Deciphering the Peptide Iodination Code: Influence on Subsequent Gas-Phase Radical Generation with Photodissociation ESI-MS

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Iodination of tyrosine was recently discovered as a useful method for generating radical peptides via photodissociation of carbon–iodine bonds by an ultraviolet photon in the gas phase. The subsequent fragmentation behavior of the resulting odd-electron peptides is largely controlled by the radical. Although previous experiments have focused on mono-iodination of tyrosine, peptides and proteins can also be multiply iodinated. Tyrosine and, to a lesser extent, histidine can both be iodinated or doubly iodinated. The behavior of doubly iodinated residues is explored under conditions where the sites of iodination are carefully controlled. It is found that radical peptides generated by the loss of a single iodine from doubly iodinated tyrosine behave effectively identically to singly iodinated peptides. This suggests that the remaining iodine does not interfere with radical directed dissociation pathways. In contrast, the concerted loss of two iodines from doubly iodinated peptides yields substantially different results that suggest that radical recombination can occur. However, sequential activation can be used to generate multiple usable radicals in different steps of an MS^n experiment. Furthermore, it is demonstrated that in actual peptides, the rate of iodination for tyrosine versus mono-iodotyrosine cannot be predicted easily a priori. In other words, previous assumptions that mono-iodination of tyrosine is the rate-limiting step to the formation of doubly iodinated tyrosine are incorrect. (J Am Soc Mass Spectrom 2009, 20, 965–971) © 2009 American Society for Mass Spectrometry

Methods for iodinating proteins have been known since the late 1800s [1]. These reactions have found many uses including: radioactive labeling [2], heavy atom addition for diffraction phasing [3], and structural studies relying on differential reactivity [4], among many others [5–7]. Surprisingly, iodination in proteins occurs with a greater degree of selectivity than one might expect and preferentially occurs at tyrosine residues, which can be singly or doubly iodinated, as shown in Scheme 1. Histidine is the primary competing iodination site, but is typically much less reactive [1]. Some investigators have suggested that doubly iodinated tyrosine is the natural endpoint for the iodination reaction and that mono-iodinated tyrosine should represent only a briefly observed intermediate in most cases [8]. This assertion has been frequently repeated, although it is not universally accepted [9]. In either case, the issue has not been satisfactorily resolved to date. Importantly, the original experiments investigating this reaction were conducted on overly simplified systems before the development of powerful analytical tools capable of examining this issue within the context of more realistic peptide models.

Recent developments in the field of mass spectrometry have made the analysis of post-translational or chemical modifications of peptides and proteins much more easily achievable. Electrospray ionization (ESI) [10] or matrix-assisted laser desorption ionization (MALDI) [11] are routinely used to obtain desolvated biomolecular ions suitable for analysis by mass spectrometry (MS). A variety of tandem mass spectrometry (MS/MS) methods are available to interrogate ion structure in the gas phase, including collision-induced dissociation (CID) [12], electron capture dissociation (ECD) [13], electron-transfer dissociation (ETD) [14], and photodissociation (PD) [15, 16], among others [17, 18]. With various strengths and weaknesses, these techniques can be used to fragment peptides and reveal structural information such as sequence. Modifications that alter mass, such as iodination, can easily be identified and frequently pinpointed with residue-specific accuracy (particularly if high throughput is not required).

Iodination has recently been implemented in MS as a means for installing photolabile iodotyrosine radical precursors into peptides and proteins [19, 20]. In these

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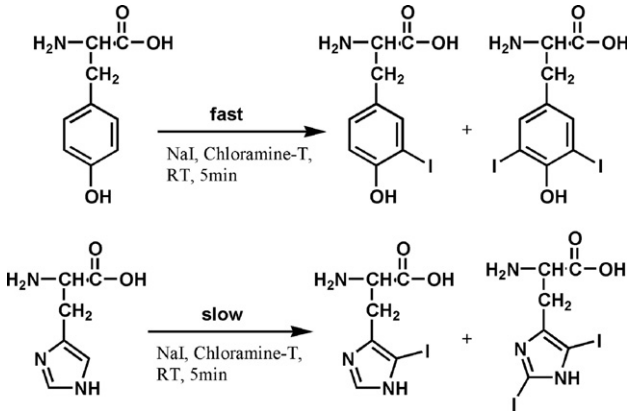

Scheme 1

experiments, absorption of a 266-nm photon by a protein containing mono-iodotyrosine results in the loss of iodine and generates radical protein in high yield. This work is part of a larger ongoing effort (highlighted by this special edition of *JASMS* honoring the contributions of Julia Laskin) in several labs to develop methods for generating gas-phase biomolecular radicals for subsequent interrogation by MS [21–30]. Although these efforts have resulted in a variety of useful techniques, the photoactivation of iodinated tyrosine is perhaps unique because of the ease with which iodination can be carried out and the possibility for generating multiple radicals on the same peptide either simultaneously or sequentially. The latter experiments obviously require the installation of multiple iodine atoms. However, to examine the feasibility of multi-step experiments, the details regarding how multiple iodination occurs in actual biological molecules must first be resolved.

It is shown herein that iodination occurs in a peptide-specific fashion. For some peptides mono-iodination of multiple tyrosine residues will occur preferentially to double iodination of any single tyrosine. For other peptides, double iodination at a particular tyrosine is favored over mono-iodination at multiple residues. These results suggest that in actual biological molecules, iodination should be examined on a case-by-case basis. The selectivity for iodination of tyrosine over histidine is found to be much less susceptible to peptide sequence or structure and strongly favors iodination at tyrosine. The behavior of multiply iodinated species as radical precursors was also examined. Doubly iodinated tyrosine behaves similarly to mono-iodotyrosine after the loss of one iodine atom and the resulting fragmentation chemistry is radical dominated. Iodinated histidine can be used to obtain radical peptides in lesser yields, but doubly iodinated histidine is not a well behaved precursor. The concerted loss of two iodine atoms from tyrosine to yield a putative diradical does not result in radical-mediated peptide fragmentation, suggesting that radical recombination occurs. Peptides that are multiply iodinated can be subjected to sequential radical activation and attack as well. In these experiments, a radical is generated by photodissociation and an even-electron fragment that contains another iodine is re-isolated and photodissociated again to regenerate the radical for further fragmentation. This capability opens the door for the stepwise gas-phase degradation of whole proteins.

Experimental

Reagents

Peptides were purchased from American Peptides Company (Sunnyvale, CA, USA). Chloramine-T, sodium metabisulfite, and sodium iodide were purchased from Fisher Chemical (Fairlawn, NJ, USA). Water was purified to 18.2 MΩ resistance using a Millipore Direct-Q (Millipore, Billerica, MA, USA). All reagents and peptides were used without purification unless otherwise noted.

Peptide Iodination

The method used is based on previously developed procedures [31]. Peptides and reagents were dissolved in water. To produce mono-iodinated peptides, the mole ratio between peptides, sodium iodide, chloramine-T, and sodium metabisulfite was 1:1:2:2. To produce multiply iodinated peptides, the amounts of reagents were increased proportionally. The amount of sodium iodide is increased to two times the amount of the total tyrosine and histidine in the peptide. The chloramine-T and sodium metabisulphite amounts were adjusted accordingly. The reaction was in the range from 50 to 100 nmol in a total volume of 100 μL. To the peptide solution, sodium iodide solution was added and mixed. Chloramine-T solution was added to the mixture and vortexed. After 5 min at room temperature, the reaction was stopped by the addition of sodium metabisulfite (with a concentration equal to that of chloramine-T). The yield of iodination for histidine peptides is typically less than that observed for tyrosine-containing peptides. To avoid confusion with isoleucine, iodine is italicized throughout and indicated via italicized superscripts within peptide sequences.

Mass Spectrometry

The samples were diluted to 10 μM in 50% acetonitrile, 50% H_2O, and 0.5% acetic acid and infused at 3 μL/min into an LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with a standard ESI source. In all cases, charges on the peptides are from the addition of protons to the molecule. Collision energies for CID experiments were varied from 20 to 27% for even-electron species and 15 to 18% for radicals. Exact collision energies were not found to substantially influence the results. The posterior plate of the LTQ was modified with a quartz window to transmit fourth-harmonic (266 nm) laser pulses from a flash lamp-

pumped Nd:YAG laser (Continuum, Santa Clara, CA, USA). Pulses were synchronized to the end of the isolation step of a typical MS^2 experiment by feeding a TTL trigger signal from the mass spectrometer to the laser via a digital delay generator (Berkeley Nucleonics, San Rafael, CA, USA). The first photodissociation (PD) of iodo-peptides took place in this step. Further MS^3 experiments were performed by re-isolation and CID of the PD product. The second PD step used a burst mode where two pulses were used to photodissociate ions by careful adjustment of the delay time between the pulses. This allowed the ions produced by MS^3 CID to be further photodissociated by the second laser pulse.

Results and Discussion

Iodination

Several peptides containing multiple tyrosine residues were iodinated and subjected to CID to determine the locations of iodination. Quantitation was achieved by comparing the intensities of fragments that varied only in the degree of iodination. This method for quantitation assumes that iodination will not significantly influence ionization efficiency, which appears to be a reasonable assumption since the results do not follow a consistent trend favoring iodination or lack thereof. Representative mass spectra are shown in Figure 1a for RYYRIK. The MS^3 spectrum for $[b_4 + I]^{2+}$ on the left reveals that mono-iodination occurs preferentially, although not exclusively, at Tyr3. On the right, two iodines have been added to the peptide. MS^3 on $[b_4 + 2I]^{2+}$ reveals more intensity for the $b_2 + I$ peak than for the b_2 or $b_2 + 2I$ peaks. The $b_2 + I$ peak represents the amount of mono-iodination that occurs at both Tyr2 and Tyr3, whereas b_2 and b_2+2I represent the amount of double iodination at Tyr3 and Tyr2, respectively. It is clear from the data that mono-iodination at both tyrosines is strongly favored over double iodination at either residue. These results are clearly not consistent with previous conclusions suggesting that mono-iodination catalyzes di-iodination [8]. The raw data from the mass spectra in Figure 1a are summarized graphically in Figure 1b, where each iodination is represented as a fractional abundance of the total amount of iodine added.

The CID results for two additional peptides are shown in Figure 1c and d. For RYLGYL, the addition of a single iodine occurs preferentially at Tyr5, although not by a large amount. However, the addition of two iodines again occurs with a strong preference for mono-iodination of both tyrosine residues. This result is also inconsistent with preferential formation of di-

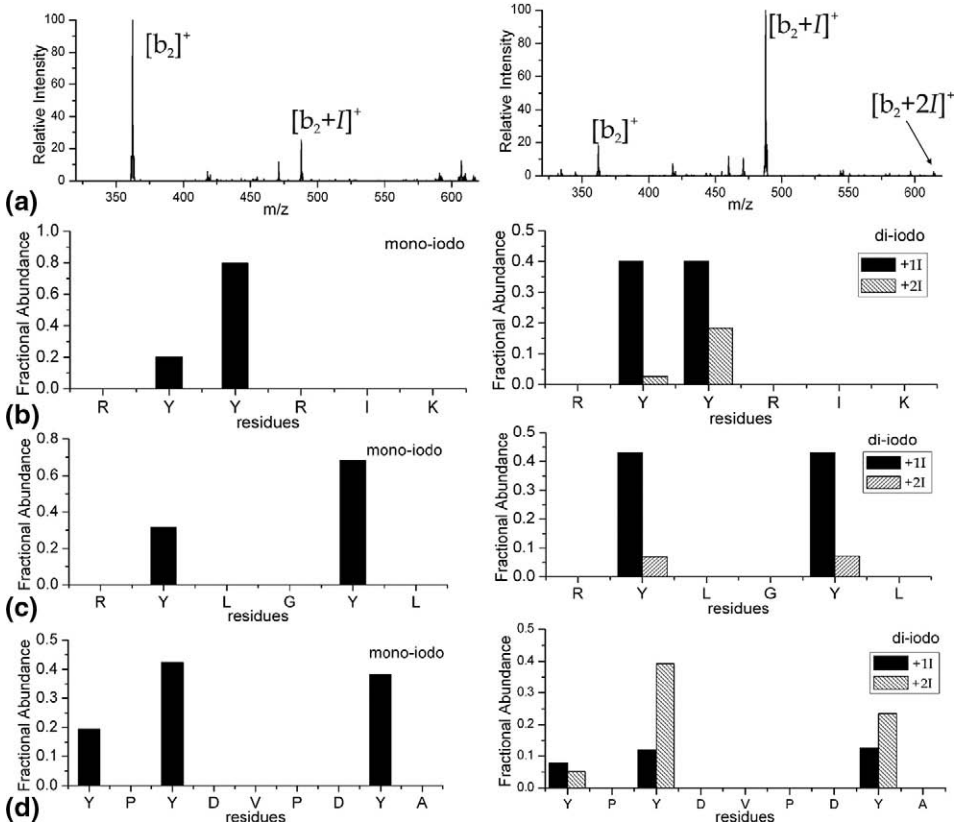

Figure 1. Iodination is tracked for several model peptides. Representative tandem mass spectra are shown in (**a**) for Ac-RYYRIK-NH_2. The fractional abundance for iodination at each location is shown for three peptides in **b–d**. The relevant sequences are indicated below each spectrum.

iodotyrosine. In Figure 1d, the peptide YPYDVPDYA is evaluated. This peptide contains three tyrosine residues that can be potentially iodinated. The addition of a single iodine occurs at all three tyrosines to varying extents. The right side of Figure 1d illustrates that double iodination of a single residue is more favorable for this peptide relative to the previous two. In particular, Tyr3 is preferentially doubly iodinated, whereas mono-iodination is competitive with di-iodination for Tyr1 and Tyr8. In summary, the results in Figure 1 clearly establish that mono-iodination is a stable end point and not merely a briefly lived intermediate on the way to double iodination. The results also demonstrate that structural effects dictated by sequence are sufficient to influence iodination even in fairly small peptides. This is manifested in the addition of both one and two iodines to these peptides. Clearly the more defined and intricate structures of proteins will have an even greater influence on relative reactivity, as has been suggested previously [4].

Iodination of Histidine

Histidine is the second most reactive amino acid toward iodination. Previous examinations have estimated that the rate of iodination at histidine is 30–100 times slower than that at tyrosine [1]. We have examined iodination of two peptides containing one histidine and one tyrosine using MS/MS. The results are shown in Figure 2. The addition of a single iodine to DRVYIHPF occurs almost exclusively at Tyr4, as shown on the left side of Figure 2a. Diagnostic ions in the mass spectrum (not shown) include b_4 and a_5 in iodinated and unmodified states. These ions are almost entirely iodinated, which indicates that histidine is not iodinated to any significant extent. The addition of two iodines is examined on the right side of Figure 2a. In this case, a more significant (although still minimal) fraction of histidine is iodinated. Although doubly iodinated Tyr4 is the major product, there is a minor amount of mono-iodinated Tyr4 and His5. This result demonstrates that tyrosine is the preferential target for iodination and it also further disputes the catalytic effect of mono-iodination on di-iodination for tyrosine since mono-iodination occurs more exclusively than does di-iodination at tyrosine. One of the lessons learned from Figure 1 is that different peptides can behave in divergent manners. Therefore, we also examined iodination of IARRHPYFL. As shown in Figure 2b, mono-iodination occurs preferentially at Tyr7 by a substantial margin. Similarly, double iodination yields primarily doubly iodinated Tyr7. Although these two peptides by no means represent a comprehensive sampling of peptide diversity, the results agree with all previous observations and suggest that iodination will occur at tyrosine before histidine. The subtle effects of sequence or structure present in peptides do not appear to be significant enough to influence the relative reactivities.

Di-iodo Radical Precursors

Given that di-iodotyrosine is a potential iodination product, it is important to determine whether this species behaves comparably to the previously examined radical precursor, mono-iodotyrosine. Photoactivation of $RGY^{I}ALG$ results in the loss of iodine to yield the radical peptide $[RGYALG\cdot]^{+}$. Reisolation of this peak, followed by collisional activation leads to the spectrum shown in Figure 3a. Backbone fragmentation occurs primarily at tyrosine, accompanied by several side-chain losses from tyrosine (−106) and leucine (−43, −56). The comparable experiment with RGY^{II} ALG yields the spectrum shown in Figure 3b. The results are nearly identical with the exception that certain peaks are mass shifted as a result of the presence or the absence of the additional iodine. For example, the loss of the tyrosine side chain represented by the −106 peak in Figure 3a corresponds to the −233 peak in

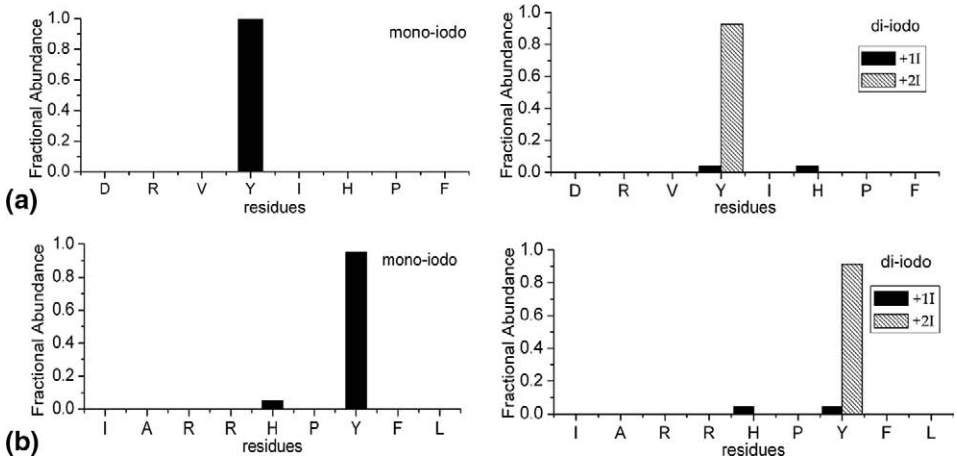

Figure 2. Iodination of tyrosine versus histidine is compared for two model peptides. The fractional abundance of iodination for both the addition of one and two iodines indicates that tyrosine is preferentially modified over histidine.

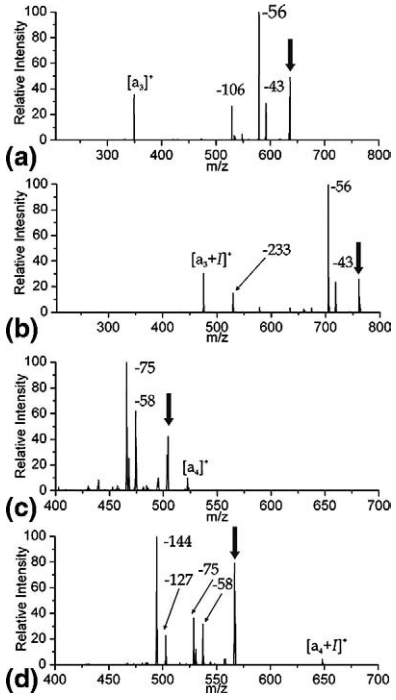

Figure 3. Doubly iodinated tyrosine and histidine are examined in comparison with mono-iodinated species as radical precursors. (**a**) $[RGYALG\cdot]^{+}$ is subjected to CID. (**b**) $[RGY^{I}ALG\cdot]^{+}$ is subjected to CID, yielding very similar results. The peak at −233 corresponds to the iodinated version of the −106 in (**a**). (**c**) $[KRQHPGKR\cdot]^{2+}$ is subjected to CID. (**d**) $[KRQH^{I}PGKR\cdot]^{2+}$ is subjected to CID, yielding dissimilar results to the non-iodinated radical. Bold down arrows indicate the parent ion.

Figure 3b where the side chain is still iodinated. Examination of other peptides yielded similar results. Therefore, the additional iodine in di-iodo tyrosine does not appear to interfere in any way with the resulting fragmentation chemistry, if a single iodine is ejected during photoactivation (the case of double iodine loss is examined in the following text). Di-iodo tyrosine is thus a suitable radical precursor and, should a particular site be more easily doubly iodinated, it could still be used to generate a radical.

Histidine can also be iodinated or doubly iodinated, but suitability as a radical precursor has not been evaluated previously. In Figure 3c, the results from subjecting $KRQH^{I}PGKR$ to photoactivation followed by collisional activation of the resulting radical are shown. Side-chain losses dominate the spectrum and are accompanied by minor fragmentation at the histidine. The yield of radical produced by this method is significantly less than is generated by tyrosine. Furthermore, comparable experiments on the doubly iodinated histidine do not generate similar results. Rather, several additional losses are observed and the iodine appears to play a more active role in the fragmentation, perhaps arising from less inherent stability. In all, these results suggest that histidine is not the target of choice for iodination if a good radical precursor is desired.

Double Radicals

Doubly iodinated species, irrespective of whether on the same residue, can potentially lose two iodines to form double radicals [32]. In Figure 4a, $RGY^{II}ALG$ was subjected to photodissociation and the peak resulting from the loss of two iodines was re-isolated and collisionally dissociated. The resulting fragments are substantially different from those shown in Figure 3a. The spectrum is more nearly similar to the CID spectrum for the unmodified, even-electron protonated peptide (See supplemental information, which can be found in the electronic version of this article).

These results suggest that radical recombination occurs, which would result in the formation of a new bond (potentially at multiple locations). Based on the observed fragments, apparently the new bond does not interfere substantially with fragmentation of the peptide relative to the unmodified molecule. Although recombination of the originally formed radicals offers the most direct explanation of the results, the highly strained product that would be formed is probably unlikely. Further light can be shed by examination of doubly iodinated systems where the iodines are primarily present on different residues. $RY^{I}LGY^{I}L$ can also yield a diradical following the loss of two iodine atoms. Subsequent collisional activation of this species yields the spectrum shown in Figure 4b, which is very similar to the CID spectrum for the unmodified, even-electron peptide. In this case, the radicals primarily begin on separate residues and direct recombination would cyclize a significant portion of the peptide. Although

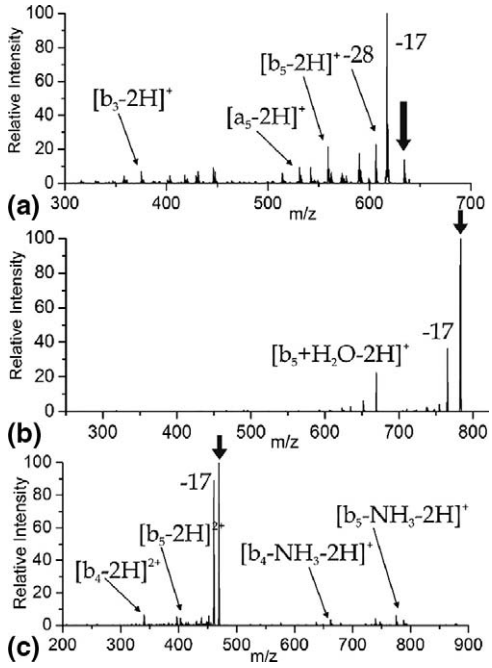

Figure 4. Double radicals do not yield fragments via radical-mediated pathways, suggesting recombination dominates. Results are shown for (**a**) $[RGYALG\cdot\cdot]^{+}$, (**b**) $[RYLGYL\cdot\cdot]^{+}$, and (**c**) $[RYYRIK\cdot\cdot]^{+}$. Bold down arrows indicate the parent ion.

significant features of the spectrum in Figure 4b are similar to those from CID on the unmodified peptide (See supplemental information), all of the fragments occurring between the two tyrosine residues are notably missing. This suggests that direct recombination and cyclization may occur in this case. The fragmentation spectrum for the doubly protonated diradical generated from doubly iodinated RYYRIK is shown in Figure 4c. It should be pointed out that contributions to the spectrum in Figure 4c from di-iodotyrosine species (as indicated in Figure 1) cannot be excluded. For this peptide, the fragmentation of the diradical is nearly identical to that observed for the even-electron ion. If this peptide adopts the conventional structure, then the tyrosine side chains should be directed away from one another, making direct recombination of the radicals improbable. Nevertheless, the results suggest that radical recombination occurs. This likely stems from the facility with which radicals can migrate within peptides [22, 20]. It is noteworthy that these experiments with diradical species contrast sharply with the results obtained with single-radical peptides. For single-radical peptides, fragmentation is almost always dominated by radical chemistry and typically bears little resemblance to dissociation of the nonradical peptide.

Sequential Double Radicals

The installation of multiple iodines in combination with the MS^n capabilities of an ion trap enables the possibility for experiments where multiple radicals are generated in different MS^n steps. To simplify the discussion, we will label both photodissociation and CID steps within the same MS^n framework. These experiments are conducted as follows. Photodissociation is used to generate a radical in MS^2. This radical is re-isolated and subjected to collisional activation in MS^3. Selection of an even-electron fragment (typically an a-type ion) that contains iodine is accompanied by another round of photodissociation in MS^4. The resulting radical can be fragmented by collisional activation in MS^5 and so forth. In conjunction with the selective fragmentation that is frequently observed in the dissociation of radical peptides, this method can be used to dissect a biomolecule in a stepwise fashion. Furthermore, the combination of iodination with photoactivation provides the easiest route to this type of chemistry because: (1) multiple modifications are facile, (2) they are frequently spatially distributed along the sequence, and (3) photoactivation of one iodine can be carried out without perturbing another or heating the entire molecule. Furthermore, iodination at tyrosine withstands the rigors of subsequent collisional activation (although C—*I* bonds are more susceptible in very small molecules) [33], leaving additional radical precursors intact.

Sequential radical activation is demonstrated in Figure 5 with $RY^{I}LGY^{I}L$. Initially, a radical is generated on Tyr5 or Tyr2. As shown in Figure 5a, the yield is quite good even with a single laser pulse. Figure 5b shows

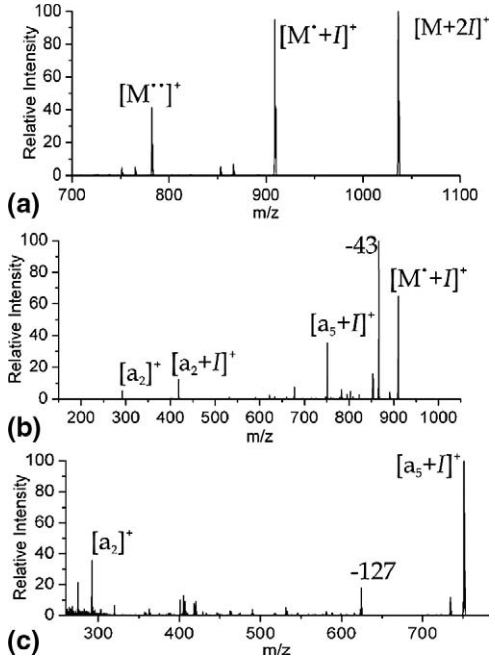

Figure 5. Sequential radical activation is demonstrated with $RY^{I}LGY^{I}L$. (**a**) Photodissociation of $RY^{I}LGY^{I}L$ yields both single and double radical species. (**b**) Isolation of the single radical followed by CID yields several a-type ions. (**c**) Photoactivation of $a_5 + I$ yields another radical that fragments spontaneously to yield the a_2 ion.

that subsequent fragmentation of the peptide radical yields the protonated $a_5 + I$ ion, which is an even-electron ion. In this step of the experiment a significant fraction of the total ion intensity is lost to other channels. Nevertheless, sufficient ions remain for photoactivation of $[a_5 + I]^+$ to yield the a_5 radical, which subsequently fragments to yield primarily the even-electron a_2 ion as shown in Figure 5c. In this case, the peptide is sufficiently small that the final photoactivation step imparts sufficient energy to the molecule to yield spontaneous fragmentation and subsequent collisional heating is not required. These results demonstrate that the combination of iodination and photoactivation can be used to generate multiple radicals in a sequential fashion similar to MS^n-type experiments conducted with collisional activation.

Conclusions

New applications based on the iodination of peptides and proteins continue to be discovered. The present results clearly demonstrate that iodination at tyrosine is strongly influenced by local structure even in small peptides. As a result, the degree of iodination that will occur at a particular tyrosine cannot be easily predicted. Regardless of iodination state, iodotyrosine is a suitable precursor for generating radicals in the gas phase by photoactivation for subsequent fragmentation by collisional activation. Diradicals generated from di-iodo peptides are subject to rapid recombination and do not

yield radical-mediated fragmentations. The unique properties of iodotyrosine, however, enable experiments where multiple radicals can be generated sequentially. In this manner, if fragmentation yields an even-electron species that contains iodotyrosine, subsequent photoactivation can generate another radical for further analysis. The combination of iodination chemistry with photoactivation to yield radical species holds substantial promise for interesting future experiments.

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