HPLC Characterization of Ag^+ and Cu^+ Metal Exchange Reactions with Zn- and Cd-Metallothioneins†

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ABSTRACT: Anion exchange HPLC methods were developed to study the metal exchange reactions of ZnMT and CdMT with Ag^+ and Cu^+. The kinetics of these reactions revealed the existence of a slow interprotein metal redistribution process that follows initial rapid displacement of metal from Zn_7MT or Cd_7MT by Ag^+ or Cu^+. Kinetically-favored products of the reaction of Ag^+ with Zn_7MT in the presence of Chelex resin include Zn_4Ag_6MT and Ag_{12}MT. Subsequent slow reaction between Zn_7MT and Ag_{12}MT leads to the thermodynamically-favored product, Zn_4Ag_6MT. Analogous behavior was observed for Cd_7MT titrated with Ag^+ and Zn_7MT with Cu^+. The use of Chelex resin to remove unbound metal ions was found to influence the reactions of Zn_7MT and Cd_7MT with Ag^+. Upon addition of Ag^+ to Zn_7MT in the absence of Chelex, a Ag_{18}MT species forms as the kinetically-favored product in addition to Zn_4Ag_6MT and Ag_{12}MT. Subsequently, Ag_{18}MT was found to donate Ag^+ to Zn_7MT and Zn_4Ag_6MT *via* slow interprotein metal exchange reactions. Chelex resin influences the titration reaction either by limiting formation of Ag_{18}MT and/or by binding the positively charged Ag_{18}MT species and removing it from solution. The formation of Ag_{18}MT in the absence of Chelex explains why more than 12 equiv of Ag^+ is required to displace all of the Zn^{2+} or Cd^{2+} from Zn_7MT and Cd_7MT. The HPLC methods developed in this study allow homogeneous preparations to be made of metal-hybrid MT species containing Ag^+ in the β-domain and Cd^{2+} or Zn^{2+} in the α-domain.

Metallothioneins (MTs)[1] are a class of small proteins with extremely high metal and sulfur contents. Mammalian MTs have molecular masses of 6−7 kDa and usually contain 61 or 62 amino acids, 20 of which are conserved cysteine residues whose thiolate sulfur atoms serve as the metal ligands (Kägi, 1993). Divalent metal ions such as Zn^{2+} and Cd^{2+} are bound to MT with a stoichiometry of 7 g-atoms per mole of protein. Three of the metals occupy a Me_3Cys_9 cluster in the N-terminal β-domain (residues 1−31), and the other four are coordinated in a Me_4Cys_{11} cluster in the C-terminal α-domain (Otvos & Armitage, 1980; Winge & Miklossy, 1982; Robbins et al., 1991). Native MT is usually heterogeneous in metal composition, often containing monovalent Cu^+ in addition to Zn^{2+} and Cd^{2+} (Ettinger, 1984; Funk et al., 1987; Cherian & Chan, 1993). The fact that all three of these metal ions induce the biosynthesis of MT is consistent with the postulated role(s) of the protein in the metabolism and detoxification of both essential and nonessential trace metals (Winge et al., 1975; Cherian & Goyer, 1978; Johnson & Foulkes, 1980; Cherian & Chan, 1993).

Cu^+ is known to bind differently than divalent metal ions to both the α- and β-domains of MT, but the structures of the complexes have yet to be determined. Part of the reason is the susceptibility of CuMTs to air oxidation, which makes them more difficult to study. An alternative is to employ Ag^+ as an air-stable, NMR-active analog of Cu^+, since numerous studies have indicated that Ag^+ and Cu^+ bind similarly, if not identically (Nielson et al., 1985; Winge, 1987; Stillman et al., 1988; Zelazowski et al., 1989; Narula et al., 1991). To measure the stoichiometry of Cu^+ or Ag^+ bound to MT, the following approaches have been used: (1) determining the point in a Cu^+ or Ag^+ titration of apo-MT or apo-fragments of the α- and β-domains where resistance to proteolysis is maximal (Nielson et al., 1985; Nielson & Winge, 1984); (2) determining the point at which Zn^{2+} and Cd^{2+} are completely displaced from Zn_7MT or Cd_7MT by the tighter-binding Cu^+ or Ag^+ ions using atomic absorption spectroscopy (Winge, 1987); (3) measuring the metal composition of apo-MT reconstituted with Ag^+ or Cu^+ after purification by gel filtration chromatography (Byrd et al., 1988); and (4) monitoring changes induced in UV, circular dichroism, magnetic circular dichroism, luminescence, and Cd NMR spectra during titration of ZnMT, CdMT, or apo-MT with Ag^+ or Cu^+ (Rupp et al., 1974; Gasyna et al., 1988; Rupp & Weser, 1978; Stillman et al., 1987; Zelazowski et al., 1989; Zelazowski & Stillman, 1992; Stillman et al., 1988; Li, 1991; Otvos et al., 1993; Green & Stillman, 1994). Although stoichiometries reported in these studies range from about 8 to 20, there is now a clear consensus that both Cu^+ and Ag^+ bind to form Cu_{12}MT and Ag_{12}MT species containing six metal ions each in the α- and β-domains. However, adding Ag^+ or Cu^+ to MT in stoichiometries exceeding 12 g-atoms per mole has been shown to induce changes in UV, CD, and luminescence spectra consistent with formation of distinct "over-metalated" species, such as Ag_{18}MT and Cu_{20}MT (Gasyna et al., 1988; Zelazowski et

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[1] Abbreviations: AAS, atomic absorption spectroscopy; CD, circular dichroism; HPLC, high-performance liquid chromatography; Me, divalent ion, e.g., Zn^{2+} and Cd^{2+}; MT2, metallothionein isoform 2; Tris, tris(hydroxymethyl)aminomethane.

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al., 1989; Stillman et al., 1987; Zelazowski & Stillman, 1992; Green & Stillman, 1994).

Using ^{111}Cd NMR to monitor the products of sequential displacement of Cd^{2+} by Ag^+ from Cd_7MT, we showed that Cd^{2+} is preferentially displaced from the β-domain 3-metal cluster in a cooperative fashion (Li & Otvos, 1996). Only after all of the Cd^{2+} in the β-domain is displaced by Ag^+ does additional Ag^+ displace Cd^{2+} from the α-domain 4-metal cluster. This evidence suggested the existence of only two Ag-containing species: $(Cd_4)^{\alpha}(Ag_6)^{\beta}$MT and $(Ag_6)^{\alpha}(Ag_6)^{\beta}$MT. A limitation of the use of ^{111}Cd NMR to study these reactions is that kinetically-favored intermediates might not be detected because of the long time (several hours) required to obtain the spectra. Possible differences between kinetically-controlled and thermodynamically-controlled product distributions have been suggested by temperature-dependent changes observed in circular dichroism spectra of Ag-reconstituted MT (Zelazowski et al., 1989). Another limitation is that only the products of reaction of Ag^+ with CdMT, but not ZnMT, can be inferred from Cd NMR data, and conclusions drawn about the structures of the Ag-containing complexes are necessarily indirect since NMR signals are not being observed from the Ag-containing clusters themselves. We therefore felt it was important to develop additional "assays" for the intermediates and products of reaction of Ag^+ and Cu^+ with both CdMT and ZnMT. This was accomplished by developing anion exchange HPLC methods to efficiently separate the different metalated species produced during these reactions. What was found by using HPLC to monitor the reaction time courses were significant differences in kinetically- and thermodynamically-determined product mixtures resulting from direct interprotein metal exchange reactions.

Also reported are experiments that demonstrate that the use of Chelex-100 resin to scavenge free or weakly bound metal ions has an unexpected influence on the outcome of the reactions. This information is relevant to the interpretation of many previous spectroscopic studies of metal displacement reactions of MT, some of which employed Chelex treatment prior to acquiring the spectral data (Winge, 1987; Nielson et al., 1985; Li & Otvos, 1996) and some of which did not (Stillman et al., 1987, 1988; Zelazowski et al., 1989; Zelazowski & Stllman, 1992; Rupp et al., 1975; Gasyna et al., 1988; Rupp & Weser, 1978; Green & Stillman, 1994).

MATERIALS AND METHODS

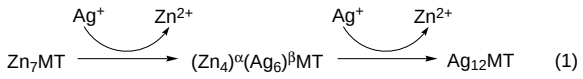
Zn_7MT was isolated from rabbit liver after induction by subcutaneous injections of $ZnSO_4$ as previously described (Li & Otvos, 1996), and only MT2 was used in this study. Cd_7MT was obtained by direct displacement of the Zn^{2+} from Zn_7MT by addition of stoichiometric amounts of $CdCl_2$ in the presence of Chelex-100 resin (Bio-Rad Laboratories) (Li & Otvos, 1996). Prior to use, the Chelex was washed successively with 1 M HCl and 1 M NaOH, and then extensively with distilled deionized water before vacuum filtration to remove most of the water. Zn_7MT and Cd_7MT concentrations were measured spectrophotometrically at 220 nm in 0.01 M HCl using an extinction coefficient of 47 300 M^{-1} cm^{-1} (Bühler & Kägi, 1979). Zn, Cd, Ag, and Cu concentrations were determined using atomic absorption spectroscopy (Perkin-Elmer model 3100). Protein cysteine sulfhydryl concentrations were measured by reaction with 5,5′-dithiobis(nitrobenzoic acid) (DTNB) in 6 M guanidine hydrochloride (Ellman, 1959). UV absorption spectra were recorded using a Beckman DU-70 spectrophotometer, and CD spectra were acquired on a Jasco model J600 spectropolarimeter.

Metal titration reactions were carried out by adding defined amounts of Ag^+ or Cu^+ to separate solutions of Zn_7MT or Cd_7MT at pH 8.0 in the presence or absence of Chelex resin (*ca.* 50 mg). Displacement of Zn^{2+} from Zn_7MT by Ag^+ detected using Zincon reagent (Rush & Yoe, 1954; Shaw et al., 1990) indicated that the reaction was completed within 1−2 min (Li, 1991). Ag^+ was supplied as $AgClO_4$, and Cu^+ was supplied as the stable $[Cu(CH_3CN)_4]ClO_4$ complex (2 mM in 50% H_2O/50% CH_3CN) synthesized according to the procedure of Hemmerich and Sigwart (1963). With Chelex present, reaction mixtures were incubated for 30 min before the Chelex was removed by centrifugation or filtration through a 0.2 μm membrane. Changes in total protein concentration during the titration of Zn_7MT2 with Ag^+ were monitored using a colorimetric dye-binding assay employing Rose Bengal (Elliott & Brewer, 1978).

HPLC experiments were performed at room temperature using an ISCO HPLC system consisting of a pump (model 2350), solvent programmer (model 2360), and UV/vis detector (model V4). The injector was equipped with a 500 μL sample loop. DEAE anion exchange HPLC columns were supplied by Bio-Rad (Bio-Gel DEAE-5-PW, 75 × 7.5 mm). The mobile phase consisted of Tris-HCl buffer, pH 8.5, ranging in concentration from 5 to 300 mM. Buffers were prepared from distilled deionized water, filtered through a 0.2 μm pore membrane, and degassed by vacuum. Separations were carried out at a flow rate of 1 mL/min using a linear gradient formed between buffer A (5 mM Tris-HCl, pH 8.5) and buffer B (300 mM Tris-HCl, pH 8.5) and were monitored at 240 nm. Typically, the following gradient was used: 0−50% buffer B within 30 min, followed by washing with buffer B for 5 min and reequilibration with buffer A for 15 min.

RESULTS

Ag^+ Titration of Zn_7MT in the Presence of Chelex Resin. By analogy to the behavior of Cd_7MT deduced from ^{111}Cd NMR studies (Li & Otvos, 1996), the following products are expected to be formed upon titration of Zn_7MT with Ag^+:



(1)

In the initial stages of the titration, the Zn^{2+} in the β-domain 3-metal cluster is expected to be selectively and cooperatively displaced by Ag^+ to produce the metal-hybrid protein species, $(Zn_4)^{\alpha}(Ag_6)^{\beta}$MT. Additional Ag^+ will then displace the Zn^{2+} in the α-domain 4-metal cluster to give Ag_{12}MT. As shown in Figure 1, these predictions were able to be tested by using anion exchange HPLC to characterize the product mixtures generated during the course of a Ag^+ titration. The experiment was conducted by adding defined amounts of Ag^+ to separate solutions of Zn_7MT (100 μM) containing Chelex resin, and allowing the solutions to incubate for 30 min prior to removing the Chelex and analyzing the product distribu-

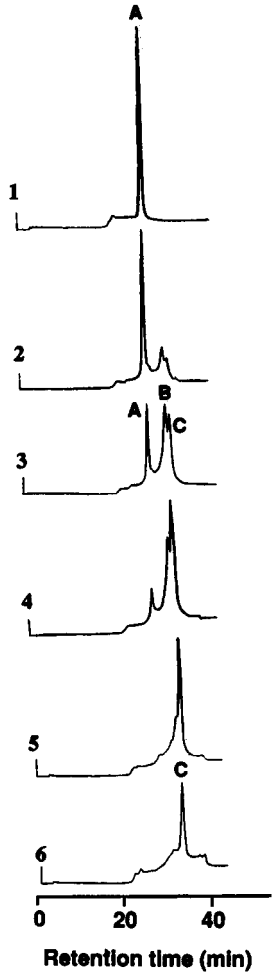


FIGURE 1: Ag^+ titration of Zn_7MT *with* Chelex treatment monitored by HPLC. Separate samples of Zn_7MT (100 μM) were incubated for 30 min with varying amounts of Ag^+ in the presence of Chelex-100 resin. 100 μL aliquots were analyzed by anion exchange HPLC. Mol equiv of Ag^+ added: (1) 0, (2) 3, (3) 6, (4) 8, (5) 13, and (6) 15. Retention times (min) and peaks: (A) 28.7, Zn_7MT; (B) 32.5, Zn_4Ag_6MT; and (C) 33.3, $Ag_{12}MT$.

tion by HPLC. Trace 1 indicates that before any Ag^+ was added, the Zn_7MT starting material elutes as a single peak (labeled A) with a retention time of 28.7 min. Samples to which increasing amounts of Ag^+ were added showed a progressive reduction in the size of peak A. The peak was no longer detectable following addition of 13 equiv of Ag^+ (Trace 5). Coincident with the reduction in size of the Zn_7MT peak was the progressive appearance of two new peaks with retention times of 32.5 and 33.3 min labeled B and C, respectively. Under different conditions where peak B was produced in the absence of peak C (see below), peak B was collected and analyzed by AAS and found to contain a Ag/Zn ratio of 1.5. Similarly, peak C was found to contain only Ag. The metal compositions of the protein species giving rise to peaks B and C are consistent with their assignment to Zn_4Ag_6MT and $Ag_{12}MT$, respectively. The retention time of peak C was observed to be exactly the same no matter whether Ag^+ was reacted with Zn_7MT or Cd_7MT (see below), which further supports its assignment as $Ag_{12}MT$. In addition, peaks B and C were both seen to increase as Ag^+ was added up to *ca.* 6 equiv (Trace 3). Beyond this point, addition of Ag^+ caused a continued increase in the size of peak C, but a decrease in the size of

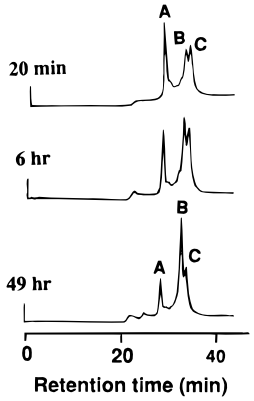


FIGURE 2: Time dependence of Zn_4Ag_6MT formation following Ag^+ addition to Zn_7MT. 6 equiv of Ag^+ was added to a 100 μM Zn_7MT sample in the presence of Chelex-100. After 30 min, the Chelex was removed and 100 μL aliquots were withdrawn for HPLC analysis at the indicated times. Retention times and peak identities are the same as given in Figure 1.

peak B. After 13 equiv of Ag^+ was added (Trace 5), the only species detectable was $Ag_{12}MT$.

Although the species detected by HPLC appear to be exactly the same as those predicted by Cd NMR studies (Li & Otvos, 1996), the stoichiometries observed at different points during the titration do not agree. The Cd NMR data predict that $Ag_{12}MT$ should only begin to be formed after all of the Zn in the β-domain has been displaced by Ag^+. Thus, at any stage of the titration, no more than two HPLC peaks are expected to be present: those from Zn_7MT (peak A) and Zn_4Ag_6MT (peak B) when less than 6 equiv of Ag^+ is added and those from Zn_4Ag_6MT (peak B) and $Ag_{12}MT$-(peak C) when more than 6 equiv is added.

A possible explanation for the discrepancy between the product distributions determined for reactions of Cd_7MT by NMR and those of Zn_7MT measured by HPLC is that the former were conducted under conditions of thermodynamic equilibrium (Li & Otvos, 1996), while the 30 min reaction times and low protein concentrations used in the HPLC experiments might not have allowed the reactions to reach equilibrium. To determine if this was true, the time dependence of the reaction between Zn_7MT and Ag^+ was monitored using a sample of Zn_7MT to which *ca.* 6 equiv of Ag^+ had been added. Figure 2 shows HPLC profiles obtained as a function of time after the initial addition of Ag^+. These results show that the sizes of peaks A (Zn_7MT) and C ($Ag_{12}MT$) slowly decrease over a period of two days, while that of peak B (Zn_4Ag_6MT) increases. These observations suggest that initial rapid displacement of Zn^{2+} by Ag^+ is followed by a much slower interprotein metal exchange reaction between Zn_7MT and $Ag_{12}MT$ to produce Zn_4Ag_6MT:

$$(Zn_4)^{\alpha}(Zn_3)^{\beta}MT + (Ag_6)^{\alpha}(Ag_6)^{\beta}MT \rightleftharpoons (Zn_4)^{\alpha}(Ag_6)^{\beta}MT \quad (2)$$

To verify this conclusion directly, we mixed solutions of Zn_7MT with $Ag_{12}MT$ at room temperature and observed by HPLC the slow production of Zn_4Ag_6MT over a 2 day time period, after which no further changes in concentration were seen (data not shown).

The presence of significant equilibrium concentrations of Zn_7MT (peak A) and $Ag_{12}MT$ (peak C) in the bottom trace of Figure 2 indicates that the equilibrium constant for eq 2 is not sufficiently large to convert Zn_7MT completely to

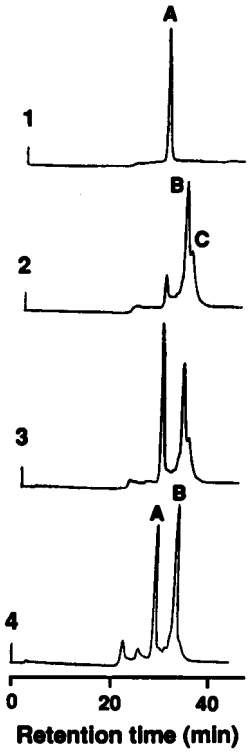


FIGURE 3: Time dependence of metal exchange reaction between Zn_7MT and $Ag_{12}MT$. Zn_7MT (Trace 1) and Zn,AgMT, prepared by adding 6 equiv Ag^+ to Zn_7MT in the presence of Chelex-100 and incubating for 5 days at 4 °C (Trace 2), were mixed at room temperature to give final concentrations of 80 and 90 μM, respectively. 80 μl aliquots were withdrawn for HPLC analysis after 1 h (Trace 3) and 57 h (Trace 4). Retention times and peak identities are the same as given in Figure 1.

Zn_4Ag_6MT when 6 equiv of Ag^+ is added to the protein. Therefore, to obtain a pure, homogeneous sample of Zn_4Ag_6MT, a sufficiently large excess of Zn_7MT must be present to drive the equilibrium to a point where all of the $Ag_{12}MT$ is consumed. Once this is accomplished, the Zn_7MT can be separated from the Zn_4Ag_6MT by preparative anion exchange HPLC. Such an approach is illustrated in Figure 3, where excess Zn_7MT was added to a sample containing an equilibrium mixture of Zn_7MT, Zn_4Ag_6MT, and $Ag_{12}MT$. After sufficient time for a new equilibrium to be established was allowed (57 h), it was clearly seen that all of the $Ag_{12}MT$ had been converted to the desired Zn_4Ag_6MT mixed-metal product.

Ag^+ Titration of Zn_7MT in the Absence of Chelex Resin. The replacement of Zn^{2+} in MT by Ag^+ induces a large change in absorbance at 260 nm that offers a convenient means of monitoring the stoichiometry of the reaction (Li, 1991; Stillman, 1992). As shown in Figure 4A , we obtained very different results when the reaction was carried out in the presence and absence of Chelex resin. In the absence of Chelex, addition of Ag^+ up to about 12 equiv caused a linear increase in absorbance, with additional Ag^+ up to 27 equiv inducing continued, but much smaller, increases. When the same experiment was conducted in the presence of Chelex to remove unbound metal ions prior to measuring the UV spectra, the absorbance increased progressively with addition of the first 14−15 equiv of Ag^+ but then decreased dramatically as more metal ion was added. The decrease in 260 nm absorbance was not due to a spectral shift, but rather to a gradual disappearance of the entire UV spectrum

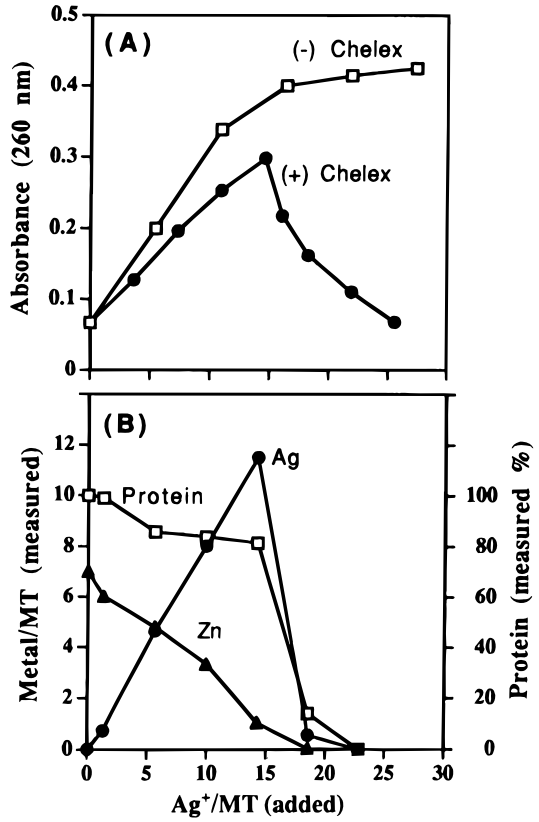


FIGURE 4: (A) Ag^+ titration of Zn_7MT (4 μM) *with* (+) and *without* (−) Chelex resin treatment monitored by absorbance at 260 nm. (B) Ag^+ titration of Zn_7MT (15 μM) in the presence of Chelex monitored by metal and protein analysis.

consistent with a progressive reduction in the amount of protein in solution. To confirm this, another experiment was conducted in which the amounts of Ag^+, Zn^{2+}, and protein remaining in solution after Chelex treatment were measured directly. The results in Figure 4B show that addition of Ag^+ up to about 14 equiv induced the progressive displacement of Zn^{2+} from MT while most, but not all, of the MT remained in solution. It is interesting to note that not all of the added Ag^+ ends up bound to the MT remaining in solution after removal of the Chelex, nor is all of the Zn^{2+} completely displaced from the protein. After addition of 14 equiv of Ag^+, only about 11.5 equiv was able to be accounted for in solution as MT-bound metal, while about 1 equiv of Zn^{2+} still remained bound to the protein. Adding Ag^+ beyond 14 equiv caused a dramatic decrease in the amount of both protein and protein-bound Ag^+, suggesting that an "over-metalated" AgMT species was being produced that is removed from solution by the Chelex resin. Consistent with this explanation are the results shown in Figure 1 of the late stages of the Ag^+ titration of Zn_7MT in the presence of Chelex monitored by HPLC (Traces 5 and 6), where addition of Ag^+ much beyond 12 equiv was seen to lead to a reduction in the amount of $Ag_{12}MT$ (peak C).

A direct DEAE HPLC analysis of the products of Ag^+ reaction with Zn_7MT in the absence of Chelex is shown in Figure 5. Compared to the results of the same experiment performed with Chelex treatment (Figure 1), there were two notable differences. First, a new peak with a retention time of 3 min (labeled D) appeared whose size increased progressively as more Ag^+ was added. Second, the amounts produced of Zn_4Ag_6MT and $Ag_{12}MT$ were much lower than when Chelex treatment was employed. The retention time

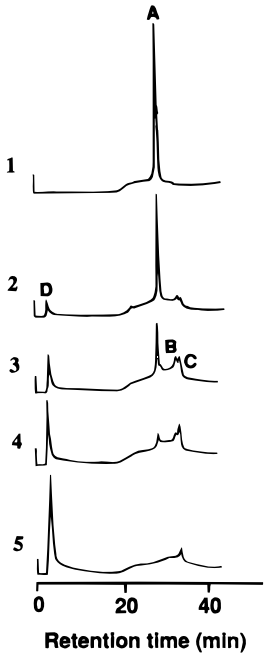


FIGURE 5: Ag^+ titration of Zn_7MT without Chelex treatment monitored by HPLC. Separate samples of Zn_7MT (100 μM) were incubated with varying amounts of Ag^+ for 25 min, and 50 μL aliquots were analyzed by HPLC. Mol equiv of Ag^+ added: (1) 0, (2) 4, (3) 8, (4) 13, and (5) 15. Retention times and peak identities are the same as given in Figure 1.

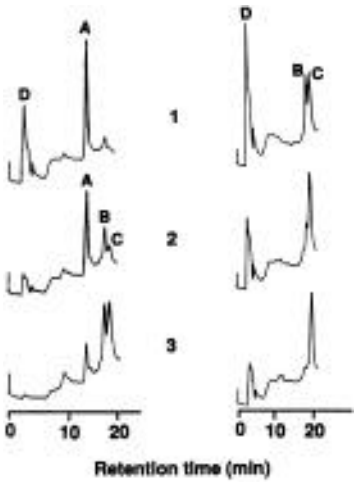


FIGURE 6: Time dependence of metal exchange reactions between Ag_{18}MT and Zn-containing MTs. *Left column*: Zn_7MT and Ag_{18}MT solutions were mixed to give final concentrations of 270 and 340 μM, respectively. 20 μL aliquots were withdrawn for HPLC analysis at the following times (h): (1) 0, (2) 0.5, and (3) 2.5. *Right column*: A solution containing a mixture of Zn_4Ag_6MT and Ag_{12}MT, prepared by incubating 350 μM Zn_7MT with 8.5 equiv of Ag^+ for 5 days at 4 °C, was mixed with Ag_{18}MT at room temperature. The final concentrations of Zn_4Ag_6MT/Ag_{12}MT and Ag_{18}MT were 220 and 340 μM, respectively. 40 μL aliquots were withdrawn for HPLC analysis at the following times (h): (1) 0, (2) 1, and (3) 2.5. Elution was with a 15 min linear gradient of 20−50% B (A, 5 mM Tris-HCl, pH 8.5; B, 300 mM Tris-HCl, pH 8.5). Peak identities are the same as given in Figure 1.

of peak D was identical to that of material having no affinity for the column (*i.e.*, lacking a net negative charge), suggesting that the protein eluting in this position is neutral or positively-charged. Analysis of the contents of peak D by AAS indicated that it contained Ag, but no Zn. A CD spectrum (data not shown) of the material revealed a distinct positive band at 293 nm, which closely resembles the appearance of the spectrum reported for the discrete Ag_{18}MT species generated by Ag^+ titration of apo-MT (Zelazowski et al, 1989; Scheuhammer & Cherian, 1986). The net charge of Ag_{18}MT at pH 8 is calculated on the basis of its amino acid composition to be +1, whereas the charges carried by Zn_7MT and Ag_{12}MT are −3 and −5, respectively. Thus, unlike the latter two MT species, Ag_{18}MT would not be expected to bind to a DEAE anion exchange column. The net positive charge carried by Ag_{18}MT would also make it susceptible to being bound by the negatively-charged carboxymethyl functional groups of Chelex 100. All of the available evidence therefore points to the identification of the so-called "over-metalated" AgMT species and the material in peak D as Ag_{18}MT.

Since interprotein metal exchange reactions were demonstrated to occur between Ag_{12}MT and Zn_7MT to form Zn_4Ag_6MT (Figure 2, see above), we wondered whether Ag_{18}MT could also serve as a donor of Ag^+ to Zn-containing MT. To investigate this question, Ag_{18}MT was first prepared preparatively by adding 13 equiv Ag^+ to Zn_7MT in the absence of Chelex resin, and then separating the reaction products by anion exchange HPLC. The material eluting at 3 min (Ag_{18}MT) was collected and mixed with either Zn_7MT or Zn_4Ag_6MT and monitored as a function of time by HPLC. Figure 6A shows HPLC profiles obtained upon mixing Zn_7MT and Ag_{18}MT. The peak intensities of Ag_{18}MT (peak D) and Zn_7MT (peak A) decreased while those corresponding to Zn_4Ag_6MT (peak B) and Ag_{12}MT (peak C) appeared and increased with time, indicating that interprotein metal transfer occurred between Ag_{18}MT and Zn_7MT. This reaction is complete in under 3 h and is thus significantly faster than the exchange between Ag_{12}MT and Zn_7MT, which requires about 2 days under similar conditions to reach equilibrium. When Ag_{18}MT was mixed with a Zn,AgMT sample containing Zn_4Ag_6MT and Ag_{12}MT and almost no Zn_7MT (Figure 6B), the peaks corresponding to Ag_{18}MT and Zn_4Ag_6MT decreased in size while that of Ag_{12}MT increased with time. This reaction was also quite rapid, with all of the Zn_4Ag_6MT disappearing in less than 3 h. The results in Figure 6 thus indicate that Ag_{18}MT can donate Ag^+ to both Zn_7MT and Zn_4Ag_6MT.

Ag^+ Titration of Cd_7MT. The reaction of Ag^+ with Cd_7-MT was monitored by UV spectroscopy, AAS, and HPLC using the same approach described above for Zn_7MT. The HPLC profiles for the reaction of Cd_7MT with increasing amounts of Ag^+ in the presence of Chelex are shown in Figure 7. Each HPLC trace was acquired about 30 min after addition of Ag^+ to the protein. Trace 1 shows the Cd_7MT solution before any Ag^+ was added and indicates the presence of a single species giving rise to a peak with a retention time of 30.2 min (labeled A). As Ag^+ was added, a new peak with a retention time of 33.3 min (labeled B) appeared, and its intensity increased progressively while the size of peak A decreased. After the complete disappearance of peak A brought about by addition of 7 equiv of Ag^+ (Trace 4), further addition of Ag^+ produced a gradual decrease in the size of peak B (Traces 5 and 6). AAS analysis of peak B indicated the presence of both Ag and Cd with the Ag/Cd ratio increasing in proportion to the amount of Ag^+ added. Under conditions of thermodynamic equilibrium (see below), AAS analysis of peak B in samples with Ag/MT ratios of about 6 gave a Ag/Cd ratio of 1.5. Addition of more Ag^+ caused this ratio to increase until finally, after a total of about

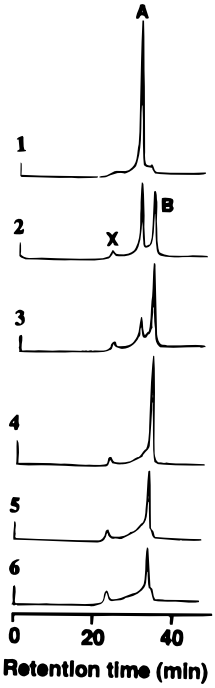


FIGURE 7: Ag^+ titration of Cd_7MT *with* Chelex treatment monitored by HPLC. Separate samples of Cd_7MT (30 μM) were incubated with varying amounts of Ag^+ in the presence of Chelex-100 resin. 100 μL aliquots were analyzed by HPLC. Mol equiv of Ag^+ added: (1) 0, (2) 3.5, (3) 5, (4) 7, (5) 9, and (6) 12. Retention times (min) and peak identities: (A) 30.2, Cd_7MT; (B) 33.3, $Cd_4Ag_6MT/Ag_{12}MT$; and (X) 22.4, an uncharacterized Ag- and Cd-containing MT.

12 equiv had been added, no Cd could be detected in peak B. These results are consistent with the assignment of peak B to both Cd_4Ag_6MT and $Ag_{12}MT$, which apparently cannot be resolved from one another under these conditions. The gradual decrease observed in the size of peak B upon addition of more than 7 equiv of Ag^+ (Traces 5 and 6) is most likely the result of $Ag_{18}MT$ formation, followed by its removal by Chelex resin. A third, small peak (labeled X) whose identity is currently unknown also appears in Figure 7 at a retention time of about 22 min. Its Ag/Cd ratio was measured to be about 0.7 by AAS. The size of peak X generally was found to increase upon prolonged incubation of the sample at room temperature (data not shown).

Except for the fact that Cd_4Ag_6MT cannot be resolved by HPLC from $Ag_{12}MT$, the results of the Ag^+ titration of Cd_7MT are qualitatively similar to those obtained with Zn_7MT. To determine if the time-dependent metal rearrangement reaction that was observed with Zn_7MT also occurs with Cd_7MT, HPLC analyses were conducted on a Cd_7MT sample at various times after *ca.* 4 equiv of Ag^+ had been added (data not shown). The results clearly showed a gradual decrease in the size of peak A (Cd_7MT) and a corresponding increase in the size of peak B ($Cd_4Ag_6MT/Ag_{12}MT$), indicative of slow intermolecular metal exchange between Cd_7MT and $Ag_{12}MT$ according to eq 3:

$$(Cd_4)^{\alpha}(Cd_3)^{\beta}MT + (Ag_6)^{\alpha}(Ag_6)^{\beta}MT \rightleftharpoons (Cd_4)^{\alpha}(Ag_6)^{\beta}MT \quad (3)$$

The amount of time required to achieve equilibrium for this reaction was about 10 h, significantly less than the 2−3 days required to reach equilibrium for the analogous reaction of Zn_7MT (eq 2).

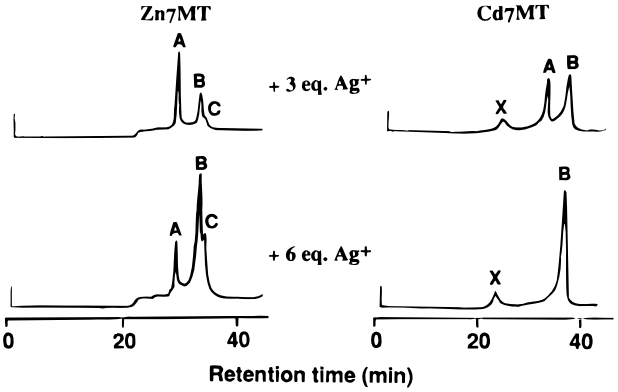


FIGURE 8: Comparison of equilibrium product distributions resulting from addition of Ag^+ to Zn_7MT (left) and Cd_7MT (right). 3 and 6 equiv of Ag^+ were added to separate solutions of Zn_7MT (100 μM) and Cd_7MT (30 μM) in the presence of Chelex-100. After 30 min, the Chelex was removed and the samples incubated at 4 °C for 10 days prior to HPLC analysis. Retention times and peak identities of the Zn_7MT and Cd_7MT products are the same as given in Figures 1 and 7, respectively.

In Figure 8 are shown HPLC traces comparing the equilibrium product distributions resulting from addition of 3 or 6 equiv of Ag^+ to Zn_7MT and Cd_7MT. As seen in the bottom two traces resulting from addition of 6 Ag^+, the original Cd_7MT peak (peak A, right) can no longer be detected, whereas the peak corresponding to Zn_7MT (peak A, left) is still present. From this result, it may be concluded that the equilibrium constant for the metal exchange reaction involving Cd_7MT (eq 3) is greater than that for the corresponding reaction of Zn_7MT (eq 2). In fact, there appears to be sufficient driving force to ensure that any $Ag_{12}MT$ formed by the addition of ≥6 equiv of Ag^+ will be converted to Cd_4Ag_6MT if sufficient time is allowed for it to react with the residual Cd_7MT left in solution.

Cu^+ Titration of Zn_7MT. Reactions of Zn_7MT with Cu^+ were carried out in a similar way to those described above with Ag^+. $[Cu(CH_3CN)_4]ClO_4$ was used as the Cu^+ source (Lontie et al., 1965; Nair & Mason, 1967; Rupp & Weser, 1974; Beltramini & Lerch, 1983; Winge et al., 1986; Gasyna et al., 1988), and additions were made to separate aliquots of Zn_7MT under a N_2 atmosphere in an anaerobic chamber. UV spectra were obtained anaerobically and HPLC profiles were obtained on samples immediately after they were removed from the chamber. HPLC measurements performed on Cu-containing MT exposed to air for varying times indicated that oxidation of the Cu-containing proteins was negligible during the short time required to perform the analyses (data not shown). Because the results were quite similar to those obtained with Ag^+, they are not shown here and only some significant differences will be discussed.

Metal displacement reactions of Zn_7MT with Cu^+ in the presence of Chelex resin gave HPLC profiles similar to those shown in Figure 1 for reaction with Ag^+. However, Zn_4Cu_6MT could not be resolved from $Cu_{12}MT$ (peak B in Figure 9). Just as with the reaction with Ag^+, time-dependent product redistribution was observed after the initial Cu^+ displacement reaction (Figure 9). However, the reaction rates appeared to be significantly faster for the Cu^+ system, with equilibrium being reached in about 7 h at room temperature.

Several other differences were noted between the results of Ag^+ and Cu^+ titration. Addition of more than about 14

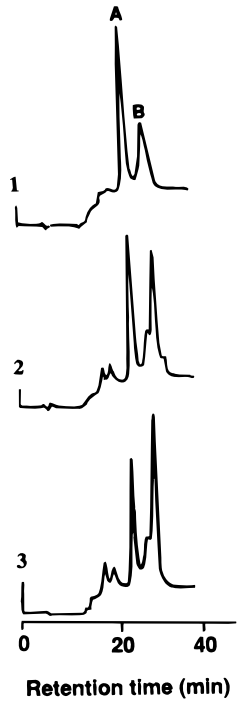


FIGURE 9: Time dependence of the product distribution resulting from Cu^+ addition to Zn_7MT. Cu^+ (4 equiv) was added to Zn_7MT (90 μM) in an anaerobic chamber at room temperature, and 35 μL aliquots were withdrawn at 0 h (1), 1.5 h (2), and 6.5 h (3) for HPLC analysis. Elution was with a 15 min linear gradient of 0 to 35% B (A, 5 mM Tris-HCl, pH 8.5; B, 300 mM Tris-HCl, pH 8.5) at a flow rate of 1 mL/min. Retention times (min) and peak identities: (A) 22.0, Zn_7MT, and (B) 24.0, Zn_4Cu_6MT/Ag_{12}MT.

equiv of Cu^+ to Zn_7MT or Cd_7MT led to the removal of some of the protein by Chelex as monitored by UV, AAS, and HPLC measurements, but the effects of "over-metalation" were much less dramatic than with Ag^+. In reactions between Zn_7MT and Cu^+ carried out in the absence of Chelex, no peaks with very short retention times similar to peak D in Figure 5 were observed in DEAE HPLC profiles. This observation suggests that excess Cu^+ might not produce a stable Cu_{18}MT species analogous to Ag_{18}MT. A difference between "over-metalated" forms of CuMT and AgMT has previously been noted on the basis of UV, CD, and luminescence spectroscopic evidence that suggested the existence of a Cu_{20}MT species (Stillman et al., 1987; Gasyna et al., 1988).

DISCUSSION

Reverse phase and anion exchange HPLC methods have proven very useful for separating the numerous isoforms and subisoforms of MT found in mammalian tissues (Klauser et al., 1983; Hunziker & Kägi, 1985). To our knowledge, however, there have been no reports of the use of HPLC to separate the various mixed-metal MT species produced during metal exchange reactions of a single isoform. After testing a variety of size exclusion, reverse phase, and ion exchange chromatographic conditions, we found that DEAE HPLC provides a rapid and effective means of monitoring the production of the several species that are produced upon reaction of Zn_7MT and Cd_7MT with Ag^+ or Cu^+. With this tool, it was found that product distributions are more complex than previously thought and are affected significantly by experimental conditions.

Metal Exchange Reactions of Zn_7MT or Cd_7MT with Ag^+. Previous studies have demonstrated that Ag^+ binds to MT many orders of magnitude more tightly that Zn^{2+} or Cd^{2+} (Nielson et al., 1985). When added to Zn_7- or Cd_7MT, Ag^+ displaces Zn^{2+} or Cd^{2+} almost instantaneously in a ratio of about one Zn^{2+} displaced to two Ag^+ ions added (Zelazowski & Stillman, 1992). When this occurs, there is no indication from the results of the present study or from Cd NMR studies (Li & Otvos, 1996) that any stable products are formed other than those with completely Ag-containing and Zn (Cd)-containing clusters. In other words, it appears that the metal displacement reaction proceeds in each domain *via* rapid, cooperative destruction of the existing divalent metal ion-containing cluster with concomitant formation of an all-Ag cluster. The question of which of the two domains of MT reacts preferentially with Ag^+ was thought to be answered by Cd NMR when it was found that addition of 6 equiv of Ag^+ to Cd_7MT led exclusively to the formation of $(Cd_4)^{\alpha}(Ag_6)^{\beta}$MT (Li & Otvos, 1996). It is now clear that the binding of the first six Ag^+ ions to the β-domain does not reflect a kinetic preference, but is instead the result of thermodynamic influences.

The key observation which led to this conclusion is that DEAE HPLC profiles of the product mixtures resulting from Ag^+ titration of Zn_7MT and Cd_7MT change over time. Although divalent metal ions (Me^{2+}) such as Zn^{2+} and Cd^{2+} are displaced extremely rapidly from the protein by Ag^+, it is obvious that at least some of the newly-formed Ag-containing MT species are metastable and undergo a series of much slower interprotein metal exchange reactions that redistribute Me^{2+} and Ag^+ from their kinetically-favored locations to those of greater thermodynamic stability. The reactions believed to be responsible for these events are the following:

$$Me_7MT \xrightarrow[\text{Fast}]{Ag^+ \;\; Me^{2+}} (Me_4)^{\alpha}(Ag_6)^{\beta}MT + Ag_{12}MT + Ag_{18}MT \qquad (4)$$

$$Me_7MT + Ag_{12}MT \underset{\text{Slow}}{\rightleftharpoons} (Me_4)^{\alpha}(Ag_6)^{\beta}MT \qquad (5)$$

$$Me_7MT + Ag_{18}MT \underset{\text{Slow}}{\overset{Me^{2+}}{\rightleftharpoons}} Ag_{12}MT + (Me_4)^{\alpha}(Ag_6)^{\beta}MT \qquad (6)$$

$$(Me_4)^{\alpha}(Ag_6)^{\beta}MT + Ag_{18}MT \underset{\text{Slow}}{\overset{Me^{2+}}{\rightleftharpoons}} Ag_{12}MT \qquad (7)$$

The first very rapid reaction (eq 4), which is complete within seconds, involves direct displacement of Me^{2+} from MT by Ag^+. As reflected by the data in Figure 5 for the reaction of Zn_7MT, addition of even small amounts of Ag^+ leads to the initial production of three different products separable by DEAE HPLC. The first of these, $(Zn_4)^{\alpha}(Ag_6)^{\beta}$MT, results from Ag^+ binding exclusively to the β-domain. The other two, Ag_{12}MT and Ag_{18}MT, represent species arising from displacement of Zn^{2+} from both the α- and β-domains. The predicted net charges of the three Ag-containing species are -3, -5, and $+1$, respectively, which accounts for their HPLC elution order. The apparent absence of a fourth species, $(Ag_6)^{\alpha}(Me_3)^{\beta}$MT, suggests that Ag^+ reacts more rapidly with the β-domain than the α-domain. The degree of kinetic discrimination must be small, however, since addition of less than 12 equiv

of Ag^+ to Zn_7MT leads to the production of comparable amounts of the Ag_6MT, $Ag_{12}MT$, and $Ag_{18}MT$ species (Figure 5). An important consequence of the kinetic nonselectivity of Reaction (4) is that significant amounts of Zn_7MT and Zn_4Ag_6MT remain in solution after addition of 6 and 12 equiv of Ag^+, respectively. As a result, Ag^+ in excess of 12 equiv needs to be added to displace all of the Zn^{2+} from the protein (Figure 4). The same observation was made by Zelazowski and Stillman (1992), but their assumption that Zn_4Ag_6MT, $Ag_{12}MT$, and $Ag_{18}MT$ form sequentially, rather than simultaneously, during a Ag^+ titration did not allow them to satisfactorily explain this behavior.

Although $Ag_{18}MT$ has a distinctive CD spectral signature (Zelazowski et al., 1989; Zelazowski & Stillman, 1992) which was useful in helping make the assignment of peak D in the DEAE HPLC traces in Figures 5 and 6, little is known about its structure. Unlike the two-domain, trigonally-coordinated complexes proposed for $Ag_{12}MT$ (Winge, 1987; Zelazowski et al., 1989), it has been suggested that the Ag^+ ions in $Ag_{18}MT$ are coordinated in a single metal-binding domain *via* linear two-coordinate cysteine ligation (Zelazowski & Stillman, 1992). Although the net charge predicted for the metal-thiolate cluster(s) of $Ag_{18}MT$ is -2, the overall charge of the protein is expected to be $+1$ owing to the contributions made by the multiple lysine residues. It is presumably for this reason that $Ag_{18}MT$, unlike the other species containing trigonal or tetrahedral metal complexes, does not bind at all to the positively-charged DEAE column matrix. It may also explain the unexpected affinity of $Ag_{18}MT$ for negatively-charged Chelex resin (see below).

Following the rapid metal displacement reaction depicted in eq 4, a series of much slower interprotein metal exchange reactions (eqs 5−7) are initiated that gradually change the initial kinetic product distribution to the thermodynamically-controlled equilibrium product mixture. These slow reactions, whose rates are dependent on protein concentration, are analogous to those described previously between Cd_7MT and Zn_7MT that are responsible for formation of several mixed-metal Cd,ZnMT species *in vivo* (Nettesheim et al., 1985; Otvos et al., 1987).

Just as Cd_7MT and Zn_7MT cannot coexist in solution without undergoing metal interchange, the same is true for $Ag_{12}MT$ and Me_7MT (eq)). The driving force for the reaction is apparently the relative thermodynamic stability of β-domains containing all-Ag^+ clusters and α-domains containing all-divalent metal ion clusters. As indicated by the data in Figure 8, the equilibrium constant for the reaction between $Ag_{12}MT$ and Zn_7MT is significantly smaller than that for the same reaction involving Cd_7MT. As a practical consequence, $(Cd_4)^{\alpha}(Ag_6)^{\beta}MT$ can be quantitatively prepared simply by adding 6 equiv of Cd^{2+} to $Ag_{12}MT$ and allowing sufficient time for the reaction to reach equilibrium. At the high protein concentrations (1−2 mM) employed in the Cd NMR titration studies (Li & Otvos, 1996), this requires much less time (<1 h) than when lower concentrations are used. Homogeneous samples of $(Zn_4)^{\alpha}(Ag_6)^{\beta}MT$ cannot be prepared in the same way, since some Zn_7MT and $Ag_{12}MT$ persist at equilibrium under these conditions. Instead, at least 8 equiv of Ag^+ must be added to produce enough $Ag_{12}MT$ to drive the reaction (eq 5) far enough to the right to use up all of the Zn_7MT remaining in solution after the initial rapid Zn^{2+} displacement reaction (eq 4).

Similar metal interexchange reactions were shown to take place between $Ag_{18}MT$ and Zn_7MT (eq 6) and $Ag_{18}MT$ and Zn_4Ag_6MT (eq 7). In the former case, the six "extra" Ag^+ ions in $Ag_{18}MT$ preferentially displace the Zn^{2+} from the β-domain cluster of Zn_7MT to form $(Zn_4)^{\alpha}(Ag_6)^{\beta}MT$, which leaves $Ag_{12}MT$ as the other product. In the latter case, the six "extra" Ag^+ of $Ag_{18}MT$ displace the α-domain Zn^{2+} from $(Zn_4)^{\alpha}(Ag_6)^{\beta}MT$ to leave $Ag_{12}MT$ as the only product. The occurrence of these reactions indicates that Ag^+ is bound most tightly to the β-domain of MT, next most tightly to the α-domain cluster, and least tightly to the six "extra" binding sites of $Ag_{18}MT$. Furthermore, there appear to be accessible kinetic pathways by which both divalent and monovalent metal ions can redistribute themselves to achieve more stable binding configurations.

Influence of Chelex Resin. Chelex-100 resin has been used widely over the years in studies of metallothionein as a convenient scavenger of free or adventitiously bound metal ions. Chelex was assumed to be benign in its action because it does not remove divalent or monovalent metal ions from their protein binding sites. In this study, we discovered that Chelex can exert a profound influence on experimental results by virtue of its unexpected binding affinity for $Ag_{18}MT$. The effect is seen most directly in Ag^+ titrations of Zn_7MT in the presence and absence of Chelex treatment (Figure 4). Up to about 14 equiv of added Ag^+, only about 20% of the protein is removed by the Chelex, reflecting the relatively small amounts of $Ag_{18}MT$ formed under these conditions. Additional Ag^+ produces much larger amounts of $Ag_{18}MT$ and Chelex appears to remove it quantitatively from solution. The origin of the binding affinity of Chelex for $Ag_{18}MT$ is not known, but we speculate it may be due to an electrostatic interaction between the positively-charged protein and negatively-charged resin. Alternatively, Chelex may bind to some or all of the six "extra" Ag^+ ions of $Ag_{18}MT$, but not sufficiently strongly to remove them from the protein.

A clear demonstration of the significant effect Chelex can have on product distributions of metal exchange reactions that are not given sufficient time to reach thermodynamic equilibrium is given in Figures 1 and 5. In the Ag^+ titration of Zn_7MT conducted in the absence of Chelex (Figure 5), large amounts of $Ag_{18}MT$ (peak D) are formed immediately after Ag^+ addition, so much so that relatively little Zn_4Ag_6MT and $Ag_{12}MT$ are produced. It is only subsequently *via* interprotein metal exchange (eqs 6 and 7) that the latter two products build up at the expense of $Ag_{18}MT$. In contrast, by having Chelex present initially, much higher concentrations of Zn_4Ag_6MT and $Ag_{12}MT$ are generated at short times after Ag^+ addition (Figure 1). In effect, the Chelex appears to be functioning as a sort of catalyst by redirecting the Ag^+ from the most kinetically-favored product ($Ag_{18}MT$) to those of higher thermodynamic stability.

With the new appreciation gained of the influence Chelex exerts on reactions of MT involving Ag^+ (and possibly Cu^+), plus the likelihood of forming complex product mixtures whose compositions may change with time and depend on such variables as temperature and protein concentration, care should be taken in the design and interpretation of related experiments. Some results of previous studies addressing the binding of Ag^+ and Cu^+ to metallothionein should also be reevaluated in light of the findings reported here.

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