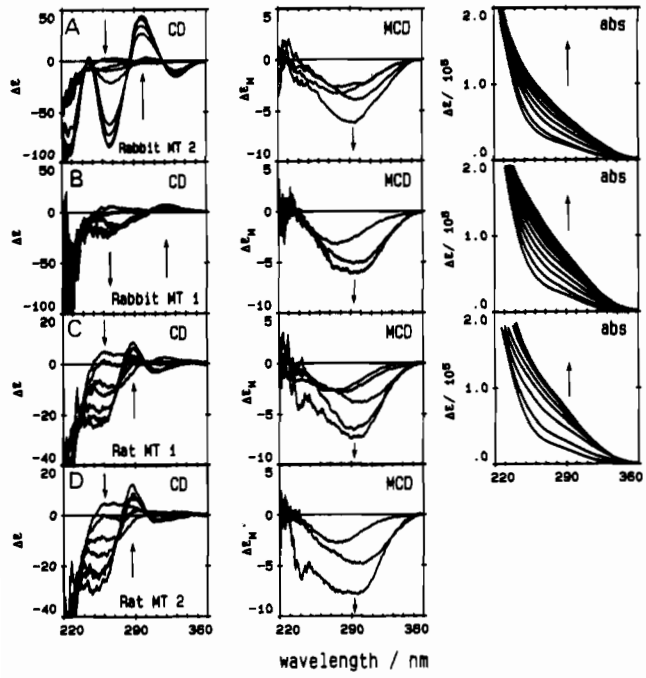

Figure 6. CD, MCD, and absorption spectra recorded during titrations of apo-MT isoforms 1 and 2 with Hg^{2+} at pH 2. (Row A) Rabbit apo-MT 2: CD and absorption spectra are with 10.0–18.0 Hg^{2+}, and MCD spectra are with 10.0, 12.0, 14.0, and 18.0 Hg^{2+}. (Row B) Rabbit apo-MT 1: CD spectra are with 12.0–17.0 Hg^{2+}; MCD spectra are with 10.0, 14.0, and 18.0 Hg^{2+}; absorption spectra are with 10.5, 11.2, 11.9, 12.6, 13.3, 14.0, 14.7, 15.4, 16.1, 16.8, and 17.5 Hg^{2+}. (Row C) Rat apo-MT 1: CD spectra are with 10.0, 11.7, 13.3, 15.0, 16.7, 18.4, and 20.0 Hg^{2+}; MCD spectra are with 10.0, 12.0, 14.0, and 18.0 Hg^{2+}; absorption spectra are with 10.0, 10.7, 12.3, 13.8, 15.4, 16.7, 18.4, and 20.0 Hg^{2+}. (Row D) Rat apo-MT 2: CD spectra are with 10.0, 10.9, 12.7, 14.5, 16.3, 18.2, and 20.0 Hg^{2+}; MCD spectra are with 11.0, 15.0, and 21.0 Hg^{2+}. The arrows indicate the direction of the changes in intensity as a function of increasing Hg^{2+} concentration. The units of $\Delta\epsilon_M$ are L mol^{-1} cm^{-1} T^{-1}; the corresponding zero-field CD spectrum has been subtracted from the field-on spectrum; field was 5.5 T. Conditions: 10 μM protein, in 10 mM HCl, at 23 °C.

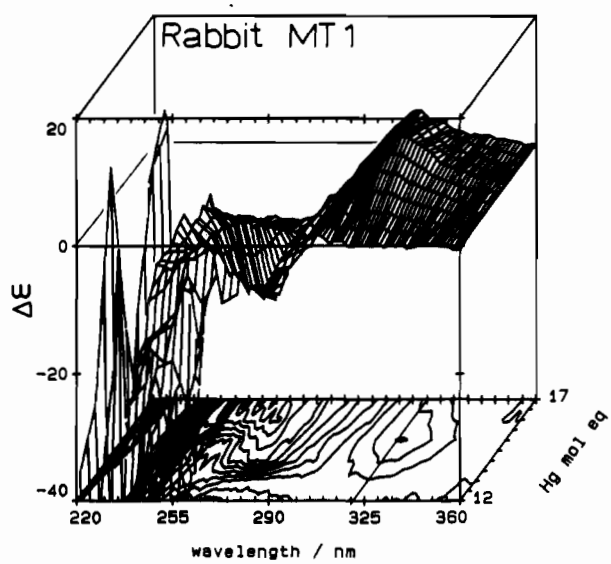

Figure 7. CD spectra recorded during a titration of rabbit liver apo-MT 1 with Hg^{2+} at pH 2. The z axis is mole equivalents of Hg^{2+}. The grid line in the contour diagram parallel to the z axis indicates 320 nm. Conditions: as in Figure 1.

The four sets of CD spectra are quite different. For rabbit apo-MT 2, as described above, the CD signal with maxima at 240 nm (+), 265 nm (–), 298 nm (+), and 330 nm (–) intensifies to an overall maximum at the 18 Hg^{2+} point. *A similar spectral envelope is not observed for the other isoforms examined.* For

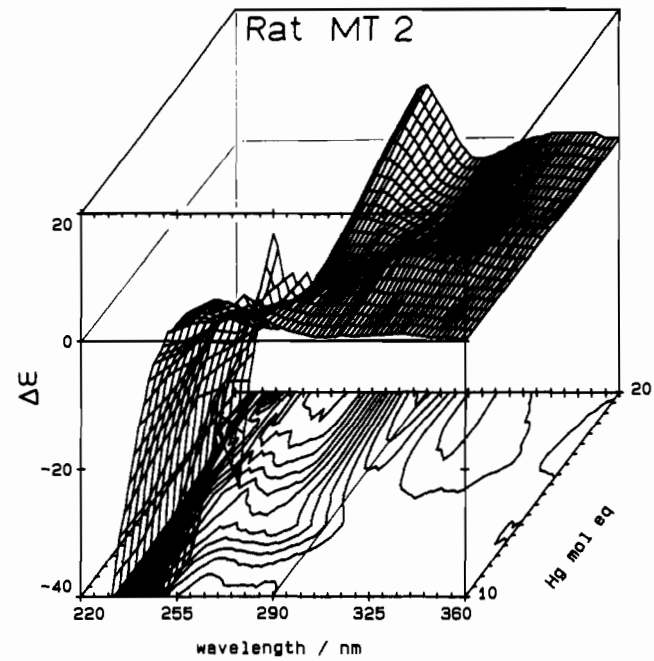

Figure 8. CD spectra recorded during a titration of rat liver apo-MT 2 with Hg^{2+} at pH 2. The z axis is mole equivalents of Hg^{2+}. The grid line in the contour diagram parallel to the z axis indicates 290 nm. Conditions: as in Figure 1.

rabbit apo-MT 1 (Figure 7), only a very weak positive band at 320 nm forms, which reaches a maximum intensity with 17 Hg^{2+}. For rat apo-MT 1 and 2, two sets of spectra develop at high molar ratios of Hg:MT that are very similar (although, note, the $\Delta\epsilon_{max}$ span for the rat MT data is 60, compared with 150 for the rabbit MT data in Figure 6). Both of the rat isoforms exhibit the same spectral features, which include a negative band at 310 nm and a positive band at 290 nm (Figure 8). *Significantly, the band centers are not the same as in the CD spectrum from rabbit apo-MT 2 with 18 Hg^{2+}.* Clearly, under low-pH conditions there are distinct spectroscopic differences between the metallothionein isoforms. Although rabbit apo-MT 1 in 10 mM HCl with 18 Hg^{2+} does not exhibit a strong CD signal, dissolving previously lyophilized rabbit liver Hg_{18}–MT 1 in 10 mM HCl results in the appearance of a CD signal that is identical to that observed for rabbit Hg_{18}–MT 2. No CD spectrum was observed for a second portion of the rabbit Hg_{18}–MT 1 powder which was dissolved first in distilled water (or D_2O), followed by acidification to pH 2 with 1 M HCl.

Spectroscopic Similarities between MT Isoforms. Figure 9 shows Hg^{2+} titration data for isoforms 1 and 2 of rat and rabbit liver Zn_7–MT carried out at neutral pH. The CD spectrum of the starting Zn–MT protein is indicated by a dotted line. Each of the isoforms exhibits a similar CD spectrum as Hg^{2+} is added to form Hg_7–MT. The CD spectral envelopes are characterized by bands at 260 nm (+), 280 nm (–), and 300 nm (+). Addition of Hg^{2+} at neutral pH in excess of 7 mol equiv results in collapse of each CD spectral envelope to the flat lines seen for Hg_{12}–MT. At very high Hg^{2+} loadings, some broad and poorly-defined bands appear for rat MT 1 and 2, labeled as Hg_{20}–MT. The only difference between the spectral properties of these MT isoforms is that the CD intensities for the rabbit MT complexes are much greater. The similarity in the CD spectra for Hg_7–MT in each of the panels in Figure 9 shows that Hg_7–MT forms with very much the same overall structure within the metal-binding sites. This pattern of bands is more intense when Hg^{2+} binds to apo-MT 2 at neutral pH.[26]

Addition of stoichiometrically correct aliquots of Zn_7–MT (of the appropriate isoform) at pH 2 to a solution of Hg_{18}–MT (1 or 2), re-forms the Hg_7–MT spectrum exactly as formed initially. The experiment tests the reversibility of the following overall

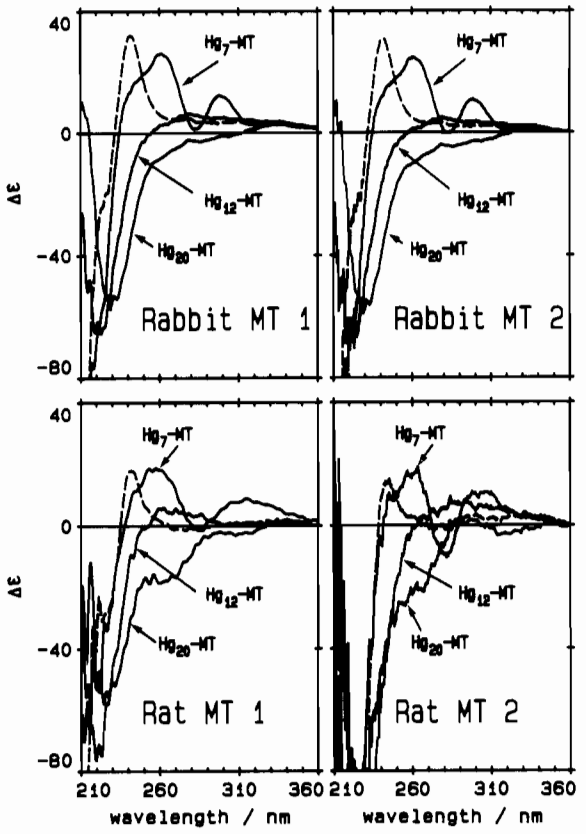

Figure 9. CD spectra recorded during titrations of zinc metallothionein isoforms 1 and 2 with Hg^{2+} at pH 7. The spectra shown are for the sequence (0, 7, 12, 20) of additions of Hg^{2+} to different metallothioneins. They are characterized by bands at 260 nm (+), 280 nm (-), and 300 nm (+). At pH 7, there is no indication that a species forms at very high Hg:MT molar ratios. Note: Between 7 and 11 Hg^{2+} at pH 7, a new species is formed, which has been assigned previously as involving a trigonal Hg^{2+} coordination; the band maximum for this Hg_{11}–MT species is near 300 nm.[26] Conditions: 10 μM protein, H_2O as solvent, at 23 °C.

reaction expressed as a series of separate steps. The observation of Hg_7–MT forming when Zn_7–MT is added to Hg_{18}–MT suggests that rearrangement of the mercury and the zinc between metallothionein molecules is a facile process.

$$[Zn_7\text{–MT}]^{6-} + 7Hg^{2+} \rightarrow [Hg_7\text{–MT}]^{6-} + 7Zn^{2+}$$

$$[Hg_7\text{–MT}]^{6-} + 11Hg^{2+} \rightarrow [Hg_{18}\text{–MT}]^{16+}$$

$$[Hg_{18}\text{–MT}]^{16+} + {}^{11}/_{7}[Zn_7\text{–MT}]^{6-} \rightarrow {}^{18}/_{7}[Hg_7\text{–MT}]^{6-} + 11Zn^{2+}$$

Clearly, metallothionein is not chemically altered in the presence of the high Hg^{2+} loading and no oxidation could have taken place. Similar evidence is obtained from the XANES study using the S L-edge.[12] Although the metallothionein isoforms that bind 18 Hg^{2+} at pH 2 exhibit different CD spectra, under the same conditions, the MCD and UV absorption spectra for each of these isoforms are very similar, as shown in Figure 6.

Discussion

The CD spectrum of metallothionein is highly dependent on the nature and stoichiometric ratio of the metal bound to the protein.[1,7] We have interpreted changes in the CD spectra measured during titrations of metallothionein with cadmium,[6,39] mercury,[7,26] and silver[10] at neutral pH in terms of changes in the binding-site structure as one of two different structural motifs, characterized by either tetrahedral or trigonal coordination of the metals, is adopted. CD spectral intensity is well-known to be extremely sensitive to the formation of chiral structures in biological molecules. We interpret the distinctive CD signals observed for metallothionein, which contains no aromatic amino acids and has no natural tendency to adopt extensive regions of α helix or β pleated sheet, in terms of metal–thiolate binding that cross-links regions of the peptide chain into a chiral binding-site cage that encloses the metals.[4-7,13,14,40] The observed chirality is a property of the whole domain, not just of the metal–thiolate bond. The key to these studies lies in the use of an optical spectrum that owes its electric dipole intensity (its absorption intensity) to charge-transfer transitions between the coordinating thiolate and the metal atom. The CD intensity arises when this transition takes place in a chiral electric field formed from a highly asymmetric environment.[13] The value of the CD technique for our present studies is that formation of complexes that exhibit 3-dimensional structures will result in changes in the CD signal in the wavelength region of metal-related transitions, that is, from metal–thiolate groups in the metal-binding sites. *Therefore, the CD spectrum selectivity reports the asymmetry in the metal-binding site, a property which is directly dependent on the coordination geometry around each metal.* The new CD envelopes observed at high metal loadings for Hg^{2+} and Ag^{+} [10] may well arise from metal–thiolate-induced "supercoiling" of the peptide chain in a manner similar to that described for both DNA[41] and copper(I) helicates.[42]

Two structural motifs have been described previously for mammalian metallothioneins. Cd,Zn–MT is based on tetrahedral coordination geometry around the metals within M_4S_{11} (α) and M_3S_9 (β) domains.[4,5] The second motif is also based on a two-domain structure but involves trigonal geometry around the metals and has been proposed for M_{12}–MT,[7-10,26,43] where M = Cu^+, Ag^+, and, recently also, Hg^{2+}. Chemical[8] and spectroscopic[10,44,45] studies support the formation of both M_6S_9-β and M_6S_{11}-α domains for Cu^+ and Ag^+. In the smaller yeast metallothioneins, a similar trigonally-based structure has been proposed on the basis of EXAFS measurements,[43] with a single domain based on a Cu_8S_{12} cluster. For Hg_{18}–MT, the question remaining concerns the coordination geometry adopted between the 18 Hg^{2+} and the 20 cysteinyl thiolates: linear, trigonal, or tetrahedral?

Inorganic Model Compounds with Mercury–Thiolate Bonding: Available Coordination Geometries. Synthetic mercury–thiolate complexes mimic the metal-binding site in proteins and provide examples of possible coordination geometries in metallothionein. The coordination chemistry of Hg^{2+} with oxide and thiolate ligands is complicated with structures with different coordination numbers being adopted.[17,19,46-49] Of particular interest are the structures involving Hg^{2+} and cysteine;[19] in $Hg[SCH_2CH(NH_3)COO]$-

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$[SCH_2CH(NH_3)COOH]Cl{\cdot}0.5H_2O$, the mercury atom is coordinated to the sulfur atoms of two cysteine molecules (average Hg–S bond length 234.2 pm) in an approximately linear array with chloride present as an essential counterion (the Hg–Cl length is 323.2 pm). A similar structural dependence on chloride has been reported for $HgCl_2[SCH_2CH(NH_3)COOH]$ synthesized from $HgCl_2$ and L-cysteine in aqueous acidic alcohol.[19] This molecule is a sulfur-bridged polymer with each mercury atom coordinated in a pseudotetrahedral fashion by two cysteinyl sulfur atoms and two chloride ions. The Hg–S distance averaged 247.2 pm, shorter than the average Hg–Cl bond length (261.4 pm).

A number of compounds with trigonal coordination of mercury have been reported;[18,46,49] among these, structures of $[Et_4N][Hg(SBu^t)_3]$[18] and Hg-merR and its models[49] most closely relate to the structures adopted in Hg_n–MT (with n = 7, 11, and 18). Because mercury can bind with a range of coordination numbers (2–4 and 6), it is not unexpected that as the Hg:MT molar ratio increases from 1:20 to 18:20 during titrations of MT with Hg^{2+}, different coordination geometries are adopted. Because the available coordination geometries for cadmium and zinc are more limited, both Cd–MT and Zn–MT apparently involve *only* tetrahedral coordination.

Formation and Properties of Hg_{18}–MT 2 Species. The spectrum observed for Hg_{18}–MT 2 is in the region of RS → Hg^{2+} charge-transfer transitions. The CD intensity observed for Hg_{18}–MT 2 must arise from a strongly chiral environment for the Hg–S bonds, which means that *a 3-dimensional structure is a necessary condition.* The temperature and concentration dependences and the molar mass determination are together strong evidence against dimer formation. (i) There is no $\Delta\epsilon$ change over a concentration range of 1–40 μM. (ii) Rabbit Hg_{18}–MT 2 forms preferentially at higher temperatures rather than at low temperatures. (iii) Hg_{18}–MT 2 elutes on the low-mass side of cytochrome *c* on Sephadex G-50 with a molar mass of about 5500 Da. Our XANES study of Hg_{18}–MT[12] indicates that Hg_{18}–MT 2 has a structure unlike M_7–MT and no disulfide bond formation occurs. Reports for both Cd_7–MT[7] and Co_7–MT[50] studied with EPR, CD, and MCD techniques support the view that metal-related transitions are reliable markers for the presence of well-defined metal–thiolate structures.

The Cl^- dependence indicates that the Hg_{18}–MT structure, like the inorganic models,[19,34,51] requires chloride ion in coordination sites on the Hg^{2+}.

Selectivity in Hg^{2+} Binding to Isoforms 1 and 2 of Rabbit and Rat Liver MT. Formation of Hg_{18}–MT is dependent on the metallothionein isoforms used. Between pH 2 and 6, Hg_{18}–MT forms selectively with rabbit MT 2 only. Isoforms of rabbit metallothionein eluted from Sephadex DEAE columns, as well as separated by polyacrylamide electrophoresis, can be identified from their distinctive CD spectra in the presence of excess Hg^{2+}. Clearly, this arises from a structural requirement of the RS–Hg–RS bond formation in Hg_{18}–MT. A similar spectral discrimination between two isoforms has been observed for copper binding to fetal bovine liver and was ascribed to differences in the amino-terminal parts of the sequence.[25]

Proposed Model. Our model for the species that forms at high Hg:MT ratios must account for (i) the spectroscopic differences between the different metallothionein isoforms at low pH when metallothionein binds 18 Hg^{2+}, (ii) the similarities between the spectra of each metallothionein isoform at neutral pH, (iii) the reason that Hg_{18}–MT 1 forms following lyophilization when the powdered protein is redissolved in HCl, and, finally, (iv) the reason that Hg^{2+} but not Cd^{2+} or Zn^{2+} reacts to form this type of complex.

Rabbit MT 2 binds Hg^{2+} at both *neutral pH* and *low pH* with a Hg:MT molar ratio of 7 to form complexes characterized by unique CD spectral patterns. Because the CD spectrum is highly pH dependent, we cannot conclude that exactly the same two-domain structure at neutral pH is adopted at low pH, but we do propose that the M_7–MT (M_7S_{20}) structure involves tetrahedral coordination in two domains; that is, there is a minimum-energy complex for tetrahedral coordination of the metal by the cysteinyl thiolate groups. Figure 10 illustrates the stabilization energy for each molar ratio of Hg:MT; with Hg:MT = 1–8, the Hg_7–MT structure is the most stable.

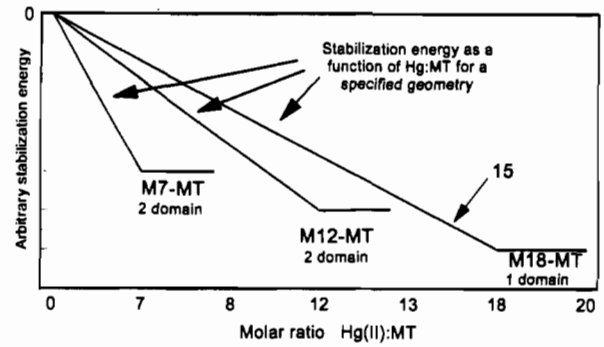

Figure 10. Energies of the M_7, M_{12}, and M_{18}–MT structures. Diagramatic representation of the energies of the three complexes that form for Hg–MT: Hg_7–MT, Hg_{12}–MT, and Hg_{18}–MT. The diagonal lines represent the free energy as a function of Hg^{2+} added for each structural geometry. Initially the tetrahedrally-based M_7 geometry provides the greatest stabilization, then as the molar ratio Hg:MT increases, so the M_{12} structure with trigonal coordination around Hg^{2+} becomes lower in energy, and finally with >14 Hg^{2+} the Hg_{18} geometry is most stable. We associate the changes in geometry with a series of accessible coordination geometries around mercury. The structures are reversibly connected.

At the 10–12 Hg^{2+} point of low pH, we find isodichroic formation of a spectrum that exhibits a very broad and weak CD envelope. This behavior suggests that a species does form but that it has a very open structure, quite unlike the Hg_7–MT structure. By contrast, when Hg^{2+} is added to Zn–MT, a new structure forms at the 11 Hg:MT point, with a proposed trigonal coordination geometry.[26] Again, we propose that the M_{12}–MT ($M_{12}S_{20}$) structures are always based on a trigonal geometry in two domains. A significant working assumption is that when CD spectral intensity is quenched by the addition of further Hg^{2+}, a new structure forms that binds all the Hg^{2+} in the solution.

Addition of up to 18 Hg^{2+} results in the new and intense CD spectrum appearing at low pH. The very steep increase in CD spectral intensity past the Hg:MT = 14 point and the inability of the Hg_{18}–MT structure to form at low temperatures strongly suggest that a major rearrangement of the peptide chain is necessary. We interpret these experimental findings to mean that a change from a two-domain structure to a one-domain structure takes place. This extensive rearrangement breaks up the two-domain structure, and so the activation energy is greater than that for an M_7–MT to M_{12}–MT transition. The Hg_{18}–MT structure requires the presence of most of the mercury necessary to fill all coordination sites before the structure is formed, which means that the structure (and, therefore, the CD spectrum) only forms near the saturation point of 18 Hg^{2+}.

In this single domain we will find either digonally-coordinated Hg^{2+}, involving bridging sulfurs with RS–Hg–RS units cross-linking the structure, or, we believe most likely, a pseudotetrahedral geometry involving two bridging thiolates and two chloride ions.

The similarities between the UV absorption and MCD spectra for the different Hg-containing MT isoforms support the view that the major differences between rabbit Hg_{18}–MT 2 and the Hg_{18}–MT complexes of the other isoforms (and sources) must result from the requirements of the 3-dimensional structure on the folding of the peptide chain. The very different CD spectral envelope means that the asymmetry of the *whole* metal-binding-site region, which for 18 Hg^{2+} means for the whole protein molecule, must be very different.

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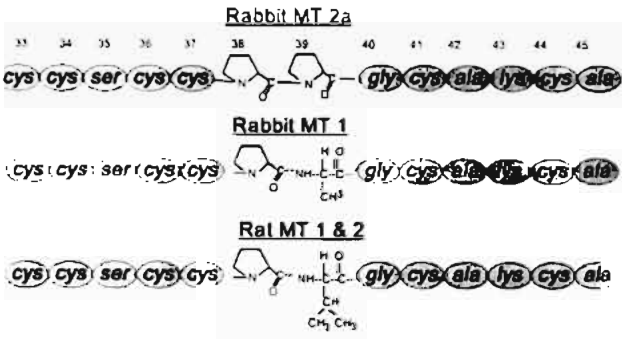

Figure 11. Part of the sequence for rabbit liver MT 2a and MT 1 and rat liver MT 1 and 2. The diagram shows a portion of the sequence for the rabbit and rat liver metallothioneins that lies in the α domain between residues 33 and 45. The data were taken from ref 1b. The three primary sequences exhibit differences at residue 39 that we propose result in the inability of rabbit MT 1 and rat MT 1 and 2 to fold to establish the single domain required by the Hg_{18}–MT structure.

The requirement that Hg_{18}–MT 2 forms only below pH 7 suggests that protonation of hydrogen-bonded groups probably controls the formation of this structure. The results of lyophilization experiments for rabbit Hg_{18}–MT 1 support this view that a specific amino acid sequence plays an important role in formation of the Hg_{18}–MT species. (Lyophilization dehydrates the protein, breaking solvent–protein hydrogen bonds, which we believe allows the protein conformation to relax when redissolved in acidic solution.) We propose that the sequence in the region 38–40 as outlined in Figure 11 accounts for the differences in binding between the isoforms. If this is the case, then we can speculatively describe the sequence of complex formation as the Hg:MT ratio increases (as described in Figure 10): At neutral pH, the tetrahedrally-based Hg_7–MT gives way to a trigonally-based Hg_{11}–MT.[26] At pH 2, following formation of the tetrahedrally-based Hg_7–MT, a less structured complex forms between 8 and 13 Hg^{2+}, before the protein adopts the new, tight molecular structure of Hg_{18}–MT. It appears that only the amino acid sequence of rabbit liver MT 2a can satisfy the requirements for the twist angles necessary to accommodate the peptide folding caused by the coordination geometry of the mercury.

Acknowledgment. We wish to thank Prof. Kagi for providing us with a sample of rabbit liver MT 2a, Prof. P. A. W. Dean at UWO for very helpful discussions, and Anthony Presta for carrying out experiments on separation of the protein isoforms. We wish to thank Drs. Annie Law and Jadwiga Szymanska for preliminary studies on mercury binding. This work was supported by the Natural Sciences and Engineering Research Council of Canada under the Operating and Strategic Grants programs.