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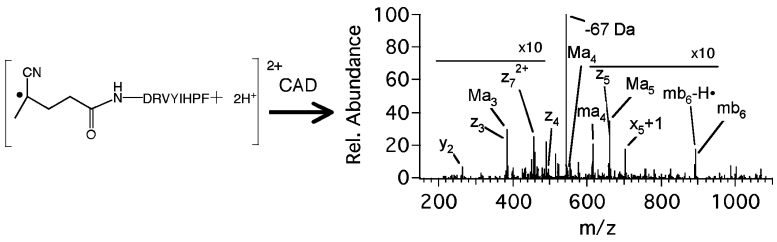

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Bioconjugates for Tunable Peptide Fragmentation: Free Radical Initiated Peptide Sequencing (FRIPS)

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Mass spectrometry is an invaluable technique for the analysis of small peptides produced by enzymatic proteolysis of proteins. Proteolytic peptides are most often sequenced using collisionally activated dissociation (CAD)[1] or electron capture dissociation (ECD)[2] of the cationized species. The ability to selectively cleave the protein backbone at specific amino acid residues in the gas phase might provide a viable alternative to enzymatic digests and could result in significantly higher throughputs for proteomic analyses.

Other researchers have investigated the fragmentation patterns arising from collisional activation of peptide radicals generated by dissociation of Cu−peptide complexes[3−6] and through ECD. The fragments that arise from collisionally activated dissociation of the radical peptides are typically *c* and *z* fragments, rather than the *b* and *y* fragments most commonly seen in the CAD of nonradical protonated peptides.[7] ECD typically cleaves many more backbone sites than collisional activation, resulting in more complete coverage of a peptide sequence.[8]

Porter et al. have modified lysine residues in solution to convert them to peroxycarbamates and find that CAD of species complexed with Li^+, Na^+, K^+, and Ag^+ results in loss of −C(O)OO*t*Bu to give a radical amine at the lysine side chain.[9] They postulate that the radical amine results from an initial free radical dissociation of the peroxide bond followed by decarboxylation. While CAD of the radical peptide results mainly in fragmentation of the lysine side chain, in some instances, the peptide backbone is also cleaved.[9]

We have begun investigating the use of free radical reactions as an alternate means of peptide and protein structure determination. To achieve this goal, the water-soluble free radical initiator Vazo 68 (DuPont) is conjugated to the N-terminus of a peptide (e.g., **1**). Conjugation is accomplished through standard peptide coupling techniques using dicyclohexylcarbodiimide. The conjugate is electrosprayed into an ion trap mass spectrometer, where CAD results in free radical formation at the azo moiety. Subsequent collision-activated dissociation fragments the peptide radical, producing mostly *a*- and *z*-type fragments, also observed in ECD experiments. However, unlike ECD, singly charged peptides can be examined using this technique. We refer to this as free radical initiated peptide sequencing, or FRIPS.

An advantage of this approach is the ability to finely alter the reactivity of the radical generated by altering the structure of the radical initiator conjugated to the peptide. Theoretically, different initiators could produce radicals with different reactivities. Highly reactive radicals will indiscriminately abstract hydrogen atoms from different sites on the peptide, leading to nonselective fragmentation useful for peptide sequencing. More stable radicals will abstract more weakly bound hydrogen atoms, leading to more selective fragmentation, useful in carrying out a gas-phase digest of a large protein. The radical produced in the decomposition of Vazo 68 is relatively stable and hence unreactive, when compared to many carbon-centered radicals. Isobutyronitrile, a model for the radical derived from Vazo 68, has a C−H bond energy at the 3° carbon of 361.9 kJ/mol.[10] This is much less than the 1, 2, and 3° C−H bond energies of ethane (423.0 kJ/mol),[10] propane (412.5 kJ/mol),[11] and isobutane (403.8 kJ/mol).[11]

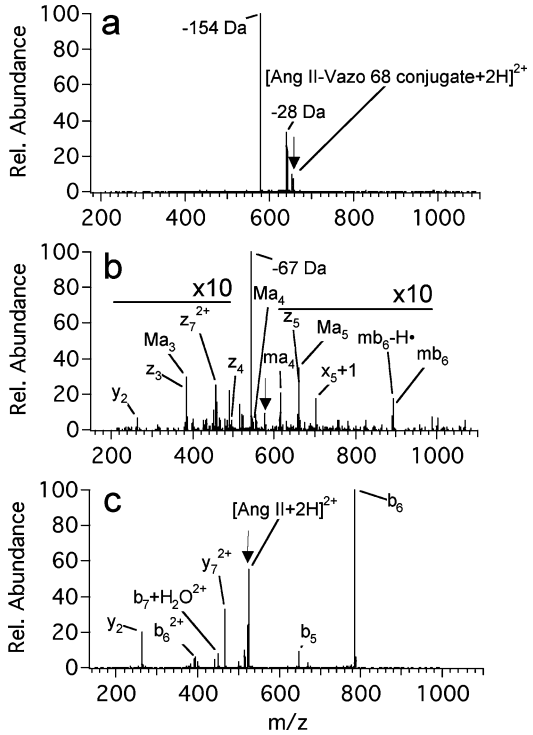

Figure 1. (a) Spectrum resulting from collisional activation of the doubly protonated Angiotensin II−Vazo 68 conjugate. (b) Spectrum resulting from collisional activation of the radical ion species produced in spectrum a. (c) Spectrum resulting from collisional activation of doubly protonated Angiotensin II. Arrows indicate the peaks being collisionally activated. Modifications to the N-terminus are denoted M for $-COCH_3$ and m for $-COCH_2CH_2CH(CH_3)(CN)$.

Spectra resulting from CAD of an Angiotensin II−Vazo 68 conjugate are shown in Figure 1. Figure 1a presents the spectrum obtained from CAD of the doubly protonated conjugate of Angiotensin II (DRVYIHPF) with Vazo 68 (**1**), obtained by electrospray ionization with an LCQ Deca ion trap mass spectrometer. Collisional activation produces two major products: a fragment corresponding to the loss of 28 Da (N_2), and another fragment ion corresponding to the loss of 154 Da. The latter ion is the free radical species generated by cleavage at the azo carbon (**2**), as shown in the first step of Scheme 1. These processes are observed with all peptide−Vazo 68 conjugates we have studied. Symmetrical azoalkanes, such as Vazo 68, tend to decompose by simultaneous loss of N_2 and generation of two alkyl radicals.[12] MS^3 experiments on the N_2 loss peak show a loss of 126 Da, corresponding to the 4-cyano-4-methylbutyric acid radical, which appears to form a noncovalent adduct with the peptide following initial dissociation of the azo moiety.

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Scheme 1. CAD of Angiotensin II−Vazo 68 Conjugate

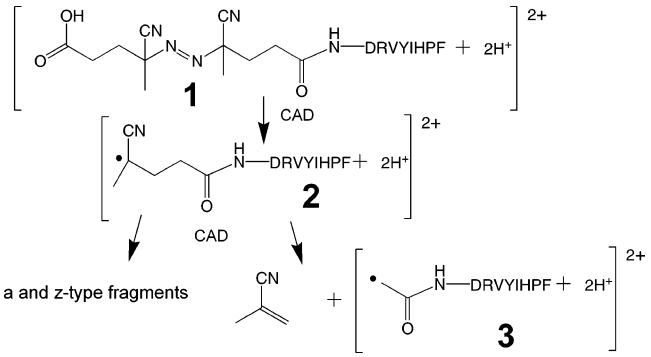

Figure 1b is the spectrum obtained by collisional activation of the radical ion (**2**) from Figure 1a. The major product is another radical ion (**3**), formed by the loss of 2-methylacrylonitrile (67 Da), as shown in the second step of Scheme 1. Also produced are a number of fragment ions, including many *a* and *z* ions.[13] As previously mentioned, formation of *c*- and *z*-type fragments is prevalent in ECD spectra of multiply charged peptides.[7] Figure 1c is the spectrum obtained by collisional activation of doubly protonated Angiotensin II. In contrast to the results in Figure 1b, this spectrum consists primarily of *b*- and *y*-type ions, as is normal for CAD fragmentation. Figure 2 contrasts the location of the backbone cleavages for CAD fragmentation and FRIPS fragmentation. The ECD spectrum of Angiotensin II has been published. Fragments are observed that arise from many peptide backbone cleavages (a_2, c_2, c_3, y_4, c_4, c_5, a_6, c_7).[14] These fragments are distinct from those observed using FRIPS.

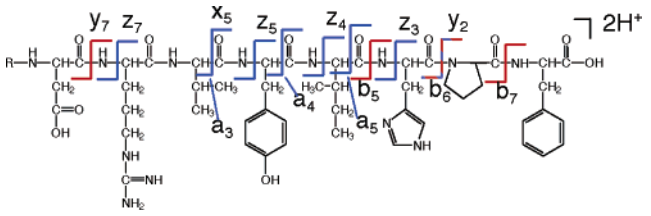

Figure 2. Backbone cleavages for the Angiotensin II−Vazo 68 conjugate. Red: CAD fragmentation, R = −H. Blue: FRIPS fragmentation, R = $-COCH_2CH_2C(CH_3)(CN)^{\bullet}$.

It is reasonable to assume that peptide fragmentation is initiated by abstraction of a hydrogen atom from the peptide by the Vazo 68 radical, followed by β-cleavage. More weakly bound hydrogen atoms should be abstracted preferentially, resulting in selective fragmentation. An examination of the homolytic bond dissociation energies (BDEs) is useful. Calculations suggest that along the peptide backbone, the α-carbon hydrogens are least strongly bound, with BDEs ranging from 326 to 369 kJ/mol,[15] depending on the side chain. Secondary structure, such as an α-helix, can increase these BDEs by as much as 40 kJ/mol[15] and may be important in determining the selectivity of radical induced fragmentation. However, abstraction of the α-carbon hydrogen cannot explain the majority of fragments shown in Figure 1b since β-cleavage would not generate the observed fragments.

Hydrogen atom abstraction may also occur from amino acid side chains. Abstraction of hydrogen from a methylene attached to the α-carbon followed by β-cleavage along the backbone would produce both *c*- and *z*-type fragments and *a*- and *x*-type fragments. The α-carbon hydrogen in ethanol, a model for the serine side chain, has a BDE of 389 kJ/mol,[10] making abstraction by the Vazo 68 radical only slightly endothermic.

While BDEs are useful to gain a qualitative understanding of possible hydrogen abstraction sites, these numbers are not necessarily reflected in the activation parameters and represent only one of the factors likely to affect reactivity. The influence of secondary and tertiary structure will also play a role in altering the actual reactivity of any specific hydrogen.

Many of the hydrogen atoms in the peptide, both backbone and side chain, are bound weakly enough that abstraction by the Vazo 68 radical is exothermic, or only mildly endothermic. Thus, a more stable radical species may yield more specific bond cleavages. For example, the 3° C−H bond in 1,1-diphenylethane has a BDE of 338.9 kJ/mol[10] and would yield a more stable radical than Vazo 68. Our current work focuses on determining the mechanisms of free radical induced fragmentation and on the design of new radicals for increased selectivity.

Free radical initiator−peptide conjugates show promise as a means for the selective fragmentation of peptides. By using a carbon-centered radical, the reactivity of the radical can be tuned to maximize selectivity or fragmentation. This is a useful step toward a completely gas-phase approach to protein sequencing, as more stable radicals could be used to selectively cleave the protein in analogy to a tryptic digest. This experimental methodology can also be used to study biologically relevant reactions of free radicals with peptides and proteins in the gas phase, removing the complications of environmental effects.

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Supporting Information Available: FRIPS and CAD spectra of other peptides, possible FRIPS mechanism, fragment nomenclature. This material is available free of charge via the Internet at http://pubs.acs.org.

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