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Fragmentation chemistry observed in hydrogen deficient radical peptides generated from N-nitrosotryptophan residues

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ABSTRACT

The N-nitrosation reaction has been utilized to add an NO group to the indole nitrogen of tryptophan in several peptides. These peptides can be electrosprayed and then subjected to collision-induced dissociation (CID). The input of CID energy causes the homolytic cleavage of the labile nitrogen–nitrogen single bond resulting in removal of the NO group and generation of a hydrogen deficient peptide radical. This N-nitrosation reaction serves as a simple way to create peptide radicals in the gas phase. Here we examine features of CID for several N-nitrosopeptides and give mechanisms which explain the observed chemistry. In particular, tryptophan side chain loss is frequently an abundant fragmentation channel. Interestingly dissociation of the N–NO bond occurs concomitantly with CO_2 loss for peptides with C-terminal tryptophan residues. The location of the nascent radical is an important factor in both of these dissociation pathways. Other fragmentation channels are observed to occur via radical- or proton-catalyzed pathways, depending on the mobility of available protons. Theoretical calculations were also performed to study the energetics of the proposed mechanisms.

1. Introduction

The molecule nitric oxide (NO) plays a key role in the physiological processes of smooth muscle relaxation [1], vascular homeostasis, neutrotransmission, and host defense [2]. The NO molecule is generated by NO synthase enzymes and can undergo addition to the thiol group of cysteine in proteins through the S-nitrosation reaction [3]. This posttranslational protein modification has been shown to modulate the functions of a diverse number of proteins [4]. It has also been discovered that the N-nitrosation of proteins occurs commonly in vivo and is linked to the redox state of cells [5]. N-nitrosation typically occurs via addition at the indole nitrogen of tryptophan and can be carried out in vitro without the need for NO synthase [6–8]. N-nitrosation at tryptophan has also been used previously to create radical peptides by breaking the labile nitrogen–nitrogen single bond with collisional activation in gas phase mass spectrometry-based experiments [9].

Radical chemistry is being increasingly used to study the sequence, fragmentation, and modification sites of peptides and proteins. There are numerous methods that can be used to generate a radical on a peptide or protein with photodissociation (PD), collision-induced dissociation (CID), or electron transfer processes. For PD, procedures include iodination of tyrosine residues in proteins [10], noncovalent complexation by iodonaphthyl crown ether to lysine or arginine [11], and addition of naphthalenethiol to phosphorylated serine or threonine residues [12]. Each of these reactions creates a bond that can be broken homolytically following absorption of a UV photon, while avoiding substantial heating of the rest of the molecule. CID-based methods include use of the free radical initiator Vazo 68 [13] or peroxyl groups [14]. Cobalt, copper, and iron complexes have also been used to generate radical peptides via collisional activation [15–17]. Electron transfer processes include the use of xenon radical cations [18] and azobenzene radical anions [19] to generate radical sites on peptides. It has also been demonstrated that radical peptides form upon electron transfer during collisions with cesium atoms [20]. Electron capture dissociation (ECD) typically yields peptide fragments which contain radicals [21]. Results frequently differ somewhat between radicals generated by PD and CID, presumably due to the fact that PD affords radicals via dissociative excited state chemistry while CID-based methods yield radicals through vibrational excitation of the ground state. Heating of the entire molecule in CID-based experiments is therefore unavoidable. The aforementioned N-nitrosation reaction generates a very labile N–N bond; therefore, very mild collisional activation could potentially yield a radical on the tryptophan residue while minimizing heating of the remaining molecule. This property makes N-nitrosation the most

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likely CID-based method to generate PD-like spectra; however, this possibility has not been fully explored.

Peptide fragmentation, even in radical peptides, can be influenced by proton-catalyzed dissociation pathways which yield distinct products from those initiated by radicals. The mobile proton model can be used to describe peptide fragmentation that is charge-directed [22]. In this model a proton on the charged peptide moves along the backbone in order to facilitate bond cleavage. Surface-induced dissociation has been used to study peptide fragmentation and examine the relationship between peptide basicity and collisional energy required to cause dissociation [23]. It was discovered that peptides with more basic amino acid residues required a larger amount of collisional energy in order to fragment. The most basic amino acid is arginine, followed by lysine, then histidine. As the most basic residue, arginine is able to sequester the proton most tightly and requires the most energy to move it to the backbone and cause fragmentation. The migration of the charge leads primarily to amide bond cleavage which yields b- and y-type ions. In contrast, radical-directed dissociation leads to various side chain losses and backbone fragmentation yielding a, x, c and z ions [11,24]. These differing fragmentation patterns indicate the presence of a radical and provide information about the composition of the peptide. O'Hair and co-authors have demonstrated previously that charge-directed processes involving mobile protons can compete directly with radical-driven dissociation for some peptides [25].

The present work focuses on studying the fragmentation of various radical peptides generated by loss of NO from tryptophan which has undergone N-nitrosation. Collisional activation is used to remove the NO group, leaving a radical on the tryptophan side chain. Peptides of varying basicity were examined to determine the effect of proton mobility on the competition between radical- and charge-directed dissociation. As expected, peptides with higher proton mobility exhibit more proton-catalyzed fragmentation. Nevertheless, tryptophan side chain loss (which results from radical chemistry) is still observed regardless of proton mobility. This loss is favored because the initial radical can produce side chain loss directly, while other fragments require migration of the initial radical. Peptides with C-terminal tryptophan residues spontaneously lose CO_2 with the loss of NO. A mechanism is proposed to explain this interesting observation. Theory is used to evaluate the proposed mechanisms for both side chain and CO_2 loss.

2. Experimental methods

2.1. Materials

Peptides RRPWIL, DLWQK, KWDNQ, FVQWLMNT, MEHFRW, TRSAW, and DYMGWMDF were purchased from Sigma–Aldrich (St. Louis, MO) or American Peptide Company (Sunnyvale, CA). All other chemicals and reagents were purchased from Sigma–Aldrich.

2.2. Sample preparation

The N-nitrosation reaction described by Hao and Gross [9] was followed with a few modifications. Each peptide was dissolved in a 0.1% trifluoroacetic acid (TFA) solution to obtain a peptide concentration of 10 μM. Sodium nitrite ($NaNO_2$) was added to the 1 mL peptide solution to obtain a concentration of 1 mM. The samples were then incubated in the dark in a 39 °C water bath for 30 min. A 0.5–50 kDa protein concentration and desalting trap (Michrom Bioresources, Inc., Auburn, CA) was then used to desalt each sample. The peptide was eluted from the trap into 1 mL of 90% acetonitrile/0.1% TFA solution. The nitrosopeptide that is created is sensitive to light and only stable for up to 1 week, so it should be used promptly.

2.3. Instrumentation

Peptide samples were injected into the electrospray ionization source of an LTQ linear ion trap mass spectrometer (Thermo Electron, San Jose, CA). Multiple steps of collision-induced dissociation (CID) were used to generate and then fragment the radical peptides.

2.4. Theoretical calculations

Gaussian 03 Version 6.1 Revision D.01 was used to perform calculations for the mechanisms proposed herein at the B3LYP/6-31G(d) level of theory. Input files were generated with Gauss View 3.0. The QST3 method was utilized to find the transition states for the mechanisms and determine the energy barrier. The energy optimizations and transition states were verified by calculating frequencies and checking for spin contamination.

3. Results and discussion

The full mass spectrum for the peptide RRPWIL following N-nitrosation is shown in Fig. 1a. The peptide is present in both the +1 and +2 charge states. This spectrum was obtained after adjusting the nitrosation reaction time to 30 min and temperature to 39 °C which gave the best yield of nitrosopeptide. These conditions were used for all subsequent nitrosation reactions. The relative abundance of nitrosopeptide is approximately 7% compared to the sum of contributions from unmodified peptide. Gentle electrospray conditions must be employed in order to keep the nitrosopeptide intact. This is evident in the fact that there is no peak observed for nitrosopeptide in the +2 charge state. The harsher conditions experienced by the higher charge state appear to cause loss of the NO group in the source of the instrument. CID of the nitrosopeptide easily cleaves the N–NO bond homolytically to generate a hydrogen deficient radical peptide as shown in Fig. 1b. The energy required to cleave NO was ~15% less than that subsequently required to fragment the radical peptide in Fig. 1c. The extreme lability of the NO modification is a disadvantage in terms of yield which can be achieved, but does offer very gentle radical formation, as desired.

RRPWIL fragments to give a_4 and a_5 peaks which correspond to dissociation at tryptophan and the adjacent isoleucine. There is also significant loss of CO_2 and side chains. The losses of 86 and 99 Da correspond to partial and complete arginine side chain losses, respectively. These peaks are labeled with the mass loss and one-letter amino acid abbreviation. The most dominant peak in the CID spectrum of the radical RRPWIL is the loss of the tryptophan side chain which has a mass of 129 Da. These results are similar in several respects to the fragmentation observed when the radical is generated through PD, which is shown in Fig. 1d. In this case, an iodonaphthyl radical precursor was used to create a hydrogen deficient peptide [11]. Complete and partial arginine side chain losses occur along with tryptophan side chain loss. Notably, the amount of side chain loss is significantly less than that observed for Fig. 1c. The a_4 and a_5 backbone fragments are generated in similar amounts in both spectra. CO_2 loss, which is a major feature in Fig. 1c, is a minor peak in Fig. 1d.

For the N-nitrosation method, the radical is originally located on the nitrogen of tryptophan. Abundant tryptophan side chain loss is common to most of the peptides studied herein. A proposed mechanism for the tryptophan side chain loss is given in Scheme 1. The radical is transferred to the α-carbon of the peptide backbone through electron rearrangement. The side chain product that results from this has a mass of 129 Da which agrees with experiment. The consistently high observed abundance of tryptophan side chain loss is consistent with this mechanism because disso-

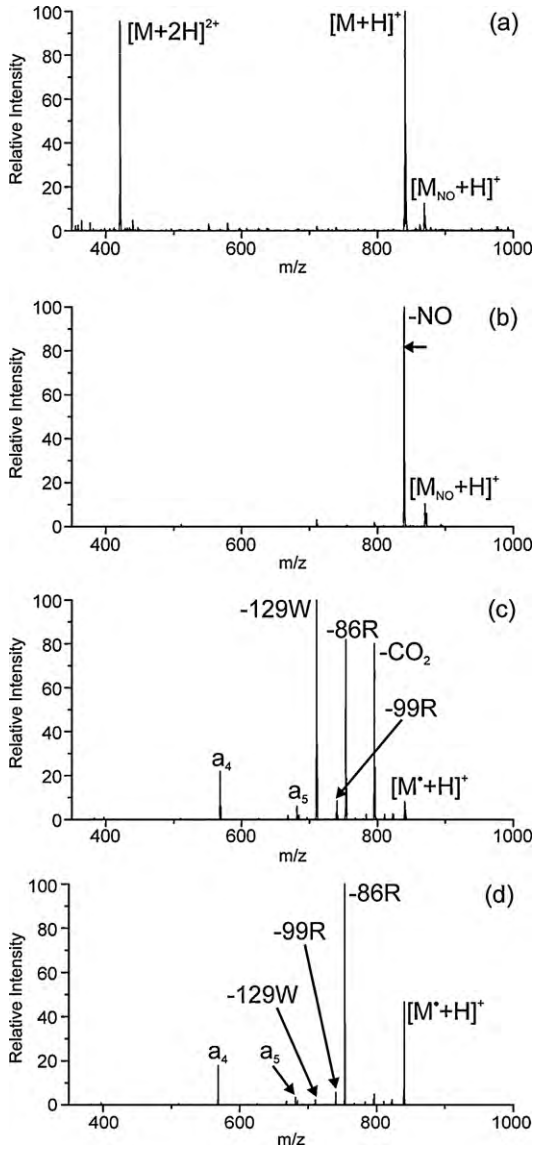
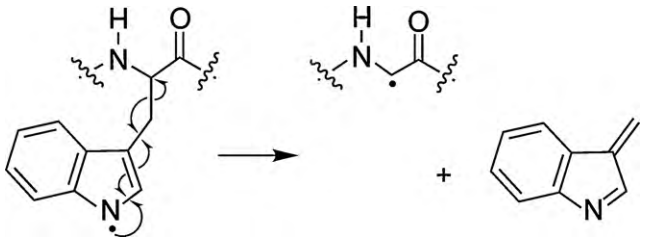


Fig. 1. (a) Full mass spectrum for $RRPW_{NO}IL$ after N-nitrosation. (b) MS^2 on $[RRPW_{NO}IL+H]^+$ removes the NO group and generates a hydrogen deficient radical peptide. (c) MS^3 spectrum for $[RRPWIL•+H]^+$. (d) Collisional activation of $[RRPWIL•+H]^+$ generated by photodissociation is shown for comparison.

ciation can occur directly without the need for radical migration involving rearrangement of any atoms.

The results for collisional activation of several radical peptides generated by loss of NO from tryptophan are presented in Fig. 2. The peptides in Fig. 2a and b both contain lysine while the peptide in Fig. 2c does not contain any of the basic amino acids. The spectrum in Fig. 2a is dominated by tryptophan, lysine, and leucine side chain losses with a relatively small amount of y-type fragments.



Scheme 1.

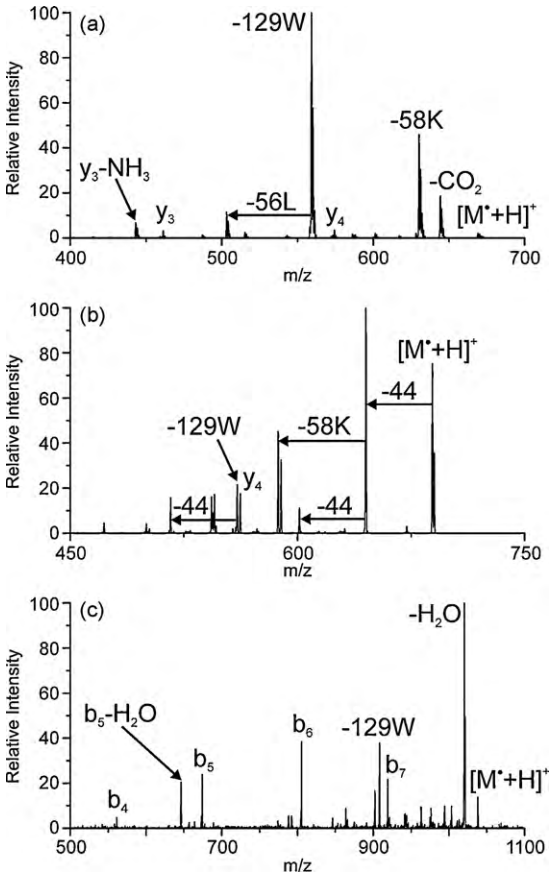


Fig. 2. (a) MS^3 spectrum for $[DLWQK•+H]^+$. (b) MS^3 spectrum for $[KWDNQ•+H]^+$. (c) MS^3 spectrum for $[FVQWLMNT•+H]^+$.

Similar results are obtained for another lysine-containing peptide, KWDNQ, shown in Fig. 2b. In addition, losses of 44 Da are observed from the aspartic acid side chain and asparagine side chain or C-terminus. The CID spectrum for an arginine-containing peptide, RRPWIL, has already been shown in Fig. 1c and has no b- or y-type fragments because the proton is even less mobile than for lysine. The only peaks seen for RRPWIL are side chain losses, CO_2 loss, and a-type fragments which all result from movement of the radical. The lysine in DLWQK is able to sequester the proton which primarily allows the fragmentation in Fig. 2a to be directed by the radical. The peptide FVQWLMNT in Fig. 2c does not contain any of the basic amino acid residues and, therefore, has a much more mobile proton. This causes fragmentation into mostly b-type ions with significant loss of water; however, the radical-directed tryptophan side chain loss is still present and abundant.

Two of the peptides used in this study contain tryptophan at the C-terminus. These peptides, TRSAW and MEHFRW, lose both CO_2 and NO simultaneously upon collisional activation as shown in Fig. 3a and c. In Fig. 3b, a second step of CID following the simultaneous loss in TRSAW yields a and c backbone fragments along with loss of H_2O. MS^3 yields substantial methionine side chain loss and minor backbone fragmentation (a_5 and c_5 fragments) for MEHFRW as shown in Fig. 3d. This simultaneous loss is likely due to the close proximity of the tryptophan radical to the carboxylic acid at the C-terminus. If the tryptophan radical formed by loss of NO is well positioned to abstract hydrogen from the C-terminus, prompt loss of CO_2 would be anticipated following hydrogen atom transfer.

Theoretical calculations were performed to determine the energies involved in the common tryptophan side chain loss and unique CO_2 loss at the B3LYP/6-31G(d) level of theory. For both situations, calculations were carried out on the model tryptophan residue

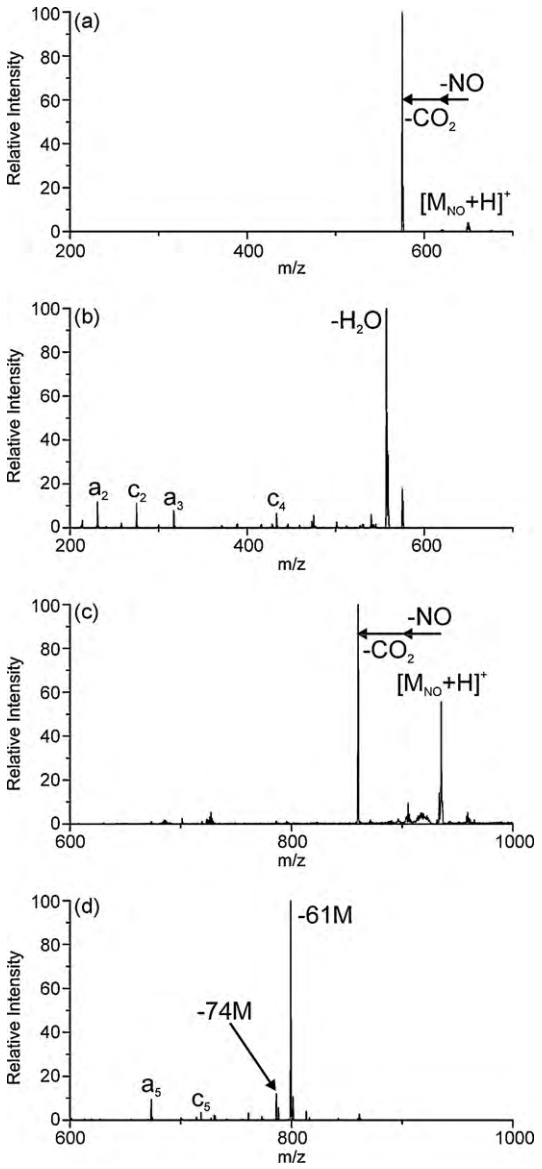


Fig. 3. (a) Collisional activation of $[TRSAW_{NO}+H]^+$ removes CO_2 and NO simultaneously. (b) MS^3 spectrum for the product ion from (a). (c) Similar results are obtained for collisional activation of $[MEHFRW_{NO}+H]^+$ which also contains a C-terminal tryptophan. (d) MS^3 spectrum for the product ion from (c).

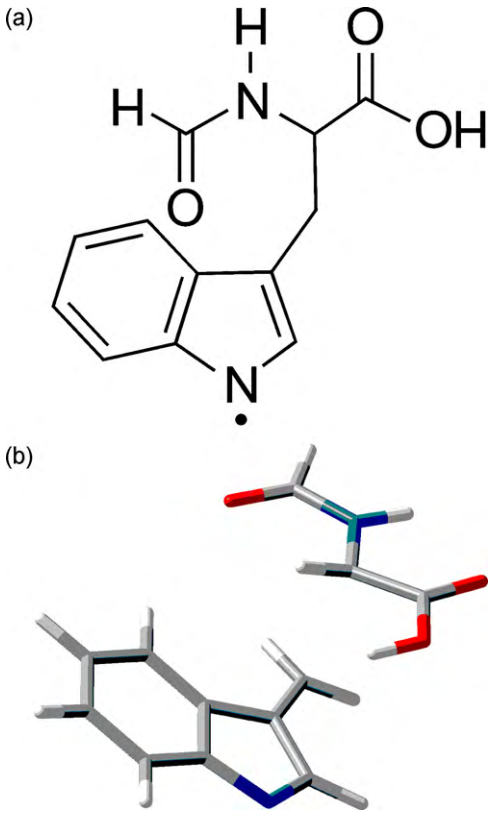
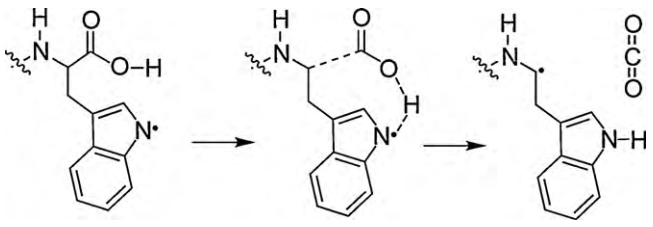


Fig. 4. (a) Tryptophan radical model used for theoretical calculations. (b) Calculated transition state structure for tryptophan side chain loss.

in Fig. 4a which contains the radical that is generated upon NO removal. This structure was energy minimized and used as the starting point for both sets of calculations. The QST3 method, in which the reactant, product, and estimation of the transition state structure is required, was utilized for evaluating the energy barrier to the tryptophan side chain loss. A transition state with a single imaginary frequency and minimal spin contamination was obtained. The transition state is consistent with the mechanism in Scheme 1 and is shown in Fig. 4b. The transition state energy was calculated to be 24 kcal/mol above the reactant, which is in reasonable agreement with experimental observation. Other theoretical calculations and experimental data have found proton initiated transition state energies for amide backbone fragmentation in peptides to be between 20 and 40 kcal/mol [26]. The energy barrier for tryptophan side chain loss is at the low end of this range which explains why the loss of 129 Da is observed even in the presence of fully mobile protons.

A proposed mechanism for the CO_2 loss observed for the peptides TRSAW and MEHFRW is shown in Scheme 2. The CO_2 is probably lost through a concerted mechanism in which there is elongation of the carbon–carbon single bond along with hydrogen transfer to the nitrogen of tryptophan. However, attempts to model this transition state were unsuccessful, likely due to the difficulty in optimizing two coordinates simultaneously. Searching along either single coordinate does not yield a true transition state. Nevertheless, the energy barrier for the concerted CO_2 loss is most likely less than 24 kcal/mol (the value obtained for tryptophan side chain loss) because tryptophan side chain loss is not observed in competition with the concerted loss (as shown in Fig. 3). The proposed mechanism for CO_2 loss results in transfer of the radical from tryptophan to the peptide backbone. This means that subsequent rearrangement to give side chain loss as in Scheme 1 is no longer likely to occur, and it is not observed. To further experimentally examine this mechanism, TRSAW and MEHFRW were examined as anions and the results are presented in Fig. 5. The largest peak in the spectrum for CID of $[TRSAW_{NO}-H]^-$ in Fig. 5a is loss of the NO group. There are also smaller peaks observed for tryptophan side chain loss and CO_2 loss from the peptide radical. This spectrum is significantly different from that obtained in positive ion mode in Fig. 3a. Having a negative charge on the C-terminus should interfere with the mechanism presented in Scheme 2, preventing simultaneous loss



Scheme 2.

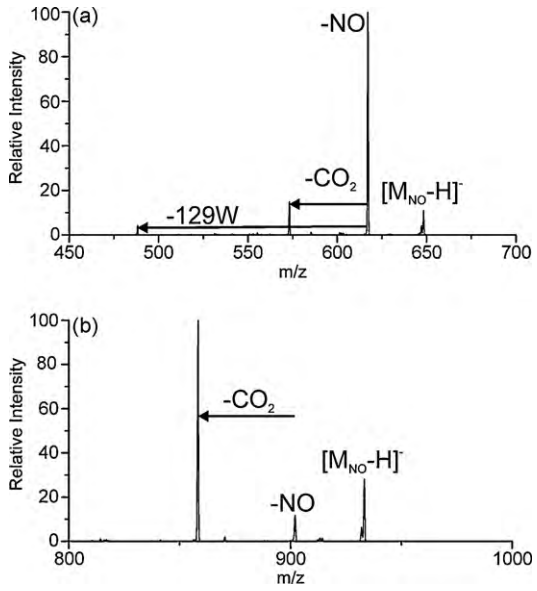


Fig. 5. (a) Collisional activation of $[TRSAW_{NO}-H]^-$ in negative ion mode. (b) Collisional activation of $[MEHFRW_{NO}-H]^-$ in negative ion mode.

of NO and CO_2. In comparison, $[MEHFRW_{NO}-H]^-$ in Fig. 5b yields a spectrum very similar to its positive ion mode counterpart in Fig. 3c. In this case, the negative charge resides on the glutamic acid side chain and does not affect radical migration as outlined in Scheme 2. These results support the mechanism outlined in Scheme 2 and furthermore eliminate the possibility that alternative charge-directed fragmentation pathways might account for the results.

4. Conclusions

The fragmentation of various tryptophan-containing peptides using CID has been studied following N-nitrosation. This reaction creates a nitrogen–nitrogen single bond by replacing a hydrogen on tryptophan with an NO group. CID cleaves the bond homolytically to generate a radical on the tryptophan residue of the peptide. This radical plays an important role in determining how the peptides fragment. The peptides yield backbone fragments at or near tryptophan as well as side chain losses upon CID. The most common side chain loss is from tryptophan itself due to facile electron rearrangement involving the initial radical. The fragmentation observed for the peptide RRPWIL is similar when the radical is created with the N-nitrosation reaction and CID or through an iodonaphthyl crown and PD. The basicity of the peptides also plays an important role in the fragmentation. Peptides with the basic residues arginine, lysine, or histidine sequester the proton well and their CID spectra are dominated by radical-directed dissociation. Peptides that lack basic residues exhibit some side chain losses but yield mostly b- or y-type ions via typical proton-catalyzed dissociation pathways. Peptides that contain tryptophan at the C-terminus undergo simultaneous NO and CO_2 loss upon the first step of collisional activation. This is most likely due to the radical migrating to the C-terminus to cause CO_2 loss which is apparently more facile than tryptophan side chain loss that is frequently dominant in peptides with internal tryptophan residues.

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