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Nucleocapsid protein of HIV-1 and its Zn^{2+} complex formation analysis with electrospray mass spectrometry

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The Zn^{2+} binding properties of the synthetic nucleocapsid protein (Ncp7) of HIV-1, containing two zinc-binding domains, have been studied using electrospray mass spectrometry (ES-MS). ES-MS measurements revealed strong binding of Zn^{2+} by Ncp7. Its shorter fragments, Ncp7-(1–35)- and (29–55)-peptides, each containing only one zinc-binding domain, bind one equivalent of Zn^{2+} ions tightly. ES-MS studies allows these fragments to be distinguished in terms of their binding affinity: they showed stronger binding of Zn^{2+} by Ncp7-(1–35)-peptide. Surprisingly, in addition to the expected two zinc-binding domains, a third metal binding site was detected in Ncp7. However, this site appears to bind different metal ions without selectivity and most probably reflects salt formation at the C-terminal acidic residues.

Zinc finger; HIV nucleocapsid protein; Electrospray mass spectrometry; Metal ion titration; Metalloprotein

1. INTRODUCTION

The nucleocapsid protein (Ncp7) of HIV-1 is a 55 residue polypeptide containing two copies of the sequence motif Cys-X_2-Cys-X_4-His-X_4-Cys. This motif resembles zinc fingers found in various nucleic acid-binding proteins and has been proposed to function physiologically as a zinc binding domain [1]. Recently, it has been shown that bound zinc co-purifies with HIV-1 and HTLV-I virus particles in molar amounts in excess of their Nc proteins [2], and that Ncp7 samples isolated from HIV-1 particles bind two equivalents of zinc tightly and stoichiometrically [3]. Furthermore, it has been proven that Zn^{2+} coordination induces the formation of folded domains that are structurally similar to the structures observed for synthetic peptides with HIV-1 Ncp zinc finger sequences [3,4]. Using circular dichroism we showed that chemically synthesized Ncp7 also binds two equivalents of Zn^{2+} ions [5,6].

Until recently, an exact mass determination of dissolved samples of protein was not possible. Electrospray mass spectrometry (ES-MS) has made it possible to ionize and detect high molecular weight peptides [7,8]. We applied this method for mass determination of Zn^{2+} complexes with chemically synthesized Ncp7 and its fragments (Fig. 1), and for other metalloproteins.

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Abbreviations: Ncp, nucleocapsid protein; ES-MS, electrospray mass spectrometry; HPLC, high performance liquid chromatography; NH_4OAc, ammoniumacetate.

2. MATERIALS AND METHODS

The 55-residue NCp7, comprising two zinc binding domains, was prepared by Fmoc-based solid-phase peptide synthesis using the segment condensation approach. Its N- and C-terminal fragments, each harbouring one zinc binding domain, were synthesized using step-by-step synthesis. The protected peptide resins were cleaved using reagent S (3% *m*-cresol, 3% DMS, 3% EDT, 91% TFA for 2 h at 20°C) as outlined elsewhere in more detail [5,9]. The peptides were precipitated and washed with diethylether, lyophilized and purified by HPLC on a Nucleosil C_{18} reverse-phase column (10 × 250 mm) using an acetonitrile/water/0.1% trifluoroacetic acid gradient. Their homogeneity was confirmed by amino acid analysis, analytical HPLC and electrospray MS; purity was > 95% (HPLC) [5,9].

ES mass spectra and tandem mass spectra were recorded on a Sciex API III triple-quadrupole mass spectrometer with 2,400 Da mass range equipped with an ES ion source (Sciex, Toronto, Canada). Peptides were analyzed via direct injection at a flow rate of 3–5 ml/min with a microliter syringe using a medical infusion pump (Harvard Apparatus, USA). Peptide concentrations ranged from 0.1 to 0.5 mM and were determined after each titration experiment with $ZnSO_4$.

3. RESULTS AND DISCUSSION

The important feature of the ionization process during ES-MS analysis of peptides is their ability to form multiply charged ions, depending on the number of positively charged residues. Peptide analysis is usually performed in the positive mode and peptides are introduced in acidic solution, in order to get a higher number of protonated molecules. Obviously, such conditions make Zn^{2+} chelation by 'zinc finger'-like peptides impossible. At pH < 5.0 histidine residues become protonated and no longer act as Zn^{2+} coordinating ligands. Therefore, we performed our studies in NH_4OAc buffer at pH 5.0 using relatively high peptide concentrations.

The ES mass spectrum of Ncp7 (Fig. 2A) in 10 mM

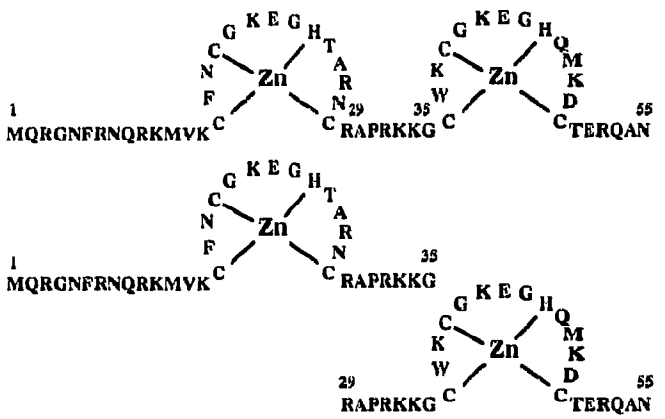

Fig. 1. Sequences of Ncp7, Ncp7-(1–35)- and Ncp7-(29–55)-peptide used for mass spectrometric complexing studies.

NH_4OAc buffer, pH 5.0, is characterized by 6 major peaks of ions possessing 10, 9, 8, 7, 6 and 5 charges (Table I) from which a molecular mass of 6444.22 + 0.4 was determined. This mass corroborates the calculated molecular mass of 6444.53 for Ncp7. From the less intense peak series (Fig. 2A) a molecular mass higher by 62 amu than the expected mass can be calculated, which corresponds to the substitution of two hydrogens by one zinc ion per peptide molecule. This indicates the presence of Zn^{2+} ions, which co-eluted during purification of synthetic Ncp7.

The Ncp7 spectra at increasing Zn^{2+} concentrations revealed a reduced pattern of peaks (Fig. 2B, C and D).

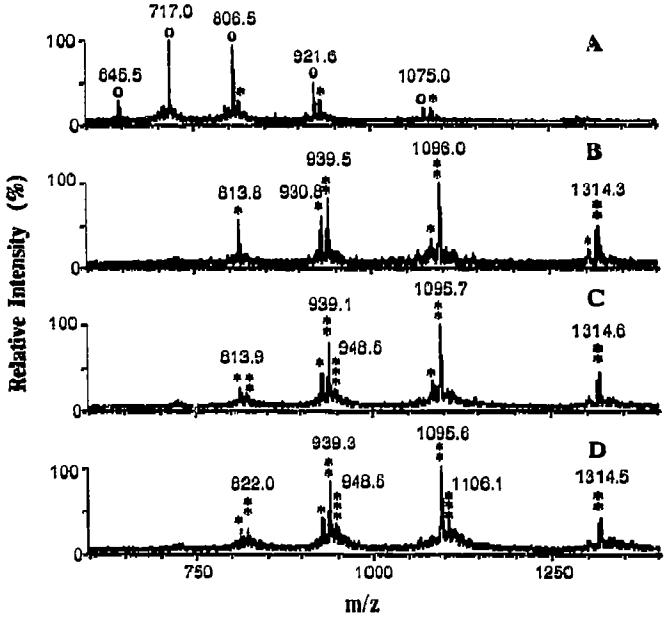

Fig. 2. ES mass spectra of Ncp7 in 10 mM NH_4OAc in the absence (A) and presence of Zn^{2+} ions: [Zn^{2+}] in (B), 0.1 mM, (C), 0.25 mM and (D), 0.5 mM. o, no Zn^{2+} bound; each bound Zn^{2+} ion is indicated by *.

The addition of Zn^{2+} to the peptide solution shifted the quasimolecular ions to lower charge numbers (higher m/z values). This could be due to the fact that Zn^{2+} bound to histidine residues results in a loss of two charges. Already at 0.1 mM concentration of Zn^{2+} the spectrum (Fig. 2B) is characterized by two series of peaks, which correspond to molecular masses of metallo-Ncp7 complexes with one and two Zn^{2+} ions. Interestingly, the relative intensity of quasimolecular ions containing one Zn^{2+} per Ncp7 decreases with increasing m/z values and is opposite to the relative intensity of peaks related to ions with 2 Zn^{2+} per Ncp7. Comparison of the experimental and calculated mass data shows good correspondence for zinc–Ncp7 complexes (Table I). Surprisingly, at the higher zinc concentrations (Fig. 2C and D), a third series of peaks could be identified, which corresponds to the NCp7 complex with 3 Zn^{2+} ions, thus indicating a third potential metal-binding site.

In order to define more precisely the regions of the Ncp7 involved in Zn^{2+} binding, two shorter fragments, each harbouring only one zinc-binding domain (Fig. 1), were used for ES-MS measurements. The ES mass spectra obtained for the Ncp7-(1–35)-peptide (Fig. 3) at increasing Zn^{2+} concentrations can be characterized by a multiply charged envelope containing 2 major series of ions possessing from 8 to 4 charges (Table I). A similar shift to lower charge numbers (higher m/z values) of the envelope with increasing Zn^{2+} molarity was observed. The results for the ion series of Ncp7-(1–35) peptide and its Zn^{2+}-complex are summarized in Table I. At 0.5 mM concentration of Zn^{2+} the spectrum is characterized by only one series of peaks corresponding to the 1:1 complex of zinc with the Ncp7-(1–35)-peptide. However, at this and higher concentrations of Zn^{2+} (data not shown) no peaks corresponding to the complex of two Zn^{2+} ions per mole of Ncp7-(1–35)-peptide could be obtained, thus indicating that the third metal-binding site is most probably located in the C-terminal region of Ncp7.

In order to obtain information concerning the stability of the Zn^{2+}–Ncp7-(1–35)-peptide complex we used

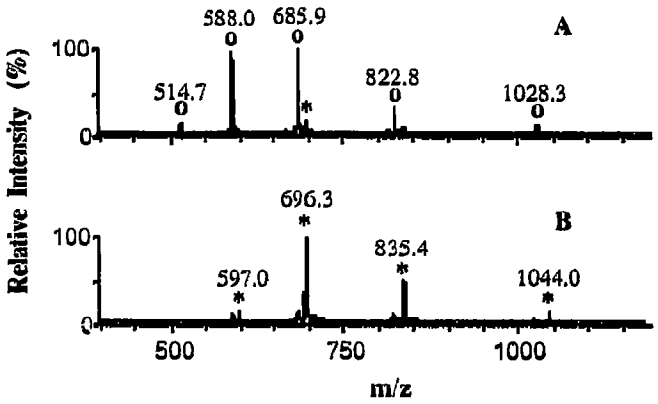

Fig. 3. ES mass spectra of Ncp7-(1–35)-peptide in 10 mM NH_4OAc in the absence (A) and presence of 0.5 mM of Zn^{2+} ions (B). o, no Zn^{2+} bound; each bound Zn^{2+} ion is indicated by *.

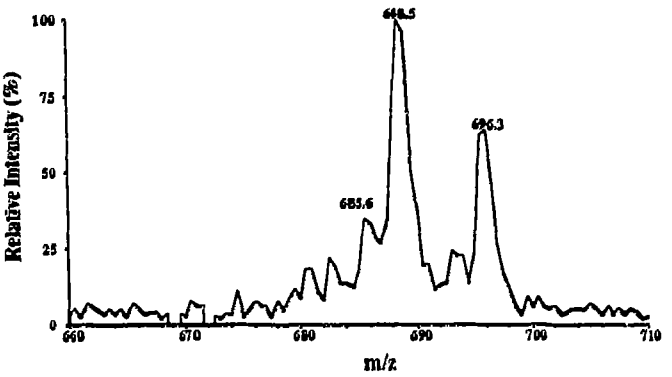

Fig. 4. Tandem mass spectrum obtained for a parent ion (696) corresponding to the 6-fold charged Zn^{2+}-Ncp7-(1–35)-peptide complex.

tandem MS techniques. A parent ion (696) possessing 6 positive charges was subjected to MS-MS, resulting in the formation of daughter ions (Fig. 4). However, under these conditions, where decarboxylation has already occurred (corresponding to peak 688.5), the Zn^{2+}–Ncp7-(1–35)-peptide complex appeared to be rather stable, and only a small amount of Zn^{2+}-free peptide could be observed (corresponding to peak 685.5).

The ES mass spectrum of Ncp7-(29–55)-peptide is characterized by two main series of peaks corresponding to 5–3 charged ions, from which the molecular masses of the Ncp7-(29–55)-peptide and its complex with Zn^{2+} ions can be determined (Table I). However, the ES mass spectrum of this C-terminal peptide differs from the one discussed for the N-terminal peptide. Even at high Zn^{2+} concentrations (1 mM) a series of peaks (Fig. 5) is observed corresponding to ions of the Ncp7-(29–55)-peptide without Zn^{2+} ions. This indicates that the affinity of the C-terminal peptide for Zn^{2+} is lower in comparison with that of the N-terminal domain. These observations are in good agreement with previously reported differences in binding affinity for Zn^{2+}

Table I

Estimated and calculated molecular masses for NCp7, its N-terminal Ncp7-(1-35)-peptide and C-terminal Ncp7-(29-55)-peptide fragments

Sample	Peak (amu)	Absolute intensity	Positive charge	Mass measured (amu)*	Final measured mass (amu)	Standard deviation	Calculated mass (amu)
Ncp7	645.5	390	10	6,444.92	6,444.22	0.40	6444.53
	717.0	1,470	9	6,443.93			
	806.5	1,380	8	6,443.94			
	921.6	670	7	6,444.15			
	1,075.0	230	6	6,443.95			
	1,289.9	80	5	6,444.46			
Ncp7 + $2Zn^{2+}$,$4H^+$ complex	731.0	85	9	6,569.93	6,567.75	0.29	6568.53
	822.3	285	8	6,570.34			
	939.7	1,015	7	6,570.84			
	1,096.1	1,215	6	6,570.55			
	1,315.2	470	5	6,570.96			
Ncp7-(1-35)-peptide	514.7	620	8	4,109.54	4,109.19	0.26	4109.86
	588.0	5,000	7	4,108.94			
	685.9	5,160	6	4,109.35			
	822.8	1,580	5	4,108.96			
	1,028.3	550	4	4,109.17			
Ncp7-(1-35)-peptide + Zn^{2+},$2H^+$ complex	597.0	600	7	4,171.94	4,171.91	0.10	4171.86
	696.3	5,420	6	4,171.75			
	835.4	3,130	5	4,171.96			
	1,044.0	990	4	4,171.97			
Ncp7-(29-55)-peptide	630.5	3,020	5	3,147.46	3,147.07	0.36	3147.66
	787.7	4,580	4	3,146.77			
	1,050.0	1,340	3	3,146.98			
Ncp7-(29-55)-peptide + Zn^{2+},$2H^+$ complex	643.0	580	5	3,209.96	3,209.54	0.44	3209.66
	803.4	3,680	4	3,209.57			
	1,070.7	850	3	3,209.08			

*amu: atomic mass unit.

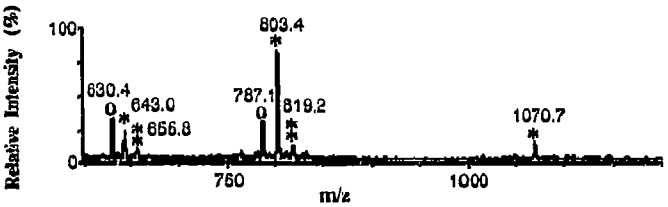

Fig. 5. ES mass spectra of Ncp7-(29–55)-peptide in 10 mM NH_4OAc in the presence of 1 mM of Zn^{2+} ions. o, no Zn^{2+} bound; each bound Zn^{2+} ion is indicated by *.

ions between proximal and distal fragments of Ncp7 [10]. More interesting is the appearance of an additional series of peaks, which seemed to result from the complexing of a second Zn^{2+} ion per mole of Ncp7-(29–55)-peptide (Fig. 5). Thus, the Ncp7 in its C-terminal fragment possesses a third attachement site. This additional Zn^{2+} ion is most probably bound through a carboxylate group of acidic residues or the C-terminus. This third Zn^{2+} ion seems to be bound 'non-specifically' as it could be easily substituted with Ca^{2+} (data not shown).

In conclusion, Ncp7 was shown for the first time by electrospray mass spectroscopy to bind two equivalents of Zn^{2+} ions. Its shorter 1–35 and 29–55 fragments, each comprising only one zinc-binding domain, bound one equivalent of Zn^{2+} ion with different affinities (higher in the case of the proximal 1–35 fragment). In addition to the expected two metal-binding sites, Ncp7 possesses a third one in the C-terminal part of the protein. Although the third ion is bound non-specifically, it could be involved in the stabilization of an α-helix, which has been suggested in our previous studies [6].

Electrospray mass spectrometry was shown to be very a convenient, fast and accurate method for direct determination of metal binding to peptides. The procedure used here can be applied to the analysis of other naturally isolated or chemically synthesized metallopeptide complexes.

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