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International Journal of Mass Spectrometry 204 (2001) 113–123

International Journal of Mass Spectrometry

Probing protein–metal ion interactions by electrospray ionization mass spectrometry: enolase and nucleocapsid protein

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Received 9 March 2000; accepted 7 May 2000

Abstract

Electrospray ionization mass spectrometry (ESIMS) was used to study the metal ion binding stoichiometry and interactions with the proteins enolase and nucleocapsid protein (NCp7). Yeast enolase is a 93-kDa homodimeric enzyme that requires divalent metal ion binding for activity. The ESIMS studies of yeast enolase show the noncovalently bound dimer as the most abundant species for the apo and holo forms of the protein. However, the Mg^{2+}- and Mn^{2+}-bound enolase dimeric enzymes exhibit enhanced stability relative to their apo- and sodium-bound counterparts. Only a small proportion of the Mg^{2+}/Mn^{2+}-bound enolase dimer dissociates to the monomer state upon collisionally activated dissociation in the atmospheric pressure/vacuum interface of the ESI mass spectrometer, whereas the majority of the enolase dimer in the apo- and sodium-bound forms can be induced to dissociate to the monomer. This characteristic of the gas phase complex is consistent with its solution phase behavior. NCp7 contains two zinc finger structures. Limited proteolysis with trypsin in conjunction with ESIMS monitoring was used to determine the binding site of an initial zinc ion exposed to NCp7. The N-terminal zinc finger was found to be the primary binding site of the first zinc ion. Similar limited proteolysis experiments on the binding of NCp7–Zn_2 with the pentanucleotide d(ACGCC) suggest the participation of both zinc finger structures. This is consistent with previous solution phase binding studies using nuclear magnetic resonance and fluorescence spectroscopy. (Int J Mass Spectrom 204 (2001) 113–123) © 2001 Elsevier Science B.V.

Keywords: Electrospray ionization; Metal binding; Enolase; Nucleocapsid protein; Proteolysis

1. Introduction

The development of bioanalytical mass spectrometry (MS), and, in particular, electrospray ionization (ESI) [1], has facilitated the study of metal ion binding to large macromolecules. Metal ions can serve as structural and/or regulatory elements for protein function. Yet the application of mass spectrometry for the determination of metal ion interactions was a difficult task for larger molecular weight proteins prior to the advent of ESI. Other commonly employed desorption/ionization methods, such as fast atom bombardment (FAB) [2] and plasma desorption (PD) [3], are able to produce gas phase molecules for 20–30 kDa proteins. However, the resolving power, sensitivity, and mass measurement accuracy of the overall mass spectrometry technique is insufficient to discern the additional mass increase contributed from metal ion binding unambiguously. The combination of the multiple charging phenomenon of ESI that produces low mass-to-charge (*m/z*) ions with efficient

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PII S1387-3806(00)00334-1

medium-to-high resolving analyzers such as quadrupole, magnetic sectors, time-of-flight, and trapping instruments (e.g. quadrupole ion trap and Fourier transform ion cyclotron resonance) allows metal ion binding to large proteins to be detected.

ESI mass spectrometry (ESIMS) has demonstrated potential for the study of noncovalent interactions [4], such as metal ion binding [5–8]. A variety of metalloproteins have been studied using ESIMS methods, such as calcium-binding EF-hand proteins, zinc finger proteins, iron–sulfur cluster containing proteins, and many others. A short list of examples is tabulated in a recent review [4]. The ESI process is considered to be a gentle process that does not disrupt weaker noncovalent bonds. These weaker interactions that are characteristic of many important biological processes in the solution phase are transferred to the gas phase, where they are analyzed by mass spectrometry. The molecular weight measurement by mass spectrometry provides a direct determination of metal ion binding stoichiometry. The mass of the apo protein can be determined by measurement from denaturing solution conditions and/or calculation from its amino acid sequence. The mass increase observed in the ESIMS measurement from neutral pH solutions yields the stoichiometry and, in cases where the metal species is unknown, the identity of the metal ion(s). This is a feature that is difficult to obtain by any other bioanalytical method with such enhanced sensitivity.

Elements of the noncovalent interaction in the solution phase are raised to the gas phase state. However, it is unclear how similar the structures and interactions are between the two phases. In some examples, peptide/protein structures can be relatively similar, as suggested by the collisionally activated dissociation (CAD) study of a cyclic peptide by Loo et al. [9]. On the other hand, several reports have suggested that the energetics of the dehydrated state of the gas phase complex may be different [4,10]. Electrostatic interactions are strengthened in the gas phase relative to their solution phase behavior [4,10,11].

In this article, two examples of metal ion binding to proteins studied by ESIMS are presented. Not only was ESIMS used to determine the stoichiometry of the interaction, but additional information about the nature of the metal ion–protein binding was provided also. Enolase is a homodimeric metalloenzyme [12]. The interaction of the protein subunits is enhanced by metal ion binding in solution and in the gas phase. Nucleocapsid protein NCp7 is a small zinc finger protein present as part of the human immunodeficiency virus (HIV) [13,14]. NCp7 is composed of two zinc finger structures. ESIMS was used to determine which of the zinc fingers is used to bind to the initial zinc ion and to study the NCp7–Zn_2 interaction with a small oligonucleotide.

2. Experimental

2.1. Materials and sample preparation

Yeast enolase was obtained from Sigma Chemical (St. Louis, MO, USA). NCp7 was synthesized and purified as previously described [15]. Oligonucleotide d(ACGCC) was synthesized by Genosys Biotechnologies (The Woodlands, TX, USA). TPCK-modified sequencing grade trypsin was obtained from Promega (Madison, WI, USA).

To remove extraneous buffer salts, the enolase protein sample was subjected to centrifugal ultrafiltration cleanup with 10 000 Da cutoff centrifugal filtration cartridges (Amicon Microcon YM-10, Millipore, Bedford, MA) and washing with 10 mM ammonium acetate. For metal binding studies, metal salts (magnesium acetate, manganese acetate, and sodium acetate) were added to the enolase solutions. Excess metal ions were removed prior to ESIMS analysis by centrifugal ultrafiltration.

Trypsin digestion of complexes NCp7-Zn_1, NCp7-Zn_2, and NCp7-Zn_2-d(ACGCC) was accomplished by adding trypsin to the protein solution at a ratio of 10:1 (w/w) in 10 mM ammonium acetate, pH 6.9.

2.2. Mass spectrometry

ESIMS and tandem mass spectrometry (MS/MS) were performed with a double focusing hybrid mass spectrometer (EB-ion trap geometry, Finnigan MAT 900 Trap, Bremen, Germany) with a mass-to-charge

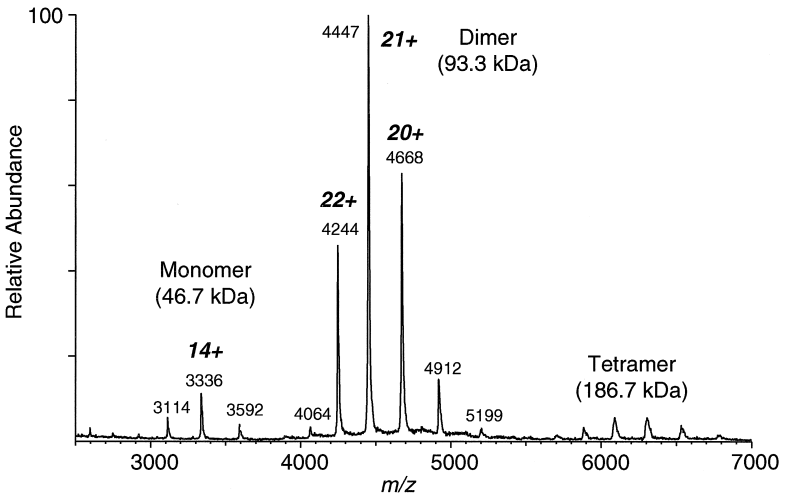

Fig. 1. ESI mass spectrum of the apo form of yeast enolase (5 μM) from a pH 6.9, 10 mM ammonium acetate solution (ΔV_{TS} = +100 V).

(*m/z*) range of 10 000 at 5 kV full acceleration potential and a PATRIC array detector [16]. The ESI interface consists of a heated metal capillary inlet with a low flow micro-ESI source [17]. Control of the analyte flow rate at 100 nL min^{-1} was accomplished by pneumatic pressure. The metal capillary temperature was maintained around 200 °C for noncovalent complex studies.

3. Results and discussion

3.1. ESIMS of yeast enolase

Enolase is a metalloenzyme involved in the glycolytic pathway, catalyzing the dehydration of 2-phospho-D-glycerate. The yeast enzyme is a 93 kDa homodimeric complex (monomer molecular mass 46.7 kDa) and requires divalent metal ions, such as Mg^{2+} and first row divalent transition metals (e.g. Mn^{2+}) for activity [12]. The ESI mass spectrum of yeast enolase in its apo form at pH 6.9 shows the dimer protein complex as the most abundant species (Fig. 1). In the presence of metal ions, ESIMS measurements show binding of a single high affinity divalent metal ion, such as Mg^{2+} and Mn^{2+}, per monomer (i.e., two per dimer, Fig. 2).

In solution at near neutral pH in the presence of excess Mg^{2+}, the equilibrium dissociation constant (K_D) for dimerization is estimated to be 15 nM [18]. In the presence of excess EDTA, K_D is 2 μM [19]. Kornblatt et al. [20] postulate that Mg^{2+}/Mn^{2+} binding is necessary for maintaining subunit interactions, and hence, activity. Enolase monomers are considered to be inactive.

The stabilization of the gas phase enolase dimer

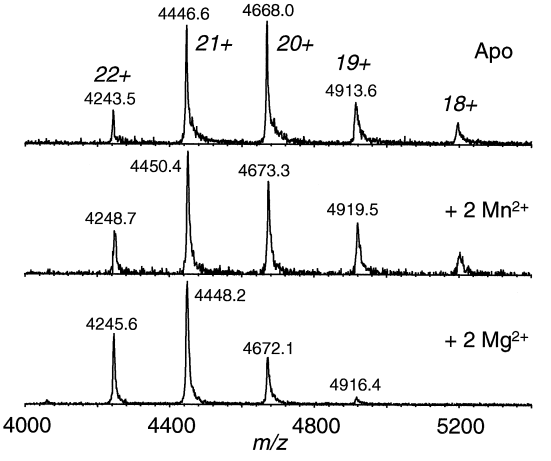

Fig. 2. Partial ESI mass spectra of yeast enolase dimer for the apo- (top, M_r 93 341.9, measured 93 342.8 ± 8.6), manganese- (middle, M_r 93 451.8, measured 93 445.9 ± 5.4), and magnesium-bound (bottom, M_r 93 390.6, measured 93 396.6 ± 15.2) forms. The data show the binding of two metal ions per dimer complex (5 μM in 10 mM ammonium acetate, pH 6.9, ΔV_{TS} = +100 V).

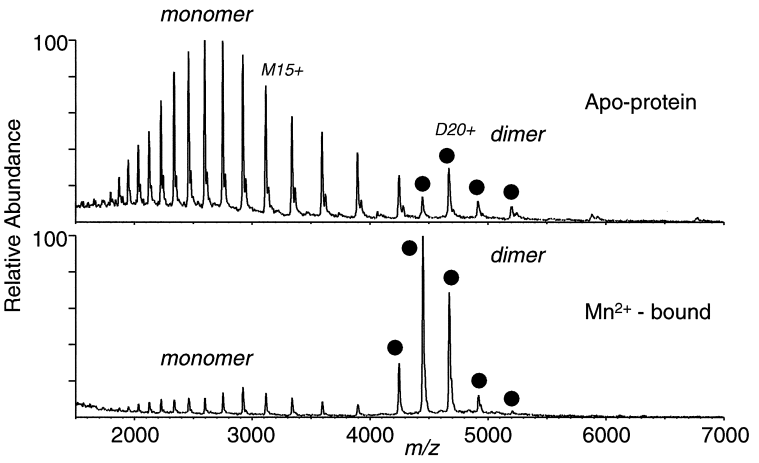

Fig. 3. ESI mass spectra of the apo-form (top) and Mn^{2+}-bound yeast enolase acquired at ΔV_{TS} of +200 V (5 μM in 10 mM ammonium acetate, pH 6.9). The filled circles denote ions representing the dimer. Compared to the mass spectra acquired at a ΔV_{TS} of +100 V (see Fig. 1), a greater proportion of the apo form of the homodimer dissociates to the monomer state relative to the Mn^{2+}-bound species.

complex is enhanced for the divalent metal-bound species as well, as demonstrated by the ESIMS measurements. By increasing the voltage difference between the tube lens and skimmer lens elements (ΔV_{TS}) of the ESI atmospheric pressure/vacuum interface, the energy of the collisions with background neutral molecules can be increased. CAD can be accomplished by this "in-source CAD" or "nozzle-skimmer dissociation" method [21,22], which results in dissociation of covalent polypeptide backbone bonds and yields sequence informative product ions for large biomolecules. However, dissociation of noncovalent bonds can be effected in this region as well. This is quite effective for stripping off water molecules from the complex to produce the dehydrated molecule. For yeast apo-enolase dimer molecules, dissociation of the noncovalent dimer to the monomer form is produced by increasing ΔV_{TS} from +100 to +200 V. At ΔV_{TS} of +200 V, most of the dimer complex has dissociated to its monomeric form for the apo-enolase protein (compare Fig. 1 with Fig. 3, top). However, for the Mn-bound enolase, most of the dimer survives intact at the elevated ΔV_{TS} ESI interface voltage (Fig. 3, bottom). This behavior is summarized in the chart in Fig. 4. Between 60% and 65% of the dimer complex survive the high collision energy conditions (+200 V relative to the +100 V conditions) for the magnesium- and manganese-bound enolase. For the apo and sodiated forms, less than 11% of the dimer species observed in the mass spectra remains intact when ΔV_{TS} is increased to +200 V.

The enhanced stability of the gas phase divalent metal-bound complexes is consistent with the solution phase structure of enolase, as binding Mn^{2+} or Mg^{2+} is necessary for maintaining subunit interactions. There are differences in conformation between Mn^{2+}/Mg^{2+}-enolase and apo-enolase that affect regions of the protein involved in subunit interactions [20]. It has been postulated that the divalent metal binding sites are near loop structures and amino acid residues

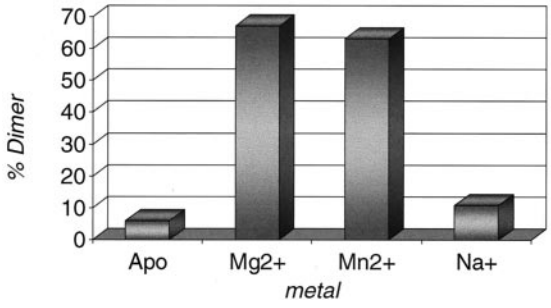

Fig. 4. Plot of relative abundance of the enolase dimer molecule at ΔV_{TS} of +200 V (5 μM in 10 mM ammonium acetate, pH 6.9) as a function of metal binding.

involved in subunit contacts. Therefore, "the loss of the metal ion disrupts this framework, resulting in either greater mobility or altered conformations for several of the loops. This in turn weakens subunit contacts" [20] in solution, and apparently, allows the gas phase protein dimer complex to dissociate to the monomer state also.

3.2. Zinc and oligonucleotide binding to nucleocapsid protein

Zinc finger domains have structural and functional roles in a number of transcription factors and are involved in the recognition of DNA [23]. The HIV-1 NCp7 protein has two CCHC zinc finger domains and is involved in the HIV life cycle through interactions with single stranded nucleic acids and viral proteins. Surovoy et al. first applied ESIMS to the study of metal ion binding to NCp7 [24]. Subsequently, mass spectrometry has been used to study the binding of zinc [15,25] and RNA [17] to NCp7. The two zinc fingers in the HIV-1 NCp7 are not functionally equivalent, as the N-terminal finger has higher affinity toward zinc [14,24]. Using peptide fragments comprising the individual zinc finger structures, Surovoy performed zinc titration experiments to demonstrate a higher zinc affinity by the N-terminal zinc finger [24]. Similarly, we used ESIMS and competitive binding experiments to demonstrate the higher affinity towards binding zinc for the N-terminal peptide [17]. Another possible experimental approach to demonstrate the differential affinity of zinc binding is through the incorporation of limited proteolysis.

By using limited, or incomplete, enzymatic digestion of protein complexes, one can obtain important information on the sites of interactions. For example, Cohen and co-workers used limited proteolysis and matrix-assisted laser desorption ionization mass measurements to gain information on the DNA-binding domain of the DNA-binding protein, Max [26]. Upon DNA binding, the interaction regions of the protein are protected from proteolysis, relative to the unbound-protein state. Mass spectrometry measurements of the proteolysis products with and without bound DNA provide information on the binding sites. A similar "footprinting" strategy can be employed to study metal binding of NCp7 by ESIMS monitoring of the limited trypsinolysis as a function of time. However, an advantage of using ESI is that the noncovalent interactions can be maintained and measured during the course of the experiment.

The ESI mass spectrum of NCp7 with one mole equivalent of zinc added is shown in Fig. 5. The relative abundances of the ions representing the apo form (M_r 6369.5) and the single zinc-binding form (M_r 6432.8) are both approximately 50% (with only a very small contribution from the two zinc-bound species also present in the spectrum). Adding two or greater mole equivalents of zinc to NCp7 yields the Zn_2-bound molecule as the only species observed [15,25]. To help address the question on which zinc finger is participating in the binding of the single zinc ion, trypsin was added to the solution composed of NCp7 with one mole equivalent of zinc (see Fig. 5) and ESIMS was used not only to monitor the degradation of the protein over the time course of the experiment, but also to determine which fragments maintain the interaction with zinc. A mass spectrum acquired from the 6.9 pH solution after 25 min of proteolysis is shown in Fig. 6. A portion of the solution was removed and diluted with an acidic acetonitrile solution to denature the protein and to induce NCp7 to release bound zinc (Fig. 6, bottom inset). Between *m/z* 700–900, three sets of ions are observed for each zinc finger region. However, only the ions representing the N-terminal zinc finger are observed to bind to zinc. This is illustrated by the comparison of mass spectra acquired at pH 6.9 and pH 3. The *m/z* positions of the ions for the C-terminal finger are the same for both pH values, suggesting that the C-terminal zinc finger in not involved in the binding of the first zinc ion. The ions for the fragments representing the N-terminal zinc finger, however, are shown to decrease in mass according to a single zinc ion upon denaturation from exposure to acid pH. (The presence of the singly charged ion at *m/z* 842.8 in the pH 3 spectrum of Fig. 6 may be due to an impurity. It does not match a peptide fragment of NCp7.) This suggests and confirms a stepwise mechanism for zinc binding to NCp7. Full occupation

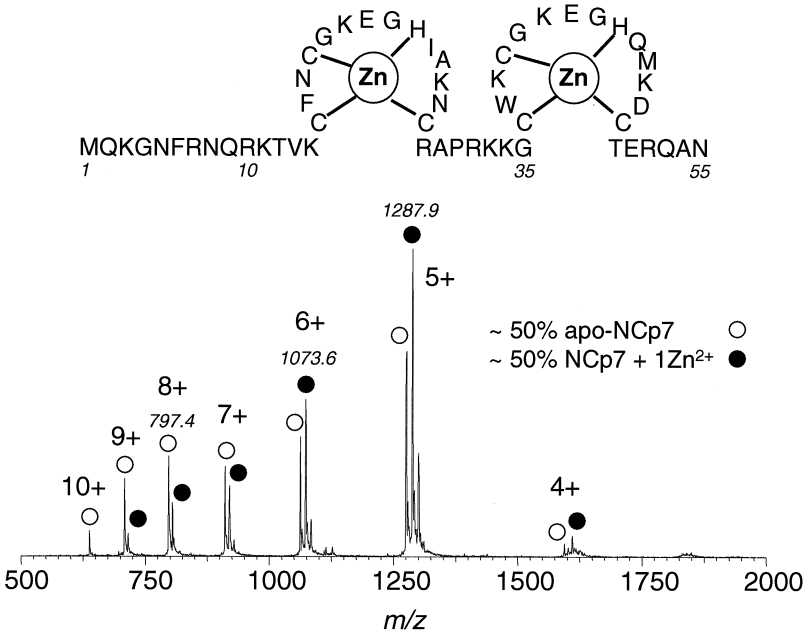

Fig. 5. ESI mass spectrum of NCp7 (1 μM) with 1 μM zinc acetate in 10 mM ammonium acetate, pH 6.9 solution. The open circles represent ions for the apo form (M_r 6369.5, measured 6369.8 $\pm$ 0.8) and the filled circles represent the ions binding 1 mol of Zn^{2+} (M_r 6432.8, measured 6433.3 $\pm$ 1.0).

of the N-terminal zinc finger occurs prior to zinc occupation of the C-terminal site. Long time exposure of trypsin to the zinc-bound NCp7 results in the complete proteolysis of the protein and disruption of metal binding. By using ESIMS to monitor the proteolysis events over the time course of the experiment, confirmation of metal binding and information on the region of the protein involved in the interaction is provided.

It should be noted that it is not possible to distinguish between fragments (residues) 11-32 and 12-33, as well as between fragments 11–33 and 12-34 by a mass measurement alone because they have equal molecular weights. However, MS/MS allows one to differentiate between the amino acid sequences. Fig. 7 shows the tandem mass spectrum acquired with the hybrid sector-ion trap system of the 3+-charged *m/z* 821 ion (see spectrum in bottom inset of Fig. 6). The data supports the assignment of the *m/z* 821 ion as fragment 11-32. Similarly, the ion at *m/z* 864 is assigned as fragment 11–33 (data not shown).

Similar limited proteolysis experiments can be carried out to study the interaction of NCp7–Zn_2 with oligonucleotides. The binding characteristics of zinc-bound nucleocapsid protein and single-stranded oligonucleotides have been studied by NMR and fluorescence spectroscopy using the pentanucleotide d(ACGCC) [27,28]. Preliminary ESIMS data on the binding of d(ACGCC) (M_r 1448.0) and NCp7–Zn_2 (M_r 6496.2) are shown in Figs. 8 and 9. The ESI mass spectrum of NCp7–Zn_2 with 1.5-fold excess of d(ACGCC) shows the 1:1 NCp7–Zn_2:d(ACGCC) noncovalent complex as the most abundant gas phase species (Fig. 8). Upon addition of trypsin, only protein regions not involved in the interaction with the single-stranded oligonucleotide are cleaved. The partial mass spectra (*m/z* 1200–1500) acquired after 15 min of trypsinolysis are shown in Fig. 9. The only cleavage products observed originate from the polypeptide tail (C-terminal of amino acid residues Arg-7, Arg-10, and Lys-11, Fig. 9) which is N-terminal from both zinc finger structures. Addition of acidified acetonitrile to the solution releases the oligonucleotide and both zinc ions from NCp7 (Fig. 9, bottom).

As a control experiment, the limited trypsin diges-

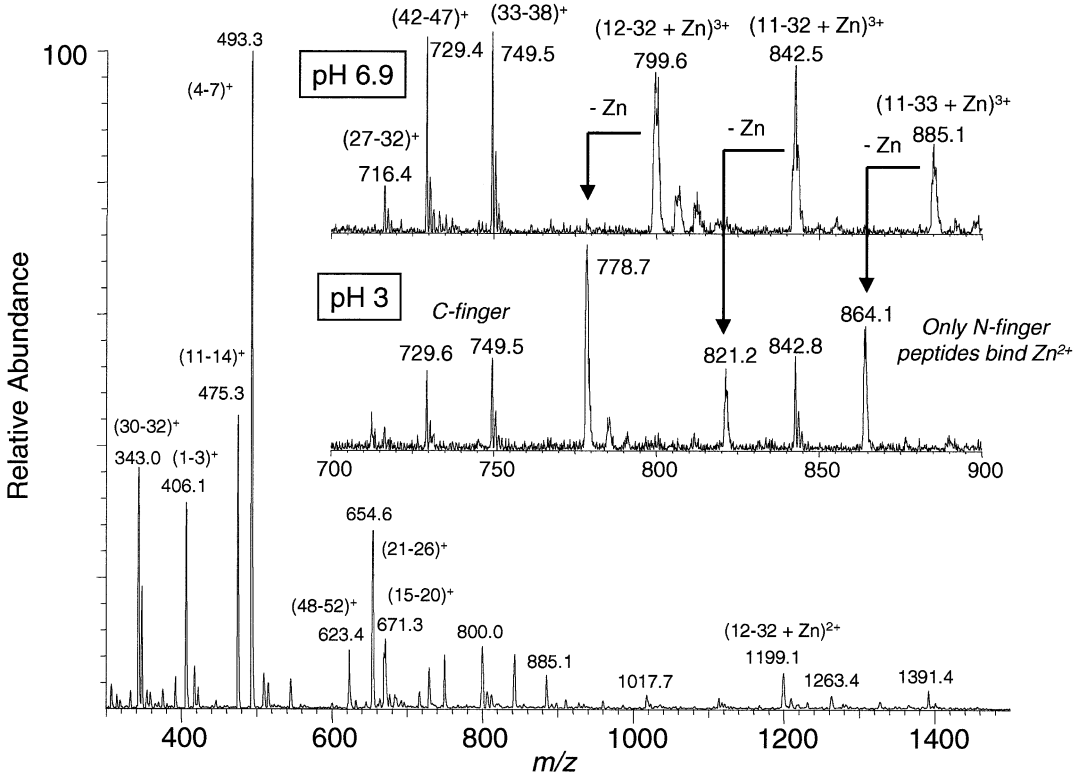

Fig. 6. ESI mass spectra of NCp7 (1 μM) with 1 μM zinc acetate in 10 mM ammonium acetate, pH 6.9 solution after 25 min of trypsinolysis. An expansion of the *m/z* 700–900 region obtained from the pH 6.9 solution is shown in the top inset spectrum. The bottom spectrum in the inset results from dilution of a portion of the solution with acidified (2.5% acetic acid) acetonitrile to 50% (*v*/*v*) to release bound zinc from the peptides. The notation above the peaks denotes the amino acid positions composing the peptide sequence of the fragments (see Fig. 5 for the sequence of NCp7). The 3+-charged ions at *m/z* 799.6, 842.5, and 885.1 represent peptides originating from the N-terminal zinc finger that are shown to bind zinc at pH 6.9, whereas the 1+-charged peptides from the C-terminal zinc finger do not bind zinc.

tion of NCp7–Zn_2 was performed. The results of the experiment are illustrated in Fig. 10. Cleavage occurs between the two zinc fingers (e.g. fragments 11-32 and 33-52). The ions at *m/z* 1263.2 and 1365.7, representing $(11\text{-}32+Zn)^{2+}$ and $(33\text{-}55+Zn)^{2+}$, respectively, observed for the trypsinolysis of NCp7–Zn_2 (Fig. 10) are not observed for the case of NCp7–Zn_2-d(ACGCC) (Fig. 9). Binding of the oligonucleotide protects the region between the zinc fingers from cleavage by trypsin.

The mass spectral data suggests that both zinc finger structures participate in the binding of d(ACGCC). This is consistent with the recent NMR and fluorescence studies, in which residues Phe-16 of the N-terminal zinc finger and Trp-37 of the C-terminal zinc finger were shown to contribute strongly to the binding interaction between d(ACGCC) and NCp7–Zn_2 [27,28]. Although very limited site-specific information on this oligonucleotide–metalloprotein interaction was gathered with the proteolysis-mass spectrometry experiment, the overall conclusions are consistent with previous solution phase structural studies: both zinc finger structures appear to be involved in the binding of d(ACGCC). Additional experiments with other specific proteolytic enzymes (with both protein- and DNA-cutting enzymes) may help further elucidate more precise contact points of the macromolecular complex.

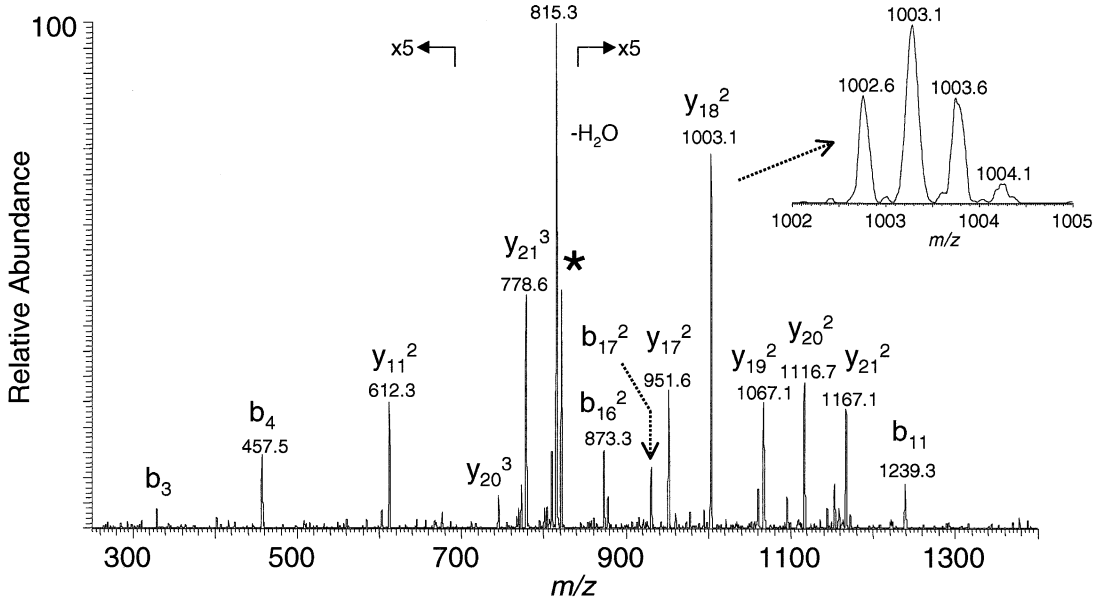

Fig. 7. MS/MS spectrum of the 3+-charged *m/z* 821.2 ion shown in the bottom inset spectrum in Fig. 6 (pH 3). The tandem mass spectrum was obtained with the sector-ion trap hybrid instrument. The inset shows a narrow region "zoom-scan" spectrum of the y_{18} product ion at *m/z* 1003 and confirms its 2+-charge state. The MS/MS spectrum confirms the assignment of the *m/z* 821.2 tryptic peptide ion as fragment 11-32, rather than identical molecular mass 12-33 fragment. (The asterisk denotes the position of the precursor ion.)

4. Conclusions

The application of bioanalytical mass spectrometry for studying metal complexation, and in particular, with proteins, will be a rich and fertile ground for future research. Prior to the development of electrospray ionization, there was not a "biochemistry-friendly" method to use the mass measurement accuracy of mass spectrometry for determining metal speciation and metal binding stoichiometry for large macromol-

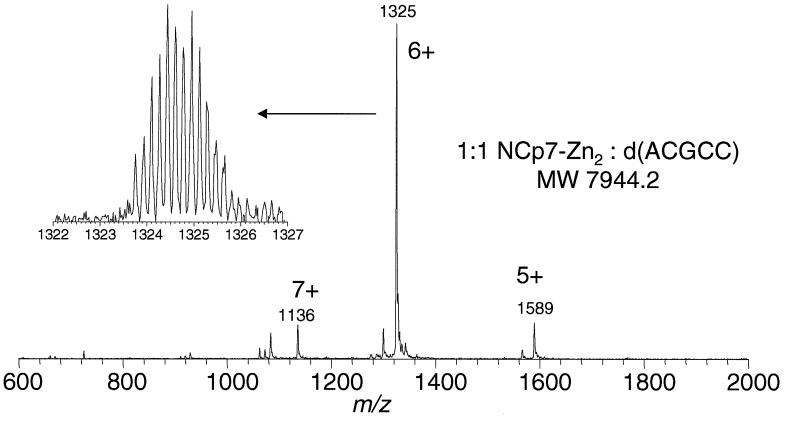

Fig. 8. Electrospray ionization mass spectrum of NCp7–Zn_2 with 1.5-fold excess of oligonucleotide d(ACGCC) in 10 mM ammonium acetate, pH 6.9. The predominant species observed in the spectrum are the 5+–7+ charged molecules of the 1:1 NCp7–Zn_2:d(ACGCC) noncovalent complex (M_r 7944.2, measured 7944.3 ± 1.0). The inset is a partial spectrum obtained at higher resolving power (16 000 FWHM), showing isotopic resolution for the 6+-charged molecule.

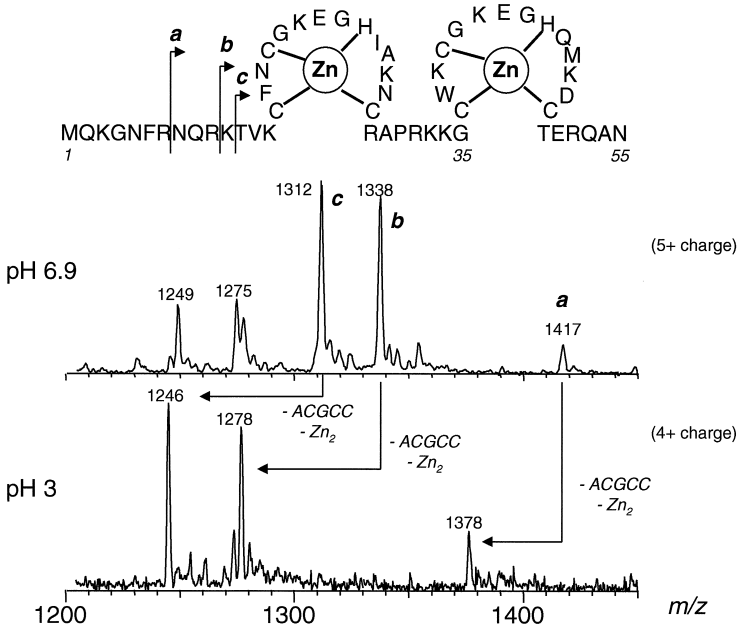

Fig. 9. Partial ESI mass spectra of NCp7–Zn_2 (1 μM) with 1.5-fold excess of d(ACGCC) in 10 mM ammonium acetate, pH 6.9 solution after 15 min of trypsinolysis. The bottom spectrum results from dilution of a portion of the solution with acidified (acetic acid) acetonitrile to 50% (*v*/*v*) to release bound zinc and oligonucleotide from the peptides. The "a, b, c" notation used in the top spectrum denote the trypsin cleavage products observed (e.g. peak c at *m*/*z* 1312 represents the tryptic peptide Thr12–Asn55 bound to $2Zn^{2+}$ and d(ACGCC) and carrying 5+ charges; see sequence at the top).

ecules. Aside from the limited molecular weight range of previous techniques, sensitivity issues also arose that limited the scope of such studies. Isolating and purifying several milligrams of protein material for a single measurement was not uncommon. However, ESIMS has allowed researchers to answer the questions, "What metal does the protein bind?" and "How many metal ions does my protein bind?" in a more sensitive and rapid fashion than other biophysical methods currently available.

However, determining the metal binding stoichiometry of a protein, although a vital piece of information for the researcher, is not typically the ultimate question posed. The more important issue in biomedical research is "What is the protein's function?" which advances in mass spectrometry may help address. Studying the gas phase protein complex may provide an important avenue for learning more about protein structure. Elements of protein structure appear to be preserved upon dehydration from the solution phase to the gaseous state. The apparent enhanced stability of the enolase dimer upon binding divalent metal ions in the solution phase [20] is transferred to its gas phase counterpart. These types of illustrations suggest the utility of mass spectrometry for structure/function studies.

Determining the binding site of a protein by mass spectrometry can be useful to structural biologists, as it provides an alternative approach to other more time and labor intensive studies using NMR and x-ray crystallography. In a drug discovery setting, rapid and sensitive mass spectrometry tools can offer the necessary information that can pre-screen protein target samples prior to more precise structural work. The limited proteolysis method described for the binding of zinc and oligonucleotides to NCp7 combines the ability of ESI to maintain noncovalent binding with the mass measurement of mass spectrometry to provide information on stoichiometry and binding site location. The protein sample is properly folded and

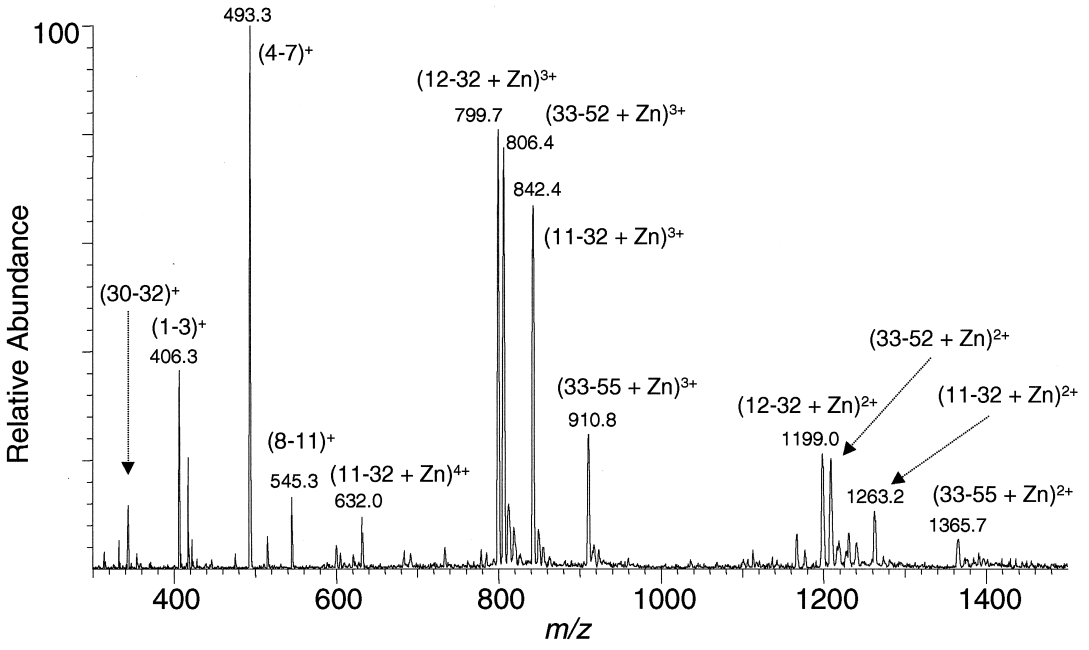

Fig. 10. ESI mass spectrum of NCp7–Zn_2 (1 μM) in 10 mM ammonium acetate, pH 6.9 solution after 15 min of trypsinolysis.

exhibits expected binding characteristics necessary for further structural work. "Bioinorganic chemistry is a leading discipline at the interface of chemistry and biology [23]." The field serves to address issues such as "which metal ions are used in living organisms, why nature might have chosen them, ... how metals bind to biopolymers, how metal binding can fold biopolymers, leading to function. . . [23]." It is clear from the many examples in the literature [4] that electrospray ionization mass spectrometry offers an important tool to answer key questions in chemistry and biology.

Acknowledgements

The author wishes to thank Dr. David Mack, Dr. Michael Reily, and Dr. Tod Holler for the supply of nucleocapsid protein.

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