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Stoichiometry in zinc ion transfer from metallothionein to zinc finger peptides

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Abstract

Electrospray and nanospray ionizations are used to study the transfer of zinc ions between Zn_7–metallothionein and apo peptides that are models for several kinds of zinc finger proteins. A membrane experiment is reported here which demonstrates that interprotein contact is required for these transfers. Analysis on a quadrupole ion trap has allowed all reactants and all products to be monitored simultaneously. Evidence is provided for the preferential transfer of a single zinc ion from each Zn_7–metallothionein complex. (Int J Mass Spectrom 204 (2001) 1–6) © 2001 Elsevier Science B.V.

Keywords: Metal ion flux; Reaction stoichiometry; Zinc finger peptides; Metallothionein; Noncovalent complexes

1. Introduction

Electrospray ionization has been used previously to monitor zinc ion flux from the zinc chaperone Zn_7–metallothionein to proteins and peptides deficient in zinc, in reactions carried out on-line [1–3]. In previous studies, only the apo-peptide reactant and resulting holo-peptide product were monitored. Because metallothionein (MT) carries seven zinc ions, questions remain about how many zinc ions are transferred from each protein and what the distribution of zinc is in the final product mixture. Zinc ions are retained in two clusters, as shown in Fig. 1, and are reported to be thermodynamically stable and kinetically labile [4].

The zinc recipients in this study are peptide models for the hundreds of zinc finger proteins that contain multiple zinc chelating centers [5,6]. Each peptide studied here contains one zinc ion complex.

2. Experimental

2.1. Materials

Rabbit liver MT-II was purchased from Sigma Chemical Co. (St Louis, MO). The consensus zinc finger peptides Cp (CCHH), Cp (CCHC), and Cp (CCCC) [7] were synthesized by the Biopolymers Laboratory, Johns Hopkins University, Baltimore, MD. The synthetic HIV-1 nucleocapsid protein [13–30] peptide [6] was a gift from Dr. Michael Summers, Howard Hughes Medical Institute, UMBC, Baltimore, MD. All structures were confirmed by electrospray ionization mass spectrometry (ESI-MS) (see the following sequences). Purity was confirmed by high-performance liquid chromatography (HPLC) and

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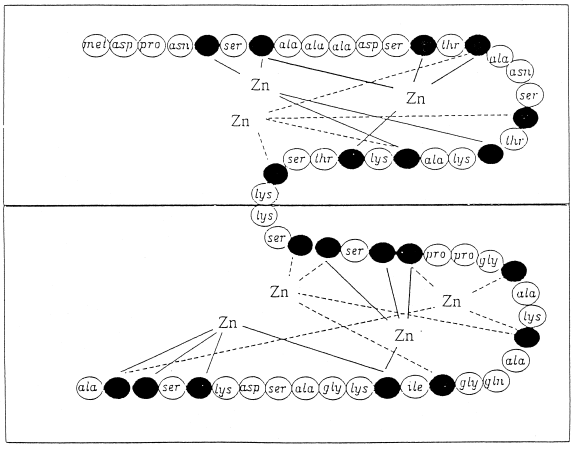

Fig. 1. Schematic representation of metallothionein showing three zinc ions in the N-terminal cluster (top) and four zinc ions in the C-terminal cluster (bottom). Solid circles are cysteine residues.

mass spectrometry. The sequences of these peptides are given, with the four zinc-chelating residues in bold type.

Cp (CCHH): PYK**C**PE**C**GKSFSQKSDLVK**H**QRT**H**TG

Cp (CCHC): PYK**C**PE**C**GKSFSQKSDLVK**H**QRT**C**TG

Cp (CCCC): PYK**C**PE**C**GKSFSQKSDLVK**C**QRT**C**TG

HIV-L Ncp7[13-30]:IK**C**FN**C**GKEG**H**IAKN**C**RA

2.2. Preparation of Zn_7–MT- II

Rabbit liver Zn–MT-II purchased from Sigma (St Louis, MO) exhibited a mixed metal species and some protein contaminants when analyzed by ESI-MS in 10 mM ammonium bicarbonate (NH_4HCO_3) pH 7.4. To prepare homogeneous Zn_7–MT, the Sigma sample (5 mg total sample) was dissolved in 2 mL of 50 mM HCl and the apo-MT was purified by HPLC using a semipreparative C18 column 250×10 mm, 10 μm particle size, 300 Å, (PrimesPhere, Phenomenex, Torrance, CA) connected to Shimadzu LC-600 pumps and a Shimadzu SDP-6A UV detector (Columbia, MD). After injection of the sample (500 μL), the metal ions were washed out by 0.1% trifluoroacetic acid (TFA) in water for 10 min at flow rate of 5 mL/min. The protein was eluted from the column with a gradient of acetonitrile containing 0.8% TFA developed from 10% to 30% in 20 min. Detection was monitored by UV at 215 nm and the peak corresponding to apo-MT was collected and assayed for purity by mass spectrometry, then freeze dried. Zn_7–MT was reconstituted according to the method previously described [7]. To avoid excess of free Zn ion in the Zn_7–MT solution, exactly seven equivalents of $ZnCl_2$ were added to the apo-MT in 50 mM HCl under argon with slow stirring. The solution mixture was then adjusted to pH 8 with 0.5 M tris base, added stepwise under argon with fast stirring. Unbound Zn^{2+} ions were removed when the reconstituted Zn_7–MT was dialyzed against degassed 10 mM ammonium bicarbonate (pH 7.4) using a centrifugal filtration device, molecular weight cutoff 5000 (Millipore, Bedford, MA). Protein concentration was determined by UV absorbance at 220 nm in 0.1 M HCl using a molar extinction coefficient of 48 200 M^{-1} cm^{-1} [8]. Aliquots with known concentrations of Zn_7–MT were prepared in 10 mM NH_4HCO_3, pH 7.4 and stored under argon at 4 °C until use. The metal ion content and stoichiometry of Zn_7–MT was confirmed by electrospray ionization mass spectrometry.

2.3. Reaction and ESI-MS analysis

The lyophilized apo-zinc finger peptide was mixed with a degassed solution of Zn_7–MT (see previous text) in a metal-free microfuge tube. Different Zn_7–MT-II/apo-zinc finger peptide molar ratios were prepared. After a 30 s reaction at room temperature an aliquot (10 μL) of each mixture was injected into an electrospray mass spectrometer. Spectra were recorded 2 min after mixing unless otherwise specified. Initial experiments were conducted using two sectors of a JEOL (Tokyo, Japan) HX110/HX110 four sector mass spectrometer. The analyte mixture was infused into the electrospray ion source in 10 mM NH_4HCO_3 solution, pH 7.4 delivered by a syringe pump at 1 μL/min. A stable sheath flow of methanol was sprayed coaxially at 1 μL/min to help maintain good spray conditions.

Measurements were also made on a Finnigan LCQ

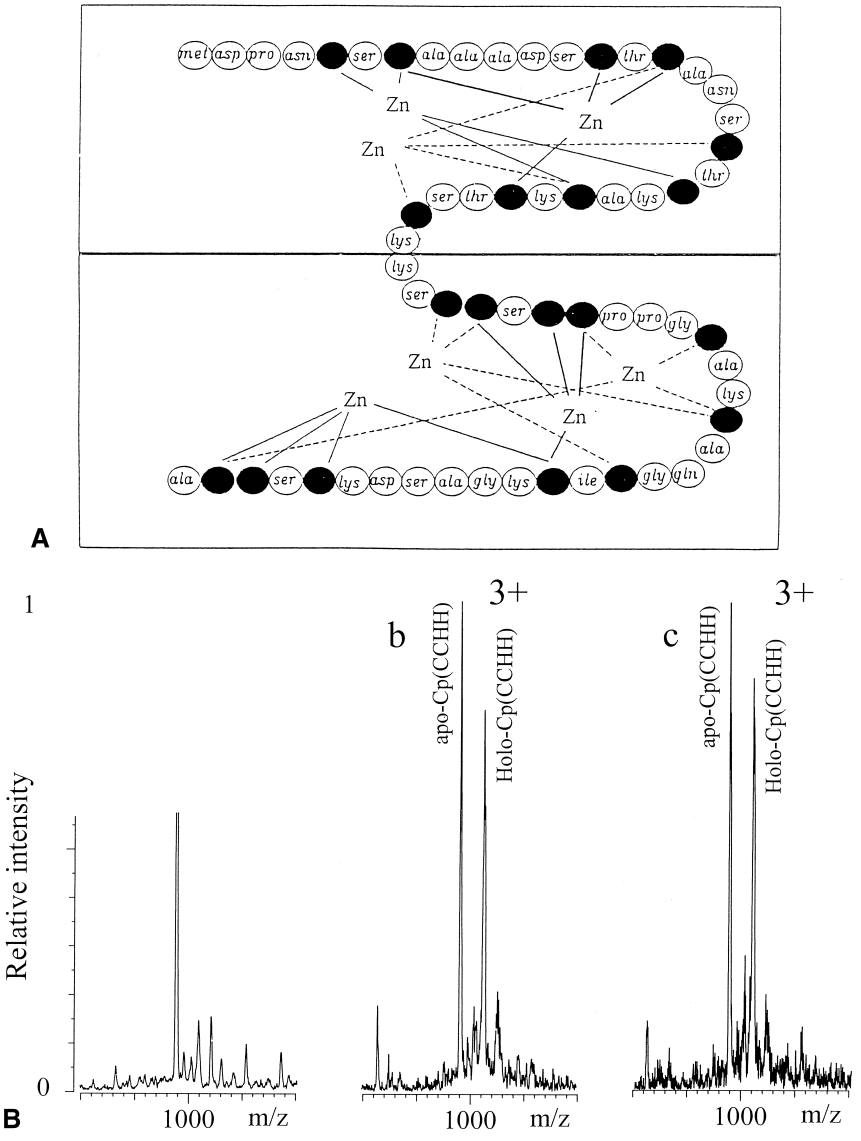

Fig. 2. ESI-MS of apo-Cp (CCHH) peptide: (a) before, (b) after 2 min interaction with Zn_7–MT, and (c) after 30 min interaction with Zn_7–MT. The molar ratio of apo-peptide/Zn_7–MT was 10:1. The reaction was carried out in 10 mM NH_4HCO_3, pH 7.4 and analyzed by ESI-MS. Spectra were acquired on the first two sectors of a JEOL HX110/HX110 mass spectrometer.

(Thermoquest, San Jose, CA) quadrupole ion trap mass spectrometer using the Finnigan nanospray inlet system. The reaction mixture in 10 mM NH_4HCO_3 solution, pH 7.4, was loaded into a disposable needle and sprayed toward the capillary orifice, which was heated at 120 °C. The voltage applied to the needle was 0.8 kV.

2.4. Dialysis chamber reaction

Three hundred microliters of 100 μM of apo-Cp (CCHH) in 10 mM NH_4HCO_3 containing 500 μM of 2-mercaptoethanol was placed in dialysis tubing (Spectra/Por Cellulose ester membrane MW cutoff 500 Da). The tube was then submerged in 50 mL of

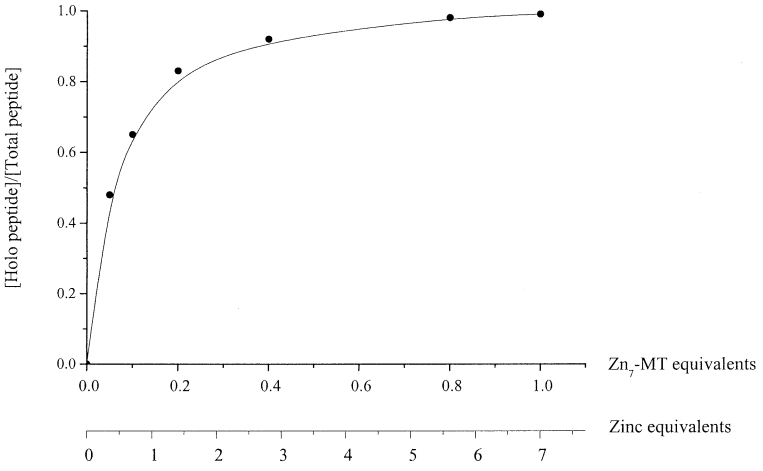

Fig. 3. Titration of 100 μM apo-Cp (CCHH) with different concentrations of Zn_7–MT. Reactions and electospray analysis were carried out as in Fig. 1. All charge values were summed. An uncertainty of $\pm$10% is estimated.

50 μM Zn_7–MT solution (10 mM NH_4HCO_3 containing 500 μM of 2-mercaptoethanol). After a 3 h reaction, the sample was dialyzed twice against 1 L solution of 10 mM NH_4HCO_3, pH 7.4 to remove the excess of 2-mercaptoethanol before ESI-MS analysis.

3. Results and discussion

It has been previously noted that zinc ions are efficiently transferred from holo-metallothionein (Zn_7–MT) to zinc finger systems [2,3]. Fig. 2 shows

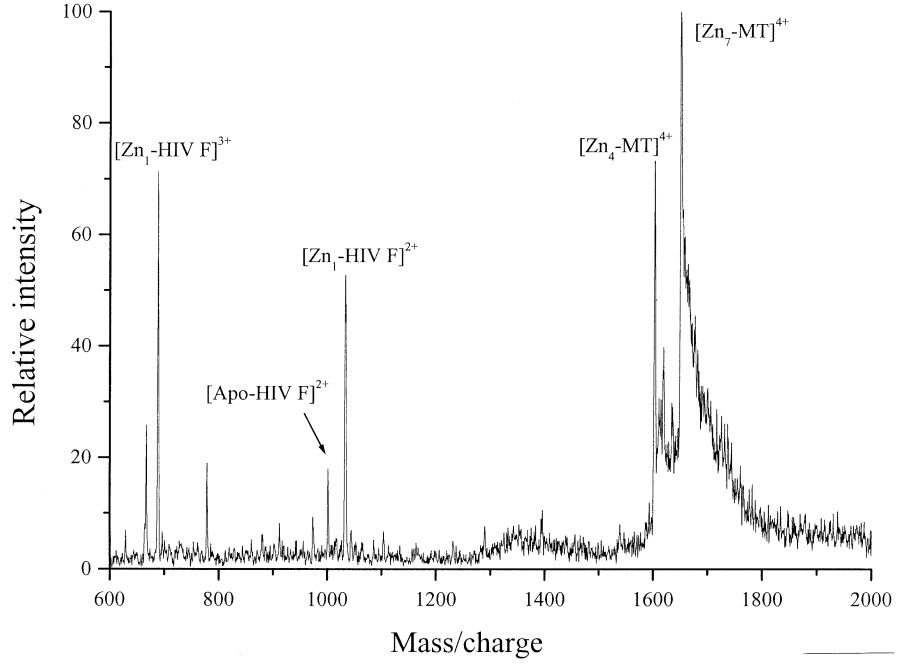

Fig. 4. ESI-MS of the products resulting from the interaction of apo-Ncp7 [13–30] peptide and Zn_7–MT at a molar rations of 10:9. The reaction was carried in 10 mM NH_4HCO_3, pH 7.4 and analyzed using nanospray on a Finnigan LCQ ion trap mass spectrometer.

the progress of zinc transfer at different times after Zn_7–MT is mixed with the apo-Cp (CCHH) finger peptide. An abundant signal for the holo-Cp (CCHH) appears in the electrospray mass spectrum of the reaction mixture after 2 min incubation [Fig. 2(b)], which is not detected in the control spectrum of the starting material [Fig. 2(a)]. Examination of the spectrum obtained after 30 min incubation [Fig. 2(c)] does not reveal any appreciable increase of holo-Cp (CCHH) over the 2 min level, suggesting that the exchange reaction was completed within a few moments after mixing. With the current instrumental setup it was not possible to start the analysis within less than 2 min after mixing the reactants, precluding kinetic measurements. Transfer was also complete in less that 2 min to the other peptides, Cp (CCHC), Cp (CCCC), and Ncp7 [13–30].

At first glance it is surprising that zinc ions are transferred so extensively. The affinity constants for the consensus peptides are all in the range of $Ka = 10^{11}\ M^{-1}$ [7] whereas the averages reported for apo-MT range from 10^{11} to $10^{12}\ M^{-1}$ [9,10]. All these values were measured as dissociation constants for zinc ions liberated into solution. In contrast, it has been proposed that the transfer of zinc ions between Zn_7–MT and other metalloproteins occurs in protein/protein complexes by way of ligand substitution [4]. This more complex mechanism allows for a series of intermediates in which the two proteins share a zinc ion, obscuring a direct comparison of *Ka* values. In addition, the dissociation constants reported for Zn_7–MT are based on the loss of multiple zinc ions.

To directly test the requirement for intermolecular contact during zinc ion transfer, an experiment was designed in which donor and recipient are separated by a dialysis membrane that allows Zn^{2+} to diffuse in both directions, but not peptide or protein. In this experiment, insignificant amount of holo-Cp (CCHH) was formed after incubation for 3 h (data not shown). This transfer occurred in less than 2 min when the apo-Cp (CCHH) and Zn_7–MT were mixed together. This is the first direct evidence for intermolecular contact for rapid transfer of metals from MT to the apo-peptides.

The extent of metal ion transfer to the apo-peptides was evaluated at different concentrations of Zn_7–MT. As illustrated for Cp (CCHH) in Fig. 3, the percentage of holo-peptide increases with the concentration of Zn_7–MT. Both zinc equivalents and Zn_7–MT equivalents are plotted on the abscissa, and it can be seen that complete reconstitution is achieved only as the amount of the Zn_7–MT protein approaches one equivalent. Similar results were also obtained for the Cp (CCCC), Cp (CCHC), and Ncp7 [13–30] apo-peptides. As for the case in Fig. 3, reconstitution is complete only when one equivalent of protein (seven total equivalents of zinc ions) is available. This suggests that each molecule of Zn_7–MT donates only one zinc ion at equimolar concentrations. A similar suggestion has been made for the transfer of zinc ions from Zn_7–MT to apo-sorbitol dehydrogenase [11]. At lower concentrations (Fig. 3) more than one metal ion is provided by each Zn_7–MT molecule.

The mass spectrum in Fig. 4 provides a snapshot of all reactant and all product ions in a reaction where about 80% of the Ncp7 [13–30] peptide is remetallated. Both apo and zinc ion containing peptide [13–30] are visible with 2^+ and 3^+ charge states. In a control reaction, analysis of fully reconstituted Zn-Ncp7 [13–30] showed only the metallated species. Four species of MT are observed, Zn_7–MT, Zn_6–MT, Zn_5–MT, and Zn_4–MT in proportions around 42:12:16:30, respectively. This is interpreted to show that after a single zinc ion is transferred from Zn_7–MT, zinc ions are rearranged within metallothionein to reconstitute Zn_7–MT and to produce Zn_5–MT and Zn_4–MT species. The latter is known to be a stable complex comprising an intact C-terminus zinc ion cluster and a denatured N-terminus cluster [12], and it has previously been characterized by electrospray ionization as a heavily populated intermediate when zinc ions are removed by EDTA [2] or by acid [13]. Based on earlier NMR studies [10], we postulate that the initial zinc ion transfers preferentially from the N-terminus domain of Zn_7–MT.

In conclusion it should be pointed out that mass spectrometry is uniquely able to provide analysis of stoichiometric populations throughout the course of metal transfer reactions. This capability is analogous to the power mass spectrometry brings to the analysis

of H/D exchange reactions, and is superior in every way except cost to the determination of metal ion protein ratios using atomic absorption, inductively coupled plasma analysis or spectrophotometric determination of released zinc ions.

Acknowledgements

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