Mass Spectrometric Studies on Noncovalent Dimers of Leucine Zipper Peptides

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Abstract: The leucine zipper, found in several DNA-binding proteins, represents a motif for noncovalent dimerization to enhance DNA binding. Solution dimerization of the leucine zipper peptides GCN4-p1 and N16V is confirmed here by on-line, size-exclusion liquid chromatography–ion spray mass spectrometry. Tandem mass spectrometry studies indicate that dimer ions also exist in the gas phase. High-resolution trapped-ion studies show that these gaseous dimers are stable for at least minutes. In qualitative agreement with their behavior in aqueous solution, N16V has a greater tendency to form dimers than does GCN4-p1, which unexpectedly forms a noncovalent heterodimer with an impurity.

Introduction

The leucine zipper motif, containing a heptad repeat of leucine, has been found in the C-termini of several DNA-binding transcriptional activator proteins.[1,2] This motif mediates protein dimerization[1,3-5] via formation of a parallel two-stranded coiled coil of α-helices[6-8] packed "knobs into holes" at the dimer interface,[9] but without interdigitating the leucine repeat at the dimer interface.[2] Dimerization of the leucine zipper motif is necessary for DNA binding[10,11] and plays an important role in DNA replication, recombination, strand scission, and transcription.[2]

Individual leucine zipper peptides also associate in the micromolar concentration range to form stable noncovalent dimers in aqueous solution, apparently through hydrophobic interactions between each α-helix. By replacing asparagine-16 with valine, a significant increase in dimer stability is achieved,[6,11] as measured by the change in melting temperature (T_m) of the dimeric coiled coil structures: for GCN4-p1 (wild type, MW = 4037), $T_m \sim 29$ °C, whereas for N16V (mutant type, MW = 4022), $T_m \sim 74$ °C.

Recently ion spray (pneumatically-assisted electrospray) mass spectrometry (MS) was applied to detect noncovalent receptor–ligand[12] and enzyme–substrate complexes[13] at physiological pH.[14] Since then, electrospray MS has been used to detect other specific, noncovalent protein association complexes of biological interest.[15-17] The ion evaporation conditions of electrospray[18-20] are sufficiently mild to detect noncovalent complexes resulting from hydrogen-bonding, ion–dipole, and electrostatic interactions, as well as different protein conformations in the gas phase.[21]

Although noncovalent leucine zipper dimers have been detected by circular dichroism (CD),[6,11] NMR,[7] and X-ray crystallography,[8] it was of interest to determine whether the weak hydrophobic interactions in such complexes would be preserved in the gas phase, absent bulk water. In fact, noncovalent dimers of leucine zipper peptides generated under physiological conditions (water, pH 7) can be detected by MS. The unexpected behavior encountered led to the additional application of tandem mass spectrometry (MS/MS), high-resolution (HR) MS, and size-exclusion chromatography to examine the relative differences between solution and gas-phase noncovalent binding.

Experimental Section

Parathyroid hormone (PTH), having a molecular weight (MW) of 9510, and porcine neuropeptide Y (NPY, MW = 4253) were purchased from Sigma Co. (St. Louis, MO) and used as standard peptide MW markers. The leucine zipper polypeptides GCN4-p1 (wild type, MW = 4037) and N16V (mutant type, MW = 4022) were obtained from Professor Peter S. Kim (Department of Biology, The Whitehead Institute, Massachusetts Institute of Technology, Cambridge, MA 02139). Amino acid sequences of these peptides are as follows, with the single-point mutation at residue 16 indicated in boldface type.

GCN4-p1:

Ac-RMKQLEDKVEELLSKNYHLENEVARLKKLVGER

N16V:

Ac-RMKQLEDKVEELLSK**V**YHLENEVARLKKLVGER

The size-exclusion chromatography column was a BioSep-SEC-S2000 (2.1 × 300 mm) purchased from Phenomenex (Torrence, CA). Separation

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of a synthetic mixture containing PTH, GCN4-p1, and NPY was accomplished using isocratic size-exclusion HPLC conditions, which consisted of 0.1% TFA as a mobile phase. At 40 μL/min, a relatively slow flow rate, the analysis time was 60 min. A sample containing PTH (2 nmol in 0.1% TFA), NPY (1 nmol in 0.1% glacial acetic acid), and GCN4-p1 (1 nmol in 10 mM NH_4OAc, pH 7) was injected directly into a Rheodyne Model 9125 injector (Cotati, CA) equipped with a 20-μL sample loop. A dual-syringe Brownlee Labs micro LC pump (Santa Clara, CA) was used as the mobile-phase delivery system for these experiments. An Applied Biosystems Inc. (ABI, Foster City, CA) Model 757 absorbance detector equipped with a 2.4-μL flow cell was used to detect the peptides at a UV wavelength of 214 nm for optimization of the chromatographic separation off-line. For on-line ion spray LC/MS the UV detector was removed from the system and the column exit connected directly to the ion spray interface. During on-line LC/MS acquisition the step size was 1 Da with a 3.3-s scan rate from m/z 450 to 2250. The total effluent of 40 μL/min was directed unsplit to the ion spray LC/MS interface.

A Sciex (Thornhill, Ontario) TAGA 6000E atmospheric pressure ionization (API) triple-quadrupole mass spectrometer upgraded to an API-III with an upper mass limit of 2400 Da was used. The peptides (1 mmol in 10 mM NH_4OAc, pH 7) were delivered at 2 μL/min at ambient temperature via an infusion pump (Model 22, Harvard Apparatus, South Natick, MA). The ion spray probe was positioned off-axis ca. 1 cm away from the orifice with nitrogen (55–60 psi) as nebulizing gas. Spraying was achieved at room temperature via charging the spray probe at 3.6 kV. Polypropylene glycol (PPG) in 80/20 CH_3CN/H_2O (3 mM NH_4OAc) was used to calibrate each mass-resolving quadrupole (Q_1 and Q_3). Experiments were performed at a declustering potential of 30 and 60 V. The collision gas (argon) was introduced into the collision cell (Q_2) for the MS/MS experiments, and a collision energy of 150 eV (laboratory frame) was used. Mass spectra were obtained at a dwell time of 2 s per scan, a step size of 0.1 Da with 10 scans summed, using a PE-Sciex Macintosh II-based data system.

For the HRMS experiments, a special Fourier-transform ion cyclotron resonance instrument[22] (Extrel FTMS, Millipore Corp., Madison, WI) was used. Aqueous solutions (50 μM) were infused at 1.5 μL/min without pneumatic nebulization, using a 120-V declustering potential. A two-second ion beam was used with pulsed-valve N_2 introduction, followed by an ion-cooling delay (2–10 min) before chirp excitation from 50 to 150 kHz at a sweep rate of 100 Hz/μs with direct mode detection of 256K data points at an acquisition bandwidth of 200 kHz. Time domain spectra were base-line corrected, hamming apodized, and zero-filled once before magnitude mode FFT.

Results and Discussion

To determine whether leucine zipper dimers could be detected by ion spray MS, their solutions were subjected to continuous introduction ("infusion") ion spray MS under physiological pH conditions (pH 7.0) in aqueous 10 mM ammonium acetate buffer. Figure 1A,B shows the corresponding full-scan ion spray mass spectra for GCN4-p1 and N16V, respectively. Only the mass-to-charge (m/z) ratio is measured for these multiply protonated ions, so that, for even charge states at this resolving power, the expected mass-to-charge ratios for dimers will overlap with the corresponding monomer charge states. For example, the MS of GCN4-p1 (Figure 1A) reveals an ion at m/z 808.2, which could result from either the $5H^+$ charge state of the monomer or the $10H^+$ charge state of the dimer. However, the relatively weak ions at m/z 1155 and 1616 correspond uniquely to the $7H^+$ and $5H^+$ charge states, respectively, of dimeric species and provide a true measure of the abundance of gas-phase dimer ions. These data were collected using "gentle" focusing conditions (declustering energy = 30 V). Higher collision voltages reduced the dimeric ion current, with none observed at 90 V, suggesting that the gas-phase dimers are held together by relatively weak (e.g. hydrophobic) interactions.

The effect of pH on dimer detection was also investigated. As the pH was varied from 3 to 9, little change in the GCN4-p1 dimer ion abundance was observed compared to Figure 1A (results not shown). Using circular dichroism, Kim *et al.* have independently shown that the melting temperature of GCN4-p1 at a concentration of 34 μM in phosphate-buffered saline is essentially independent of pH (57 °C at pH 7, 51 °C at pH 2, 49 °C at pH 11).[23] Taken together, such observations are more consistent with hydrophobic binding than with electrostatic interactions between the polypeptide chains.

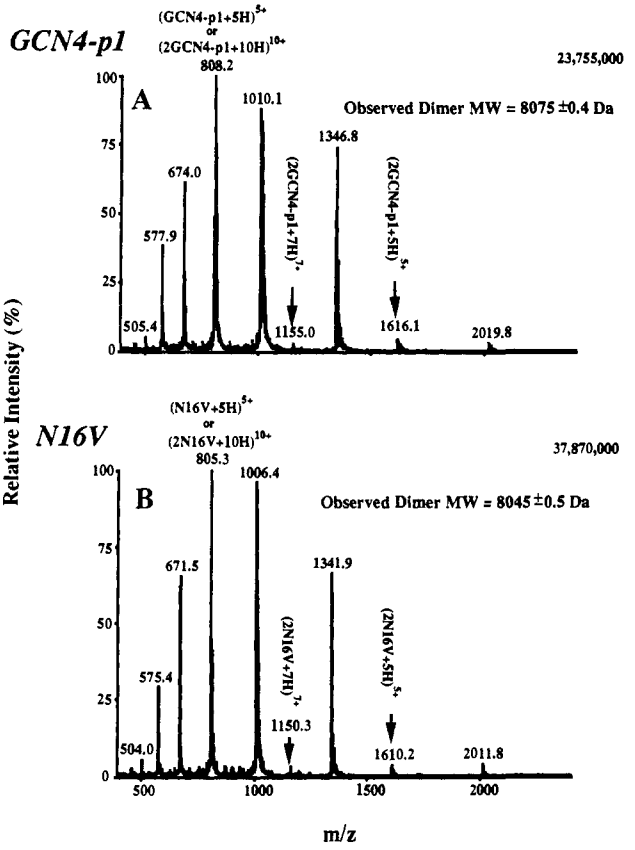

Figure 1. Ion spray mass spectra for (A) GCN4-p1 and (B) N16V.

Replacing asparagine-16 with valine in GCN4-p1 gives rise to another leucine zipper peptide, designated N16V. In water, N16V forms a more stable dimer than GCN4-p1,[6,11] as evidenced by the enhanced T_m of N16V (~74 °C vs ~29 °C for GCN4-p1). The greater aqueous stability of the N16V leucine zipper dimer is attributed to a regular heptad repeat of valine residues that completes a second motif of hydrophobic amino acids. The ion spray mass spectrum of N16V (Figure 1B), under conditions identical to those for Figure 1A, also displays principal ions (m/z 575, 671, 805, 1006, and 1342) representing mostly monomeric peptides. However, the ions at m/z 1150.3 and 1610.2 correspond uniquely to the $7H^+$ and $5H^+$ charge states, respectively, of the noncovalent dimer. Surprisingly, the two diagnostic N16V dimer ions were not correspondingly more abundant than those of the GCN4-p1 dimer, as was predicted from aqueous behavior. In an earlier study of receptor–ligand binding,[12] albeit where hydrogen bonding was the dominant noncovalent interaction, a much closer correlation was observed between solution and gas-phase association tendencies. This apparent anomaly prompted further investigation of leucine zipper dimers.

LC/MS

To confirm that the peptides were actually dimeric in the solution used, on-line microbore size-exclusion liquid chromatography/mass spectrometry (LC/MS) was applied to a synthetic mixture containing GCN4-p1, parathyroid hormone (PTH), and neuropeptide Y (NPY). Figure 2 shows the LC/MS chromato-

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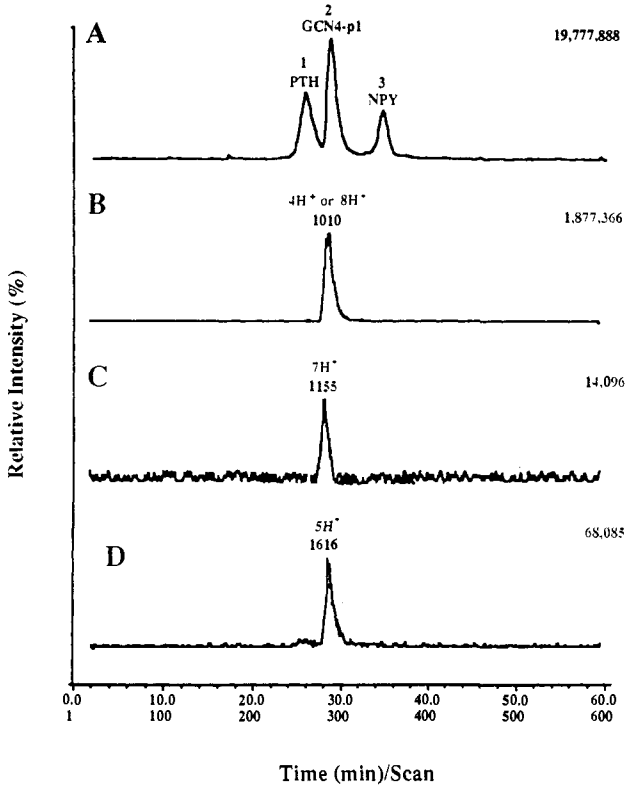

Figure 2. Size-exclusion LC/MS for a synthetic mixture of parathyroid hormone (PTH, MW = 9510), GCN4-pl, and neuropeptide Y (NPY, MW 4253): (A) total ion current profile; (B–D). Extracted ion current profiles for m/z 1010, 1155, and 1616.

gram for detection of total ions and m/z 1010, 1155, and 1616. Size-exclusion separation[24] is based upon permeation of molecules into the interstitial spaces of the silica packing material. Although peptide MW standards may vary in conformation (thus impeding quantitative correlations), large molecules generally elute more rapidly, and chromatographic peaks exhibit retention times which are approximately inversely related to each substance's MW.

The elution of GCN4-pl (peak 2, Figure 2A) between PTH (MW = 9510) and NPY (MW = 4253) confirms that GCN4-pl exists as a dimer (MW = 8047) in the solution subjected to ion spray MS. The resulting mass spectrum for GCN4-pl (not shown) is the same as that obtained by infusion experiments (Figure 1A). The chromatographic retention times for the ions at m/z 1010, 1155, and 1616 are identical, thus corroborating that they all originate from the solution dimer.

Several controls with other peptides further confirm that the gas-phase leucine zipper dimer ions reflect true solution behavior and are not artifacts of clustering in the mass spectrometer. Angiotensin II, bombesin, bradykinin, leucine enkephalin, α-melanocyte stimulating hormone, methionine enkephalin, NPY, oxytocin, ribonuclease S peptide, and thyrotropin releasing hormone range in MW from 362 to 4253, and all 10 substances are known to be monomeric in solution. Ion spray mass spectra of these peptides (not shown) reveal no dimer ions whatsoever when measured under the same experimental conditions as the leucine zipper peptides.

MS/MS

To confirm the dimeric nature of the $5H^+$ ions at m/z 1616 and 1610 in Figure 1A,B, respectively, these gas-phase ions were regenerated in the same way, separated in the first quadrupole (Q_1), and subjected to collision-induced dissociation in Q_2. In the resulting spectra (Figure 3), the principal product ions have m/z values corresponding to the expected $2H^+$ and $3H^+$ monomers. The corresponding dimer precursor ion for N16V (Figure 3B) shows less dissociation to $2H^+$ and $3H^+$ monomer product ions than does GCN4-pl under identical experimental conditions (Figure 3A). These results are qualitatively consistent with the observation that the N16V solution dimer is more strongly associated than the GCN4-pl dimer,[6,11] although the apparent difference in the gas phase is less than might be expected on the basis of solution dimerization tendencies. However, hydrophobic interactions, a key driving force for zipper peptide dimer formation in water, are known to be much less significant in a vacuum than in aqueous solution.

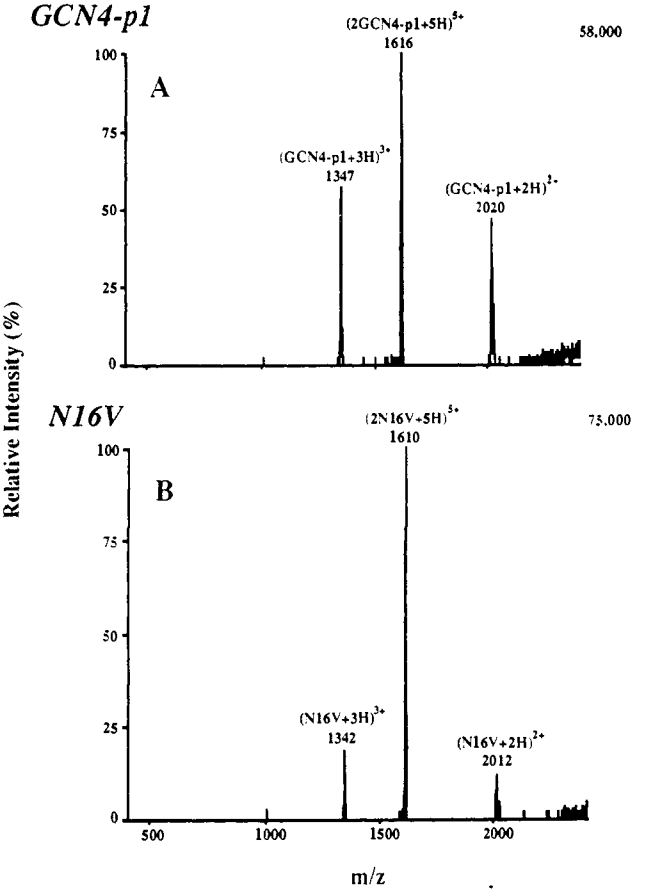

Figure 3. Collision-induced dissociation mass spectra for m/z 1616 and 1610 ions from (A) GCN4-pl and (B) N16V.

High-Resolution Studies

Loss of these hydrophobic interactions, together with the enhanced Coulombic repulsions resulting from multiple charge sites, could explain the low relative abundance of the zipper peptide dimers in the gas phase (Figure 1) in comparison to that in solution (Figure 2). Alternatively, the noncovalent complex ions observed in the quadrupole mass spectra could be thermodynamically unstable, although the millisecond time scale of this instrument would kinetically limit the degree of observable dissociation. This issue was investigated using a Fourier-transform trapped-ion instrument, varying the time between ion injection and measurement. The electrospray mass spectrum of the GCN4-pl peptide shows complete resolution of the isotopic peaks (Figure 4) and confirms that m/z 1157 corresponds to the $7H^+$ ion, since their seven isotopic peaks, each separated by the 1.0034-Da $^{12}C/^{13}C$ mass difference, require one m/z unit. The relative abundance of the $7H^+$ dimer does not change when the delay before

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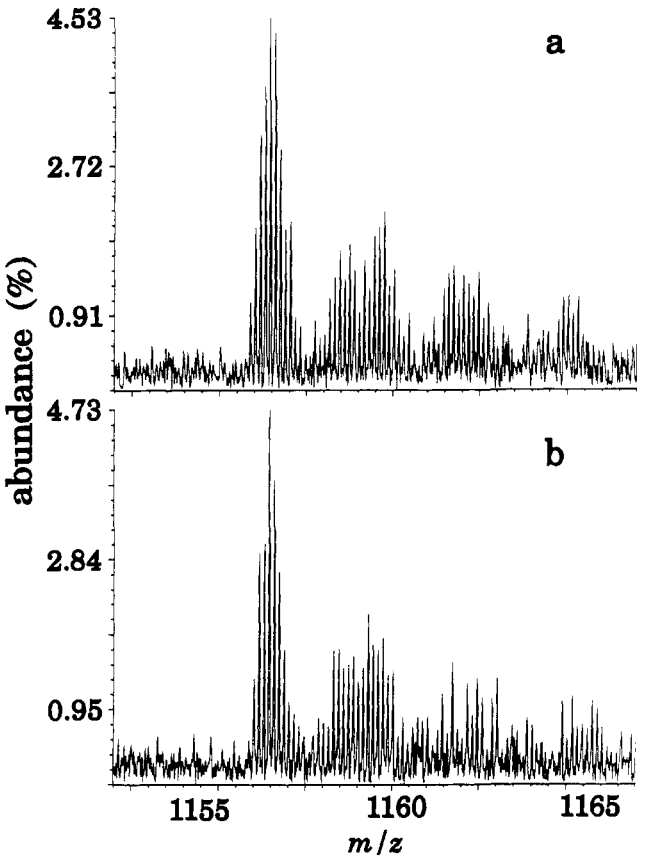

Figure 4. Fourier-transform spectra of the GCN4-p1 7H+ dimer region after (a) 2-min cooling delay and (B) a 10-min cooling delay.

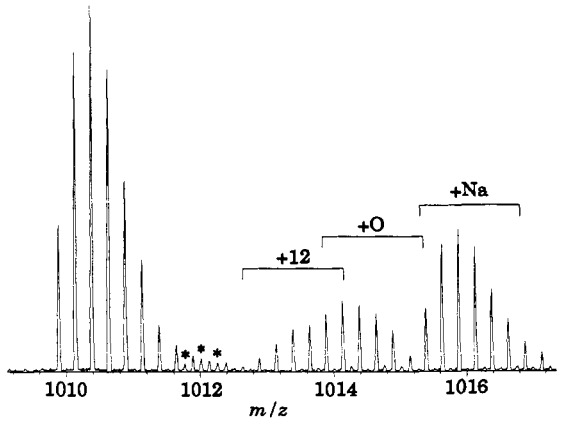

Figure 5. Fourier-transform spectrum of the GCN4-p1 4H+ monomer region showing heterodimers (*), impurities (+12 and +16 Da), and adducts (+22 Da).

measurement was extended from 2 to 10 min. This shows that dissociation kinetics have not significantly affected the quadrupole mass spectra (Figure 1) and that the forces maintaining the dimer (van der Waals and/or hydrogen bonding) are stronger than the Coulombic forces caused by the repulsion of two positively charged ions at very short range.

The HR mass spectrum of GCN4-p1 unexpectedly shows the mass value of the 7H+ dimer to be 11.98 Da higher than that predicted for the $(GCN4\text{-}p1+7H)^{7+}$ homodimer. Similarly, isotopic peaks corresponding to 8H+ (12 Da higher than that expected for the 4H+ homodimer) are visible in the expanded spectra at m/z 1012–1013 (asterisks, Figure 5). These peaks apparently correspond to heterodimers of GCN4-p1 with another peptide whose mass is 11.98 Da higher. In fact, a component higher in mass by 11.993 ± 0.009 Da is indicated by the 4H+

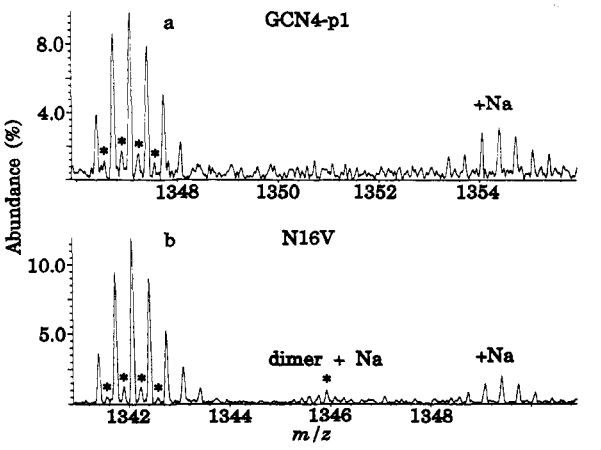

Figure 6. Expansion plots of Fourier-transform spectra of (a) GCN4-p1 and (b) N16V peptides for the m/z 1347 and 1342 ions, respectively, showing (*) homodimer isotopic peaks.

isotopic peaks at m/z 1014 (Figure 5). The unit mass assignment is compromised by overlap of isotopic peaks corresponding to another impurity whose MW is 16.012 ± 0.015 higher than GCN4-p1. Oxidation of methionine (residue 2) to its sulfoxide is common in peptides, and one extra oxygen atom (15.995 Da) would account for the heavier impurity.[25] Other 4H+ peaks near m/z 1010 correspond to (observed, theoretical mass difference) H_2O loss (−17.994 ± 0.012, −18.011 Da), monosodium adduct (21.978 ± 0.008, 21.982), and the monosodium adducts of the +12 and +16 impurities. Higher mass peaks could similarly arise from di- and trisodium adducts of these compounds and from the copper adduct of GCN4-p1.[22]

Given the amino acid composition of GCN4-p1, the only point mutation which might account for the +11.98-Da impurity is S14V (serine-to-valine; +12.036 Da). However, the accurate mass data make this possibility unlikely. Regrettably, studies consumed the sample before the impurity could be identified, and succeeding preparations of GCN4-p1 contained <1% of the contaminant, which is reflected by the 5-fold-lower dimer abundances. Direct comparison of HR mass spectra of these new samples (Figure 6a,b) by expanding the 3+ monomer charge state revealed isotopic peaks for the 6+ homodimer charge state (asterisks), thus confirming earlier findings (Figure 1A,B). Moreover, examination of the 5+, 6+, and 7+ dimer charge states by HRMS indicates that the N16V homodimer is approximately twice as abundant as the GCN4-p1 homodimer (data not shown). These results are qualitatively consistent with the stronger solution dimerization of the N16V peptide.

Conclusions

The ability to detect and characterize biologically important noncovalent complexes involving hydrogen bonding, charge-transfer complexes, ion–dipole effects, and electrostatic interactions has been established.[12-17] However, the present work suggests that the significance of various types of intermolecular forces, commonly grouped together as "noncovalent interactions", may be altered when transferred from an aqueous to a gaseous environment. In the present study, association of leucine zipper peptides into dimers is mainly due to hydrophobic interactions, which may be expected to diminish significantly in the gas phase, where water is absent. Despite this tendency, the gaseous dimer ions of two closely related leucine zipper peptides may be detected by ion spray MS, although those from GCN4-p1 are observed only with difficulty.

(25) Other 8H+ isotopic peaks are consistent with the presence of the (GCN4-p1:+16) heterodimer, along with homodimers of the +12 and +16 impurities.

In qualitative agreement with their solution behavior, the homodimer ions of N16V are more stable than those of GCN4-p1. The importance of structure on complexation ability is illustrated by a minor impurity which forms heterodimer ions significantly more abundant than those of the GCN4-p1 homodimer. Heterodimers of this nature have recently been implicated in the products of nuclear oncogenes such as *fos* and *jun*, which also bind to DNA and modulate transcription.[3] Given the possibility of such unpredictable impurity effects, the importance of high-resolution MS is evident in providing definitive information concerning the gas-phase behavior of biologically important complexation reactions.

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