On the Existence of Structurally Different Isobaric B_n Fragment Ions

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INTRODUCTION

In 2001, we reported the existence of structurally different isobaric b fragments for WHWLQL [1]. Recently, we reported on the details of the hydrogen/deuterium (H/D) exchange reactions for structurally different isobaric singly- [2] and doubly-charged b_n fragment ions [3] of various peptides during gas-phase fragmentation. H/D exchange ion-molecule reactions were used to demonstrate the presence of at least two different isomers/conformers reactions [2]. In other words, H/D exchange reactions of isolated single isotopes ($^{12}C_{all}$) of b_n fragment ions yielded bimodal distribution patterns and allowed for observation of the two resolved ion populations. Production of structurally different b_n fragment ions can play a major role on peptide/protein fragmentation and sequencing (*viz.*, sequence scrambling [4]). Presently several research groups around the world, recognizing the importance of our initial observations and the significance of different b type fragment ions in peptide/protein sequencing, are performing amino acid residue dependant and other systematic studies to explore the details of such fragmentation mechanism(s). In this study, for the first time, we report the influence of other structural (*e.g.*, N-terminus acetylation) and experimental parameters (*e.g.*, ion fragmentation energetics) on the production of structurally different b fragment ions; we also report on the use of peptide ion gas phase basicity (GB) measurements in this context. Based on our findings, *we propose the use of standard peptides and b fragment ions (as molecular/ionic thermometers) to reduce potential inter-laboratory experimental variations and enhance meaningful data comparisons.*

GOALS

Our goal is to utilize (I) gas-phase proton transfer reactions, (II) collision induced dissociation (CID), and electron capture dissociation (ECD) to examine the existence of isobaric singly- and doubly-charged b_n fragment ions. We are interested in the mechanism(s) and pathways involved in the formation of different isobaric b fragment ions. Here, we explore the influence of structural variations (*e.g.*, effect of N-terminus acetylation of model peptides) and experimental conditions on the appearance or disappearance of structurally different b fragment ions.

EXPERIMENTAL

Instrumentation: **ESI/FT-ICR mass spectra were acquired with an FT-ICR mass spectrometer equipped with an open-ended cylindrical Penning trap (former IonSpec Corp. - now a division of Varian, Inc., Lake Forest, CA) and a 9.4 tesla superconducting magnet (Cryomagnetics Inc., Oak Ridge, TN).**

Ions were generated externally using an Analytica electrospray source (Analytica of Branford Inc., Branford, CT) equipped with in-house built spraying setups. Peptide fragment ions were generated using nozzle-skimmer fragmentation technique. A pulsed-leak valve was used for controlled introduction of the reagent gas (for GB measurements and/or H/D exchange reactions) into the ICR cell. Stored waveform inverse FT (SWIFT) techniques were utilized for ion isolations [5].

Sample Preparation: **All of the chemical solvents and reagents, and peptides were purchased from commercial sources and used without any further purifications except for applying conventional freeze-dry cycles as deemed necessary (*e.g.*, for reagents used in proton transfer reactions). Micromolar concentration of individual or mixture of peptide solutions in methanol: water: acetic acid (49.5: 49.5: 1) were used for the ESI experiments.**

Data Analysis: **Normalized ion intensities were calculated from $I_i / \sum I_i$ where I is the ion intensity (*i.e.*, $^{12}C_{all}$ or D_i peak intensity) and $\sum I_i$ is the total ion intensity (*i.e.*, $I_{do} + I_{d1} + I_{d2} + ...$ in HDX and $I_{12Call} + I_{contaminant-related}$ in proton transfer reactions).**

To calculate the rate constants, an exponential curve fitting (using Origin 7.0 (OriginLab Corporation, MA)) was performed to extract the time constants (τ) for "fast" and "slow" populations. Then the rate constants were estimated using the following equation:

$k = 1 / (\tau \times [N])$

k: rate constant ($cm^3\ Mol^{-1}\ s^{-1}$)

τ: time constant (s^{-1})

[N]: number density of the neutral reagent ($Mol\ cm^{-3}$)

RESULTS and DISCUSSION

(a) Proton Affinities of Different Structures of b_{10}^{2+} Fragment ion

b_{10}^{2+} + DIPEA → b_{10}^{+} + [DIPEA + H]$^+$

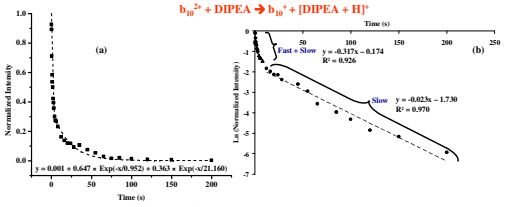

Table 1. CID products of b_{10}^{2+} fragment ion.

Fragment type	Rate Constant ($cm^3\ Mol^{-1}\ s^{-1}$)	GB (kcal mol^{-1})
b_{10}^{+2} (fast)	4.1 (± 0.3) × 10^{-10}	229.29 (± 0.06)
b_{10}^{+2} (slow)	1.8 (± 0.4) × 10^{-11}	227.30 (± 0.13)

Figure 2. **(a) Temporal and (b) semi-log plots of normalized ion intensity *vs.* proton transfer reaction time for the disappearance of $[b_{10}(^{12}C_{all})]^{+2}$. Neutral reagent: diisopropylethylamine (DIPEA) {PA ≈ 237.6 kcal/mol}, P(DIPEA) = 7.5 × 10^{-8} torr. The Calculated rate constants and gas phase basicity of fast and slow proton transferring populations are shown in Table 1.**

(b) Collision Induced Dissociation (CID) Mass Spectrum of b_{10}^{2+} Fragment ion

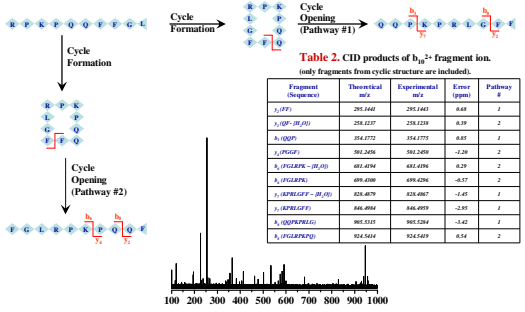

Table 2. CID products of b_{10}^{2+} fragment ion.
(only fragments from cyclic structure are included).

Fragment (Sequence)	Theoretical m/z	Experimental m/z	Error (ppm)	Pathway #
y_1 (FF)	295.1641	295.1641	0.68	1
y_2 (QF - $[H_2O]$)	258.1237	258.1238	0.39	2
b_2 (QP)	354.1772	354.1773	0.85	1
y_2 (PGF)	501.2456	501.2459	-1.20	2
b_4 (FGLRPK - $[H_2O]$)	681.4194	681.4196	0.29	2
b_4 (FGLRPK)	699.4300	699.4296	-0.57	2
b_5 (RPRLGFF - $[H_2O]$)	828.4879	828.4867	-1.45	1
b_5 (RPRLGFF)	846.4984	846.4959	-2.95	1
b_7 (QPKPRLG)	905.5315	905.5284	-3.42	1
b_8 (FGLRPKPQ)	924.5414	924.5419	0.54	2

Figure 2. **CID/FT-ICR mass spectrum of b_{10}^{2+} fragment ion illustrating the indirect fragment ion formation from cyclic structure {assignments of the fragments (with theoretical and experimental m/z) are shown in Table 2}.**

Note: The CID spectrum in Figure 2 shows the potential for cyclic structure formation for b_{10}^{2+} fragment ions and *"sequence scrambling"* in subsequent fragmentation (*e.g.*, MS^3).

(c) Electron Capture Dissociation (ECD) Mass Spectrum of b_{10}^{2+} Fragment ion

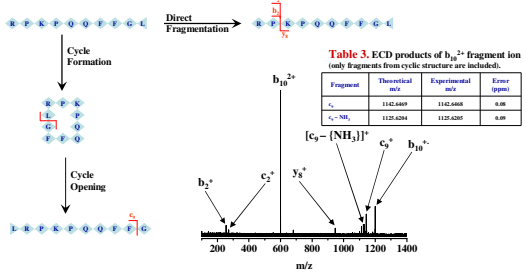

Table 3. ECD products of b_{10}^{2+} fragment ion
(only fragments from cyclic structure are included).

Fragment	Theoretical m/z	Experimental m/z	Error (ppm)
c_9	1142.6469	1142.6468	0.08
$c_9 - NH_3$	1125.6204	1125.6205	0.09

Figure 3. **ECD/FT-ICR mass spectrum of b_{10}^{2+} fragment ion showing the direct and indirect fragment ion formation from cyclic and linear structures of b_{10}^{2+}, respectively {assignments of the fragments (with theoretical and experimental m/z) are shown in Table 3}.**

Note: The ECD spectrum in Figure 3 shows the evidence for potential cyclic b_{10}^{2+} structure formation.

(d) Possibility for interconversion between "Fast" and "Slow" b_n fragment ions

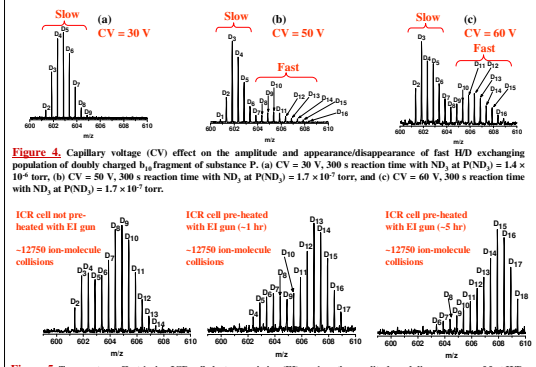

Figure 4. **Capillary voltage (CV) effect on the amplitude and appearance/disappearance of fast H/D exchanging population of doubly charged b_{10} fragment of substance P. (a) CV = 30 V, 300 s reaction time with ND_3 at $P(ND_3) = 1.4 \times 10^{-6}$ torr, (b) CV = 50 V, 300 s reaction time with ND_3 at $P(ND_3) = 1.7 \times 10^{-7}$ torr, and (c) CV = 60 V, 300 s reaction time with ND_3 at $P(ND_3) = 1.7 \times 10^{-7}$ torr.**

Figure 5. **Temperature effect {using ICR cell electron emission (EI) gun} on the amplitude and disappearance of fast H/D exchanging population of doubly charged b_{10} fragment of substance P. (a) EI gun "off", (b) EI gun "on" ~1 hr, and (c) EI gun "on" ~5 hr. $^{12}C_{all}$ isotopic peak of b_{10}^{2+} was SWIFT isolated and then reacted with ND_3 for 500 s at $P(ND_3) \sim 5.0 \times 10^{-7}$ torr.**

(e) Effect of Peptide N-terminus Acetylation on the b-type fragment Structure

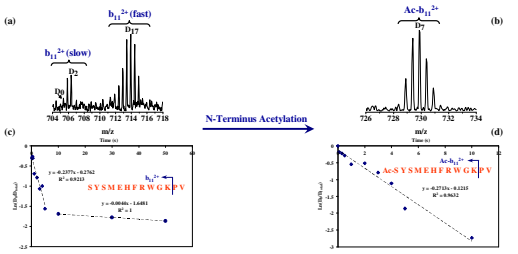

Figure 6. **ESI/FT-ICR mass spectrum of SWIFT-isolated (a) b_{11}^{2+} of des-Ac-α-Melanocyte and (b) acetylated b_{11}^{2+} of α-Melanocyte {*i.e.*, Ac-b_{11}^{2+}} after 300 s H/D exchange with ND_3 at $P(ND_3) = 1.1 \times 10^{-7}$ torr. The semi-log plots for disappearance of D_0 ion population of b_{11}^{2+} and Ac-b_{11}^{2+} are shown in Figures 1c and 1d, respectively.**

Note: While b_{11}^{2+} fragment ion shows at least two structures with different reactivities in H/D exchange reaction, Ac-b_{11}^{2+} fragment ion shows a single structure. The results in Figure 6 suggest that N-terminus acetylation potentially prevents the cyclization of b_{11}^{2+} fragment ion.

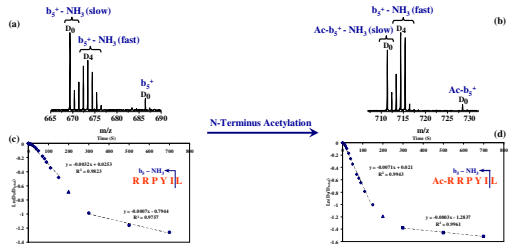

Figure 7. **ESI/FT-ICR mass spectrum of SWIFT-isolated (a) b_5^+ and b_5^+ - NH_3 of Neurotensin (b) N-terminus acetylated b_5^+ {*i.e.*, Ac-b_5^+} and b_5^+ - NH_3 {*i.e.*, Ac-b_5^+ - NH_3} of Ac-Neurotensin after 700 s H/D exchange with ND_3 at $P(ND_3) = 1.5 \times 10^{-7}$ torr. The semi-log plots for disappearance of D_0 ion population of b_5^+ - NH_3 and Ac-b_5^+ - NH_3 are shown in Figures 2c and 2d, respectively.**

Note: While niether b_5^+ nor Ac-b_5^+ fragment ions show H/D exchange reactivity, loss of NH_3 from both fragments (*i.e.*, b_5^+ - NH_3 and Ac-b_5^+ - NH_3) activates H/D exchange reaction channels.

CONCLUSIONS

- ✓ **Experimental gas phase basicity (GB) values for structurally different isobaric b_{10}^{2+} fragment ions of substance P showed ~2 kcal/mol GB difference.**
- ✓ **CID and ECD results suggest the presence of a cyclic structure for b_{10}^{2+} fragment ion of substance P.**
- ✓ **The preliminary results suggest that the relative abundances of "fast"- and "slow"-H/D exchanging ion populations can be controlled in the ion source (*i.e.*, metal capillary voltage effect) or in the ICR cell (*i.e.*, temperature effect). Although the influence of temperature variations on H/D exchange rate must be studied in detail to account for its contribution.**
- ✓ **N-terminus acetylation of des-Ac-α-melanocyte prevented the formation of "fast" exchanging b_{11}^{2+} ion population, contrary to the present of two structures for the non-acetylated b_{11}^{2+} fragment.**
- ✓ **Neither b_5^+ nor Ac-b_5^+ fragment ions of neurotensin show H/D exchange reactivities; however, b_5^+ - NH_3 and Ac-b_5^+ - NH_3 yield at least two structures with different H/D exchange reactivities/patterns.**
- ✓ **The presence of different isomer/conformer b fragment ions is not limited to small peptides. This is a significant finding and has very important consequences in all proteomics studies and gas-phase protein sequencing. To reduce experimental variabilities, we propose the use of standard peptides and b fragment ions (as molecular/ionic thermometers) in all systematic studies involving the formation of different b fragment ions.**
- ✓ **Future experimental and theoretical calculations should provide additional details on the influence of various thermochemical parameters on fragmentation mechanisms.**

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