DOI: 10.1002/anie.200705048

Peptide Mass Spectrometry

Does a Charge Tag Really Provide a Fixed Charge?**

*Yi He and James P. Reilly**

In biomolecule mass spectrometry, peptides are usually ionized by an acidic solvent or matrix. It is generally accepted that the mobilization of attached protons weakens backbone bonds and facilitates peptide fragmentation.[1] While this is often advantageous, it can lead to a multiplicity of fragment ions (both N-terminal a_n, b_n and C-terminal y_n ions). Formation of too many types of fragment ions can overly complicate spectra. Although very basic arginines tend to sequester protons and to some extent simplify spectra, charge tags, small molecules containing a permanent charge (for example, a quaternary ammonium ion), have been proposed as an even better solution to the problem.[2] Charge-tagged peptides have a localized positive charge that is assumed to be absolutely fixed. Therefore, in MS/MS experiments, only N-terminal or C-terminal fragment ions should be formed through charge-remote mechanisms[3] depending on the position of the tag. Such a simplified series of contiguous fragment ions would provide complete sequencing information and would be ideal for spectral interpretation. Sensitivity should also be greatly improved after derivatization, since charge-tagged peptides are already ionized and need not be protonated. However, the lack of a mobile proton may reduce fragmentation efficiency which would be a potential disadvantage. Most of the charge tags that have been developed label the N-terminus. Two popular examples are trimethylammonium butyric acid[4] (TMAB) and tris(2,4,6-trimethoxyphenyl) phosphonium acetic acid[5] (TMPP-Ac).

Herein, we introduce a (4-trimethylammoniumbutyryl (TMAB)) positive charge onto the N-terminus of several peptides. It has been reported that this modification improves peptide detection sensitivity, and leads to the production of N-terminal fragment ions in MALDI-PSD mass spectra.[6] However, we now demonstrate that the positive charge does not always remain fixed at the N-terminus of the peptide.

Figure 1a is the ESI mass spectrum of charge-tagged Fibrinopeptide A. The presence of only a single peak associated with the doubly charged, derivatized peptide (from the charge tag and one proton) indicates that the reaction between the labeling reagent (TMAB-NHS; NHS = *N*-hydroxysuccinimide) and peptides was complete.

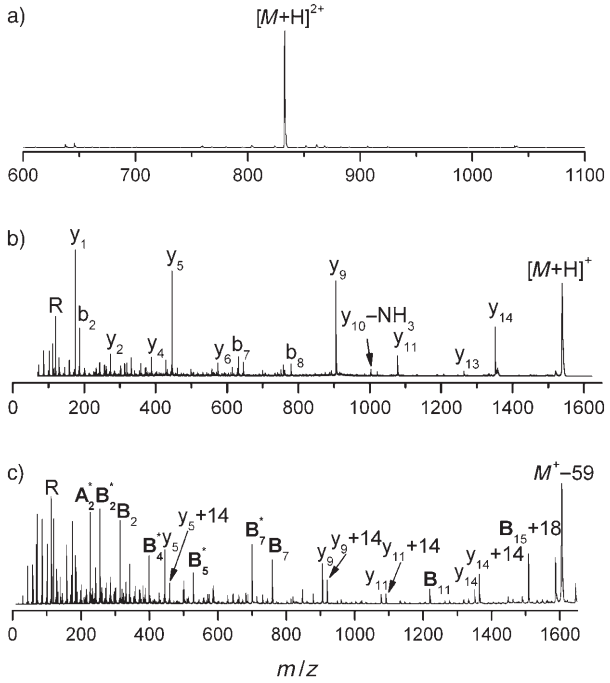

Figure 1. a) ESI-MS spectrum of doubly charged labeled Fibrinopeptide A (TMAB-ADSGEGDFLAEGGGVR), *m/z* 833.09 Da; MALDI-TOF/TOF CID (1 kV) and spectra of singly charged b) Fibrinopeptide A and c) the TMAB derivative. See text for details.

Singly-charged ions associated with Fibrinopeptide A (ADSGEGDFLAEGGGVR) and its derivative were isolated and fragmented in a MALDI TOF/TOF apparatus. Results are shown in Figure 1b and 1c. As anticipated, y_n ions are the dominant features in Figure 1b because of the presence of C-terminal arginine residue. We had expected to observe N-terminal fragment ions from the TMAB-derivatized peptide, but the spectrum is surprisingly more complex (Figure 1c). In this case, B_n represents a TMAB-labeled b ion ($B_n = b_n + 127$). A_n* and B_n* represent A_n and B_n ions that have lost trimethylamine ($A_n^* = A_n - 59$ and $B_n^* = B_n - 59$). Hines et al.[7] as well as Che and Fricker.[6b] have observed the loss of trimethylamine from this charge tag. Remarkably, although the charge is supposed to be N-terminal, signals from immonium ions,[8] y ions and even some ions 14 Da heavier than regular y ions (y + 14 ions) were still observed in the spectrum. These ions must be protonated, despite there being no mobile proton in the charge-tagged peptide. Observation of y + 14 ions is particularly remarkable. We observed similar results with derivatized peptides GSFGSAIR and FVDGSIR, indicating that these charge-tagged peptides routinely undergo unexpected complex fragmentation processes.

[*] Y. He, Prof. J. P. Reilly
Department of Chemistry
Indiana University
800 E. Kirkwood Ave., Bloomington, IN 47405 (USA)
Fax: (+1) 812-855-8300
E-mail: reilly@indiana.edu
Homepage: http://www.indiana.edu/~reillyjp/index.htm

[**] This work was supported by the National Science Foundation, grants CHE-0518234 and CHE-0431991, and by the Indiana METACyt Institute of Indiana University, founded in part through a major grant from the Lilly Endowment, Inc. We thank Prof. Krishnan Raghavachari for performing the theoretical calculations.

To explain all of these unusual results, we propose that during the collisional dissociation process, decomposition of this charge tag is initiated by an electron pair on the nearby oxygen (Figure 2a) or nitrogen (Figure 2b) atom, releasing a neutral trimethylamine (loss of 59 Da). In either case, a cyclic structure would be generated, neutral trimethylamine eliminated and the peptide's N-terminal nitrogen center protonated. What is particularly significant about this process is that the proton on the nitrogen should now be mobile.

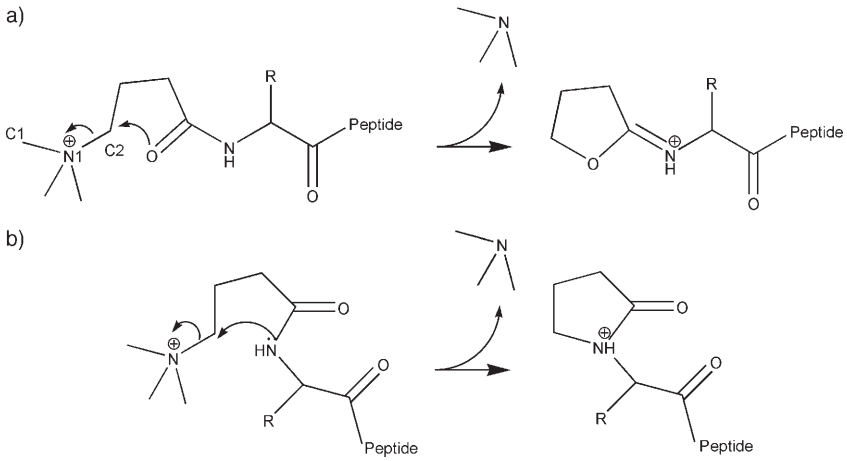

Figure 2. Proposed mechanisms associated with the loss of trimethylamine and production of a mobile proton by a) nucleophilic attack by oxygen b) nucleophilic attack by nitrogen.

To understand the relative stabilities of the two species, theoretical studies were performed using density functional theory (DFT) with the standard B3LYP hybrid functional.[9] We utilized the 6-31G(d,p) basis set[10] (containing d-type polarization functions on C, N, and O, and p-polarization functions on H) to optimize the molecular geometries and obtain relative energies. Both structures were confirmed to be local minima by a vibrational analysis. The structure shown in Figure 2a is calculated to be more stable than the one in Figure 2b by 13 kcal mol^{-1}. To confirm the DFT results, additional geometry optimizations were carried out at the MP2/6-31G(d,p) level.[10] Again, the same energy ordering was obtained, though the energy difference was computed to be smaller (6 kcal mol^{-1}). All calculations were performed with the Gaussian-03 program suite.[11]

If either of the protonated structures shown in Figure 2 were formed, the appearance of y ions in the spectrum of the TMAB-derivatized peptide would be understandable. A mobile proton presumably would move to an arginine residue if one was present, where it would become sequestered. The subsequent fragmentation would follow a charge-remote mechanism and some y ions would be formed.

The series of $y+14$ ions must be formed by another fragmentation process. To investigate their origin and structures, y_9 (FLAEGGGVR) and y_9+14 ions generated from the CID fragmentation of labeled Fibrinopeptide A were isolated in the LTQ ion trap. These ions were collisionally dissociated and the resulting spectra are displayed in Figure 3a and b. The two spectra appear very similar, except for the 14 Da shift of the y ions. More importantly, the b_8 ions and b_8-H_2O ions are unshifted. These observations imply that the 14 Da mass increment is associated with the C-terminal arginine residue. A 14 Da mass increment would be expected if the proton were replaced by a methyl cation. It has already been demonstrated that the N1–C2 bond in the tag can be cleaved in the CID process, releasing trimethylamine (Figure 2a). It should be energetically feasible to cleave the N1–C1 bond and generate a methyl cation. Therefore, we believe that during the decomposition of the charge tag, a methyl cation is produced and then transferred to the C-terminus of the peptide. Under this circumstance, a $y+14$ ion can form.

Protonated structures are also helpful in understanding the formation of immonium ions from charge tagged peptides. Mobile protons are required to generate immonium ions. To confirm this, we compared the performance of two different charge tags TMAB and TMPP-Ac. TMPP-Ac introduces a phosphonium ion at the N-terminus of the peptide. Decomposition of this tag leading to a series of unique fragments (*m*/*z* 181, *m*/*z* 533 and *m*/*z* 573)[12] has been reported. However, no evidence was observed for the production of a proton. The low mass ranges of TOF/TOF spectra of TMAB-Fibrinopeptide A (TMAB-ADSGEGDFLAEGGGVR) and TMPP-Ac-Fibrinopeptide A (TMPP-Ac-ADSGEGDFLAEGGGVR) are displayed in Figure 4a and b. Many immonium ions were produced with the TMAB-derivatized peptide but not with the TMPP analogue despite the fact that both peptide ions had a permanent positive charge on their N-terminus precursors. These results indicate that the charge introduced by TMPP stays fixed at the N-terminus of the peptide and no proton is generated in the decomposition of the tag, while for

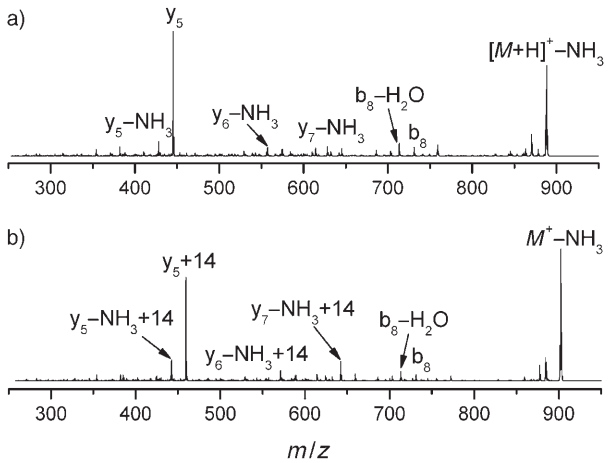

Figure 3. ESI-MS/MS spectra of a) y_9^+ (905.48 Da) and b) $[y_9+14]^+$ (919.47 Da).

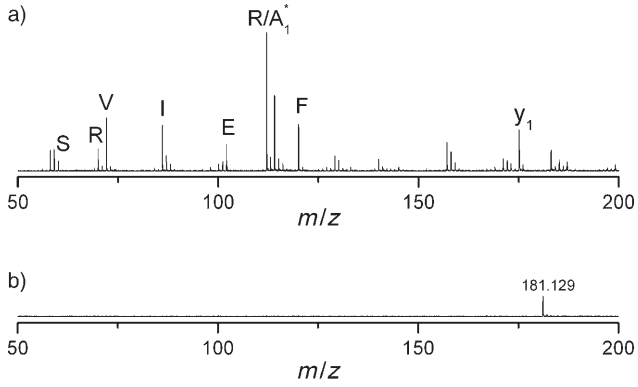

Figure 4. Immonium ions in CID spectra of singly charged a) TMAB-ADSGEGDFLAEGGGVR (1663.44 Da); b) TMPP-ADSGEGD-FLAEGGGVR (2108.85 Da).

the TMAB-derivatized peptide, the charge is no longer fixed and a mobile proton is generated.

In summary, this work has demonstrated that the fragmentation of TMAB-tagged peptides is considerably more complicated than was generally accepted. In most cases, the tag will undergo decomposition: part of the tag (trimethylamine) is lost during the CID process and a mobile proton is generated at the N-terminus. Under these circumstances, both N- and C-terminal fragment ions can be observed in the CID spectra. A comparison with TMPP-labeled peptides demonstrates that mobile protons are crucial for generating immonium ions. It is also observed that a methyl cation sometimes leaves the tag and migrates to a C-terminal arginine residue to generate $y+14$ ions.

Although our quaternary-ammonium charge tag does not effectively provide a fixed charge for peptide mass spectrometry, it might be envisioned that changing the carbon-chain length of the tag would affect the cyclization depicted in Figure 2 and thereby affect the stability of the label. However, Hines et al.[7] have found that immonium ions were still produced from a peptide with a longer tag. This result suggests that a mobile proton was still generated. In contrast, TMPP-labeled peptides appear to maintain the fixed charge after collisional activation. The only problem with TMPP is the low intensity of fragment ions which is due to the lack of the mobile proton. If charge-tagged peptides can be activated with more energy, this problem may be resolved.

Experimental Section

Synthesis of TMAB-NHS and labeled peptides has been reported elsewhere.[4a] TMPP-NHS was purchased from Sigma–Aldrich and labeled peptides are synthesized as described.[5c]

The labeled sample was diluted to 2 pmol μL^{-1} with H_2O, and then 0.5 μL of this solution was spotted on a MALDI target plate. When the spots were dry, 0.5 μL of matrix solution (10 mg mL^{-1} CHCA in 0.1% trifluoroacetic acid in 1:1 ACN(acetonitrile)/H_2O) was applied on top of it.

MALDI mass spectra were acquired using an Applied Biosystems 4800 proteomics analyzer (Applied Biosystems, Framingham, MA, USA) in the positive-ion mode.

ESI-MS experiments were conducted in positive ion mode using a Finnigan LTQ mass spectrometer (Thermo Electron, San Jose, CA, USA). All the samples were diluted to 2 pmol μL^{-1} with 1% acetic acid in 1:1 (ACN/H_2O). The sample solutions were infused into the mass spectrometer at a flow rate of 3 μL min^{-1} by the syringe pump. CID of precursor ions was accomplished by applying a resonant RF excitation waveform for 30 ms with activation, Q, of 0.25 and normalized collision energy of 35%.

Received: October 31, 2007
Published online: February 25, 2008

Keywords: charge tags · fragmentation mechanism · mass spectrometry · peptides

[1] V. H. Wysocki, G. Tsaprailis, L. L. Smith, L. A. Breci, *J. Mass Spectrom.* **2000**, *35*, 1399–1406.
[2] a) R. S. Johnson, S. A. Martin, K. Biemann, *Int. J Mass Spectrom Ion Processes* **1988**, *86*, 137–154; b) K. D. W. Roth, Z. Huang, N. Sadagopan, J. T. Watson, *Mass Spectrom. Rev.* **1998**, *17*, 255–274.
[3] a) M. L. Gross, *Int. J. Mass Spectrom Ion Processes* **1992**, *118/119*, 137–165; b) R. S. Johnson, S. A. Martin, K. Biemann, *Int. J. Mass Spectrom Ion Processes* **1988**, *86*, 137–154.
[4] a) F. Che, L. D. Fricker, *J. Mass Spectrom.* **2005**, *40*, 238–249; b) H. Mirzaei, F. Regnier, *Anal. Chem.* **2006**, *78*, 4175–4183.
[5] a) Z. Huang, J. Wu, K. Roth, Y. Yang, D. Gage, J. Watson, *Anal. Chem.* **1997**, *69*, 137–144; b) T. Shen, Z. Huang, M. Laivenieks, J. G. Zeikus, D. Gage, J. Allison, *J. Mass Spectrom.* **1999**, *34*, 1154–1165; c) W. Chen, P. J. Lee, H. Shion, N. Ellor, J. C. Gebler, *Anal. Chem.* **2007**, *79*, 1583–1590.
[6] a) M. Bartlet-Jones, W. A. Jeffery, H. F. Hansen, D. J. C. Pappin, *Rapid Commun. Mass Spectrom.* **1994**, *8*, 737–742; b) F. Che, L. D. Fricker, *J. Mass Spectrom.* **2005**, *40*, 238–249; c) B. Spengler, F. Luetzenkirchen, S. Metzger, P. Chaurand, R. Kaufmann, W. Jeffery, M. Bartlet-Jones, D. J. C. Pappin, *Int. J. Mass Spectrom Ion Processes* **1997**, *169/170*, 127–140.
[7] W. Hines, J. Peltier, F. Hsieh, S. A. Martin, *In 43rd ASMS Conf Mass Spectrom Allied Topics Atlanta*, **1995**, p. 387.
[8] a) A. M. Falick, W. M. Hines, K. F. Medzihradszky, M. A. Baldwin, B. W. Gibson, *J. Am. Soc. Mass Spectrom.* **1993**, *4*, 882–893; b) K. Ambihapathy, T. Yalcin, H.-W. Leung, A. G. Harrison, *J. Mass Spectrom.* **1997**, *32*, 209–215.
[9] a) A. D. J. Becke, *Chem. Phys.* **1993**, *98*, 5648–5652; b) C. T. Lee, W. T. Yang, R. G. Parr, *Phys. Rev. B* **1988**, *37*, 785–789.
[10] J. B. Foresman, Frisch, A. "Exploring Chemistry With Electronic Structure Methods", Gaussian Inc., Pittsburgh, PA **1996**.
[11] M. J. Frisch, G. W. Trucks, H. B. Schlegel, GAUSSIAN 03, Revision B.05, Gaussian Inc., Pittsburgh, PA, **2003**.
[12] Z.-H. Huang, T. Shen, J. Wu, D. A. Gage, J. T. Watson, *Anal. Biochem.* **1999**, *268*, 305–317.

