Silver Binding to Rabbit Liver Zinc Metallothionein and Zinc α and β Fragments. Formation of Silver Metallothionein with Ag(I):Protein Ratios of 6, 12, and 18 Observed Using Circular Dichroism Spectroscopy

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Formation of a series of complexes between Ag^+ and the cysteine thiolate groups in rabbit liver zinc metallothionein (MT) and the Zn_3–β MT 1 and Zn_4–α MT 1 fragments is reported from analysis of the circular dichroism (CD) spectral data recorded between 5 and 55 °C during titrations of the protein with Ag^+. The spectral envelopes reveal formation of Ag_{12}–MT, Ag_{18}–MT, Ag_6–α MT, and Ag_6–β MT. Silver(I)–thiolate complex formation is associated with characteristic CD spectral envelopes and depends on the stoichiometric ratio of Ag:MT, the temperature, and the pH. The presence of the tetrahedrally-coordinated Zn^{2+} in Zn_7–MT 2 inhibits formation of the Ag_6–MT and Ag_{12}–MT species previously observed when Ag^+ binds to apo-MT 2 (Zelazowski, A. J.; Gasyna, Z.; Stillman, M. J. *J. Biol. Chem.* **1989**, *264*, 17091–17099) at 20 °C, and formation of Ag_{18}–MT dominates the spectral traces. At 55 °C, the Ag_{12}–MT species does form. Addition of Ag^+ at pH 3.8 and 55 °C to Zn_7–MT 2 (nominally apo-MT 2) results in a different sequence of complexes forming in the range Ag^+:MT = 1–18. Analysis of the CD spectral data suggests that the low pH enhances formation of Ag_6–S_9 clusters in the β domain, characterized by a single band at 254 nm, inhibits formation of Ag_6–S_{11} clusters in the α domain, and saturates the binding sites with the formation of Ag_{18}–MT. The CD spectral envelopes obtained as Ag^+ was added to solutions of the Zn_4–α MT and Zn_3–β MT fragments clearly show for the first time spectral signatures associated with formation of both Ag_6–α MT 1 and Ag_6–β MT 1 complexes, respectively. The CD spectral characteristics of the Ag_6 fragments match the spectral patterns observed for Ag_n–MT (n = 6, 12) formed from Zn_7–MT 2. A new species forms at high mole ratios of Ag:MT with Zn_4–α MT 1. Tentatively written as Ag_{12}–α MT 1, this complex shows an intense CD spectrum which suggests that its structure may be similar to the "supercoil" postulated previously for Hg_{18}–MT 2 (Cai, W.; Stillman, M. J. *J. Am. Chem. Soc.* **1988**, *110*, 7872–7873). Metal analysis for titrations of Zn_7–MT 2 at pH 7 shows that each Zn^{2+} is displaced by 2 Ag^+ ions, so that stoichiometric amounts of Zn^{2+} remain bound to the protein up to the 14 Ag^+ point.

Introduction

Metallothionein (MT) is a low-molecular-weight metal-binding protein, rich in cysteine,[1] which was first isolated from equine kidneys.[1,2] MT has been found generally in mammalian tissue,[3-5] as well as in microorganisms and invertebrae.[6] While there has been continuing interest in the induction of MT by cadmium and zinc, several other metals will induce protein synthesis in either the liver or the kidneys of mammals,[2,7-10] and many metals will bind to MT in vitro.[2,10,11] Although the metal-binding reactions of zinc and cadmium have been very well studied,[2] metal-binding reactions of other metals are much less well-known. Recent studies on methods to reduce the toxicity of platinum anticancer drugs suggest that metallothionein plays a role in the clearing of these metals.[12] Preventative measures include preexposure to less toxic MT-inducing metals such as zinc or bismuth.[13] In the absence of metals, apo-MT adopts a random coil structure, whereas, in Zn_7–MT, the zinc is tetrahedrally coordinated by thiolate groups.[2] It is therefore of considerable interest to examine how a metal that adopts trigonal or linear coordination can coexist with zinc in a mixed-metal protein. In this paper, we report our analysis of CD spectral data recorded during titrations of rabbit liver zinc metallothionein, and the zinc-containing α and β fragments, with Ag^+. Formation of Ag_6–α and Ag_6–β domains within the complete protein is reported. Spectral evidence for the formation of Ag_{18}–MT and Ag_{12}–α MT is also presented.

Materials and Methods

Zn–MT 2 was isolated from the livers of rabbits injected eight times with a solution of $ZnSO_4$ (20 mg of Zn/kg of body weight) over a 2-week period and purified as previously described.[14-17] The α fragment was prepared from rabbit liver apo-MT 1 as described by Winge and Miklossy.[16] Briefly, 4 mol equiv of cadmium was added to an apo-MT 1 solution and the pH of the solution was brought up to pH 8. Partial digestion of the protein was achieved using the enzyme subtilisin as

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previously described.[16-18] In these experiments, a single metal-binding domain remains undigested so that either the α domain with 11 cysteines or the β domain with 9 cysteines can be isolated. The β fragment was prepared from apo-MT 1 after substitution with 6 mol equiv of Cu^+ and digested as described previously.[14,17] Apo-α MT was produced from Cd_4-α MT by removing cadmium on a Sephadex G-25 column, previously equilibrated with 0.01 M HCl. Apo-β MT 1 was obtained by incubation of the Cu_6-β fragment with 5 molar excess of KCN in HCl at pH 0.3 for 1 h. The solution was then separated on a Sephadex G-25 column which had been equilibrated with 0.01 M HCl. Absorptions at 220 nm and the cadmium and copper concentrations from AAS measurements were used to monitor the concentrations of the fragments. Zn_4-α MT 1 and Zn_3-β MT 1 were prepared by adding the stoichiometrically-correct amounts of Zn^{2+} to the apo fragments at pH 2.2 and then adjusting the pH to 7.5 with Trizma base.

Protein concentrations were estimated from measurements of SH groups using 5,5'-dithiobis(nitrobenzoic acid) in 6 M guanidine hydrochloride.[19] Calculations were based on the assumption that there are 20 -SH groups in the whole protein and 11 or 9 in the α or β fragments, respectively. The total -SH, plus RSSR, concentration was estimated using the method of Cavallini et al.[20] Metal concentrations were determined with a Varian 875 atomic absorption spectrophotometer. Precision in the determination of the SH and metal concentrations is estimated to be ±5%. Circular dichroism spectra were recorded on a Jasco J-500 spectrometer, controlled by an IBM 9001 computer using a modified version of the program CDSCANS.[21] Ag^+ was added to solutions of the Zn-protein under argon. The spectra were processed using the programs Spectra Manager[22a] and Plot3D[22b] and replotted on an HP 7550A plotter.

Molar ratio aliquots of Ag^+ (using $AgNO_3$) were added sequentially to a single solution of Zn_7-MT at pH 7 that had been saturated with Ar. This procedure is similar to that used for Cd^{2+} titrations of Zn-MT[14] but contrasts the use of separate solutions with our apo-MT experiments.[14,23] Ag^+ was mixed with Zn-MT at each temperature for between 5 and 10 min before the spectrum was recorded. Kuziemska[24] has shown that, unlike the reaction of MT with Hg^{2+}, reactions of MT with Ag^+ are almost instantaneous.

In the experiments described here, we identify the formation of discrete complexes from the stoichiometric ratios between silver and the protein that result in saturation of the CD intensity recorded in the wavelength region of metal-related transitions.[2b,14,23]

The CD spectra are displayed in terms of amplitude vs wavelength vs mol equiv of Ag^+ added, in the form of 3-dimensional plots in order to distinguish gradual processes more readily than is possible from 2-dimensional plots. Contour plots extracted from the 3-dimensional data provide unambiguous evidence of species formation. Throughout this paper, we refer to the mole equivalents of silver added in terms of "6 Ag^+", to stand for "6 mol equiv of Ag^+", or "a molar ratio for Ag^+:MT of 6". We use the nomenclature of Ag_6-β MT to indicate that 6 mol equiv of Ag^+ is bound to the 9 thiolates in the β fragment, Ag_6-α MT to indicate that 6 mol equiv of Ag^+ is bound to the 11 thiolates in the α fragment, and Ag_6^{β},Zn_4^{α}-MT to indicate metal distribution in the β and α domains of the holoprotein, respectively.[2]

Results

We have previously described the design of our experiments in some detail.[14,23] Briefly, we use the CD spectrum to monitor changes in the chirality of the peptide chain as Ag^+ is added to 10^{-6} M solutions of rabbit liver Zn_7-MT 2 or the Zn_4-α MT 1 or Zn_3-β MT 1 fragments. We assume in this discussion that all new bands in the CD spectrum to the red of 250 nm arise from silver(I) or silver–thiolate dependent excited states. We also assume that the development of a spectral signal that reaches a maximum intensity at a certain stoichiometry of silver:protein is related to the formation of a single species. We suggest that these well-defined, 3-dimensional structures are characterized by specific CD spectral intensity under the intrametal or metal–thiolate transitions. We also suggest that the CD spectra are a property of the metal-binding site as a whole and not of isolated metal–thiolate units, so that the CD intensity surface plotted in the figures relates structural changes that occur in the binding site with the presence of incoming silver ions.

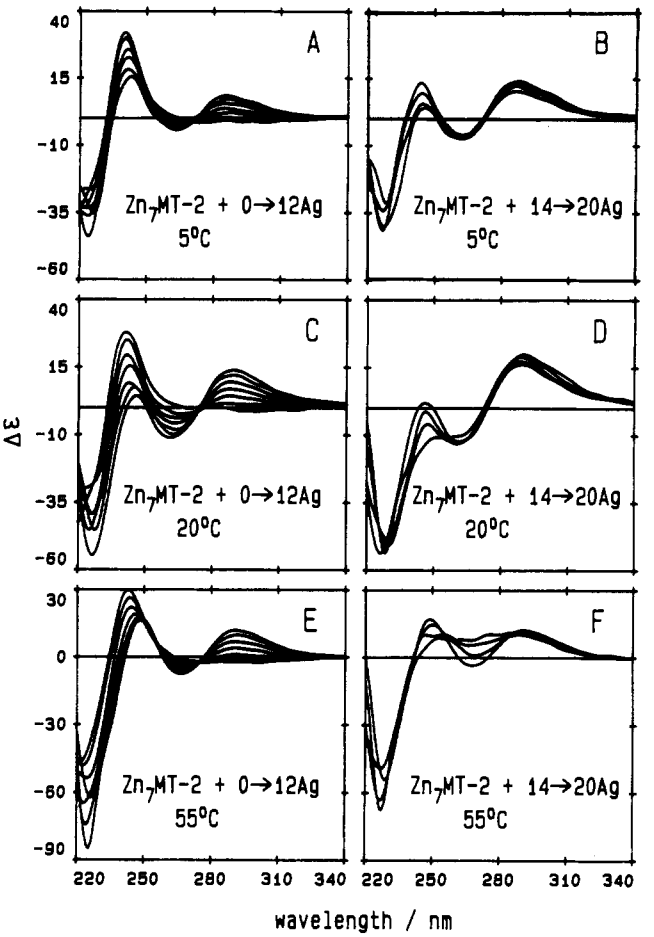

Figure 1. CD spectra recorded during titrations of Zn_7-MT 2 with Ag^+ at 5, 20, and 55 °C: 0–12 Ag^+ (left); 14–20 Ag^+ (right). 2 mol equiv aliquots of Ag^+ were added to a single solution held at the specified temperature.

Titration of Zn_7-MT 2 with Ag^+ at 5, 20, and 55 °C. Figure 1 shows CD spectral data obtained as aliquots of Ag^+ were added to single solutions of rabbit liver Zn_7-MT 2 held at the specified temperature (A and B at 5 °C; C and D at 20 °C; E and F at 55 °C). The same overall sequence of spectral changes is seen at each temperature as from 1 to 20 Ag^+ are added to a single solution of Zn-MT 2. The 243-nm band characteristic of Zn_7-MT first diminishes in intensity, while a band near 295 nm gradually intensifies. The 3-dimensional and contour representations of the spectral data obtained in these experiments (Figures 2–4) show much more detail and particularly highlight the trends in speciation that are dependent on both mole ratio of Ag:protein and temperature. The major effect of an increase in temperature from 5 to 55 °C on the spectral patterns is to resolve the formation of a species that gives rise to a negative band near 268 nm and a positive band near 293 nm.

In detail, at 5 °C (Figure 2), the CD spectra recorded as the molar ratio Ag:MT increased show a very gradual increase in the concentration of a species with a band maximum near 293 nm. Over the same range, the 243-nm band of Zn_7-MT loses intensity. A gradual red shift of the 243-nm band is indicated by the skew in the contour line maximum; this indicates that the band near 246 nm at the 18 Ag^+ point is dependent on Ag_{18}-MT rather than on Zn_7-MT. It is important to note the plateau in intensity

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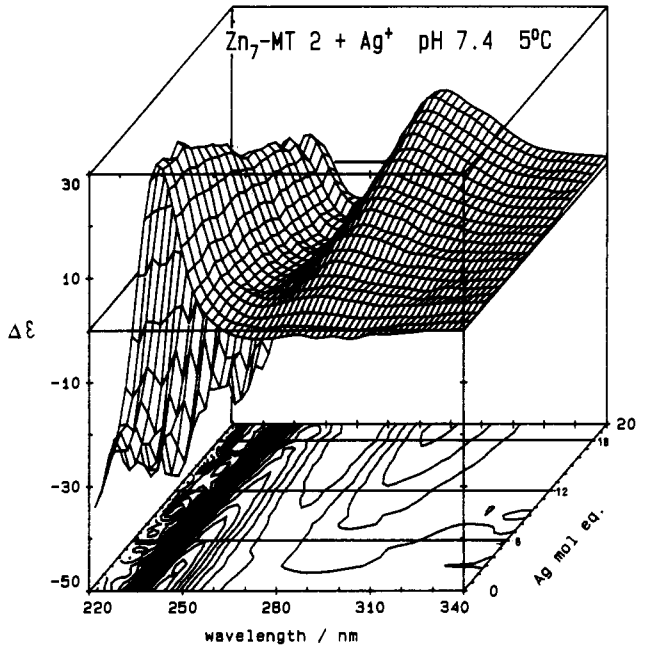

Figure 2. Titration of Zn_7–MT 2 with Ag^+ at 5 °C: 3-dimensional representation of the spectral data recorded at 5 °C.

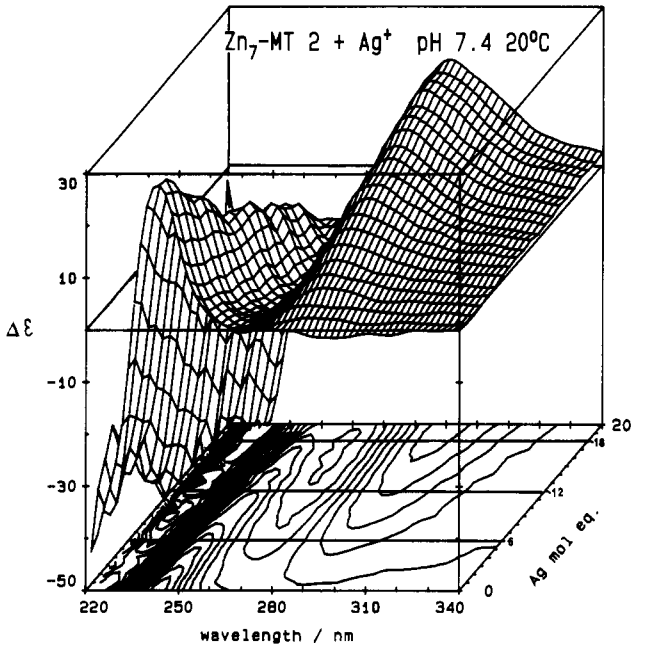

Figure 3. Titration of Zn_7–MT 2 with Ag^+ at 20 °C: 3-dimensional representation of the spectral data recorded at 20 °C.

Table I. CD Spectral Parameters of Ag_n–MT Species Formed from Zn_7–MT, Zn_4–α MT, and Zn_3–β MT

protein used	reaction conditions	species formed	CD band max/nm (sign of CD signal)
Zn_7–MT 2	pH 7; 5 °C	Zn_7–MT	243 (+)
	+12 Ag^+	no species resolved	
	+18 Ag^+	Ag_{18}–MT	246 (+), 287 (+)
Zn_7–MT 2	pH 7; 20 °C	Zn_7–MT	243 (+)
	+12 Ag^+	Ag_6^{α}, Ag_6^{β}–MT	248 (+) weak
	+18 Ag^+	Ag_{18}–MT	262 (–), 289 (+), 293 (+) sh
Zn_7–MT 2	pH 7; 55 °C	Zn_7–MT	244 (+)
	+12 Ag^+	Ag_6^{α}, Ag_6^{β}–MT	250 (+), 268 (–), 293 (+)
	+18 Ag^+	Ag_{18}–MT	293 (+)
Zn_7–MT 2	pH 3.8; 55 °C	Zn_7–MT	no resolved bands
	+3–6 Ag^+	H_{11}^{α},Ag_6^{β}–MT	255 (+)
	+18 Ag^+	Ag_{18}–MT	268 (–), 300 (+)
Zn_4–α MT 1	pH 7; 5 °C	Zn_4–α MT	246 (+)
	+6 Ag^+	Ag_6–α MT	248 (+), 270 (–), 297 (+)
Zn_4–α MT 1	pH 7; 20 °C	Zn_4–α MT	245 (+)
	+6 Ag^+	Ag_6–α MT	264 (–), 293 (+)
	+12 Ag^+	Ag_{12}–α MT	264 (+), 306 (–)
Zn_3–β MT 1	pH 7; 20 °C	Zn_3–β MT	250 (+)
	+6 Ag^+	Ag_6–β MT	252 (+), 295 (+)

near 260 nm that extends to the 12 Ag^+ point. The straightforward pattern of the contour diagram indicates that no other highly chiral species form between 0 Ag^+ and 18 Ag^+ at 5 °C.

At 20 °C (Figure 3), we see a steeper reduction in CD intensity of the Zn_7–MT chromophore at 243 nm and a strong dependence of the positive 248-nm band on the molar ratio of Ag:MT. The 262-nm plateau region extends only to near 6 Ag^+ before a new negatively-signed band begins to form, which develops into a valley in the contour diagram, reaching a maximum at 12 Ag^+. As at 5 °C, the 243-nm band of the Zn–thiolate masks the development of this Ag–thiolate 248-nm band, but unlike the data recorded at 5 °C, there is more indication that a species forms at the 12 Ag^+ point, although the spectral envelope is dominated by formation of Ag_{18}–MT. It is striking that the strong 240 (+)/260 (–) feature observed at the 12 Ag^+ point with apo-MT 2[23] is only very weakly observed during the titration of Zn_7–MT with Ag^+.

The CD data obtained at 55 °C (Figure 4) are quite unlike the data recorded at 5 and 20 °C. The Zn–S band at 244 nm

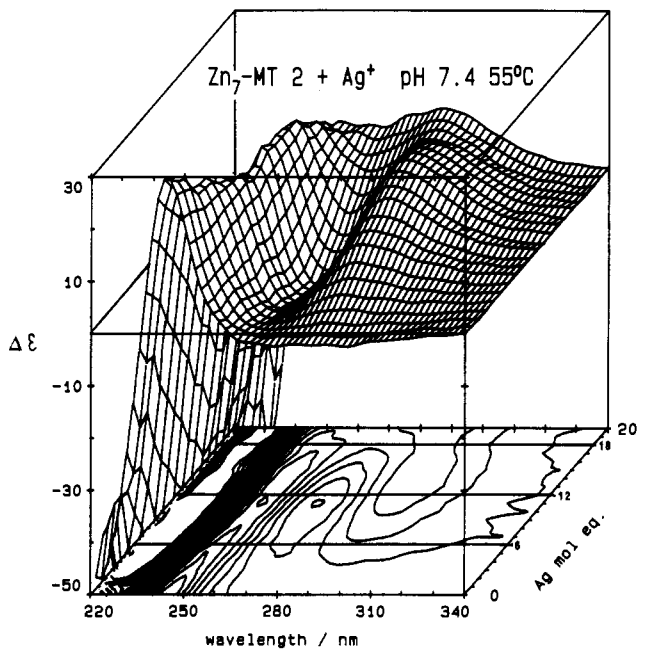

Figure 4. Titration of Zn_7–MT 2 with Ag^+ at 55 °C: 3-dimensional representation of the spectral data recorded at 55 °C.

diminishes as before, but the steeper intensification of the new 250-nm band means that the red shift is quite pronounced, with a maximum near 12 Ag^+. The development of a negative band at 275 nm, which reaches a maximum at 12 Ag^+, together with positive bands at 250 and 293 nm, strongly suggests that a distinct species described as Ag_{12}–MT forms at 55 °C. Unlike the case when apo-MT is titrated with Ag^+,[23] there is no indication of an Ag_6–MT species forming at the 6 Ag^+ point. At higher molar ratios of Ag:MT, quite complicated effects are measured. Unlike the situation with apo-MT,[23] we interpret the new broad CD band as arising from partial oxidation of the protein, catalyzed by the elevated temperatures, together with the effects of the presence of a number of different protein conformations, which lead to a much less well resolved CD intensity in this spectral region. The dependence of the CD intensity on the Ag:protein molar ratio at 20 °C is illustrated in Figure 5 for the key

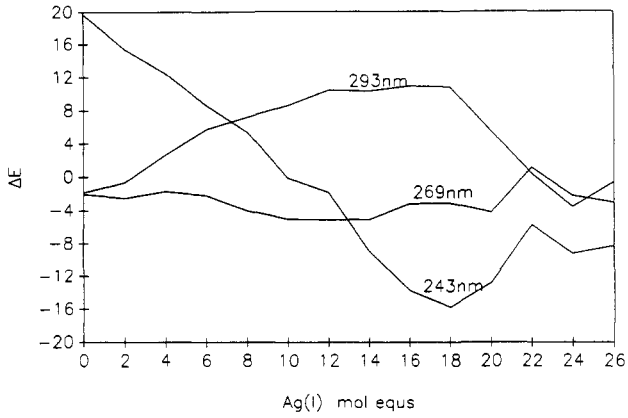

Figure 5. Changes in the CD intensities of bands at 243, 269, and 293 nm as a function of the molar ratio of Ag^+ added to Zn_7–MT at 25 °C.

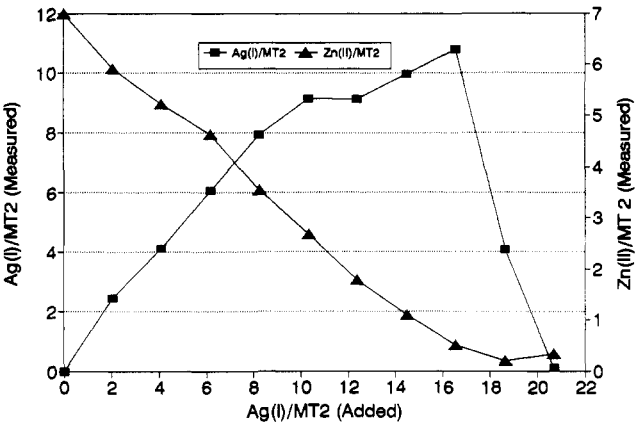

Figure 6. Molar ratios of Zn^{2+} and Ag^+ bound to metallothionein as a function of the molar ratio of Ag^+ added to a solution of Zn_7–MT 2. The method was as previously reported.[25] The concentrations of Ag^+ and Zn^{2+} were determined by flame atomic absorption spectrometry for a series of solutions each with a greater molar ratio of Ag:MT added. Chelex-100 was added to bind free Zn^{2+} 6 min after Ag^+ had been added to the protein solution. The supernatant was decanted from the Chelex-100 following 4 min of mixing. The molar ratios Ag:MT (left) and Zn:MT (right) are plotted as a 2:1 ratio. The CD spectrum was recorded before and after addition of Chelex-100. The Chelex-100 results in a collapse of the CD spectral intensity past the 12 Ag:MT point, so the values for Ag:MT (measured) between 10 and 18 Ag:MT (added) are not representative of Ag:MT (bound). Errors of ±5% are associated with each point.

wavelengths of 243 nm (Ag_{12}–MT) and 269 and 293 nm (Ag_{18}–MT).

Figure 6 shows the molar ratios of Zn^{2+} and Ag^+ bound to the protein following each addition of silver(I). Chelex-100 was used to bind free Ag^+ and Zn^{2+} according to the method described previously.[25] When the reaction is carried out at 20 °C, we find a linear relationship between Zn^{2+} loss and Ag^+ gain (note the compensation for charge in the ordinate scale for Zn^{2+}). There is an almost direct relationship between the 238-nm CD band intensity and the Zn^{2+} concentration in the early stages of the displacement of the Zn^{2+}. This means that before the 7 Zn^{2+} have been completely displaced, the Ag^+ has to share the domain with existing Zn^{2+}. The change in Zn^{2+} concentration as a function of Ag^+, shown in Figure 6, reproducibly displays a step at the 5–7 Ag^+ point, followed by a steeper loss of Zn^{2+} (on a per Ag^+ basis) past the 7 Ag^+ point. While Zn^{2+} is displaced almost linearly as a function of incoming charge (2 Ag^+ per Zn^{2+}), the molar ratio of Ag:MT bound to the protein in the presence of Chelex-100 above the 10 Ag^+ (added) point is always determined to be lower than the molar ratio of Ag^+ actually added. We associate this effect with the use of Chelex-100 to

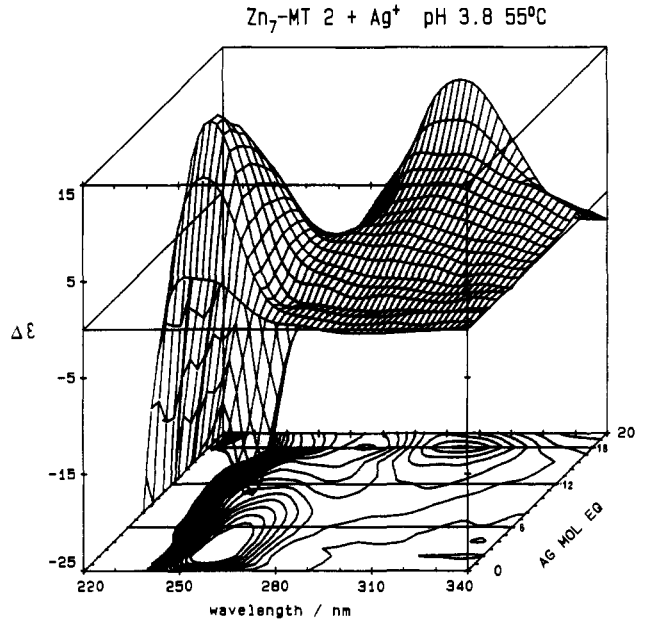

Figure 7. Change in the CD spectrum as Ag^+ is added to Zn_7–MT at 55 °C and pH 3.8.

scavenge for free metals. CD spectra recorded in the presence of Chelex-100 (not shown) are unchanged between 0 and 10 Ag^+, but above 10 Ag^+ the Chelex-100 appears to compete for the Ag^+ bound to the protein. Under these conditions, the CD spectral intensity is quenched following addition of Chelex-100, which indicates that the RS–Ag–RS–Ag–RS structure is disrupted. The data in Figure 6 show that the Chelex-100 specifically competes for the Ag^+ bound to the protein past the 10–12 Ag^+ point. These data show that the Zn^{2+} not displaced by Ag^+ does not bind to the Chelex-100 within the 4-min mixing period, a result previously reported for Zn^{2+} binding to Cd–MT.[25] We do not believe that the Ag^+ concentrations determined by the Chelex-100 method for additions of Ag^+ greater than 12 represent the actual molar ratio of Ag^+:MT bound to the protein.

Titration of Zn_7–MT 2 (Nominally Apo-MT 2) with Ag^+ at pH 3.8 and 55 °C. Titrations of MT with metal ions at a pH other than 7 are expected to result in different products. This is because differences in the ionization of the peptide chain are likely to lead to changes in the binding constants involved in the formation of the overall domain structure adopted by the Zn^{2+}- and Cd^{2+}-containing protein.[26] As the pH is lowered, protons will compete with zinc for the thiolate sites. At pH 3.8, we expect Zn^{2+} to be partially dissociated from the protein. Changes in hydrogen bonding will also lead to different structures being stabilized. Unlike our previous experiments with apo-MT,[23] in which aliquots of Ag^+ were added to single samples of the apo-MT at low pH, and the pH was then raised to 7.4 by addition of a buffer, the experiments reported here use a single sample of protein at pH 3.8. Successive aliquots of Ag^+ were added without raising the pH. The spectral data shown in Figure 7 were recorded at 55 °C. No bands appear in the 280–340-nm region until after 12 Ag^+ have been added. At pH 3.8, there is only minimal intensity near 240 nm from Zn–S, which allows the Ag–thiolate dependent 255-nm band to be observed clearly. This band is maximal from 3 to 6 Ag^+ and is then followed by formation of an intense derivative-shaped band, 268 nm (+) and 300 nm (–), with 18 Ag^+ added. We have not previously recorded a sequence of spectral envelopes like this for any other titration.

Titration of Zn–α MT 1 with Ag^+ at 5, 20, and 55 °C. These data are unusually noisy because of a combination of low CD intensities and the low concentrations of the fragments used. At

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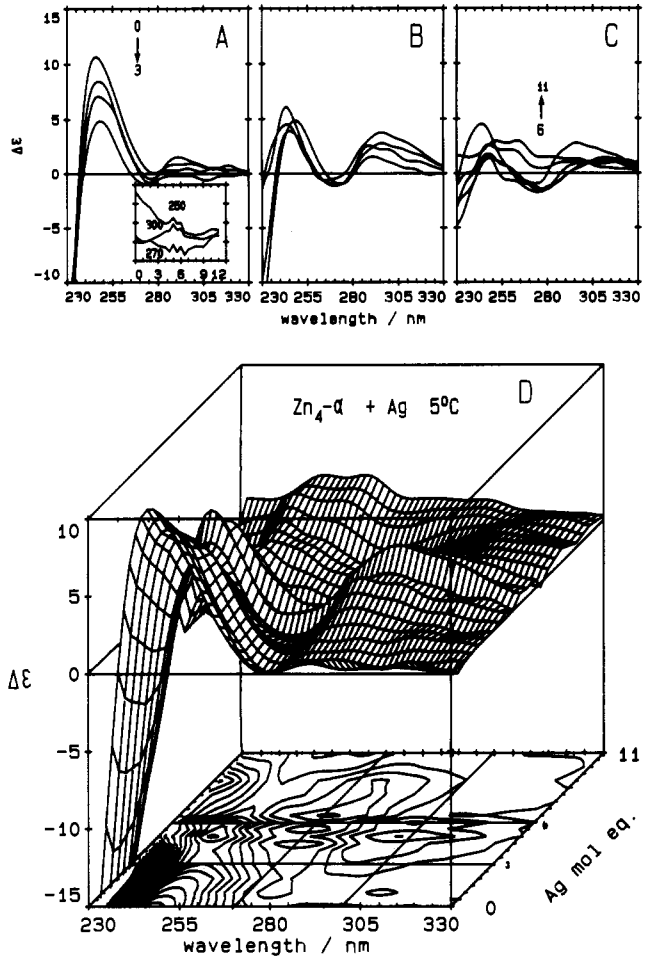

Figure 8. Titration of Zn_4–α MT 1 with Ag^+ at 5 °C (D). CD spectra at 5 °C from a single solution of Zn_4–α MT 1 as a function of increasing molar ratio of Ag^+ (A–C) depict the recordings following the addition of aliquots of 1 mol equiv of Ag^+.

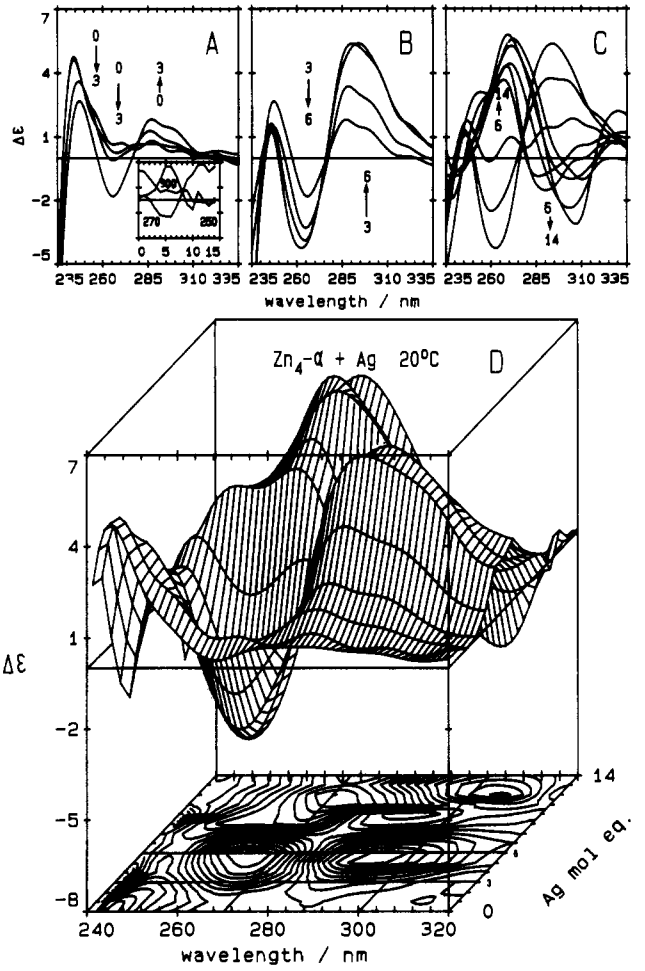

Figure 9. Titration of Zn_4–α MT 1 with Ag^+ at 20 °C (D). CD spectra at 20 °C from a single solution of Zn_4–α MT 1 as a function of increasing molar ratio of Ag^+ (A–C) depict the recordings following the addition of aliquots of 1 mol equiv of Ag^+.

5 °C (Figure 8), the lack of intensity as Ag^+ is added to the Zn_4–α MT 1 fragment suggests only partial formation of a clustered –Ag–RS–Ag– species. However, at 20 °C (Figure 9), the formation of the Ag_6–α MT species is dramatically displayed by the CD spectrum. The signal at 270 nm (–) and 297 nm (+) reaches a maximum with 6 Ag^+, which we associate with the specific formation of the filled α domain in the fragment. Addition of more Ag^+, from 6 to 12 Ag^+, results in the formation of a new species which is characterized by a quite different CD spectrum with band maxima at 264 nm (+) and 306 nm (–). This spectrum is also unlike any that we have previously obtained for Ag-containing proteins or fragments. Carrying out the titration at 55 °C appears to quench complex formation (Figure 10). A weak signal appears between 3 and 6 Ag^+. This is then replaced by a new spectrum at the 11 Ag^+ point, which could be due to the formation of the Ag_{12}–α species, as at 20 °C. In view of the startling change in complex formation between 5 and 20 °C for this fragment, it is probable that at 55 °C the Ag–thiolate complex is just too unstable to survive.

Titration of Zn_3–β MT 1 with Ag^+ at 20 °C. The set of spectral data obtained for the Zn_3–β MT 1 fragment (Figure 11) quite closely resemble the data recorded during the titration of apo-β MT 1 with Ag^+ (Figure 7 in ref 14). Zn_3–β MT itself exhibits a very weak CD spectrum, as does Cd_3–β MT.[14] Addition of Ag^+ results in a steep growth in the intensity of the 250-nm band, which reaches a maximum between 3 and 6 Ag^+. While formation of a species that we tentatively identify as Ag_6–β can be seen in the contour diagram, no other species appear to be present. Specifically, no complex forms at high molar ratios of Ag^+. The CD spectra for solutions with more than 6 Ag^+ are featureless, suggesting a random coil structure.

Discussion

Analysis of spectroscopic and structural studies that probe the metal-binding reactions of metallothionein, for example,[2,14,16,17,26] shows that specific, 3-dimensional structures form with many different metals. The exact structures adopted appear to depend on the coordination number preferences of the metal, the stoichiometric ratio of metal:MT, the temperature, and the pH. Three structural classes have been reported for mammalian metallothioneins: (i) M_7–MT, involving tetrahedral coordination of cadmium, zinc, and mercury; (ii) M_{12}–MT, involving trigonally-coordinated metals, which is supported by spectroscopic[2b,14,23] and biochemical data[27] for metals like copper and silver; and (iii) a class represented by Hg_{18}–MT,[28] which is considered to involve linear coordination of the Hg^{2+}. While the 20 thiolate groups in the protein appear to bind as two, almost independent, multidentate chelators[2,17] in M_7–MT and M_{12}–MT, we have suggested that, in Hg_{18}–MT, the peptide chain adopts a single metal-binding domain.[28] The 3-dimensional representations of the CD spectral data for Ag^+ binding to Zn–MT show that a spectacular structural chemistry exists in solution, a chemistry that is more clearly resolved at elevated temperatures and at low pH values.

We suggest that the sign and magnitude of the bands in the CD spectrum to the red of 220 nm in metallothioneins are dependent on the chirality of the metal-binding site as a whole, so

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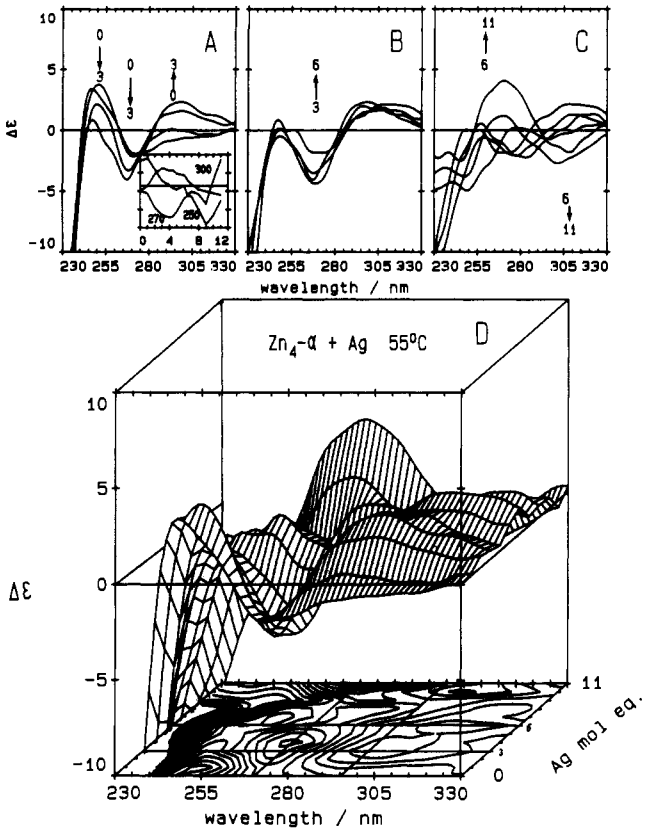

Figure 10. Titration of Zn_4–α MT 1 with Ag^+ at 55 °C (D). CD spectra at 55 °C from a single solution of Zn_4–α MT 1 as a function of increasing molar ratio of Ag^+ (A–C) depict the recordings following the addition of aliquots of 1 mol equiv of Ag^+.

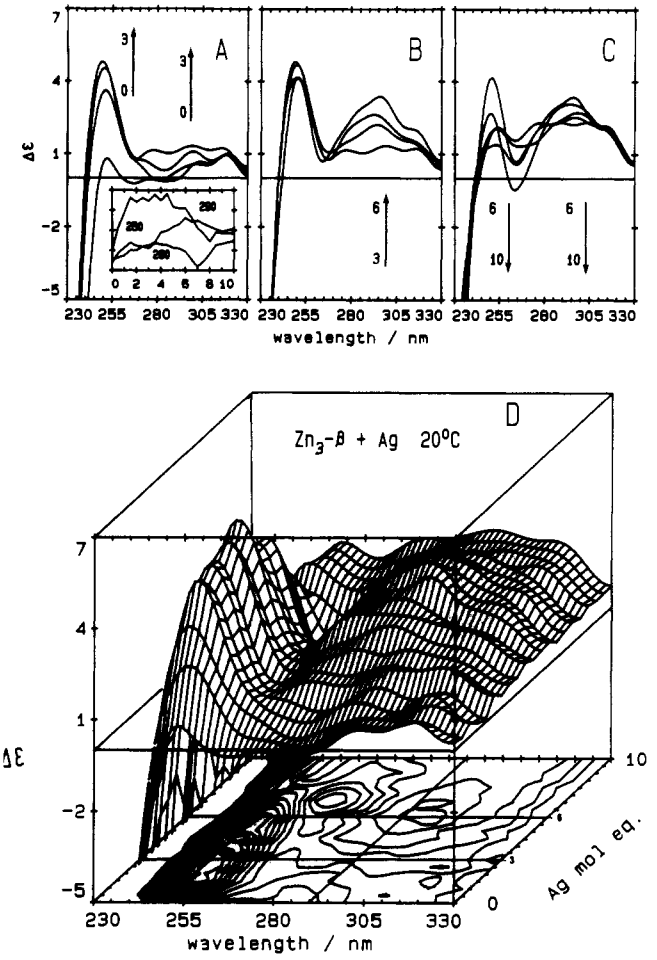

Figure 11. Titration of Zn_3–β MT 1 with Ag^+ at 20 °C (D). CD spectra (A–C) are for a single solution of Zn_3–β fragment, with increasing molar ratios of Ag^+ at 20 °C.

that the appearance and disappearance of intensity in individual bands can be associated with the formation and collapse of stoichiometrically-specific metal–thiolate complexes. The spectral data for silver binding are of considerable additional interest when compared with similar results from cadmium binding to Zn–MT because while the tertiary structure of Zn_7–MT has tetrahedral zinc atoms cross-linked by thiolates from cysteine residues in both the α and β domains, the silver atoms are expected to bind trigonally, or even linearly, in the two domains.[2b,29,30]

When Ag^+ is added to Zn_7–MT, we interpret changes in the intensity of the CD spectral envelope in terms of the formation of specific Zn_m,Ag_n–MT species, where m = 7–0 and n = 0–20. We assume that Zn_7–MT adopts the same structure as Cd_7–MT, so that there will be a $Zn_3(RS)_9$ domain and a $Zn_4(RS)_{11}$ domain.[2b] Interpretation of changes in the CD spectrum as Ag^+ is added to Zn_7–MT must take into account the decrease in the CD signal due to the $RS^- \rightarrow Zn^{2+}$ ligand to metal charge-transfer band near 243 nm and the growth of new bands due to silver–thiolate interactions. This makes the analysis more complicated than is the case when Ag^+ is added to the random coil, apo-MT.[23]

The spectral data in the 243-nm region are a superposition of at least two bands: the first is from the Zn–S chromophore, as the temperature of the titration increases from 5 to 50 °C, so the 243-nm band intensity collapses at lower Ag:MT ratios, and a new negative band grows in more steeply, reaching a maximum at 18 Ag:MT. Figure 5 shows that the band intensity at 243 nm reaches zero near 10 Ag:MT. The AAS results strongly suggest that Zn^{2+} is displaced from MT almost exactly on a charge compensation basis, so that significant mole fractions of Zn^{2+} remain even when 12 Ag^+ have been added. We find it surprising that Zn^{2+} remains so tightly associated with the protein for so long in the presence of Ag^+. If the 243-nm band arises from exciton coupling between Zn–S units, then as the Zn–S cluster structure is replaced by regions of –Ag–RS–Ag–RS, the exciton coupling should be quenched, leading to the very much reduced intensity that is observed at low Ag:MT ratios. As the Zn^{2+} is tetrahedrally coordinated, unless the Ag^+ adopts tetrahedral coordination as well,[2b,29,30] there must be considerable rearrangement within the binding site domains as Ag^+ binds adjacent to the remaining Zn^{2+} atoms. Spectroscopic reports of mixed-metal metallothioneins where the coordination preferences of the metals are not the same include Cu_{12},Cd_4–MT,[31a] Cu_6,Cd_4–α MT,[31b] Au,Cd–MT,[32] and Cd,Zn,Au–MT.[32] It is possible that that Zn^{2+} binds in the same manner as proposed for Cd^{2+} in Cu_{12},Cd_4–MT.

The spectral data we report here show the development of maxima at molar ratios (6 and 12) that appear to be reasonable for Ag–MT complexes, matching those values determined from Ag^+ binding to apo-MT.[23,27] New complexes also form at very high molar ratios of Ag:MT. Observation of complex formation with 12 Ag^+ and 18 Ag^+ implies that the cross-linked RS–M–SR units rearrange as the molar ratio of Ag:MT increases from zero to 18, and the molar ratio of Zn:MT decreases from 7 to zero. The spectral data recorded for titrations of Zn_7–MT at elevated temperatures show the development of individual species with

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much less overlap than at lower temperatures. This effect must be related to a thermally-induced rearrangement from a kinetic product formed at lower temperatures to a thermodynamic product formed at raised temperatures. We interpret some of these changes in terms of a necessary rearrangement of the peptide chain to accommodate the trigonal Ag^+ in the Ag_{12}–MT species. It seems unlikely that the peptide chain can adopt the energetically-preferred configuration during replacement of Zn^{2+} by Ag^+ at lower temperatures where thermally-induced lability is reduced. The data recorded between 5 and 20 °C for both Ag^+ and Cd^{2+} [14,23] binding to Zn–MT indicate that a temperature above 10 °C is required for new clusters to form.

Speciation in the α and β Fragments. Reactions of Ag^+ with Zn_7–MT involve Ag^+ binding to sites in either the α or β domain, as well as competition between a domain-specific mechanism, in which one domain fills first, and a distributed mechanism, in which Ag^+ binds randomly across both domains. Figures 8–10 provide evidence for the formation of Ag_6–α MT and Ag_{12}–α MT. These data suggest that the peptide chain in the region of the metal-binding domain is acting as a multidentate chelating ligand. The 20 °C data show formation of a species at 6 Ag^+ with strongly dichroic bands at 264 nm (–) and 293 nm (+), which closely match the bands observed for Ag_6–α MT from apo-α MT at 55 °C (although we believe that some decomposition had occurred with the α MT at these temperatures). Ag_6–α MT is replaced by a species that is characterized by a spectrum with reversed CD signs, which is completely formed at 12 Ag^+ (264 nm (+) and 306 nm (–)). There is no evidence for the formation of an Ag_3–α MT complex. Previous reports for the formation of Cu_6,Cd_4–α MT 2 [31b] suggest that the α-domain peptide is able to wrap metals in an even more complex manner than that characterized for Cd_4–α MT.[2b,14] Data for the β fragment are always poorly resolved. However, we can associate the band near 252 nm with formation of Ag_6–β MT. We find very little negative intensity in these spectra, which contrasts with the data obtained when Ag^+ binds to apo-β MT,[23] in which an Ag_3–β MT species clearly forms.

Speciation When Ag^+ Is Added to Zn_7–MT at pH 3.8. Even at this pH, the partially dissociated Zn^{2+} appears to provide some stability against oxidation. The spectral data (Figure 7) show unambiguously that only a single species forms before Ag_{18}–MT. The Zn–S CD band is absent at this pH, which means that growth of a band near 255 nm can be observed between 1 and 6 Ag^+. There is no sign of a negative band near 260 nm or a positive band at 290 nm, bands that are characteristic of Ag_6^{α}, the saturated α domain. We assign these spectral features to Ag_6^{β}. Between 6 Ag^+ and 12 Ag^+, the peptide chain must rearrange to allow Ag_{18}–MT to form. The structures that form between 6 and 12 Ag^+ exhibit essentially no CD intensity. These spectacular data show that, during a titration of metallothionein at pH 3.8 with Ag^+, the β domain first forms at the expense of other species but then Ag–RS–Ag cluster formation in the β domain is replaced by Ag_{18}–MT. This is the second piece of evidence that M_{18}–MT species do not involve domain structures of the type exhibited by M_{12}–MT or M_7–MT. Strong support for this view is also provided by a XANES study of M_7–MT (M = Cd(II), Zn(II), Hg(II)) and Hg_{18}–MT.[33]

Analysis of the CD Spectra Recorded between 5 and 55 °C for Zn_7–MT. We identify several features in the spectra recorded at 55 °C. The red shift of the 240-nm band toward 250 nm masks the point at which the Zn–thiolate band at 243 nm is lost. The new positive band near 250 nm is associated with the development of the Ag_6^{β} domain, while the negative band at 268 nm and the positive band near 293 nm are associated with the Ag_6^{α} domain. The formation of these species is described in Figure 12. The different reactions of Ag^+ with Zn_7–MT up to

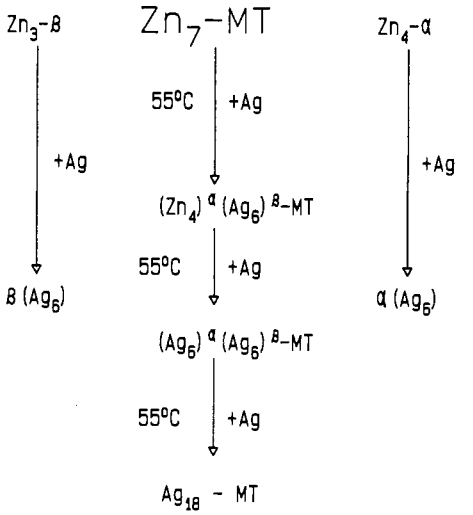

Figure 12. Series of proposed binding pathways that are followed when Ag^+ is added to Zn_7–MT 2 and to the two fragments Zn_4–α MT 1 and Zn_3–β MT 1. The formation of the domain-specific complex Ag_6^{β},Zn_4^{α}–MT is not identified in the spectral data at pH 7; a single species which is identified as a filled β domain, Ag_6^{β}–MT, forms at pH 3.8. Ag_{12}–α MT forms from Zn_4–α MT at 20 °C.

Ag_{12}–MT are not well defined at pH 7, so we have no evidence to support a domain-specific or distributed pathway at the 6 Ag:MT point for titrations of Zn_7–MT at pH 7. Unlike the emission data for Cu–MT,[2b,31a] emission data for Ag–MT do not show changes in band shape or energy over the range Ag:MT = 1–12,[23,34] which might suggest domain specificity. At pH 3.8, we do see the development of a single species with a unique spectral character that we characterize as Ag_6^{β}–MT (in which Ag^+ binds only in the β domain with protonation of the thiolates in the α domain). The spectral data recorded in this experiment strongly support the formation of Ag_{18}–MT at high Ag:MT molar ratios. This structure must involve bridging thiolates, and we suggest also linear RS–Ag–SR units. A report on EXAFS included the species "Ag_{17},Cd_2-MT",[35] for which the EXAFS data were described as being "entirely different from Cd_7–MT" and were analyzed in terms of Ag–S cluster formation with a linear coordination for the silver.[35] The occurrence of Ag_{18}–MT and Hg_{18}–MT, but not Cu_{18}–MT, suggests that a linear coordination geometry is a requirement for these high metal:MT ratio structures.

Model Compounds with Ag–Thiolate Bonding. Silver binds[2b,29,30,36–39] either trigonally or linearly with thiolate ligands and often binds in polymeric chains. Thus silver(I)–thiolate complexes lie structurally between copper(I)–thiolates, where trigonal complexation is common,[38] and gold(I)–thiolates,[37–40] which are almost exclusively linear, although there are reports of a tetrahedral gold(I)–phosphine complex.[41,42] From the X-ray structure determinations of silver–thiolate complexes by Dance et al.[30] and Tang et al.,[29] we find only trigonal coordination in dimeric $\{[AgSCH(SiMe_3)_2]_4\}_2$ and polymeric $\{Ag_4[SCH_2(SiMe_3)]_2(OMe)\}_n$. The twisted-chain structures,[2b,30] which

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involve linear S–Ag–S units, may well be models for the Ag_{12}–α MT and Ag_{18}–MT species found at high loadings of Ag^+ in Zn_4–α MT and Zn_7–MT, respectively. The zigzag segments induced by the sp^3-hybridized sulfur could result in the enhanced chirality measured in the CD spectrum.[43] Of particular note with respect to the formation of this unusual Ag_{18}–MT species are the helical strands formed from Cu^+ and poly(bipyridine) units.[44]

In summary, a series of distinct complexes form when Ag^+ is added to zinc metallothioneins. Formation of Ag_6–MT, Ag_{12}–MT, and Ag_{18}–MT at selected stoichiometric ratios, temperatures, and pH values can be observed during titrations of Zn–MT 2. The Ag–MT species observed to form from Zn_7–MT resemble those found when Ag^+ is added to apo-MT. At pH 3.8, only a β-domain cluster and the Ag_{18}–MT species form.

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