Chapter 5. Advances in Gas Phase Proteomics Techniques: Free Radical Initiated Protein Sequencing (FRIPS)

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5.1. Introduction

The human genome has been sequenced, and identifying the composition of the proteome is one of the next major challenges ahead. New generations of diagnostic tools, designed to obtain a complete understanding of what proteins normally exist in cells and how the composition of these proteins changes when a disease state is present, would benefit greatly from a fast and efficient technology to rapidly identify such proteins. Mass spectrometry (MS) is an invaluable technique for the analysis of small proteins produced by enzymatic proteolysis of proteins. Proteolytic peptides are most often sequenced via collisionally induced dissociation (CID) or electron capture dissociation (ECD) of the cationized species.[1-4] The ability to selectively cleave the backbone of proteins at specific amino acid sites in the gas phase would circumvent the need for enzymatic digests, and could result in significantly higher throughputs for proteomic analyses.

Previous work has examined several types of enhanced reactivity of proteins in the gas phase. Enhanced cleavage of the peptide backbone C-terminal to an acidic residue has been observed in both protonated and sodiated peptides.[5,6] Cleavages of peptide bonds are often enhanced when they are N-terminal to proline residues[7] and C-

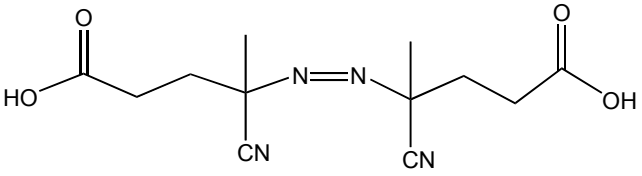
terminal to histidine residues.[8] Transition metals can also be used to facilitate cleavage at a specific amino acid; for example, Zn^{2+}, Cu^{2+}, Ni^{2+}, and Co^{2+} enhance cleavage at histidine residues,[9] while Fe^{2+} enhances cleavage at cysteine residues.[10]

Other researchers have investigated the fragmentation patterns arising from radical peptides. One method involves collisional activation of a $[Cu^{II}(L)(M)]^{2+}$ species, where L is a nitrogen donor ligand and M is an oligopeptide of interest.[11-13] Activation of such ternary complexes can result in radical peptide cations whose fragmentation patterns have been studied extensively in the case of tripeptides GXR.[12] Positively charged radical proteins have also been generated using electron capture dissociation (ECD), in which low-energy electrons are collided with a multiply charged protein ion.[14] The fragments that arise from collision-induced dissociation of the radical peptides are typically *c* and *z* fragments, rather than the *y* and *b* fragments most commonly seen in the collision-induced dissociation of non-radical peptides.[15] ECD cleaves many more backbone sites than collisional activation, resulting in more coverage of a peptide sequence.[3]

Several researchers have investigated radical peptides and their dissociation patterns in an attempt to achieve more selective fragmentation. Work in this laboratory has used the high affinity of 18-crown-6 for lysine residues to attach a diazo 18-crown-6 reagent to a peptide at the lysine residues and collisionally activate the resulting non-covalently bound complex in order to form a highly reactive carbene. The carbene reacts to form covalent bonds with the peptide, but the resulting molecule fragments readily and does not yield sequence-specific information.[16] Porter *et al.* have modified lysine residues in solution to convert them to peroxycarbamates and find that CID of species

complexed with Li^+, Na^+, K^+, and Ag^+ result in loss of –C(O)OOtBu to give a radical amine at the lysine side chain.[14] They postulate that the radical amine results from an initial free radical dissociation of the peroxide bond followed by decarboxylation. While CID results mainly in fragmentation of the lysine side chain, in some instances the peptide backbone was also cleaved at the lysine residue.

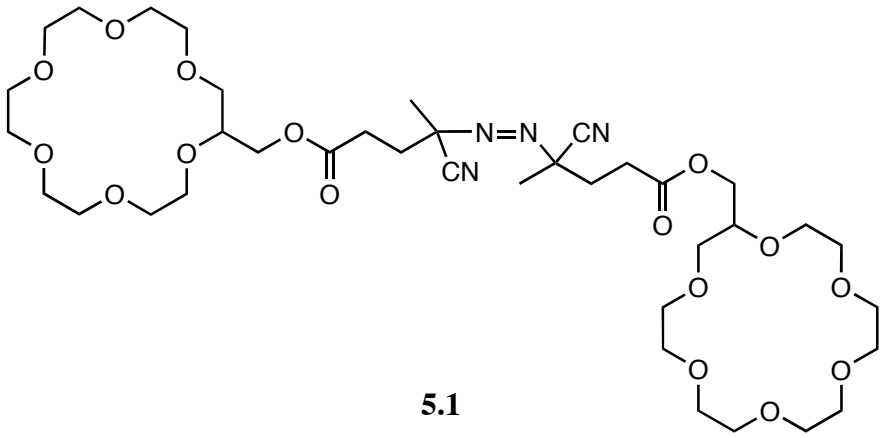
We have begun investigating the use of free radical reactions as an alternate means of peptide and protein structure determination. To achieve this goal, the water-soluble free radical initiator Vazo 68, shown in Scheme 5.1, is conjugated to the N-terminus of a peptide. The conjugate is electrosprayed into an ion trap mass spectrometer, where CID results in free radical formation at the azo moiety. Subsequent collisional dissociation fragments the peptide radical, producing mostly *c*- and *z*-type fragment ions. Generation of the peptide radical can be observed both in positive and negative ion modes. An advantage of this approach is the ability to finely alter the reactivity of the radical by altering the structure of the azo species conjugated to the peptide. Possible mechanistic pathways yielding the *c*- and *z*-type fragment ions, and a discussion of the pertinent bond dissociation energies, are presented.



Scheme 5.1. Vazo 68.

5.2. Experimental

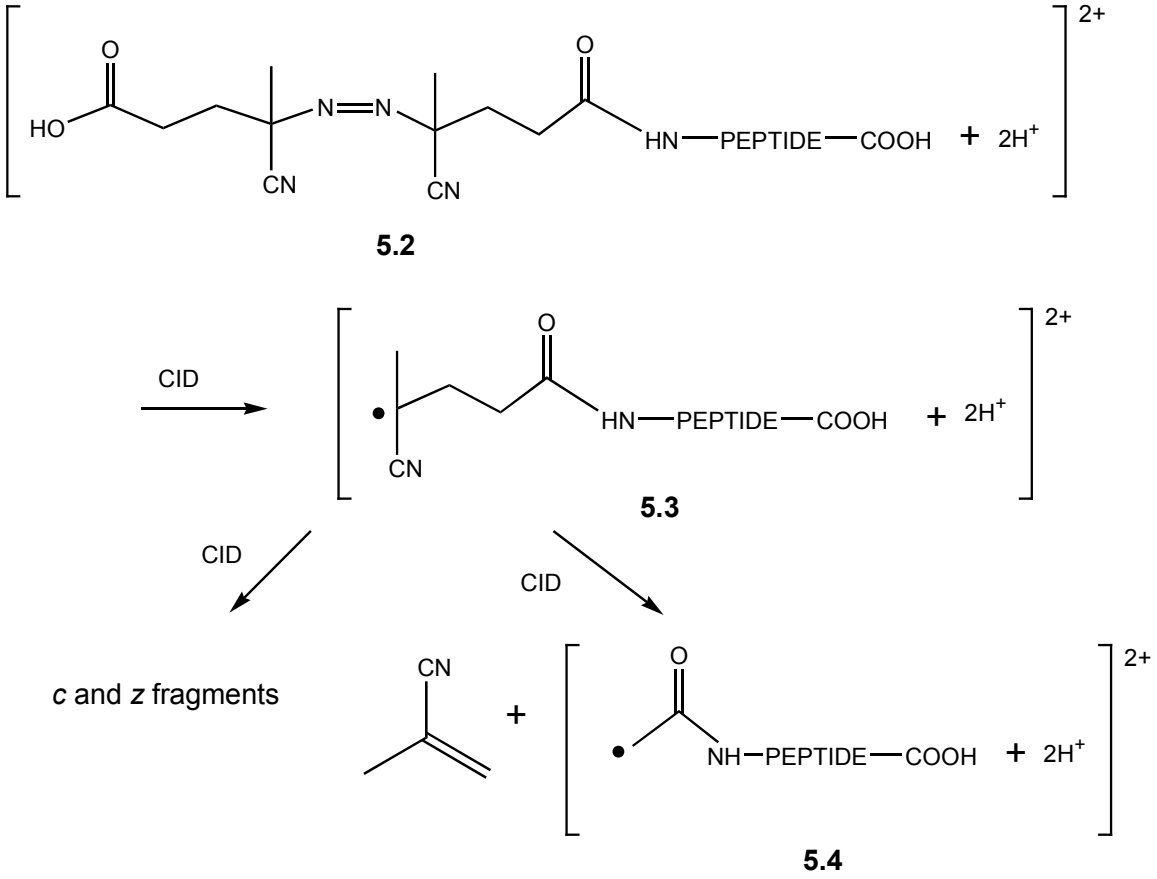
Vazo 68 was procured from DuPont (www.dupont.com). To synthesize conjugated peptides, Vazo 68 and N,N-dicyclohexylcarbodiimide (DCC) were dissolved in N,N-dimethylformamide (DMF) at concentrations of approximately 40 mM. This solution was added to an equivalent volume of 2 – 5 mg/mL aqueous solution of peptide and the reaction mixture was protected from light. All peptides were obtained from American Peptide Company (Sunnyvale, CA). The reaction was allowed to progress for one hour. This solution was then diluted by 10^5 in methanol and used in electrospray experiments.



5.1

Compound **5.1** was synthesized by Jeremy May in the Stoltz group at Caltech. Vazo 68 was transformed to the dichloride species and added to 18-crown-6-methanol to generate **5.1**.

All experiments were conducted on an LCQ Deca quadrupole ion trap instrument with a standard electrospray ion source. Solution flow rates were 3 μL/min. Ions of interest were isolated and subjected to collisional activation with helium gas until product peaks were observed.



Scheme 5.2. The observed gas phase reaction of the Vazo 68-peptide conjugate.

5.3. Results and Discussion

5.3.1. Covalently Bound Radicals

A mass spectrum of a solution of a derivatized peptide, Angiotensin II, is shown in Figure 5.1a. In this case, the derivatized, doubly charged peptide is present at an intensity 12% that of the most abundant ion intensity. In general, the Vazo 68 derivatized peptides (shown generally as **5.2** in Scheme 5.2) were detected at intensities ranging from 1% to 15% of the most abundant ion intensity. Typically the most abundant ion was the underivatized peptide.

Figure 5.1b shows the results of CID of the Vazo 68 derivatized peptide. The major peak in the spectrum corresponds to formation of a free radical species obtained from decomposition of the azo moiety, shown generally as **5.3** in Scheme 5.2. A loss of 28 Daltons is also observed, which corresponds to loss of N_2. Symmetrical azoalkanes like Vazo 68 tend to decompose by simultaneous loss of N_2 and generation of two alkyl radicals.[17] MS^3 experiments on the N_2 loss peak show *b*- and *y*-type fragment ions, as well as losses of 27 and 126, corresponding to HCN and the tertiary radical of 4-cyano-4-methylbutyric acid, respectively. The 4-cyano-4-methylbutyric acid radical corresponds to half of the Vazo 68 species after N_2 is lost and appears to form a non-covalent adduct with the peptide following initial dissociation of the azo moiety. The fragmentation pattern shown in Figure 5.1b is observed for all Vazo derivatized peptides studied and is considered to be diagnostic of the presence of the Vazo derivatized peptide.

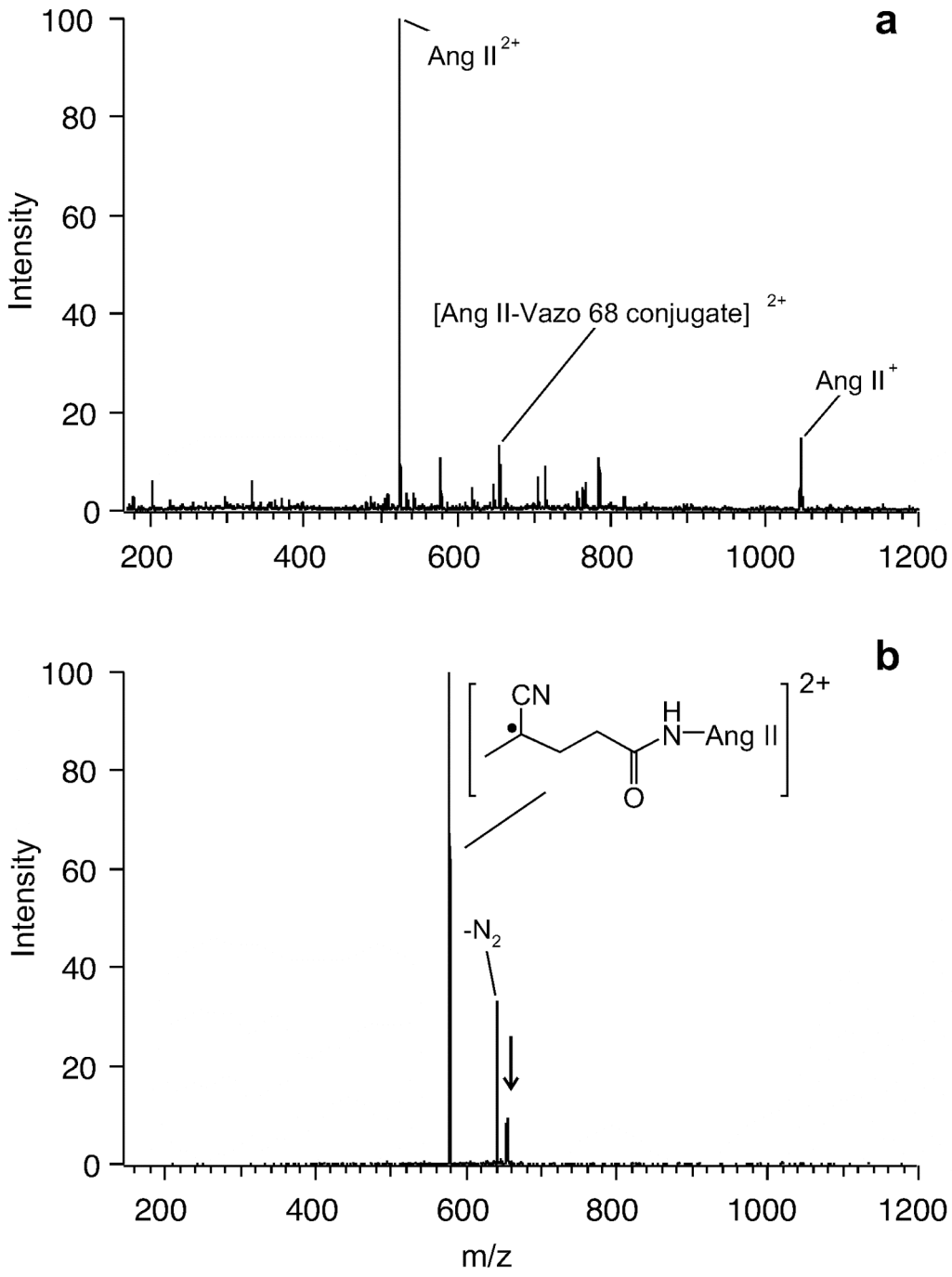


Figure 5.1. (a) Full scan showing doubly angiotensin II (Ang II) and Vazo 68 derivatized angiotensin II. (b) Spectrum resulting from CID of the derivatized peptide 5.2. Loss of N_2 and formation of a radical species are observed. Arrows point to the dissociated species.

The newly formed free radical species, generally referred to as **5.3**, can be subjected to further collisions. Figure 5.2a shows the results of isolation of **5.3** in the case of angiotensin II, DRVYIHPF. The major product ion results from the fragmentation of the remaining covalently bound Vazo 68 to give a primary free radical **5.4** (Scheme 5.2) and 2-methyl acrylonitrile. Again, this product is commonly observed with high abundance for the peptides studied. Also produced are a number of fragment ions, including the z_3, z_5, z_7, c_3, x_5, and a_4 fragments. Figure 5.2b shows the spectrum resulting from collision-induced dissociation of unmodified angiotensin II. In comparing these two spectra, we see that the unmodified peptide yields predominantly *b*- and *y*- type fragment ions under CID, while the free radical peptide **5.3** yields predominantly *x* and *z* fragments, and the fragmentation occurs at different sites on the peptide backbone. The *x* and *z* fragments are also commonly observed in electron capture dissociation experiments,[3,15] in which peptides are made into radical species by the pickup of an electron. Figure 5.3 contrasts the location of the backbone cleavages for CID fragmentation and FRIPS fragmentation. In the ECD spectrum of a similar peptide, angiotensin I (DRVYIHPFHL), fragments are observed that arise from many more peptide backbone cleavages (z_1, z_2, z_5, c_1, c_2, c_3, c_5, c_6, c_7, c_8).[18] FRIPS results in fewer fragments and may be a more selective fragmentation process, as discussed later in the chapter.

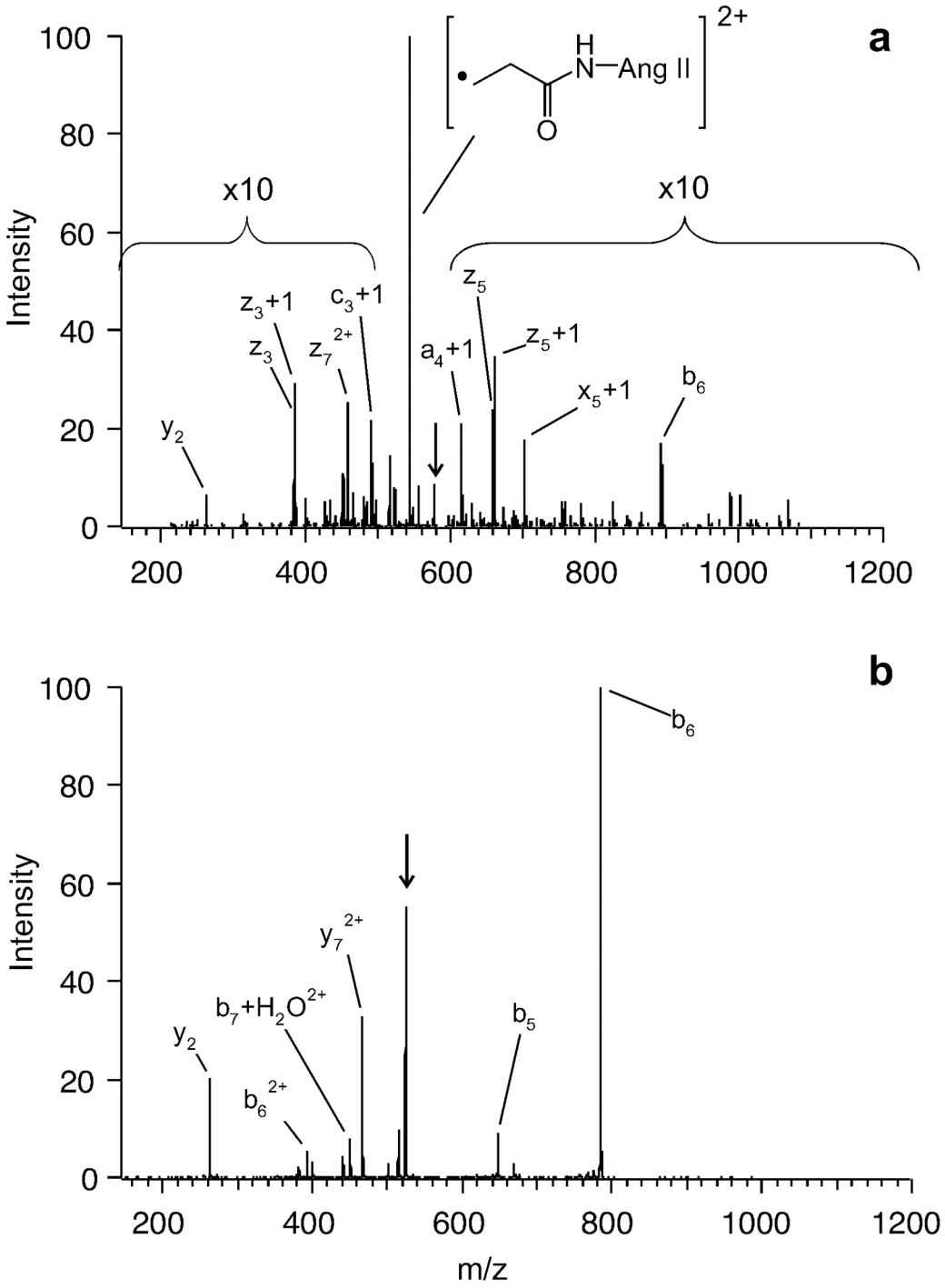


Figure 5.2. (a) Spectrum resulting from collision-induced dissociation of the doubly protonated free radical species formed in Figure 5.1. (b) Spectrum resulting from collision-induced dissociation of doubly protonated angiotensin II. Arrows point to the dissociated species.

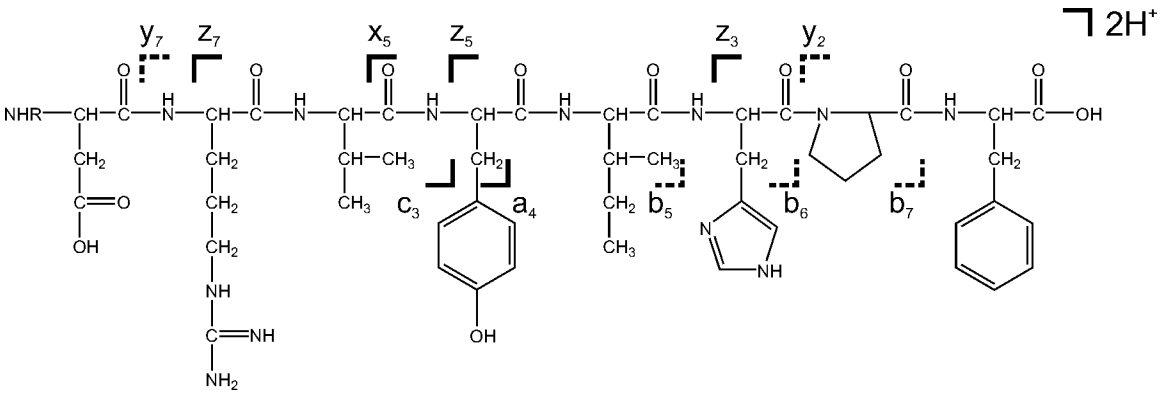


Figure 5.3. Backbone cleavages for the angiotensin II-Vazo 68 conjugate. Solid lines show products of FRIPS fragmentation; dotted lines show products of CID fragmentation. R = –COCH2CH2C(CH3)(CN)•.

Figure 5.4 and Figure 5.5 show results of collision-induced dissociation of both derivatized and unmodified free bradykinin, RPPGFSPFR, and anxiety peptide, QATVGDVNTDRPGLLDLK. In both cases, the free radical induced peptide sequencing (FRIPS) technique results in fewer identifiable backbone fragments than collision-induced dissociation of the unmodified peptide. Table 5.1 summarizes the results of the collision-induced dissociation experiments conducted on protonated, larger peptides. The peptides KA, RL, and GGH were also examined, but typically the MS^3 step resulted only in loss of 2-methylacrylonitrile and did not produce fragments resulting from peptide backbone cleavage. In addition, the large protein ubiquitin, with 77 amino acids, was also studied. While multiple CID steps were diagnostic of free radical formation, and FRIPS spectra showed differences from conventional CID spectra, the spectra were too complex to be readily interpreted.

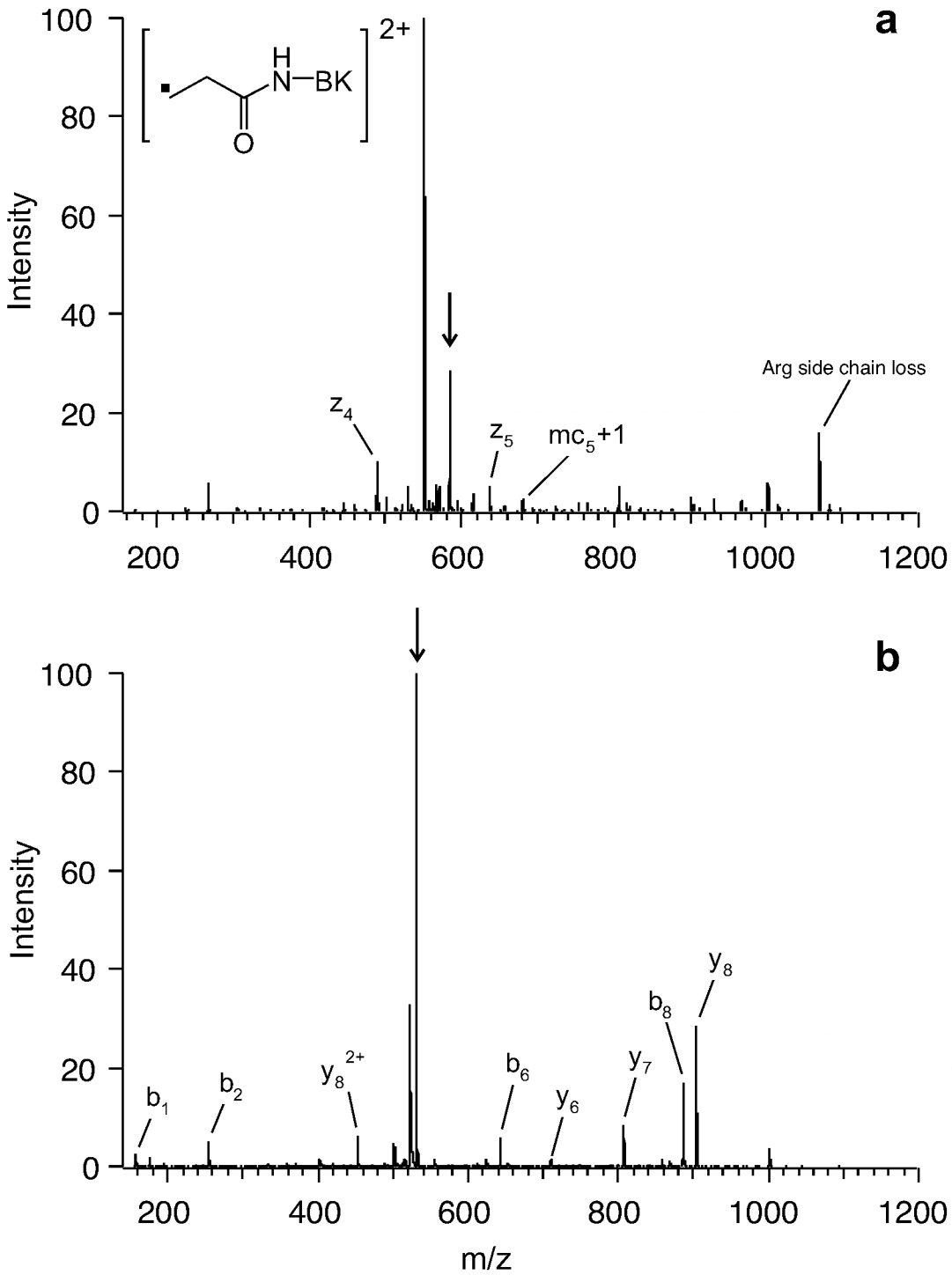


Figure 5.4. (a) Spectrum resulting from CID of doubly protonated 5.3, where the peptide is bradykinin. (b) Spectrum resulting from CID of doubly protonated unmodified bradykinin. Arrows point to the dissociated species.

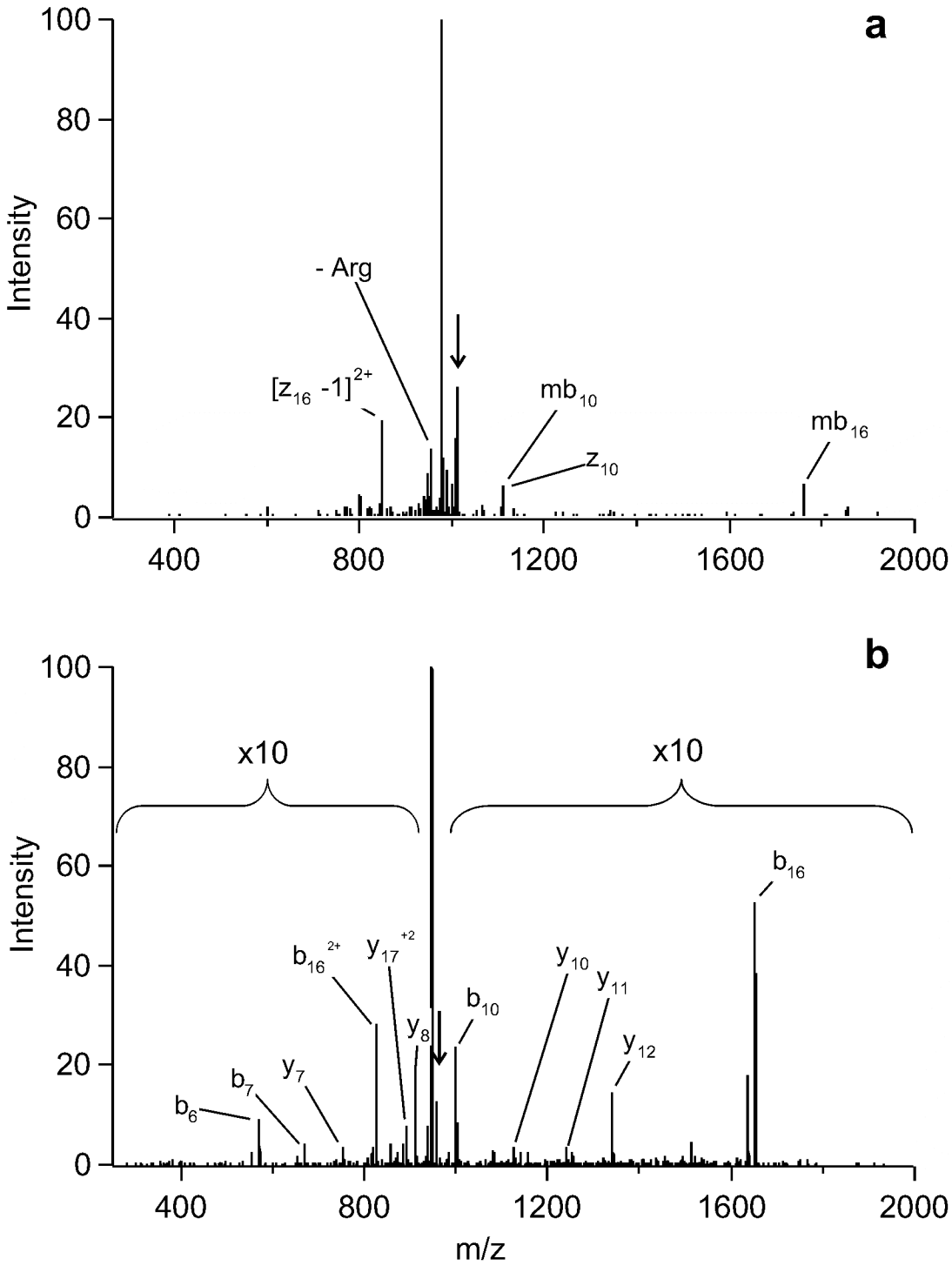


Figure 5.5. (a) Spectrum resulting from CID of doubly protonated 5.3 for anxiety peptide, QATVGDVNTDRPGLLDLK. (b) Spectrum resulting from CID of doubly protonated anxiety peptide. Arrows point to the dissociated species.

Table 5.1. Results of FRIPS experiments

Peptide	Sequence	CID Fragments	FRIPS Fragments[1]
Angiotensin II^{2+}	DRVYIHPF	b_7, y_7, b_5, b_6, y_2	z_7, x_5, z_5, z_3, y_2, b_6
Bradykinin^{2+}	RPPGFSPFR	b_1, y_8, b_2, y_7, b_6, b_8	z_5, c_5, z_4
Anxiety Peptide^{+2}	QATVGDVNT DRPGLLDLK	b_6, b_7, y_7, b_{16}, y_{17}, y_8, b_{10}, y_{10}, y_{11}, y_{12}	z_{16}, z_{10}, b_{10}, b_{16},
ACTH^{+4}	SYSMEHFRW GKPVGKKRR PVKVYP	b_2, b_8, y_{22}, b_{11}, y_{13}, y_{19}, y_{20}, y_{21}, y_{16}	z_{22}, z_{17}
Met-Enk^{-1}	YGGFM	c_2, Y_2, x_3+1, b_4	z_4, x_4, - Tyr, - Met

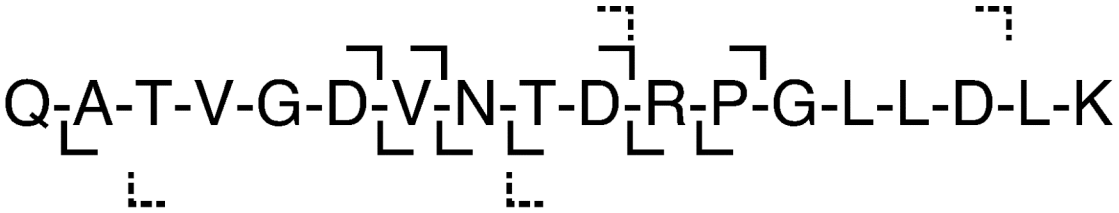


Figure 5.6. Fragmentation patterns for anxiety peptide. Solid lines show CID fragmentations, while dotted lines show FRIPS fragmentations.

Excluding angiotensin II, the peptides listed in Table 5.1 all undergo more fragmentation when subjected to CID than when the conjugated peptide is subjected to FRIPS. This is especially noticeable for larger peptides. For example, the anxiety peptide is 18 residues long. It cleaves at seven distinct sites in CID experiments, but only

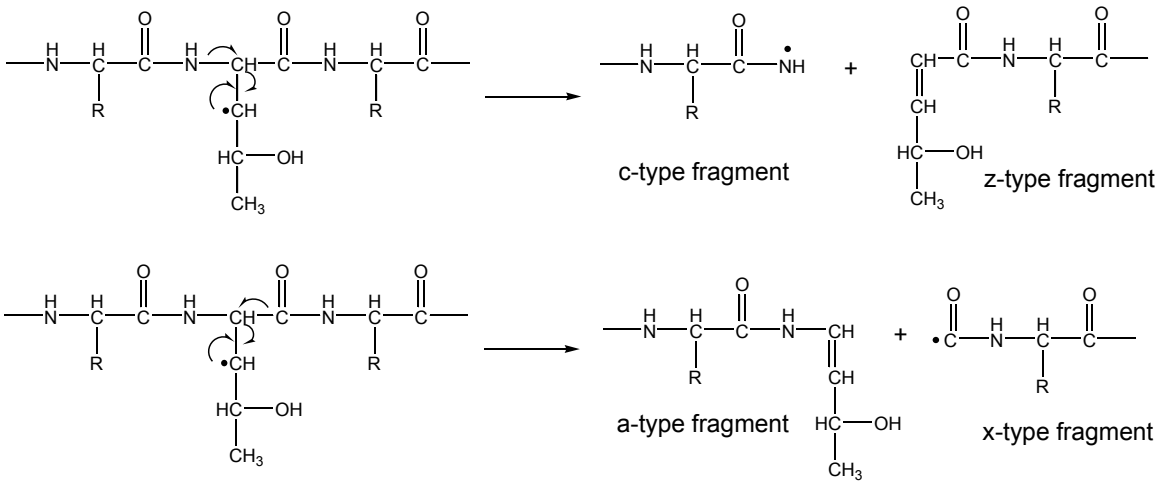
[1] Fragments are from MS3 spectra, where conjugated peptide is isolated and then activated (MS2) and then dissociated (MS3)

at four distinct sites in FRIPS experiments (Figure 5.6). The *z* fragments occur at the C-terminal side of a threonine residue, which has a hydroxyl group. It is likely that the observed *b* fragments result from CID-type excitation, as the b_{10} and b_{16} fragments are formed with relatively high yield when the underivatized peptide is dissociated, as shown in Figure 5.5.

Fragmentation of the peptide 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. Calculations of the bond dissociation energies (BDEs) of bonds along the peptide backbone suggest that the α-carbon hydrogens are most weakly bound, with BDEs ranging from 326-369 ± 10 kJ/mol depending on the side chain.[19] Secondary structure increases the BDEs, by about 10 kJ/mol for a β-sheet and 40 kJ/mol for an α-helix, and may play an important role in determining the selectivity of radical induced fragmentation. In comparison, a model for the radical derived from Vazo 68, 2,2'-azobis(2-methylpropionitrile) (AIBN), has a C-H bond energy at the 3° carbon of 362 ± 8 kJ/mol.[20] However, the abstraction of an α-carbon hydrogen cannot explain the majority of fragments seen using FRIPS, as β-cleavage would generate *b*- and *y*-type fragment ions.

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, could produce either *c* and *z* or *a* and *x* fragments, as shown in Scheme 5.3. These types of fragments are the majority of fragments observed in the FRIPS fragmentation process. The α-carbon hydrogen in ethanol, a model for the serine

side chain, has a BDE of 389 ± 8 kJ/mol, and the α-carbon hydrogen in *t*-butanol, a model for the threonine side chain, has a BDE of 381 ± 4 kJ/mol. Abstraction by the Vazo 68 radical of either of these hydrogens would be only slightly endothermic. For reference, the BDE of ethane (an alanine model) is 423 ± 2 kJ/mol.



Scheme 5.3. Mechanism for FRIPS peptide fragmentation involving abstraction of a side chain methylene hydrogen. A variety of fragments (*a*, *c*, *x*, *z*) are formed.

The FRIPS technique works in negative ion mode as well as positive ion mode, as shown in Figure 5.7a. The loss of the methionine side chain and tyrosine side chain dominate the spectrum. The O-H BDE of the tyrosine side chain may be approximated by that of phenol, 361.9 ± 8 kJ/mol,[20] making the abstraction of phenolic hydrogen thermoneutral. This species could then react to lose an OC6H4CH quinolic molecule, resulting in the peak labeled "-Tyr" in Figure 5.7. A similar side chain abstraction may result in the loss of the methionine side chain.

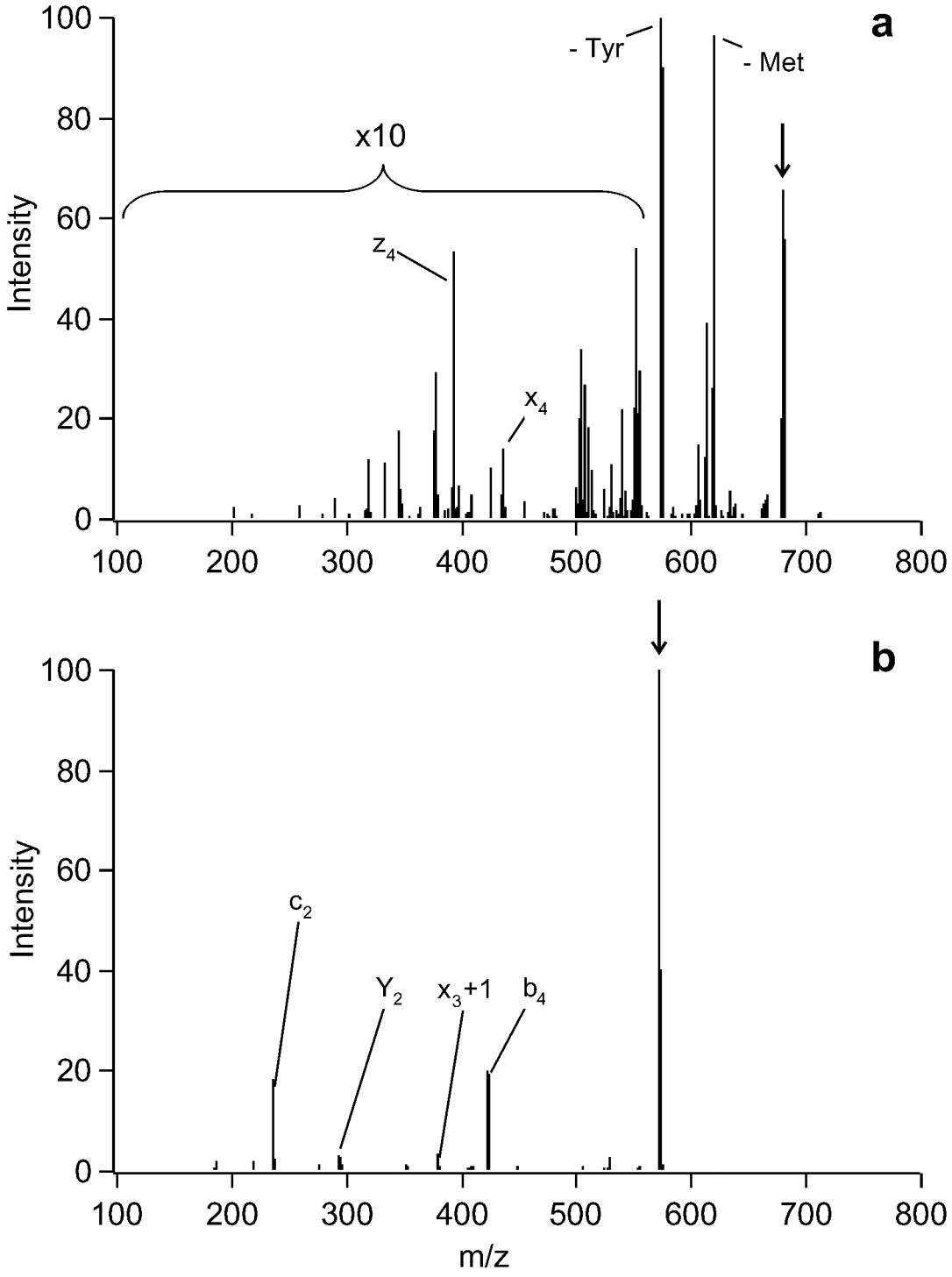
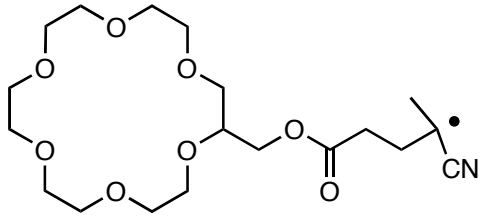


Figure 5.7. (a) Spectrum resulting from collision-induced dissociation of singly negatively charged 5.3 for met-enkephalin. (b) Spectrum resulting from CID of singly negatively charged met-enkephalin.

5.3.2. Noncovalently Bound Radicals

The crown ether 18-crown-6 (18c6) complexes readily with ammonium ions in the gas phase.[21,22] This molecule has been previously used for molecular recognition of lysine in small peptides and proteins.[23] The compound **5.1** was synthesized to combine the molecular recognition properties of 18c6 with the radical initiator Vazo 68. The compound should preferentially bind to lysines, and the radical generated from this species, **5.5**, could then cleave a peptide at sites adjacent to a lysine residue.



5.5

Figure 5.8 shows the results of an experiment using **5.1** and the peptide prodynorphin (PDN), with the sequence YGGFLRRQFKVVTRSQEDPNAYYEELFDV. This peptide has three arginine (R) residues and one lysine (K) residue. The full mass spectrum shown in Figure 5.8a shows formation of adduct peaks. When the +3 adduct is dissociated, the predominant reaction pathway leads to loss of **5.1**, and loss of N_2 is also observed, as shown in Figure 5.8b. The N_2 loss product dissociates to give +3 PDN (data not shown). Since there are three arginines and three protons, the arginines may be sequestering the charge from the lysine residue. In this case, the crown ether would associate with arginine, which binds 18c6 less strongly than lysine does.[24]

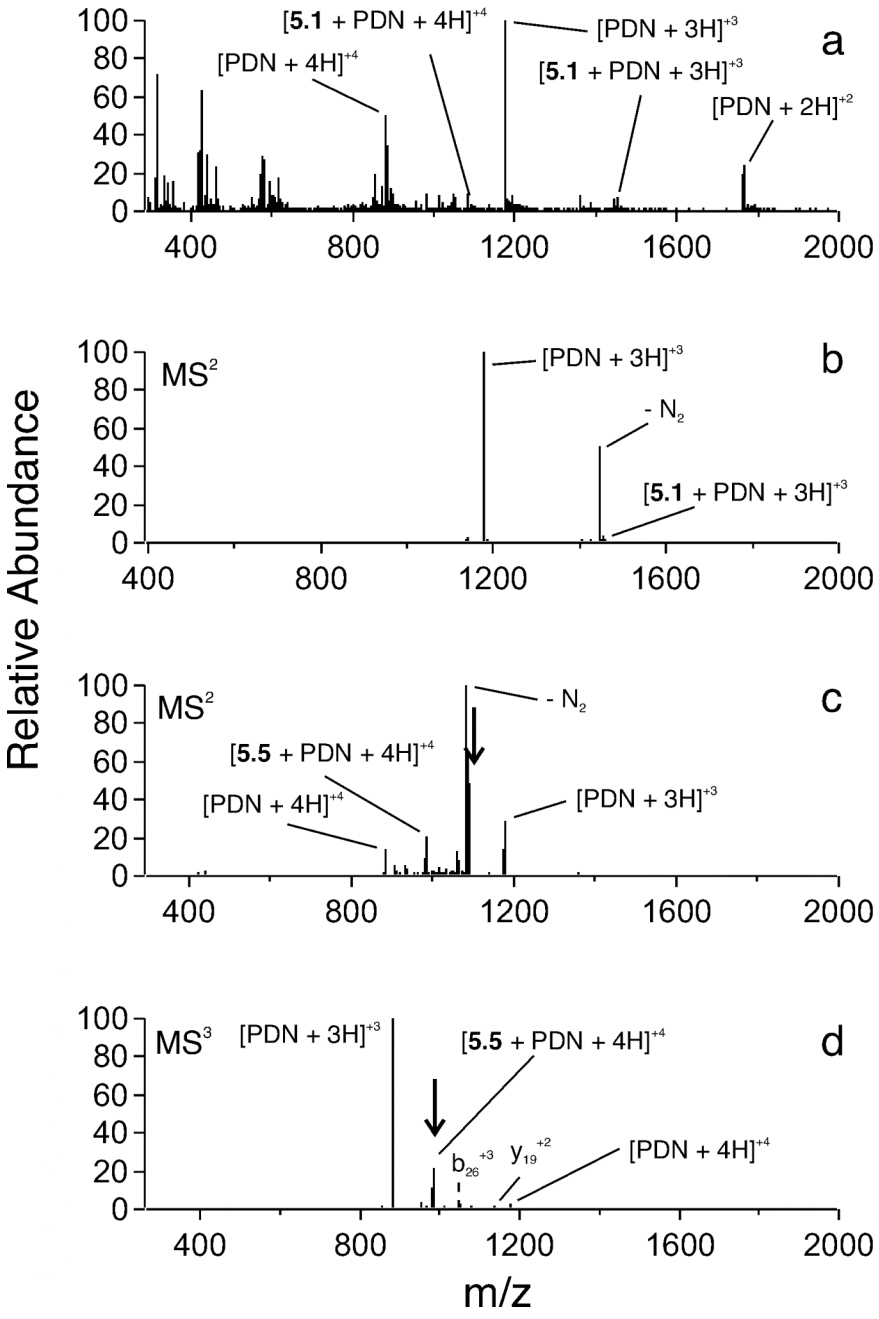


Figure 5.8. (a) Mass spectrum of a solution containing 12 μM prodynorphin (PDN) and 12 μM 5.1. Adduct peaks are observed. (b) Dissociation of the +3 adduct shows loss of N_2 and loss of 5.1. (c) Dissociation of the +4 adduct shows an activation product, $[5.5 + PDN + 4H]^{4+}$. (d) Dissociation of the activation product yields mostly PDN, but small fragmentation products are also observed.

The spectrum obtained from collisionally activating the +4 adduct is shown in Figure 5.8c. An activated radical, [**5.5** + PDN + 4H]$^{4+}$, is produced, along with a loss of N_2 and loss of **5.1**. In this case, there are enough protons to protonate all three arginine residues and the lysine residue. Subsequent dissociation of the activated radical [**5.5** + PDN + 4H]$^{4+}$ predominantly results in loss of the radical species, which abstracts a proton to give [PDN + 3H]$^{3+}$ or is lost as a neutral so that [PDN + 4H]$^{4+}$ is seen. However, some fragmentation products are also observed. The fragment tentatively assigned as y_{19}^{2+} in Figure 5.8d corresponds to a fragmentation on the C-terminal side of the lysine residue, as would be expected if the crown ether were noncovalently bound to lysine. However, the b_{26}^{3+} fragmentation site is far from the lysine residue. A different 18c6-radical initiator, that produces a less stable radical, might result in more backbone fragmentation and provide more insight into whether selective fragmentations can be achieved by combining non-covalent complexation of an 18c6 with a radical species.

5.4. Conclusion

Free radical initiated peptide sequencing (FRIPS) is a novel method of sequencing peptides in the gas phase. It is accomplished by using standard peptide coupling techniques to conjugate a free radical initiator (in this case, Vazo 68) to the N-terminus of a peptide. This conjugated species is then electrosprayed into a mass spectrometer and collisionally activated to produce a free radical species. Subsequent collisional dissociation fragments the peptide radical, producing mostly *c*- and *z*-type fragment ions. Generation of the peptide radical can be observed both in positive and negative ion modes.

In this approach, the reactivity of the radical can be changed by altering the structure of the azo species conjugated to the peptide. For example, different free radical initiators could produce 1°, 2°, or 3° radicals with different reactivities. Highly reactive radicals will indiscriminately abstract hydrogen atoms from different sites on the peptide, leading to non-selective fragmentation. More stable radicals, on the other hand, will abstract only the hydrogens that are weakly bound to the peptide, leading to more selective fragmentation. The degree of selectivity can be controlled by the reactivity of the radical conjugated peptide. The radical produced in the decomposition of Vazo 68 is relatively stable, and hence selectively reactive. Isobutyronitrile, a model for the radical derived from Vazo 68, has a C-H bond energy at the 3° carbon of 362 ± 8 kJ/mol.[20] This is much less than the 1°, 2°, and 3° C-H bond energies of ethane (423 ± 2 kJ/mol), propane (413 ± 2 kJ/mol), and isobutane (404 ± 2 kJ/mol),[25] indicating the stability of the Vazo 68 radical over these hydrocarbon radicals. The synthesis of different azo compounds offers the possibility of tuning the reactivity of the conjugated peptide to enhance or diminish reactivity. For example, the 3° C-H bond in 1,1-diphenylethane has a BDE of 338.9 ± 8 kJ/mol[20] and would be a more selective radical initiator than Vazo 68. In addition, choosing a free radical initiator with less probability of internal fragmentation (such as the loss of 2-methyl acrylonitrile observed for Vazo 68) should result in more peptide backbone fragments.

However, thermodynamics alone may not provide adequate information for truly rational design of versatile FRIPS reagents. For example, the reaction trends of thiyl radicals with peptides correlate poorly with BDEs of α-C-H bonds.[26] Kinetic data regarding the energy of activation, E_{act}, for conjugated azo species such as **1** and peptide

fragmentation processes are also necessary. The activation energy of thermolysis of AIBN, our model compound for Vazo 68, is 128 kJ/mol in benzene, an apolar solvent that should provide a reasonable approximation to gas phase conditions.[17] Blackbody infrared radiative dissociation (BIRD) experiments have measured the activation energy of fragmentation for several peptides in the gas phase. Singly charged bradykinin has an E_{act} of 125 kJ/mol, while the E_{act} of doubly charged bradykinin is 81 kJ/mol, with standard deviations of 3 – 10 kJ/mol.[27] If the conjugation of Vazo 68 to the peptide resulted in no change in the activation energy for fragmentation of the peptide, we would expect the doubly charged bradykinin conjugate **5.2** to fragment into *b*- and *y*- type ions before the Vazo 68 moiety dissociated. However, this is contrary to the experimental evidence; therefore, the conjugation of the peptide to Vazo 68 changes the dissociation pathways and their activation energies. It would be useful to carry out BIRD experiments on the conjugated peptide species to determine E_{act} of dissociation and discover whether the activation energy of dissociating N_2 is lowered by peptide conjugation. BIRD experiments on several azo-conjugated peptides could provide the framework for rational design of future FRIPS reagents.

A combinatorial peptide library would also be essential to determine the details of the FRIPS reaction mechanism and any potential enhancement of fragmentation at specific side chains. The data reported here suggest that certain amino acids, such as threonine and serine, direct FRIPS cleavage processes; however, secondary structure effects have not been ruled out. Synthesis of peptides known to have specific secondary structures (such as $(Ala)_n$, which has an α-helical structure) would help determine whether this influences FRIPS fragmentation, while synthesis of a sequence of

polyglycine peptides containing one non-glycine residue would establish whether certain amino acids enhance specific backbone cleavages.

With a finely tuned free radical initiator and a deeper understanding of the mechanism of FRIPS fragmentation, this technique could potentially act as a true gas phase enzyme, cleaving only at specific side chains in a protein. This would be a critical step toward a completely gas phase approach to peptide sequencing. This technique 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.

5.5. References

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