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Monitoring metal ion flux in reactions of metallothionein and drug-modified metallothionein by electrospray mass spectrometry

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Abstract

The capabilities of electrospray ionization mass spectrometry are demonstrated for monitoring the flux of metal ions out of and into the metalloprotein rabbit liver metallothionein and, in one example, chlorambucil-alkylated metallothionein. Metal ion transfers may be followed as the reactions proceed in situ to provide kinetic information. More uniquely to this technique, metal ion stoichiometries may be determined for reaction intermediates and products. Partners used in these studies include EDTA, carbonic anhydrase, a zinc-bound hexamer of insulin, and the core domain of bacteriophage T4 gene 32 protein, a binding protein for single-stranded DNA.

Keywords: acquired drug resistance; DNA binding protein; electrospray mass spectrometry; insulin; metal ion flux; metallothionein; stress response

Metallothioneins (MT) comprise a family of small metal binding proteins that occurs widely throughout the animal kingdom. Plants and bacteria also produce types of metallothioneins. Vertebrate MTs share essentially the same structure (Kägi, 1993), with 20 cysteines coordinating seven divalent metal cations (Schultze et al., 1988; Robbins et al., 1991). Two domains are joined by a linker region, the N-terminal or β-domain binding three metal cations and the C-terminal or α-domain binding four metal cations. Metallothioneins in invertebrate species also contain multiple cysteine side chains that bind metal cations.

Although it would seem that a protein as widespread as MT must play a biological role that is essential for the survival of organisms (Vallee, 1995), the function of MT has been a subject for debate since it was discovered 40 years ago (Margoshes & Vallee, 1957). One function often cited for MT is the detoxification of heavy metals, cadmium in particular. However, the incidence of toxic concentrations of heavy metals in the environment is sporadic; thus, the need to detoxify heavy metals does not seem to account for the ability of virtually all living cells to synthesize metallothioneins transcriptionally or enzymatically (Steffens, 1990; Lazo et al., 1995; Vallee, 1995).

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Another function proposed for MT is the intracellular regulation of zinc ion levels (Li et al., 1980; Zeng et al., 1991a, 1991b; Vallee, 1995). MT has been shown to transfer zinc ions to apo-metalloenzymes in vitro, including carbonic anhydrase (Li et al., 1980), aldolase, alkaline phosphatase, thermolysin (Udom & Brady, 1980), pyridoxal kinase (Churchich et al., 1989; Hao et al., 1993), and the estrogen receptor (Cano-Gauci & Sarkar, 1996). Phytochelatins (plant metallothioneins) are also known to reactivate apo-metalloenzymes (Thumann et al., 1991). Thionein, on the other hand, has been found to suppress the binding of several DNA binding zinc-proteins by abstracting zinc ions (Zeng et al., 1991a, 1991b).

Thionein synthesis is controlled by a metallo-regulatory element upstream from the thionein gene, which is, in turn, activated by zinc-dependent MRE binding factors (Czupryn et al., 1992; Maret, 1995; Vallee, 1995; Maret et al., 1997). According to one model, transcription of thionein is activated by the presence of elevated zinc levels in the cell, and deactivated as zinc drops below a certain level. It has been suggested that thionein deactivates its own transcription by extracting zinc ions from an MRE binding factor (Czupryn et al., 1992; Maret, 1994; Vallee, 1995). If so, such a feedback mechanism may account for the observation that MT is transiently induced by metal ion treatment.

Although transgenic mice lacking functional MT genes have been shown to grow and reproduce normally (Michalska & Choo, 1993; Masters et al., 1994), they, however, display increased sensitivity to chemical stress, compared to wild-type mice (Lazo et al., 1995; Philcox et al., 1995; Rofe et al., 1996). These types of stresses,

while absent in benign laboratory conditions, are ubiquitous in the natural environment, and might thus provide the selective pressure behind the highly conserved ability to express MT (Steffens, 1990; Lazo et al., 1995; Vallee, 1995).

Demetallation of MT in vitro occurs with rather high concentrations of oxidants such as *t*-butylhydroperoxide and glutathione disulfide (Savas et al., 1993; Maret, 1994, 1995), while the cellular environment can contain a continuum of concentrations of these and other electrophiles. Consequently, it is also important to consider the effect of lower levels of chemical modifiers on the affinity of MT for zinc. By doing so, it may be possible to shed light on the role of MT in the stress response.

MT synthesis has been found to be induced in some tumor cells exposed to alkylating chemotherapeutic agents (Kelley et al., 1988), and a causal role has been proposed for MT in the acquired drug resistance that interferes with long-term chemotherapy in many patients. Covalent sequestration is proposed as a mechanism, triggering induction of further synthesis (Yu et al., 1995; Zaia et al., 1996). Electrospray mass spectrometric monitoring and molecular dynamics calculations support the hypothesis that the drug molecules associate specifically with the MT molecule in noncovalent fashion and then form a covalent bond (Zaia et al., 1996).

It has been shown that MT retains a full complement of seven metal ions when covalently modified with one or two molecules of CHB (Zaia et al., 1996). These results showed that the MT structure is not destabilized by covalent modification to the extent that demetallation of a domain occurs in the absence of competing ligands. It is, therefore, important to study the metal ion transfer phenomenon directly, so that the effect of covalent modification by drugs and other reagents can be determined.

The present work evaluates electrospray ionization mass spectrometry to investigate metal ion transfer between MT and other ligands in vitro. This technique allows the metal ion transfer in aqueous solution to be monitored on-line. The transfer of zinc to EDTA is presented as a model for small molecule ligands. The effect of covalent binding of chlorambucil (CHB) with MT on the transfer of zinc from MT to apo-carbonic anhydrase is assessed kinetically. Transfers of zinc ions to MT from the zinc ion-binding hexamer of insulin and from a single-stranded DNA binding protein (bacteriophage T4 gene 32 protein) (Karpel, 1990) are also demonstrated.

Experimental

Materials

Bovine erythrocyte carbonic anhydrase (lot 15H0897), chlorambucil, ethylenediaminetetraacetic acid (EDTA), and dipicolinic acid (DPA) were obtained from Sigma Chemical Co. (St. Louis, Missouri), and bovine insulin sodium salt (lot 405598) from Calbiochem Inc. (LaJolla, California).

Cd/Zn rabbit liver metallothionein 2a was from a single lot (34H95161) from Sigma Chemical Co., selected from a half dozen lots screened by HPLC and electrospray mass spectrometry. This material was demetallated, purified, and reconstituted as the all zinc complex for the studies reported here. First, thionein was prepared from the Cd, Zn–MT complex by de-salting and demetallating twice on a 2.1 × 30 mm reversed-phase column (Poros II R/H, PerSeptive Biosystems, Cambridge, Massachusetts). MT (5 mg) was dissolved in 1 mL H_2O, 0.1% trifluoroacetic acid (TFA). After 30 min the solution was applied to the HPLC column, which was then washed with 20 column volumes of H_2O, 0.1% TFA. The column was then eluted with 20% H_2O, 80% acetonitrile, 0.1% TFA, and two isoforms of thionein 2a were collected together. This purified fraction was dried using a centrifugal evaporator and reapplied to the reverse phase column for a second demetallation/purification step. This dry thionein was used in metal transfer reactions with insulin hexamer and bacteriophage T4 gene 32 protein, immediately following redissolution in 10 mM NH_4OAC pH7 purged under argon. The electrospray spectrum of purified thionein 2a and 2a′ in Figure 5A shows the presence of small amounts of Zn_1 and Zn_4 species. Fragmentation, for example, loss of the acetyl group and loss of acetylmethionine from the N-terminus in a small percentage of the protonated apo and holo protein molecules, has been reported previously (Yu et al., 1993), and results from the electrospray process. This is the source of the small peaks at masses just below that of thionein 2a.

Zn_7–MT was prepared from purified thionein using a published method (Vašák, 1991). The metal content of the reconstituted Zn_7–MT was confirmed using electrospray mass spectrometry (Yu et al., 1993). Metallothionein concentration was confirmed using the molar absorptivity of rabbit MT (48,200 mol^{-1} cm^{-1}) at 220 nM (Vašák et al., 1987).

The reaction between Zn_7–MT and chlorambucil was performed as described previously (Zaia et al., 1996), and the sample was used immediately for mass spectrometric studies.

Apo-carbonic anhydrase was prepared from carbonic anhydrase using dipicolinic acid (DPA) to chelate zinc (Hunt et al., 1977). DPA (50 mM) was solubilized with NaOH to give a solution with pH 7.5. Carbonic anhydrase (CA) (25 mg) was dissolved in the DPA solution (4 mL) and concentrated using a Centricon-10 concentrator (Amicon, Inc., Beverly, Massachusetts) to a volume of 0.5 mL, to which was added 2 mL of DPA solution. The solution was concentrated and diluted a total of 12 times with DPA and then 8 times with H_2O. Electrospray mass spectrometric analysis of the resulting solution (see below) clearly indicated that the carbonic anhydrase had been demetallated. The concentration of the apo-CA solution was determined using the Pierce Chemical Co. (Rockford, Illinois) BCA protein assay reagent. Apo-CA (4.5 μM) was incubated with Zn_7–MT or CHB–Zn_7–MT (4.5 μM) in 10 mM NH_4OAc pH 7.5 in the syringe attached to an electrospray ionization source.

Insulin hexamer was prepared (Brader & Dunn, 1991) by incubating 330 μM insulin with 330 μM $ZnCl_2$ in HEPES buffer 0.1 M, pH 7.0 for 15 h at 4 °C. The solution (total volume 500 μL) was placed into a Centricon 10 ultrafiltration device (Amicon, Beverly, Massachusetts), diluted to a volume of 2 mL with NH_4OAc (10 mM, pH 7.5), and concentrated by centrifugation. The filtration was repeated two more times using the NH_4OAc solution. The concentration of insulin in the retentate was determined after diluting 10 μL of the solution into 1.0 mL guanidine hydrochloride (6 M), by measuring the optical absorbance at 276 nM using a molar absorptivity of 6,010 (Pace et al., 1995). The sample was diluted to 65 μM insulin hexamer and characterized by electrospray mass spectrometry using an external standard (average molecular mass calculated for $I_6 \cdot Zn_2 \cdot 4H_2O$ 34,604.4 Da observed 34,601.4).

The core domain of bacteriophage T4 gene 32 protein was isolated from extracts of *Escherichia coli* cells transformed with the overproducing *E. coli* plasmid pYS55, obtained from Dr. Yousif Shamoo, which encodes amino acid residues 17 to 253 of 32 protein. pYS55 is derived from pYS6, which encodes full-length 32 protein (Shamoo et al., 1986). Induction was achieved by using

the nalidixic acid-sensitive strain of *E. coli*, AR120, and the protein was purified by chromatography on DEAE-Sephacel and single-stranded DNA cellulose (Bittner et al., 1979). The purified protein was ≥98% pure, as assessed by SDS-polyacrylamide gel electrophoresis. Concentrations were determined spectrophotometrically, using $\epsilon_{280}^{M} = 3.7 \times 10^4\ M^{-1}\ cm^{-1}$ (Jensen et al., 1976). The protein was filtered three times through a Centricon-10 cell with NH_4OAc (10 mM, pH 7.0). It was characterized by electrospray mass spectrometry as chelating one Zn ion (average molecular mass calculated for core domain [16–253] 26,762.5, observed 26,762.9 bracketed by three charge states of α-chymotrypsinogen).

In incubations with thionein, insulin hexamer (50 μM in 10 mM NH_4OAc, pH 7.5), or 32 protein core domain (50 μM in 10 mM NH_4OAC, pH 7.5) was added to dry aliquots of thionein to make a final thionein concentration of 50 μM. The solution was allowed to sit at ambient temperature for 30 min and then infused into the mass spectrometer. Control reactions were carried out with thionein alone.

Electrospray mass spectra were collected using the first mass spectrometer of a JEOL (Tokyo, Japan) HX110/HX110 instrument fitted with a thermally assisted electrospray source. Aqueous reaction or analyte solutions were infused into the source at 1 μL/min using a syringe pump and the interfacing capillary was heated to 120 °C. A stable sheath flow of methanol was sprayed coaxially to the analyte solution at 1 μL/min. The spray needle was kept at ground potential and the counter electrode at 4 kV. Linear scans were performed over the appropriate mass range with a typical duty cycle between 9 and 12 s. Resolution was set to 500 (10% valley) by adjusting the slit widths. Data acquisition was started 2 to 5 min after the solution was mixed, and the mass range was then scanned continuously for the duration of the experiment. The data set consists of a series of mass spectra that represent time points of the reaction. Each spectrum shown was the averaged profile of several scans recorded by the JEOL MP7000 data system. Spectra were routinely calibrated by the data system against a fast atom bombardment spectrum of cesium iodide clusters. Accuracy in the on-line measurements is ±2 Da. All reactions were carried out in aqueous solutions buffered at 7.5 pH.

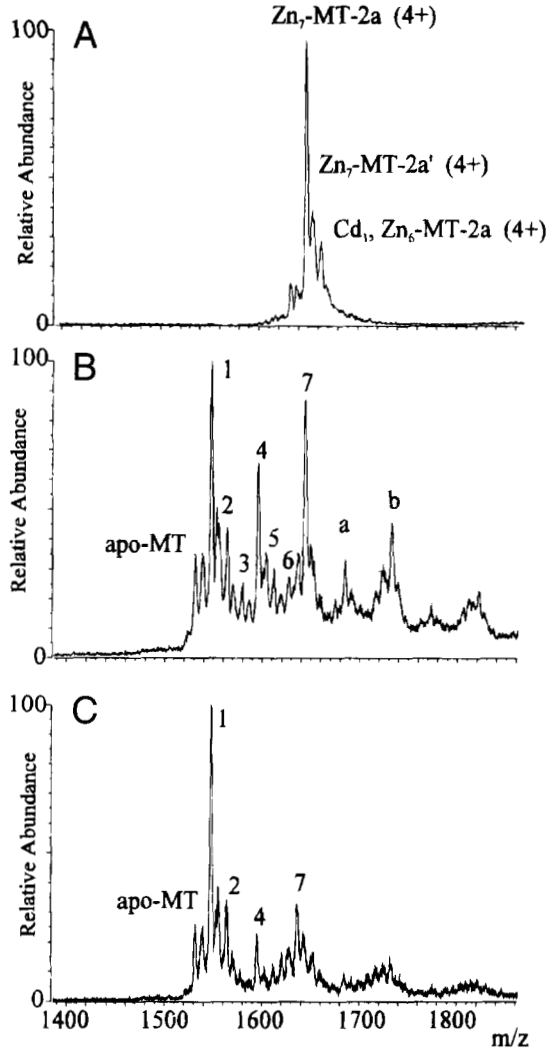

Fig. 1. ESI mass spectra showing the demetallation of Zn_7–MT by EDTA at 1:10 MT:EDTA ratio. **A:** The 4+ ion of Zn_7–MT before the addition of EDTA: MW Zn_7–MT 6,572. **B:** Five-minute reaction with EDTA: MW Zn_1–MT 6,189; Zn_4–MT 6,380; Zn_7–MT 6,572; a 6,735; b 6,928. **C:** Twenty-five minute reaction with EDTA: MW Zn_1–MT 6,189. Numbers 1 through 7 represent chelated zinc ions.

Results

Fully metallated MT is detected in aqueous solution at pH 7.5 using electrospray mass spectrometry, with the 4+ charge state by far the most abundant (Yu et al., 1993). Partially metallated MT species also produce predominantly the 4+ charge state in these on-line experiments. Figure 1A shows the spectrum of Zn_7–MT, which was collected before the addition of EDTA as a competing ligand. The m/z value of the most abundant ion (1,644.1) corresponds well with the expected value for the 4+ charge state of Zn_7–MT2a (1,643.23). The next most abundant ion (m/z 1,650.0) corresponds to the expected m/z of the 4+ charge state of Zn_7–MT2a′, an MT2a isoform (1,650.72) (Vašák et al., 1987). The two small shoulder peaks (m/z 1,655.4 and 1,662.9) correspond to $CdZn_6$–MT2a and $CdZn_6$–MT2a′ with 4+ charge states (calculated m/z values 1,654.97 and 1,662.48, respectively). These masses are expressed as deconvoluted molecular weights in the figure legend. The accuracy of these measurements was sufficient to identify the isoforms and metal compositions without ambiguity.

Figure 1B shows the spectrum produced after Zn_7–MT was reacted with EDTA at a 1:10 molar ratio for 5 min. Ions corresponding to Zn_1–, Zn_7–, and Zn_4–MT are the most abundant in the spectrum, in that order. Figure 1C shows the spectrum collected after 25 min of reaction. The ion corresponding to Zn_1–MT is clearly the most abundant in the spectrum. An analysis of the products of Zn_7–MT reacted with EDTA (1:10 molar ratio) at 5, 15, and 25 min is provided in Table 1.

Figure 2 shows the decrease in Zn_7–MT ion abundance as a function of time for three MT:EDTA ratios. The data are expressed as the abundance of the Zn_7–MT species divided by the total ion abundances of both reactant and product MT species.

Figure 3 shows spectra from an on-line monitoring experiment for the transfer of zinc ions from Zn_7–MT to apo-CA recorded at 3.5 and 43.5 min. The two most abundant charge states in the ESI-MS spectrum of CA at neutral pH were determined to be the 9+ and 10+. The resolution of the instrument (set to $m/\Delta m = 500$, 10% valley) was sufficient to resolve apo- and holo-CA as is shown in the Figure 3 insets for the 9+ ion (calculated m/z for

Table 1. *Abundances of MT^{4+} ions at various times in the reaction between Zn_7–MT and EDTA monitored on-line*

	Zn_7–MT:EDTA 1:10		
Zn_n–MT	5 min	15 min	25 min
Zn_0	0.07[a]	0.12	0.09
Zn_1	0.22	0.34	0.31
Zn_2	0.1	0.17	0.13
Zn_3	0.05	0.05	0.03
Zn_4	0.13	0.05	0.1
Zn_5	0.07	0.05	0.06
Zn_6	0.05	0.07	0.07
Zn_7	0.18	0.12	0.13
Zn_7–MT-EDTA·Zn	0.11	0.05	0.06

[a]Calculated as percent of total abundance for all MT species.

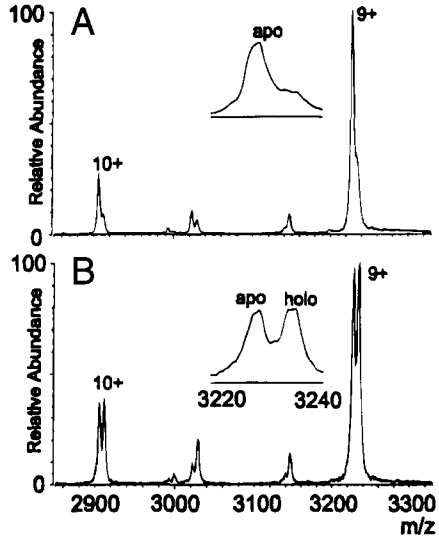

Fig. 3. Transfer of zinc ion from Zn_7–MT to carbonic anhydrase. **A:** Partial mass spectrum of the apo-CA and Zn_7–MT reaction mixture at time = 3.5 min. **B:** Partial spectrum of the reaction mixture at 43.5 min.

apo-CA 9+ = 3,225.9, observed 3,226; calculated for holo-CA = 3,234.9, observed 3,235). The latter mass reflects chelation of one Zn ion and the addition of either a water molecule or hydroxide ion as one ligand in the zinc complex (Huheey et al., 1993). The mass range of the on-line scans was set to bracket these two ions to maximize the number of scans that could be collected. The reaction was monitored for 120 min, during which 75% of the apo-CA was converted to holo-CA by accepting a zinc ion from Zn_7–MT. On-line data were collected continuously for 45 min, and then for 5 min intervals at 90 min and at 120 min reaction time.

The relative increases in ion abundance for the holo-CA species were plotted to represent the reaction time course. In Figure 4, this time course is compared to one determined under the same conditions for the transfer of zinc ion from chlorambucil-modified Zn_7–MT. The lower curve represents the transfer of zinc ions from unmodified Zn_7–MT, and the upper curve that from CHB-modified Zn_7–MT. The data points were calculated by dividing the summed abundances for the holo-CA 9+ and 10+ ions by the summed ion abundances for apo- and holo-CA. At time = 120 min (not shown on the plot), the relative ion abundances for the holo-CA species were the same for the two reactions, as would be expected because the amount of zinc available was the same in the two cases. Zinc ion complexes were not found when holo-CA and thionein were incubated under the same conditions.

Figure 5 illustrates the detection by electrospray mass spectrometry of transfer of zinc ions from two zinc-containing proteins to thionein. Figure 5A shows the spectrum of a control solution of thionein in 10 mM NH_4HCO_3, pH 7.5. In this spectrum, reduced thionein is free of metal. (The sources of several small peaks in the spectrum are detailed in the Experimental section.) Figure 5B shows the spectrum of thionein after a 30 min incubation with a zinc–

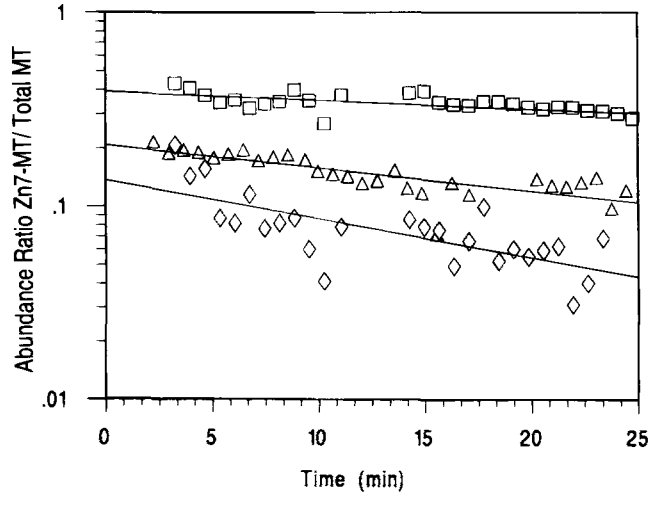

Fig. 2. Disappearance of Zn_7–MT produced by reaction with three ratios of EDTA. MT:EDTA 1:3, open square; MT:EDTA 1:10, open triangle; MT:EDTA 1:30, open diamond. Correlation coefficients (r^2) for the exponential fits are 0.66206, 0.58497, and 0.50598, respectively.

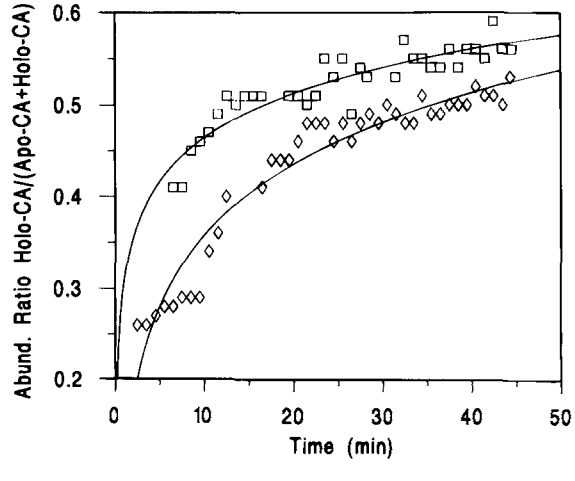

Fig. 4. Time course for the transfer of zinc ion from Zn_7–MT and chlorambucil-modified Zn_7–MT to CA. Zn_7–MT reaction, open diamond; CHB-modified Zn_7–MT reaction, open square.

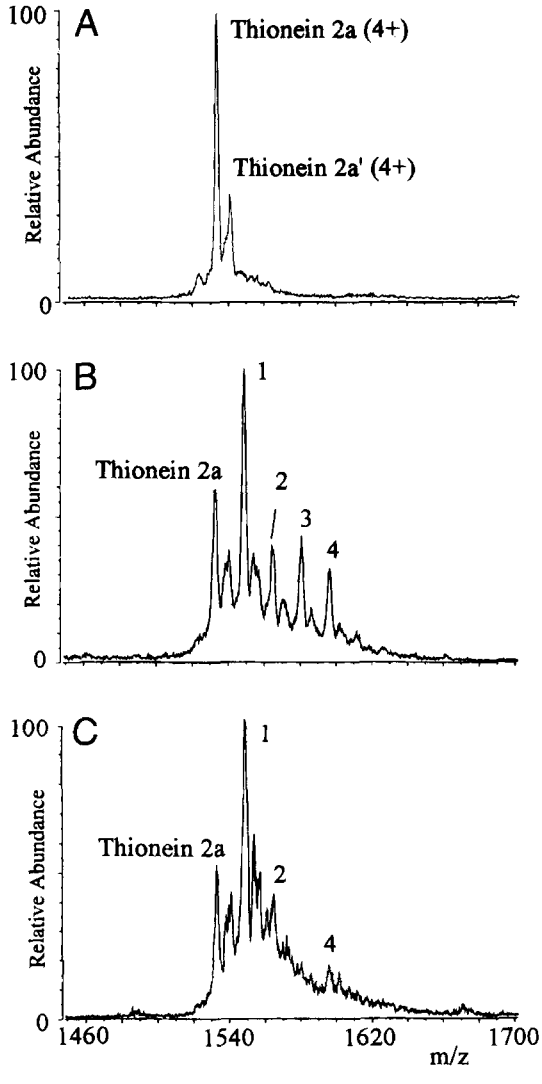

Fig. 5. ESI mass spectra showing zinc ion transfer to thionein. **A:** Spectrum of thionein in 10 mM NH_4OAc, pH 7.5: MW thionein 6,127. **B:** Thionein in the presence of insulin hexamer (7:2 Zn equivalents), 30 min: MW Zn_1–MT 6,188; Zn_2–MT 6,254; Zn_3–MT 6,318; Zn_4–MT 6,381. **C:** Thionein in the presence of a DNA binding protein (7:1 Zn equivalents), 30 min: MW Zn_1–MT 6,191.

insulin hexamer preparation. The m/z values for the ions in this spectrum, assigned using the data system calibration, are consistent with 0, 1, 2, 3, and 4 zinc ions bound to MT, 6,127, 6,188, 6,254, 6,318, and 6,381. Figure 5C shows the spectrum of thionein reconstituted with a solution containing bacteriophage T4 gene 32 protein, core domain (Williams et al., 1981; Karpel, 1990). In this case, the mass of the new ions (6,191Da) indicates that thionein has accepted one zinc ion from the limited amount of zinc equivalents in the g32 protein used.

Discussion

This kind of mass spectrometric study on Zn_7–MT provides information that is complementary to information provided by experiments using cadmium nuclear magnetic resonance (NMR). Mass spectrometry can be used to follow in situ the reaction in aqueous solutions at any pH between MT or thionein and a competing ligand for time periods ranging from 2 min to several hours. It is also possible to detect and differentiate partially metallated species (Zn_n–MT), which exist in solution simultaneously. These spectra can be used to generate a reaction time course. It is not possible, however, to directly determine which metals are removed from the MT structure. NMR can be used to follow the demetallation using various ratios of competing ligands, essentially by monitoring the end point of the reaction (Gan et al., 1995; Nicholson et al., 1987; Vazquez & Vašák, 1988) and determines which metals are being removed, based on the chemical shifts of the corresponding peaks. Resonances for metals that are exchanged rapidly on the NMR time scale are severely broadened, however, and this especially appears to be a problem in the analysis of MT when only one or two metals remain.

Some points should also be made about the electrospray technique used here. Electrospray is a popular technique for studying biochemical complexes because it appears to transmit some complexes into the mass spectrometer without disruption. It can, however, also introduce artifactual clusters, and it is important to design control reactions to distinguish between the two cases. The most widely accepted approach to analysis of protein complexes is to analyze the complex in aqueous solution at physiologic pH, and to carry out control reactions under denaturing conditions, for example, high pH, low pH, or other relevant conditions (Ganguly et al., 1993; Yu et al., 1993; Loo, 1997). It should also be pointed out that both susceptible samples and metal capillaries can undergo redox reactions in the conditions used to electrospray (Blades et al., 1991; Van Berkel & Zhou, 1995).

Electrospray studies of zinc- and cadmium-containing metallothionein were initiated in this laboratory with experiments at several pH values, and by comparison of the metal ion determinations with parallel analyses made using graphite furnace atomic absorption (Yu et al., 1993). Care was taken in all the experiments reported here to exclude artifactual clustering by using low concentration, aqueous solutions, and spray conditions that provide steady ion currents. The syringe and electrospray needle were also kept at ground potential to avoid possible artifactual effects of a high electric field on the metal-exchange process. Oxidation can often be detected by changes in molecular weight. However, this is not always the case. Recent studies in our lab of copper-containing MT prepared on-line from both apo MT and Zn_7–MT showed a distribution at pH 7.5 of 8 to more than 20 copper ions per protein molecule, in sharp contrast with the ratio of 12:1 determined by several optical methods (Li & Weser, 1992; Green et al., 1994) at the same pH. One explanation is that oxidation has taken place of some cupric to cuprous ions, and/or some thiolate to disulfide bonds. One final limitation of the electrospray technique, at least in our apparatus, is that the signals for one set of charge states is often optimized at the expense of other charge states. Thus 9+ and 10+ ions of carbonic anhydrase could not be monitored optimally in the same experiment as 4+ metallothionein ions. With these concerns in mind, electrospray is recommended as a dynamic technique to monitor metal ion flux.

The spectra in Figure 1 are consistent with cooperative loss proposed by others (Nielson & Winge, 1983) of the three zinc ions in the β-domain of MT. This can be seen from the pattern of ion abundances in Figure 1B, which was produced after Zn_7–MT had been reacted with 1:10 EDTA for 5 min. In this spectrum, the ions corresponding to Zn_1–, Zn_4–, and Zn_7–MT are the most abundant.

The relation of the electrospray spectrum to the composition of the aqueous solution has been tested previously by comparison to atomic absorption measurements and variation of the pH (Yu et al., 1993). After 25 min of reaction (Fig. 1C), the ion corresponding to Zn_1–MT is clearly the most abundant. These results appear to reflect a two-step process. The faster step is the loss of three metals, leaving a Zn_4–MT. The fact that abundant intermediates corresponding to Zn_2– and Zn_3–MT are not detected suggests that the second slower process is a cooperative loss of three metal ions from the α-domain. Removal of the last Zn ion is very slow, a result consistent with observations made by others (Vallee, 1995). Peaks a and b in Figure 1B have masses consistent with assignment as Zn_4–MT–EDTA·Zn and Zn_7–MT–EDTA·Zn. Observation of putative EDTA complexes (Table 1) in the electrospray spectrum suggests a mechanistic intermediate for zinc ion transfer; however, artifactual formation in the electrospray process has not yet been ruled out.

Transfer of zinc from MT to other metalloproteins is likely to be part of the biological activity of MT. Figure 3 shows electrospray spectra produced from an on-line experiment monitoring the transfer of zinc from Zn_7–MT to apo-carbonic anhydrase. These spectra were used to generate a reaction time course. The experiment was repeated using CHB-modified Zn_7–MT, and the two time courses are compared in Figure 4. The results indicate that zinc ion is transferred more rapidly from CHB-modified Zn_7–MT than from unmodified Zn_7–MT. Thus, it appears that modification of a single cysteine (Zaia et al., 1996) in the MT structure (Cys48 or Cys33) increases the availability of zinc ion to other zinc metalloproteins. This perturbation-induced increase in zinc availability has implications with regard to the mechanism of acquired drug resistance. Specifically, the results show that alkylation at the level of even one adduct per MT molecule measurably increases the availability of zinc, thus producing a potential effect on the function of other zinc metalloproteins. The role proposed for the MT/thionein equilibrium in controlling its own transcription and synthesis (Czupryn et al., 1992; Maret, 1994; Vallee, 1995) has important implications for the mechanism of acquired drug resistance (Kelley et al., 1988; Yu et al., 1995; Zaia et al., 1996), because zinc made more biologically available by modification of MT may act to increase the rate of transcription of thionein. Increasing levels of thionein and MT would be expected to increase drug sequestration and inactivation.

The affinity of thionein for zinc has been postulated to be high enough that it can extract this metal from some other metalloproteins (Zeng et al., 1991a, 1991b; Cano-Gauci & Sarkar, 1996; Maret et al., 1997). In the present work this possibility has been studied using a zinc chelated insulin hexamer and a single-stranded DNA binding protein. Incubation of thionein with the Zn_2-insulin hexamer at a concentration too low to provide a full complement of zinc ions for thionein leads to MT species carrying zero to four metal ions, presumably filling the carboxy terminal domain first as has been demonstrated in other metal ion transfer reactions (Nielson & Winge, 1983). The high abundance of Zn_1–MT in Figure 1B is consistent with the stability of this species reported by others (Vallee, 1995). It should be noted that a role has recently been proposed for MT in maintaining glucose levels in blood and liver (Rofe et al., 1996). Finally, the idea that thionein can deactivate its own transcription by extracting zinc ions from proteins that bind DNA was tested here using another DNA binding protein, the bacteriophage T4 gene 32 protein core domain. The shift in mass in Figure 5C, relative to thionein (Fig. 5A), is consistent with chelation by thionein of zinc from the DNA binding protein. This reaction was carried out with a ratio of zinc equivalents of 7:1, thionein:DNA binding protein, and under these zinc-limited conditions the major thionein product is again found to be the monozinc chelate.

We note that the nucleic acid binding activities (Karpel, 1990), autotranslational control (Shamoo et al., 1991; Green et al., 1994), and resistance to proteolytic breakdown (Nielson & Winge, 1983; Giedroc et al., 1986) of gene 32 protein are all very dependent on the presence of the bound metal. The results presented herein raise the possibility that these activities might be regulated in vivo by metal transfer to or from metallothionein or analogous proteins.

Acknowledgments

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