Higher Order Structure in the Gas Phase Reflects Solution Structure

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As new methods for volatilizing and ionizing biological molecules for mass spectrometry (MS) analysis are developed, the conformation of gas-phase biomolecule ions and how it relates to solution-phase reactivity and structure has been generating considerable interest.[1] The conformations of gas-phase ions of large polypeptides formed by electrospray ionization (ESI) have been probed by a variety of means, including ion−molecule reactions,[2] collision cross-section measurements,[3] and hydrogen−deuterium-exchange (H/D).[4] The number of exchangeable protons reflects the openness of the protein conformation. Ion mobility measurements have resolved gas-phase structural isomers for a number of proteins.[5] The charge state distribution produced by ESI as a function of pH,[6] solvent content,[7] and metal-binding properties[8] has also been related to the protein conformation in solution. Another recent application of ESI-MS includes the study of noncovalently bound complexes,[9,10] for which MS has unique advantages in determining complex stoichiometry of the binding partners. Characteristics of the native solution state of proteins and oligonucleotides and their interactions with other biomolecules can be also captured by MS measurements.

Tandem mass spectrometry (MS/MS) with collisionally activated dissociation (CAD) has been used also to explore the reactivities of large ions. Mass-analyzed kinetic energy (MIKE) spectrometry was used to study the stability of secondary structure in the absence of solvent for the peptide melittin.[11] The CAD mass spectra of peptide−metal ion complexes show that relatively selective bond cleavages can be induced, as specific complexation with metal ions can direct specific dissociation pathways.[12] Smith and co-workers' CAD study of 12 kDa cytochrome *c* proteins suggests that the fragmentation pattern could be influenced by the protein's higher order structure.[13] However, from these studies, it is not clear whether these three-dimensional structures in a solvent-less environment bear any resemblance to the conformation in the solution phase. We present an example in which the gas-phase MS/MS-based measurements of a polypeptide are consistent with its known solution-phase structure.

RES-701-1 (**I**), isolated from *Streptomyces* sp. RE-701, is a potent and selective endothelin ET_B receptor antagonist.[14] The hexadecapeptide possesses a unique linkage between the β-carbonyl carbon of Asp-9 and the α-amino group of Gly-1, forming a 9-residue cyclic "head" structure and a 7-residue "tail" (see Scheme 1). However, a synthetic version of the peptide (**II**) is characterized by an affinity for the ET_B receptor reduced by nearly 3 orders of magnitude (IC_{50} of 10 nM for the natural peptide and 6.5 μM for the synthetic peptide).[15,16] High-resolution, exact mass measurements with ESI show the two peptides to be within ±0.004 mass units (±2 ppm) of the expected value (measured monoisotopic mass of **I** was 2041.8520, and a mass of 2041.8492 for **II**; expected mass from sequence is 2041.8536).[17]

Dramatic differences between the natural and synthetic peptides are shown by CAD of ESI-generated singly and doubly protonated molecules.[18] MS/MS of the $(M + H)^+$ and $(M + 2H)^{2+}$ ions for both peptide forms yields primarily cleavage of the peptide backbone in series of a_n- and b_n-type product ions (where n = 9−15), but fragmentation from within the cyclized structure was not observed for either peptide. However, as shown in Figure 1, the relative abundances of the product ions differ for **I** and **II**. CAD of the singly protonated molecule corresponding to the naturally isolated peptide **I** shows increasing abundance of the product ions from b_9 to b_{12}; the relative abundances of b_{13-15} are greatly reduced compared to the b_{12} product ion (amide bond cleavage between Phe-12 and Asn-13, see Scheme 1). The a_{9-15} and b_{9-15} fragment ions for the synthetic peptide **II** are more nearly equal in abundance (Figure 1b). The same trend is observed for the dissociation of the 2+-charged molecule (data not shown).

H/D-exchange experiments confirmed the different structures in the solution phase.[19] Exchange in the corresponding molecular ions and product ions generated by CAD induced in the

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(17) Amino acid analysis of both peptide forms show the same amino acid composition, as described in refs 15 and 16.

(18) ESI mass spectra were acquired with a Finnigan MAT 900 double-focusing mass spectrometer. The ESI interface is based on a heated glass capillary design. Analyses were performed at full accelerating potential (5 kV), except for linked scan at constant *B*/*E* experiments of 2+-charged parent ions. MS/MS spectra were acquired by scanning the magnet and the electrical analyzer simultaneously at a constant *B*/*E* ratio. CAD was performed in the first field-free region by introducing helium gas sufficient to reduce the precursor ion abundance to 50%. Sample solutions in acetonitrile/water/acetic acid (49:49:2, *v*/*v*/*v*) were infused through the ESI source at 1 μL/min.

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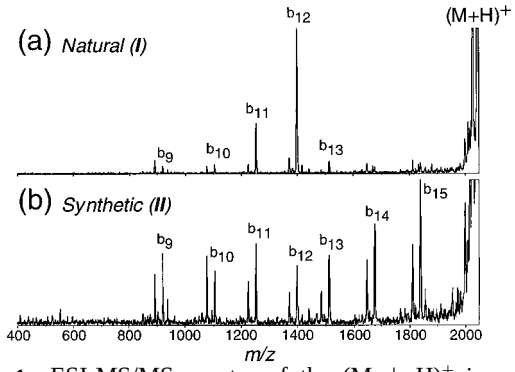


Figure 1. ESI-MS/MS spectra of the $(M + H)^+$ ions of (a) naturally isolated RES-701-1 peptide **I** and (b) the synthetic peptide **II** of same primary structure. Conventional notation for fragmentation of polypeptides is used,[28] with amide bond cleavage yielding b-type and y-type ions containing the NH_2- or COOH-terminus, respectively.

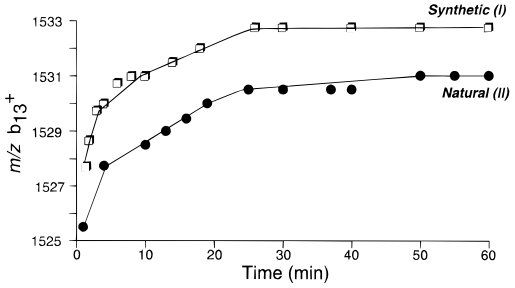
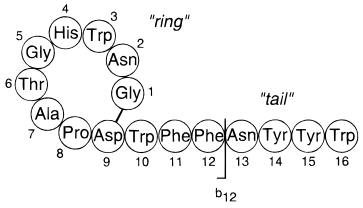


Figure 2. ESI-MS H/D-exchange of peptides **I** and **II** for the b_{13} product ion. Mass spectra were acquired over the time course of the experiment with $\Delta V_{TS} = +220$ V to induce peptide dissociation in the ESI interface.

Scheme 1



atmospheric pressure−vacuum interface of the ESI source was monitored over a 60 min time course by ESI-MS. For **II**, all of the product ions and the molecular ions increased in mass to values consistent with complete H/D-exchange (27 total exchangeable hydrogens) over the period of the experiment, while peptide **I** showed less than complete H/D-exchange for all ions monitored; the molecular ion exchanged only 24 hydrogens and the product ions from the tail region showed correspondingly less exchange than its synthetic peptide counterpart (Figure 2). Moreover, the a_9/b_9 product ions composing the ring sequence exchanged to the same extent for both **I** and **II**, indicating that the tail structure differentiates the two peptides. Thus, the solution-phase structures appear to be different, as the naturally isolated peptide has a structure which is more protected from H/D-exchange than the synthetic peptide.

The MS/MS data and the H/D-exchange results are consistent with the recently published NMR structure of RES-701-1 by Yoshida and co-workers.[20,21] The natural peptide adopts a highly ordered structure in which the "tail" passes through the "ring" region. The Phe-12−Asn-13 portion of the peptide tail lies above the Gly-1−Trp-3 of the ring. The Tyr-14−Tyr-15 segment continues below the Gly-5−Ala-7 portion of the ring. A hydrogen bond between the amide proton of Asn-13 and the CO of Asn-2 is key to stabilizing this "lasso-type" structure.[20,21] This type of structure is not accessible synthetically. CAD-MS of natural RES-701-1 (**I**) shows the b_{12} fragment as the most abundant product ion with much lower relative abundances observed for products resulting from cleavages C-terminal from Phe-12. The tail portion C-terminal from Phe-12 that resides within the ring region is protected from gas-phase CAD and solution-phase H/D-exchange processes. The synthetic peptide shows an unordered structure by NMR analysis.[16] By CAD-MS, it shows that products b_{13-15} are relatively abundant because the tail is not embedded in the ring and is more accessible to CAD.

Previously, CAD of disulfide-containing proteins such as ribonuclease A,[22] proinsulin,[23] and albumin[24] showed different fragmentation behavior for their disulfide-reduced and oxidized forms; in general, the reduced forms yielded greater product ion yields and fragmentation channels. However, it was not clear if those results truly reflected solution structure or the increased efficiencies for dissociation of only one bond (reduced form) vs multiple bonds (at a minimum two bonds must be broken to liberate product ions in a disulfide-bonded or cyclic region).

This peptide is a clear example in which the higher order structure of a gas-phase ion relates directly to its solution-phase structure. The natural and synthetic peptides share the same primary structure, but they have distinct and unique conformations to which tandem mass spectrometry and H/D-exchange−CAD have been applied as tools for establishing their higher order structure. RES-701-1 belongs to a unique structural peptide family prepared by bacteria (or prokaryotes).[16,21,25] Its bioactive form adopts the "lasso-type" structure in which the tail is "locked" in the ring and the tail cannot be extricated once the ring has been formed.

The details of noncovalent folding interactions can affect the gas-phase fragmentation process. Whether this is generally true for all ESI-generated ions remains a question to be addressed. Noncovalent protein−oligonucleotide complexes in which electrostatic forces dominate can survive the solution-phase−gas-phase transition and are typically extremely stable toward CAD processes.[10,26] However, complexes driven together in solution by hydrophobic forces may not exhibit similar stabilities in vacuo.[27] Although RES-701-1 may represent an usually stable structure, this example demonstrates that methods used to probe the structure of a gas-phase molecule such as collisionally activated dissociation can be used to differentiate solution-phase conformers.

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(19) The deuterated solvent of acetonitrile/D_2O/CD_3CO_2D (49:49:2, *v*/*v*/*v*) was added to the dried peptide and the solution was introduced into the ESI source in less than 25 s. Interface CAD experiments were accomplished by increasing the voltage difference between the metallized exit of the glass capillary and the first skimmer element of the interface (ΔV_{TS}) from +120 to +220 V. The experimental procedure is similar to that reported by Anderegg et al. (Anderegg, R. J.; Wagner, D. S.; Stevenson, C. L.; Borchardt, R. T. *J. Am. Soc. Mass Spectrom.* **1993**, *5*, 425−433).

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