FOCUS: MS/MS PEPTIDE IDENTIFICATION: RESEARCH ARTICLE

Relative Stability of Peptide Sequence Ions Generated by Tandem Mass Spectrometry

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Abstract

We report the use of unimolecular dissociation by infrared radiation for gaseous multiphoton energy transfer to determine relative activation energy ($E_{a,laser}$) for dissociation of peptide sequence ions. The sequence ions of interest are mass-isolated; the entire ion cloud is then irradiated with a continuous wave CO₂ laser, and the first order rate constant, k_d , is determined for each of a series of laser powers. Provided these conditions are met, a plot of the natural logarithm of k_d versus the natural logarithm of laser power yields a straight line, whose slope provides a measure of $E_{a,laser}$. This method reproduces the E_a values from blackbody radiative dissociation (BIRD) for the comparatively large, singly and doubly protonated bradykinin ions (nominally y_9 and y_9^{2+}). The comparatively small sequence ion systems produce $E_{a,laser}$ values that are systematic underestimates of theoretical barriers calculated with density functional theory (DFT). However, the relative $E_{a,laser}$ values are in qualitative agreement with the mobile proton model and available theory. Additionally, novel protonated cyclic-dipeptide (diketopiperazine) fragmentation reactions are analyzed with DFT. FT-ICR MS provides access to sequence ions generated by electron capture dissociation, infrared multiphoton dissociation, and collisional activation methods (i.e., b_n , y_m , c_n , z_m^+ ions).

Key words: Peptide fragmentation, Ion structure, IRMPD, Fourier transform, Ion cyclotron resonance, ICR, FT-ICR, FTMS

Introduction

Product ions formed by tandem mass spectrometry (MS/MS or MS²) provide the basis for peptide sequencing, sequence algorithms, and ion structure determination. Once formed, the most stable sequence-informative ions are most likely to be detected, and thus contribute to the sequence assignment of the peptide/protein under investigation. Conversely, labile sequence-informative ions are more likely to experience secondary fragmentation, so are less likely to

be detected. Thus, it is important to establish the stability of MS/MS candidate sequence ions.

Gas-phase ion stability is ultimately determined by ion structure and the method of formation. Consequently, the gas-phase ion structures that make up each class of sequence-informative ion (i.e., b_n , y_m , c_n , z_m^+ ions from an N-residue peptide in which $N=n+m$) coupled with the distribution of energies within the ion population determine which ions are detected and thus influence sequence assignment. Concomitant instrumental discrimination (activation and/or ionization method, reaction time scale, detection efficiency, etc.) also affects the resulting mass spectrum [1].

Ion structure has been investigated by IR “action” spectroscopy [2–7], gas-phase hydrogen/deuterium exchange [8–11], neutralization-reionization experiments [12],

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and theory [2–7, 11–13]. These combined methods have improved our understanding of the ion structure(s) in a mass spectrometer. Precursor protonated peptides [14–17], proteins [18], and sequence ions produced in collision-induced dissociation experiments [2–7] have been investigated.

y_m ions have been shown to behave indistinguishably from linear protonated peptides with the same sequence and charge state [9, 19–21], in agreement with theory [22–24]. Thus, the two kinds of ions have identical linear, protonated peptide structure and, therefore, identical gas-phase chemistry. This finding enables direct comparison between precursor protonated peptides (nominally y_N) and those y_m ions generated in the MS² experiment.

In contrast, the complementary b_n and a_n ions adopt quite different structures depending on the amino acid sequence, ion size (n), and fragmentation conditions [2–11]. Most small b_n ions ($n=2, 3$) have a five-membered oxazolone ring at the C-terminus [3, 5, 7, 11, 25], although some b_2 ions containing histidine, generated from non-tryptic peptides, are a mixture of protonated oxazolone and oxygen-protonated diketopiperazine structures (six-membered rings) [26]. Larger b_n ions ($n \geq 4$) show an increasing tendency (with n) to rearrange and adopt carbonyl-oxygen protonated macrocyclic structures in the gas phase [10, 13, 27–29]. a_n ions adopt a mixture of cyclic and linear forms [4, 6, 30]. Unlike product ions generated by collisional activation, electron capture/transfer dissociation product ions (c_n and z_m^+) have received far less attention [31], presumably because of the difficulty in integrating those methods into existent instrumentation at free electron laser facilities. None of these useful methods provides any experimental determination of the energetic barrier to fragmentation of the analyte ion. In this work, we present preliminary measurements of the relative activation energy of gas-phase sequence ion unimolecular dissociation by infrared multiphoton energy transfer [32, 33], in an attempt to provide relative fragmentation barriers [32–34] for comparison with the putative ion structures and theory. Specifically, we seek to (1) determine if this technique is viable for sequence ions generated via CID, ECD, IRMPD, or any combination of those approaches; (2) establish if this approach is sensitive to ion type, charge state, and composition; (3) more narrowly, are predictable differences present within particular ion types [e.g., does the b_2 (YG oxazolone structure) ion have a lower E_a than the b_5 (YGGFL cyclic peptide structure from theory and experiment)? A higher E_a than the analogous YI b_2 ion?]; (4) to evaluate the correlation between the limited quantum calculations of sequence ion transition structures and the relative E_a values determined with this technique. Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) [35, 36] provides the ultrahigh vacuum and stable trapping conditions necessary to perform these experiments. Moreover, our instrument is equipped for electron capture dissociation (ECD) [37], which enables investigation of c_n and z_m^+ sequence ions.

Theory

Our approach is a modification of an earlier technique [32, 33], based on the work of Dunbar [38]. Dunbar showed that following a short induction period (<1 s), monochromatic infrared (IR) laser irradiation [with a continuous wave (CW) CO₂ laser in this case] can closely approximate the effect of steady-state blackbody irradiation, especially if the molecule has a strongly absorbing vibrational mode near the laser frequency [39]. The approach relies on “bathing” the analyte ion cloud in photons continuously (Figure 1), to produce an internal energy distribution that is almost indistinguishable from a Boltzmann distribution [32, 33, 38, 39].

Following analyte ion isolation [40, 41] in the ICR cell, the entire analyte ion cloud is irradiated with a continuous wave CO₂ (10.6 μ) laser. The slope of a plot of $\log_e(\text{precursor ion mass spectral peak relative magnitude})$ versus irradiation period yields a unimolecular dissociation rate constant (k_d) for each laser power. The slope of a plot of $\log_e(k_d)$ versus $\log_e(\text{laser power})$ provides a measure of relative activation energy, $E_{a,\text{laser}}$ [32, 33, 38], required to fragment each analyte ion according to Equation (1):

$$E_{a,\text{laser}} = qh\nu(\partial \ln(k_d)/\partial \ln(I_{\text{laser}})) \quad (1)$$

in which q is the partition function for the fundamental vibrational mode that absorbs the incoming radiation, h is Planck’s constant, ν is the frequency of the normal mode absorbing the incoming radiation, and I_{laser} is the laser power [38]. In practice, q is fixed at 1.05 for all $E_{a,\text{laser}}$ calculations because over the expected range of internal energy (300–580 K), the partition function, q , for a single vibrational mode at 943.4 cm^{−1} varies between 1.01 and 1.1 [32, 33].

This technique is related to blackbody induced radiative dissociation (BIRD) [42–44], in which a vacuum heated to a specified temperature acts as a blackbody source of IR photons [45], in which the ion population equilibrates. This

Figure 1. Top: Schematic presentation of the apparatus; bottom: ICR cell, showing trapping potentials and overlap between the analyte ion cloud and the infrared laser beam

method has been applied successfully by the Dunbar, McMahon, and Williams groups to determine threshold energies of clusters [6, 42, 43, 46] and biomolecules [44, 47, 48] but not sequence ions.

Equation (1) has been found to underestimate the value of E_a [32, 34, 49]. For large systems (proteins), it was initially attributed to multiple modes absorbing photons from the laser (rather than a single mode) [32] and that the *relative* values were correct. Williams and co-workers [49] argued that this approximation, namely, laser-heated ions emit only at the laser frequency (943.4 cm^{-1} in this case), was the root cause of the underestimation; the emission in fact occurs over a wide frequency range. The model predicts that “unimolecular dissociation rate constants depend significantly on vibrational transitions not at the laser frequency” and proposes correction factors to the original equation [49]. Equation (2) illustrates the result that “does not depend on the absolute laser power” [49].

$$E_{a,\text{laser}}/sk_B = (\partial \ln(k_d)/\partial \ln(I_{\text{laser}})) \quad (2)$$

in which k_B is the Boltzmann constant and s is a proportionality factor that depends on the relative intensities of an analyte ion's vibrational transitions. Furthermore, for a given class of analyte ion (polymer), the frequency and relative intensity distributions should change very little as the number of modes varies, so that s should be essentially the same for entire classes of molecules [49]. A value of 4.8 was determined for $(sk_B)^{-1}$ from a selection of peptides and proteins (standard deviation was $<4\%$) [49, 50]. Here we test to see if the same proportionality factor for (the comparatively small) sequence ions matches *ab initio* calculations (i.e., Equation (2) is used for all comparisons).

Experimental

Sample Preparation

Standard peptides were purchased from Sigma Aldrich (St. Louis, MO, USA) (RPPGFSPFR, AAAAA, and RPKPQQFFGLM-NH₂) or American Peptide Company (Sunnyvale, CA) (YGGFL-NH₂, YGGFLR, DSDPR, YIGSR, and GGYR), and used as received. We chose peptides for which structures of likely sequence ions had previously been investigated by spectroscopy, MS/MS, HDX, and/or theoretical calculations [e.g., YIGSR (b_2, y_3, y_5^{2+}), YGGFLR (b_2, y_4, y_6^{2+}), DSDPR (b_2, b_3, y_3, y_5^{2+}), AAAAA (b_3, b_4, y_5), YGGFL-NH₂ (b_5)]. Prior experience and the literature [51–53] also guided our choices (cyclo-FL, Cyclo-WL, GGYR, YGGFL-NH₂, RPPGFSPFR, and RPKPQQFFGLM-NH₂). The experiments were performed with a custom-built 9.4 T ESI FT-ICR mass spectrometer recently described in detail [54]. Samples were infused into a tapered 50 μm i.d. fused silica micro-ESI needle [55, 56] at a rate of 300–500 nL min⁻¹ at a concentration of $\sim 10\text{ }\mu\text{M}$. ESI conditions were needle voltage 2 kV and heated capillary current 4.0 A. 10 V was applied to the end cap and

compensation electrodes of the ICR cell to minimize the axial extent of the ion cloud [57]. The analyte ions were isolated by stored waveform inverse Fourier transform (SWIFT) [40, 41] mass-selective ion ejection, and irradiated with a Synrad (Mukilteo, WA) CW CO₂ laser ($\lambda=10.6\text{ }\mu\text{m}$).

The laser is mounted off-axis ($\sim 3.5^\circ$) due to the need to accommodate an axially-mounted electron capture dissociation cathode [58]. The laser beam diameter was taken as the factory-reported value of 3.5 mm. To ensure complete irradiation of the ion cloud throughout the course of the experiment, the laser beam diameter was expanded to $\sim 9\text{ mm}$ by means of a Synrad 2.5-fold beam expander (Mukilteo, WA, USA). Careful alignment produced linear plots of $\log_e(\text{precursor ion mass spectral peak relative magnitude})$ versus irradiation period, from which a unimolecular dissociation rate constant, k_d , was determined for each of several laser intensities. Precursor ions of various m/z were completely eliminated under extended irradiation periods. Precursor ion mass spectral peak relative magnitude is defined as the ratio [precursor ion magnitude/(sum of precursor and fragment ion magnitudes)]. The fragment ion signal is used to correct for any fluctuation in ion current over the course of the experiment. Typical (uncorrected) base pressure for the instrument was $\leq 2 \times 10^{-10}$ Torr, measured by a Bayard-Alpert ionization gauge.

Instrument control, data acquisition, and data analysis were carried out with a modular ICR data station [59]. Data acquisition was automated by use of a tool command language script [60]. A typical experiment (consisting of eight different irradiation periods for each of eight different laser intensities) was completed in $\sim 3\text{--}5\text{ h}$. Each time-domain ICR signal (sum of 20 transients) was Hanning apodized [61], zero-filled once [61], and Fourier transformed to generate a magnitude-mode spectrum that is converted to mass-to-charge ratio by a two-term calibration equation [62].

The b_n and y_m ions were generated by either quadrupole collision-induced dissociation (Q-CID) or (in-cell) infrared multiphoton dissociation (IRMPD) prior to isolation. No significant difference was found between the slopes obtained from the small sequence ions generated by these approaches, indicating that the analytes were equivalently cooled/thermalized during the $\sim 0.25\text{ s}$ SWIFT isolation step prior to initiation of fragmentation. Consequently, either a single structure is formed or the structures formed by the two fragmentation techniques are energetically so similar as to make no measurable difference in $E_{a,\text{laser}}$. The conditions for generating the sequence ions with the laser were deliberately as gentle as possible (least laser power and shortest irradiation period), but sufficient to isolate the needed sequence ions for subsequent analysis.

As y_m ions are known to be linear protonated peptides; additional protonated peptides, y_N ions, were also examined for comparison. FT-ICR mass resolving power ($\sim 10^5$ at m/z 400) and mass accuracy ($<1\text{ ppm}$) enabled confident identification of the stoichiometry of the products of dissociation generated by unimolecular dissociation (despite

being far from optimal conditions). Electron capture dissociation was performed in multipass mode [63], with nominal electron energy of 0.5–5 eV (50–100 ms irradiation period). The ^{12}C peak was SWIFT-isolated prior to irradiation (i.e., no $z_{\text{m}}+1$ ions were inadvertently analyzed. A detailed summary of the events performed in each experiment is given provided below:

CID

Accumulate ions in first octopole; mass select precursor with quadrupole; transfer to second octopole to collide with collision gas; hold ions in second octopole (collisional cooling); transfer ions to the ICR cell; SWIFT isolate analyte m/z of choice; irradiate with CO_2 laser for a period, t ; excite ions to higher radius; detect.

IRMPD

Mass selective accumulation and cooling of ions in second octopole (i.e., by use of a mass-selective quadrupole); transfer ions to the ICR cell; irradiate ions for a short period; SWIFT isolate analyte m/z of choice; irradiate with CO_2 laser for period, t ; excite ions to higher radius; detect.

ECD

Mass-selectively accumulate and cool ions in second octopole (i.e., by use of a mass-selective quadrupole); transfer ions to the ICR cell; ECD; electron clean-up event; SWIFT isolate analyte m/z of choice; irradiate with CO_2 laser for period, t ; excite ions to higher radius; detect.

Calculations

Theoretical Calculations

Transition structures for the b_2 - a_2 reaction were calculated for the oxazolone b_2 ions with the sequences YG and YI (a_2 was the predominant and also first detected fragment ion). The initial minimum-energy structures were taken from earlier work [7, 11]. Due to the system size and considerable prior experience with these reactions, major molecular dynamics searches were not needed to generate the necessary candidate transition state (TS) geometries for subsequent TS optimization. Intrinsic reaction coordinate (IRC) calculations were run to identify the minimum energy path that connects the reactant and the product geometries, thereby ensuring that the structures are indeed the correct transition structures.

The b_5 - a_5 reaction was also calculated for the comparatively simple b_5 GGGGG sequence to provide a second point of comparison for the b_5 (YGGFL) sequence ion investigated experimentally. Unfortunately, calculating the full potential energy surface for this ion from scratch is

impractical due to at least 20 types of minima (10 oxazolone minima families; 10 macrocycle minima families) and >15 types of TS calculation required (at least 10 cyclization/re-opening TS families; at least 5 b_5 - a_5 reaction families). Our calculations were therefore based on initial geometries generated directly from manipulation of structures taken from the literature [27]. Additional structures generated by manipulation of structures from reference [64] (i.e., replacement of the A, Y, F, and L side chains with a hydrogen atom) produced identical, or less energetically favorable TSs following optimization. The lack of side chains other than the hydrogen atom greatly simplifies these calculations. IRC calculations were again performed.

The protonated diketopiperazine (cyclo-LF and cyclo-WL dipeptide) b_2 ion structures were calculated from initial geometries generated directly by adaptation of similar sequences and structures taken from prior work [7, 11]. Multiple conformers were optimized for the b_2 ion minima, transition structures, and product geometries. Prior experience guided selection of critical bond lengths and angles before TS optimization. The number of possible conformations is limited by the comparatively rigid six-membered ring and the bulk of the peptide side chains, significantly simplifying these calculations and rendering molecular dynamics unnecessary. IRC calculations were again run. Transition structure barriers determined for the cyclic- b_2 - a_2 reaction were consistent with prior unpublished work on other cyclic dipeptide systems (Bythell, Paizs, Harrison, unpublished work).

Multiple conformers of the z_2^+ (LR) ion minima were optimized. Transition structures for the loss of C_3H_7^+ with concomitant formation of the w_2 ion were calculated. IRC calculations were again run. All calculations were undertaken by use of density functional theory (DFT) at the B3LYP/6-31+g** level of theory, with zero-point energy corrections calculated at the same level of theory. The Gaussian suite of programs was used for all calculations [65]. The results are used in conjunction with literature values (calculated at the same level of theory) for comparison to experimental $E_{\text{a,laser}}$ values.

Results and Discussion

The slope of a plot of $\log_e(\text{precursor ion mass spectral peak relative magnitude})$ versus irradiation period provides a unimolecular dissociation rate constant (k_d) for each laser power [illustrated for a y_3^+ ion (sequence, GSR) in Figure 2]. The slope of a plot of $\log_e(k_d)$ versus $\log_e(\text{laser power})$ is also linear [illustrated for y_5^{2+} (YIGSR), b_2 (YI), and y_3 (GSR) in Figure 3]. The relative $E_{\text{a,laser}}$ values (and uncertainties) determined from Equation (2) and the pertinent slopes of a $\log_e(k_d)$ versus $\log_e(\text{laser power})$ plots are listed in Table 1. The barriers determined for singly and doubly protonated RPPGFSPFR are consistent with the earlier laser dissociation work of Williams and coworkers [49, 50]; 1.4 ± 0.15 versus 1.3; 0.93 ± 0.06 versus 0.9 (values in eV for direct comparison, 1 eV = 97 kJ mol $^{-1}$). These

Figure 2. Semilog plot of y_3^+ precursor ion (sequence, GSR) relative magnitude versus IR laser irradiation period for each of several laser powers

barriers are the same (within experimental uncertainties) as those determined by BIRD [48] for the same ions.

In the following, we initially discuss the results for the different classes of sequence ions. A more general discussion of the underestimation of $E_{a, \text{laser}}$ in comparison to the DFT calculations and its potential causes follows.

CID Fragments

b_n Ions

Of the six b_n ions examined to date, five are linear b_n ions ($n=2-4$) [3, 7, 11, 66] and one is a macrocyclic b_5 ion [27, 64]. A guide to b_n sequence ion fragmentation is provided in Scheme 1. Our calculations for the b_2 - a_2 fragmentation barrier for the oxazolone b_2 ion structure, supported by

spectroscopy [7], theory [7, 11], and gas-phase hydrogen-deuterium exchange [11] experiments, produce theoretical barriers ($\Delta E_{\text{el+ZPE}}$) of 130.1 kJ mol⁻¹ and 144.3 kJ mol⁻¹ for the YI and YG sequences, reflecting the increased stabilization of the YI b_2 - a_2 transition structure provided by the charge-donating isoleucine alkyl side chain, as opposed to the H atom of the YG analogue. An increasingly stabilized transition structure corresponds to a smaller barrier to fragmentation, thus a less stable ion.

Our experimental data reproduce the order of relative activation energies, but with $E_{a, \text{laser}}$ values of 75.2 ± 6.2 and 89.1 ± 11.6 kJ mol⁻¹ (Table 1), with overlapping 95% confidence intervals. The experimental values of 66.1 ± 4.8 and 76.0 ± 7.8 for the linear b_3 and b_4 (AAA and AAAA oxazolone) ions are similar to the b_2 ion experimental barriers, consistent with similar structure. The remaining b_3 ion (linear DSD sequence) also falls in that experimental range. Theory predicts [13, 27, 64] that these smaller, linear ions are able to fragment directly or at least with very minimal proton mobilization, whereas the energetically more stable macrocyclic b_5 ion requires major structural rearrangement to regenerate a linear form from which it can fragment (Scheme 1). Such structural rearrangements require energy [13, 27, 64], which is consequently reflected in higher measured $E_{a, \text{laser}}$; the b_5 - a_5 TSs calculated by Paizs and co-authors [64] for the linear permutations of the similar YAGFL sequence that ranged from 152.7 to 188.3 kJ mol⁻¹ with the AGFLY b_5 sequence providing the lowest barrier. That result is consistent with the logic of the largest charge-donating R group (tyrosine) providing additional stabilization to the b_2 - a_2 transition structure. For comparison, calculations for the b_5 - a_5 TS of the much simpler sequence, GGGGG, gave a barrier of 177.8 kJ mol⁻¹. A barrier closer to that of the YAGFL sequence would be expected for our YGGFL b_5 ion sequence.

The experimental value of 120.3 ± 17.0 kJ mol⁻¹ (b_5 initial sequence, YGGFL) is clearly distinct from the oxazolone b_n ions and, thus, generally agrees with the mechanistic proposal and theory (Scheme 1). To test this hypothesis further, we examined two smaller cyclic peptides experimentally and with density functional calculations. These protonated diketopiperazines [20]; protonated cyclo-FL and cyclo-WL are potential b_2 ions [52]. These ions are most energetically favorably protonated at the amide oxygen of the phenylalanine and tryptophan residues respectively (Scheme 2 for the protonated cyclo-FL, Scheme 3 for the protonated cyclo-WL). For cyclo-FL, the lowest energy fragmentation is loss of CO, the b_2 - a_2 pathway. The lowest energy pathway to this reaction is complicated and involves several proton transfers, rotational barriers, and transition structures of similar energy (Scheme 2). This produces the comparatively large theoretical barrier of 212.2 kJ mol⁻¹. The alternative pathway involves a direct four-center proton transfer [22, 23, 67] from the global minimum phenylalanine O-protonated structure to the adjacent leucine amide nitrogen to weaken the amide bond, thereby enabling

Figure 3. Log-log plot of dissociation constant versus laser power, for each of three precursor peptide ions

Table 1. Summary of Experimental and Calculated (where available) $E_{a,laser}$ Values a: Reference [66]; b: Reference [64] for YAGFL-NH₂, b_5 , and this Work; c: Reference [71]; d: Reference [11]. The Error from the Slope ($\pm$ kJ mol⁻¹ at the 95% Confidence Limit) is Indicated Following each $E_{a,laser}$ Value. Calc. Barrier values in parentheses are for analyte ions with similar sequence (YAGFL)

Peptide	Sequence Ion	Degrees of Freedom	$E_{a,laser}$ /kJ mol ⁻¹	R ²	Calc. Barrier/kJ mol ⁻¹
<i>b_n</i> ions					
[YIGSR+2H] ²⁺	<i>b</i> ₂	117	75.2 $\pm$ 6.2	0.993	130.1
[YGGFLR+2H] ²⁺	<i>b</i> ₂	81	89.1 $\pm$ 11.6	0.986	144.3
[DSDPR+2H] ²⁺	<i>b</i> ₃	111	72.0 $\pm$ 7.2	0.990	-
[AAAAA+H] ⁺	<i>b</i> ₃	87	66.1 $\pm$ 4.8	0.995	132.6 ^a
[AAAAA+H] ⁺	<i>b</i> ₄	117	76.0 $\pm$ 7.8	0.990	126.8 ^a
<i>Cyclic-b_n</i> ions					
[cyclo-(FL)+H] ⁺	cyclo- <i>b</i> ₂	114	136.6 $\pm$ 16.8	0.989	212.2
[cyclo-(LW)+H] ⁺	cyclo- <i>b</i> ₂	126	119.9 $\pm$ 17.0	0.985	169.7
[YGGFL-NH ₂ +H] ⁺	<i>b</i> ₅	219	120.3 $\pm$ 17.0	0.980	(152.7) ^b
<i>y_m</i> ions					
[GGYR+2H] ²⁺	<i>y</i> ₂	138	152.2 $\pm$ 24.1	0.979	-
[DSDPR+2H] ²⁺	<i>y</i> ₂	117	136.2 $\pm$ 4.7	0.999	-
[YIGSR+2H] ²⁺	<i>y</i> ₃	132	119.7 $\pm$ 17.3	0.982	-
[AAAAA+H] ⁺	<i>y</i> ₅	156	97.1 $\pm$ 7.1	0.995	135.6 ^c
[YGGFL-NH ₂ +H] ⁺	<i>y</i> ₅ amide	231	93.2 $\pm$ 11.2	0.993	(141.0) ^b
[RPPGFSPFR+H] ⁺	<i>y</i> ₉	447	137.5 $\pm$ 14.2	0.993	-
<i>y_m</i> ²⁺ ions					
[GGYR+2H] ²⁺	<i>y</i> ₄ ²⁺	183	114.5 $\pm$ 10.7	0.991	-
[YIGSR+2H] ²⁺	<i>y</i> ₅ ²⁺	252	88.4 $\pm$ 6.2	0.993	132.6 ^d
[DSDPR+2H] ²⁺	<i>y</i> ₅ ²⁺	234	87.0 $\pm$ 5.2	0.993	-
[YGGFLR+2H] ²⁺	<i>y</i> ₆ ²⁺	300	122.0 $\pm$ 10.4	0.991	-
[RPPGFSPFR+2H] ²⁺	<i>y</i> ₉ ²⁺	450	90.7 $\pm$ 5.9	0.993	-
<i>c_n</i> ions					
[RPPGFSPFR+2H] ²⁺	<i>c</i> ₄	183	149.3 $\pm$ 17.3	0.987	-
[RPKPQQFFGLM-NH ₂ +2H] ²⁺	<i>c</i> ₄	213	82.8 $\pm$ 6.6	0.994	-
[RPKPQQFFGLM-NH ₂ +2H] ²⁺	<i>c</i> ₅	264	95.5 $\pm$ 11.3	0.986	-
[RPKPQQFFGLM-NH ₂ +2H] ²⁺	<i>c</i> ₆	324	105.8 $\pm$ 12.4	0.989	-
<i>z_m</i> ⁺ ions					
[YGGFLR+2H] ²⁺	<i>z</i> ₂ ⁺	123	52.7 $\pm$ 6.9	0.987	99.1
[RPKPQQFFGLM-NH ₂ +2H] ²⁺	<i>z</i> ₉ ⁺	462	71.2 $\pm$ 9.2	0.984	-

subsequent amide bond cleavage and CO expulsion (Scheme S1, Supporting Information). This proton transfer barrier (226.5 kJ mol⁻¹ for protonated cyclo-FL; 230.3 kJ mol⁻¹ for protonated cyclo-WL) is rate-limiting for the *b*₂-*a*₂ reaction as the barrier is much higher than that of the subsequent amide bond cleavage (170.5 kJ mol⁻¹ for protonated cyclo-FL; 177.7 kJ mol⁻¹ for protonated cyclo-WL). That proton transfer barrier prevents this pathway (Scheme S1) from being competitive with the more complicated one (Scheme 2).

Experimentally, the protonated cyclo-FL ion produces a concomitantly large $E_{a,laser}$ value of 136.6 $\pm$ 16.8 kJ mol⁻¹. In contrast, the protonated cyclo-WL ion is able to fragment via a still energetically demanding, but lower energy residue-specific side-chain loss reaction (*m/z* 130.0651; C₉H₈N⁺) from the tryptophan residue (Scheme 3). This reaction comes with a concomitantly lower experimental $E_{a,laser}$ value of 119.9 $\pm$ 17.0 kJ mol⁻¹.

This significant energy difference for two very similar sequence ion structures illustrates the practical difficulty in making general statements of the “ion type 1 *always* has barriers of x kcal mol⁻¹” variety. Seemingly small structural changes can have a large impact on the E_a for fragmentation both in terms of barriers for individual reactions (e.g., *b_n*-*a_n*) and in terms of how competitive

different fragmentation pathways are. Obviously, any comparison with theory has to be with the energetically lowest barrier to be worthwhile.

y_m Ions

Singly protonated *y_m* ions containing one or more strongly basic arginine residues may be distinguished from those lacking a strongly basic residue (i.e., *y_m* ions with sequestered ionizing protons versus *y_m* ions with comparatively “mobile” protons [51, 68, 69]). Recent theoretical work shows clear energetic, mechanistic [67, 69], and dynamic [69, 70] differences between those two broad classes. Our experimental data support the “mobile proton model” [51, 68] rationale that more energy is necessary to fragment arginine-containing *y_m* ions than those lacking arginine ($E_{a,laser}$ 119.7–152.2 versus 93.2–97.1 kJ mol⁻¹). Although presence or absence of arginine does not solely determine *y_m* ion fragmentation threshold [e.g., water loss from serine in the *y*₃ ion, (GSR+H)⁺ is the most facile fragmentation pathway], our findings agree with Paizs and Suhai [71], who calculated the lowest fragmentation pathway of [A₅+H]⁺ (*y*₅) to require at least 135.6 kJ mol⁻¹ (experimental $E_{a,laser}$ = 97.1 $\pm$ 7.1 kJ mol⁻¹). In contrast, calculations for [G_nR+H]⁺ ions [69] indicate barriers of $\geq$ 161.9 kJ mol⁻¹ to

Scheme 1. **(a)** Summary of b_n - a_n fragmentation chemistry of small protonated oxazolone b_n ions; **(b)** Summary of b_n - a_n fragmentation chemistry of larger protonated oxazolone b_n ions ($n \geq 5$)

cleave amide bonds. In agreement with proton mobility arguments, the doubly protonated y_m^{2+} ions have generally lower relative $E_{a,\text{laser}}$, 87.0–122.0 kJ mol⁻¹ than the y_m ions, consistent with the theoretical barrier calculated for [YIGSR + 2H]²⁺ of 132.6 kJ mol⁻¹ [11] ($E_{a,\text{laser}}$, 88.4 ± 6.2 kJ mol⁻¹).

ECD Fragments

The potential utility and the inherent complications of applying this approach to ions formed via ECD (or ETD) ions will require further investigation.

Scheme 2. The most energetically favorable, protonated diketopiperazine (cyclo-FL) fragmentation pathway; the b_2 - a_2 reaction

Scheme 3. The most energetically favorable, protonated diketopiperazine (cyclo-LW) fragmentation pathway; loss of the W side-chain to produce m/z 130.0651

c_n and $z_m^\bullet$ Ions

The present c_n ions were compositionally similar, each having an N-terminal arginine residue and other basic residues (K, P). Although one would thus expect larger relative $E_{a,\text{laser}}$ values with presumably similar structures and fragmentation chemistries, the present results span a comparatively wide range ($82.8\text{--}149.3 \text{ kJ mol}^{-1}$), i.e., approaching the entire range spanned by all other $E_{a,\text{laser}}$ values. Thus, significant structural or residue specific chemistries may be involved. The problem is potentially complicated further by the issue of cooling of these ions in the ICR cell which at present has not been investigated in any detail (unlike the CID/IRMPD generated fragments). Comparisons with ECD/ETD generated and then collisionally cooled ions are planned. Additional experimental and theoretical data should help elucidate the reason(s).

Our current $z_m^\bullet$ ion data are confined to two systems: $z_0^\bullet$ from ECD of $[\text{RPKPQQFFGLM-NH}_2+2\text{H}]^{2+}$ and $z_2^\bullet$ from ECD of $[\text{YGGFLR}+2\text{H}]^{2+}$. Their $E_{a,\text{laser}}$ values (52.7 ± 6.9 and $71.2\pm 9.2 \text{ kJ mol}^{-1}$) are among the lowest obtained despite the presence of a basic residue in each case (K and R), potentially because of the open-shell nature of the radical cations enabling relatively facile electron movement and, therefore, lower bond cleavage barriers [72–74]. For example, radical initiated fragmentation from a radical cation is often substantially more feasible energetically than proton mobilization followed by fragmentation from a compositionally equivalent closed-shell system (with the same caveats as the preceding paragraph). This contention is supported by the calculated barrier for side-chain fragmentation of the $z_2^\bullet$ ion; transition structures for the loss of $\text{C}_3\text{H}_7^\bullet$ with concomitant formation of the w_2 ion (Scheme S2, Supporting Information) are the lowest calculated thus far at 99.1 kJ mol^{-1} .

Comparison of Sequence Ion $E_{a,\text{laser}}$ with Density Functional Theory

Equation (2) yields activation energies that are systematically lower than the DFT calculated barriers. A plot of experimental barrier versus DFT barrier using those data points for which we have a direct (DFT) barrier produces a R^2 value of 0.88. Forcing the intercept through zero reduces R^2 to 0.75 and yields a slope of 1.57. Ergo, a smaller value of $(sk_B)^{-1}$ is necessary to fit the experimental data to the calculated barriers from density functional theory; approximately 3, rather than 4.8.

Why does the present approach for the comparatively small systems investigated underestimate the values from theory? This is potentially due to one of, or a combination of several reasons. Williams notes that the laser produces a distribution slightly narrower than a Boltzmann internal energy distribution of temperature, T [49, 50]. The disparity is a little larger at higher laser power, and rate constants for a given laser power are progressively larger than they should be. This underestimation of the laser power necessary to bring molecules to an “effective” temperature T will result in an underestimation of the activation energy. The practical problem remains that estimation depends on the individual analyte and its rate of energy exchange [49].

A second source of the underestimation relative to Equation (2) is probably the decreased ability of smaller systems with less degrees of freedom to fully meet one of the assumptions inherent in Equation (2), namely, that the net absorption of the steady-state dissociating ion population is zero [49]. That assumption is valid in the rapid energy exchange limit, in which the rates of activation/deactivation are substantially greater than the rate of dissociation [49, 75]. Peptides have been described as having increased

“brightness” in the IR and, thus, greater rates of energy exchange than other types of molecule [76], enabling fewer degrees of freedom (by a factor of ≥ 3) for the rapid exchange limit to be accessed. Nevertheless, barriers are lower than from DFT theory, so some of the underestimation results from all/some of the ions not satisfying the rapid exchange limit. However, we have no obvious degrees of freedom effect for our data, so either it is a sample size issue, or the underestimation is fairly similar, or something else is responsible.

A third possible factor is the question of how effectively the DFT calculations agree with BIRD [i.e., the standard to which current fragmentation barriers (experimental and theoretical) for larger ions are compared]. A different yardstick is potentially necessary for DFT, but not easy to test, because most of the peptide systems studied with BIRD are enormous from a DFT calculation point of view. Anecdotally, BIRD provides the $[\text{YGGFL} + \text{H}]^+$ barrier as $106.7 \text{ kJ mol}^{-1}$ [48], which is substantially lower than barriers determined for the fragmentation of $[\text{AAAPA} + \text{H}]^+$ by DFT ($115.9 \text{ kJ mol}^{-1}$ [77] (same level of theory as used here) and the lowest barrier available in the literature for such calculations). That P-containing peptide has a well-established low barrier residue specific bond cleavage (for comparison $[\text{AAAAA} + \text{H}]^+$ is $135.6 \text{ kJ mol}^{-1}$ [71]) unlike YGGFL, but the barrier is still higher than that from BIRD. In contrast, recent guided ion beam studies are in good agreement with DFT theory [78, 79] but, again, for comparatively small systems. Bottom line: this issue is too complex to be resolved (or even discussed at length) in the present paper.

Practical Experimental Limitations

In the present instrument, the analyte ions and, consequently, the range of accessible relative activation energies is limited by the available laser power. Not all analyte ions examined could be fragmented to a sufficient extent in a reasonably available time. The most problematic analyte ions were medium-sized singly charged y_m ions with arginine at the C-terminus (e.g., y_4 sequences GGYR and GFLR). Those ions likely require more energy to fragment due to the lack of easily mobilizable protons [51, 68]. A minimum precursor ion signal-to-noise (S/N) ratio of approximately 100 following SWIFT isolation is desirable; greater S/N increases confidence and reduces the need for signal averaging.

Conclusions

Measurement of the relative activation energy for gas-phase peptide sequence ion unimolecular dissociation by infrared radiation for gaseous multiphoton energy transfer provides a useful guide to relative fragmentation thresholds in a collision-free environment. In its present implementation, the approach provides relative $E_{a, \text{laser}}$ values for sequence

ions generated by collisional-activated dissociation, IRMPD, and possibly ECD. Although not quantitative (consistently lower threshold energy than theoretical predictions), the current data agree qualitatively with the mobile proton model [51, 68] and its logical extrapolation to sequence ions.

Comparisons within ion types for which the likely range of absorption modes are most similar show differences based on charge state and constituent amino acids for y_m and c_n ions, and on structure for b_n ions. Preliminary $E_{a, \text{laser}}$ values for z_m^+ ions are low, possibly reflecting the comparative instability of open-shell radical cations. Additional experimental and theoretical work will establish the generality of these findings.

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