Characterization of the Primary Thermal Degradation Processes of Peptides Using the Mass Spectrometric Technique K^+IDS, K^+ Ionization of Desorbed Species

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The mass spectrometric technique of K^+ ionization of desorbed species, K^+IDS, is used here to characterize the primary thermal degradation chemistry of small peptides. In this technique, a small amount of a compound is rapidly heated in the condensed phase. Desorption of the intact molecule can occur. Also, thermal degradation products are formed which quickly desorb as well, rather than remain on the surface and undergo subsequent chemistry. The desorbed molecules form adducts with gas phase K^+ ions, and a mass spectrum is obtained. Deuterium labeling experiments, and the use of derivatizing reagents, allows for the thermal degradation chemistry of small peptides to be elucidated. Apparently, skeletal bond cleavages are accompanied by H-shifts, although the hydrogen atoms shift from "remote" sites, brought into close proximity with the fragmenting skeletal bond via secondary interactions. Experimental results are presented that allow for correlations between thermal degradation chemistry and the resulting K^+IDS mass spectra to be made. *(J Am Soc Mass Spectrom 1994, 5, 564–575)*

The pyrolysis/thermal degradation of organic molecules is a process that has been used both for the production of chemicals and for analysis [1]. In the latter, mass spectrometry has been an important tool in characterizing products of pyrolysis (Py) of organic compounds, both directly in techniques such as in Py/MS, and with a separation step such as in Py/gas chromatography/MS. In pyrolysis studies, temperatures of 600–800 °C are frequently used. The characterization of thermal degradation processes can be challenging since, under some conditions, primary degradation products decompose further and undergo subsequent chemistry, yielding complex mixtures of products. To date, little work has been reported on the thermal stability/thermal degradation of peptides in general; however, information is available on the pyrolysis of small dipeptides and amino acids [2].

In this article, we present K^+ ionization of desorbed species (K^+IDS) for the characterization of the primary thermal degradation chemistry of peptides. K^+IDS is a desorption/ionization technique for mass spectrometry that was introduced in 1984 [3]. It is based on the use of rapid heating of an analyte to produce, in the gas phase, intact analyte molecules and thermal degradation products. These desorbed species interact with gas-phase K^+ ions, generated by thermionic emission, to yield K^+ adducts of the desorbed neutral molecules, for subsequent mass spectrometric analysis [4]. Rapid heating is used due to the temperature dependence of vaporization and decomposition [5]. It has been shown that, at sufficiently high temperatures, vaporization of thermally labile molecules and degradation chemistry can occur at competitive rates [5]. Here, the K^+IDS technique is presented as a tool for investigating the primary thermal degradation processes of uncharged peptides.

The strength of the K^+IDS technique is in the simplicity of both the experiment and the resulting mass spectra. Since each thermal degradation product gives one peak in the mass spectrum, K^+IDS mass spectra reflect a molecular weight distribution of the thermal degradation products of the analyte molecules, which, in combination with analogous studies of derivatized and isotopically labeled compounds, can be used for the study of unimolecular, condensed phase thermal degradation chemistry. However, the simplicity of the mass spectra is also a complication since *only* the molecular weight of each degradation product is directly determined. The approaches presented here are designed to provide insights into the structures of the thermal degradation products, and to demonstrate a unique and simple tool for characterizing thermal degradation processes for biomolecules, as well as other thermally labile compounds.

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There are a number of reasons why the thermal degradation chemistry of peptides is of current interest. The first is a process in mass spectrometry called charge-remote chemistry. When mass spectrometry is used for structural analysis of peptides, the analyte molecules undergo desorption and ionization via techniques such as fast-atom bombardment (FAB). When a peptide, M, is converted into a gas-phase cation in the FAB ion source, it appears as a protonated molecule, MH^+, which subsequently fragments (or is induced to fragment), giving structural information. There are two types of processes through which protonated peptides can fragment. The first is that most commonly considered in mass spectrometry for even-electron ions—the chemistry occurs at the site of protonation, and is *charge-initiated*. A second possibility is a *charge-remote* [6–8] mechanism. In the latter, bonds are broken far from the charge site, and are unaffected by the charge. They are driven by excess energy in the protonated molecule (as are charge-initiated processes). It is very difficult to distinguish between these two mechanisms because, while the precursor to all fragment ions is the protonated peptide molecule in positive ion FAB, the initial site of protonation is not known and cannot be controlled. The advantage of the K^+IDS technique is that the molecules undergo fragmentation (thermal degradation) before a charge is placed on the molecule. Thus, for mass spectrometrists it may represent a model for charge-remote chemistry. Molecular fragmentation occurs due to energy deposition in the unionized molecule. It is hoped that the chemistry of bond cleavage in peptides will be sufficiently different for these two cases, charge-initiated and charge-free, such that the contributions from each in the fragmentation of protonated peptides can be deduced.

Secondly, there have been a number of experimentally simple, direct probe approaches for the mass spectrometric analysis of compounds such as small peptides. In such experiments, solid samples are introduced into an electron impact (EI) or chemical ionization (CI) source and heated. Mass spectra are generated, which are either simple mass spectra of desorbed molecules, or complex spectra, resulting from the simultaneous ionization of desorbed molecules and desorbed thermal degradation products [9, 10]. The results of the experiments presented here provide direct information on such processes, to assist in the evaluation of the results of such experiments.

Experimental Section

All mass spectrometric analysis were performed on an unmodified HP5985 GC/MS/DS quadrupole mass spectrometer (Hewlett-Packard, Avondale, PA) with a mass range of 10–1000 Da equipped with a modified direct insertion probe, similar to that reported previously by Röllgen and co-workers [11] and by Kassel and Allison et al. [12]. The probe places two wire filaments into the ion source. One filament serves as the thermionic emitter, generating K^+ ions when resistively heated. The other filament serves as the sample holder from which the analyte molecules undergo thermal degradation and desorption. The sample holder is radiatively heated from the ionic emitter or resistively heated with a separated power supply (direct heating). The analyte molecules and their degradation products are thermally desorbed from the second filament and form adducts in the gas phase with the potassium ions emitted from the first filament.

The peptides *H*-GP-*OH*, *H*-GGFL-*OH*, cyclo-GP, *H*-YGGFL-*OH*, and *H*-FGGF-*OH* were obtained from the Sigma Chemical Co. (St. Louis, MO) and used without further purification. *H*-AGGFL-*OH*, *H*-(β-A)GGFL-*OH* (β-A is β alanine), *H*-VGGFL-*OH*, *H*-GGVFL-*OH*, *H*-VGVAPG-*OH*, *H*-VGVA$^{\alpha d}$PG-*OH*, *H*-V$^{\beta d}$GV$^{\beta d}$APG-*OH*, and *H*-AF$^{\beta d2}$LA-*OH* were synthesized in the laboratory of Dr. Phil Andrews at the University of Michigan. The three partially deuterated peptides listed above were synthesized with the corresponding deuterated amino acids as the starting materials. N-terminally acetylated peptides such as Ac-VGVAPG-*OH* are formed from the free peptide using acetic anhydride and a small amount of pyridine as a catalyst by the procedure described by Knapp [13].

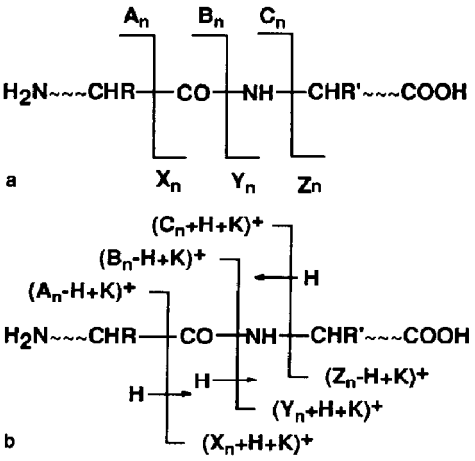
All of the samples used in K^+IDS experiments were dissolved or suspended in methanol to allow transfer of 1–2 μL of sample onto the tip of the sample holder. The solvent was evaporated off before the probe was inserted into the mass spectrometer.

Results and Discussion

Nomenclature for Sequence Ions

To precisely designate the neutral species and ions formed in K^+IDS, a variation of the original Roepstorff nomenclature [14] is used. For a peptide $NH_2 \sim\sim CHRCO\text{-}NH \sim\sim COOH$, if the interior amide bond cleaves, two fragments (radicals) will be formed. The N-terminal fragment, $NH_2 \sim\sim CHRCO\cdot$, is called a B_n fragment and the C-terminal fragment, $\cdot NH \sim\sim COOH$, is called a Y_n fragment. The ions formed in the K^+IDS experiment are K^+ adducts of even-electron species. Skeletal bond cleavages are accompanied by an H-shift or an OH-shift in thermal degradation processes for peptides. Thus, ionic designations such as $[Y_n + H + K]^+$, $[B_n - H + K]^+$, $[C_n + H + K]^+$, and $[B_n + OH + K]^+$ will be used, where n is the number of amino acid residues (or number of side chains) contained in that fragment. From this designation scheme, the mass-to-charge ratio value of each ion under discussion is unambiguous. The details of the nomenclature scheme, indicating the types of ions observed (the types of thermal degradation products generated), are given in Scheme I. The K^+ adduct of each intact peptide will be designated as $[M + K]^+$.

In peptide chemistry, one-letter designations are frequently used for amino acid residues. To designate



Scheme I. Nomenclatures for peptide fragments and ions. (a) Roepstorff nomenclature [14] for peptide fragments; (b) nomenclature for ions in K^+IDS.

a peptide completely, the H at the N-terminus and the OH at the C-terminus will be specifically indicated. To avoid confusion between histidine (H) and hydrogen (*H*), individual atoms will be indicated in italics; that is, *H* designates a hydrogen atom and H designates a histidine residue.

The K^+IDS Experiment

In the K^+IDS experiment, submicrogram quantities of an analyte are placed on a rhenium wire, and the temperature is increased from room temperature to approximately 300 °C in 10 s. The temperature is then held at this final value until the sample is depleted. During the experiment, K^+ ions attach to desorbed neutral molecules, the mass spectrometer is repetitively scanned, and a set of mass spectra are collected. For some samples, there is a time (temperature) dependence of the resulting mass spectra since each degradation process has a unique activation energy, which leads to the differences in the relative intensities of mass spectral peaks in different scan files. However, what is frequently observed is that a number of processes begin to occur at approximately the same temperature. This might be expected for peptides since, even though each amino acid residue has a unique side chain, peptides consist of only three types of skeletal bonds.

If the tetrapeptide *H*-FGGF-*OH* is subjected to rapid heating, and K^+ ions are attached to molecular species that desorb into the gas phase, typical experimental results are shown in Figure 1a. As shown, the spectra collected as desorption and thermal degradation occur are summed, and a single K^+IDS spectrum is reported, which will provide information on the low-energy thermal degradation processes. Note that, as the thermal degradation chemistry occurs, some of the peptide

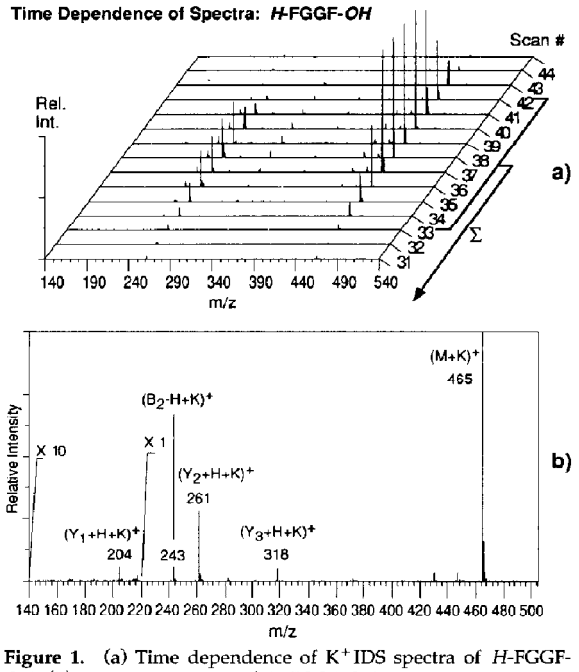


Figure 1. (a) Time dependence of K^+IDS spectra of *H*-FGGF-*OH*; (b) average spectrum of *H*-FGGF-*OH*.

molecules desorb intact. A temperature is achieved at which desorption and thermal degradation are competitive. If a peptide decomposes on the surface to two smaller molecules, they appear to promptly desorb rather than remain on the surface and decompose further. Thus, the experiment allows for the primary degradation processes to be investigated. Following such primary unimolecular processes, prompt desorption of thermal degradation products occurs because of the temperatures used.

The spectrum shown in Figure 1b is a typical K^+IDS mass spectrum for a small peptide. All of the ions detected are K^+ adducts. For example, the peak in the spectrum at m/z 465 represents a K^+ adduct of the intact analyte molecule, which weighs 426 Da (K^+ weighs 39 Da). The peak at m/z 261 represents a K^+ adduct of a thermal degradation product with a mass of 222 Da. A fragment of the peptide can be generated with this mass by cleaving the central amide bond, with one additional hydrogen atom, to yield (Y_2 + H), which can also be written as *H*-GF-*OH*. Thus, the ions at m/z 261 are designated as $[Y_2 + H + K]^+$ or [*H*-GF-*OH*]K^+. By correlating K^+IDS mass spectra with the structures of many peptide analyte molecules, the trends in the degradation chemistry become clear, and ionic designations become apparent. Note that the $[Y_n + H + K]^+$ ion series in the spectrum (n = 1–3) provides information on the amino-acid sequence of the peptide and K^+ adducts of the intact, desorbed peptide provide the molecular weight information. Thus, K^+IDS is a useful mass spectrometric method as well

as a tool for understanding thermal degradation chemistry.

Figure 2a shows the K^+IDS mass spectrum of *H*-VGVAPG-*OH* as a second example. Again, the spectrum shows that both the intact molecule and its thermal degradation products can be formed simultaneously. All of the ions observed are K^+ adducts of closed-shell molecules that can be related to the parent molecule, formed by skeletal bond cleavages with accompanying hydrogen atom shifts.

The spectra shown in Figures 1b and 2a are typical of the K^+IDS results obtained to date in the following respect. The dominant ions are K^+ adducts of degradation products that result from cleavage of amide bonds, even though these bonds are the strongest skeletal bonds. While two neutral products are generated (simultaneously and in equal quantities) when an amide bond is broken, $(Y_n + H)$ and $(B_m - H)$, the $[Y_n + H + K]^+$ ions are more intense than the corresponding $[B_m - H + K]^+$ ions. The information which follows will provide insights into these observations.

The Primary Thermal Degradation Processes of Peptides

The thermal degradation chemistry for peptides, involving skeletal bond cleavage with an accompanying hydrogen atom shift, had been proposed [15] to follow a 1,2-elimination process, Scheme II. However, additional experimental results suggest an alternative. A 1,2-elimination mechanism would suggest that the probabilities of cleavage of any of the amide bonds would be similar since the process involves only amide bonds and adjacent H atoms. In our experiments, however, the rates for cleaving the various amide bonds differ dramatically throughout a single peptide, as well as from one peptide to another. Also, this mechanism cannot explain the formation of $[C + H + K]^+$ ions, which are occasionally observed, since there is no hydrogen atom at the required position (on the carbonyl group) to participate.

Since the 1,2-elimination mechanism is inconsistent with the experimental results for peptides, alternative degradation mechanisms were considered. Since H-shifts are frequently involved in the thermal degradation of peptides, as well as other thermally labile molecules [3, 15], determining the source of the shifting H is key to determining the mechanism. This can be done using deuterium labeling.

Deuterium-Labeling Experiments

Two deuteration approaches for peptides have been successfully used. The first method involves deuterium exchange in a deuterated solvent such as CH_3OD and D_2O. Hydrogen atoms that are bound to the N-terminal nitrogen, amide nitrogens, and the C-terminal carboxylate group can be exchanged. To demonstrate the utility of H/D exchange, consider the dipeptide *H*-GP-*OH*. When this small peptide is subjected to rapid heating, desorption of the intact molecule dominates. A fraction of the molecules dehydrate, and the $(M-H_2O)$ molecules desorb as well. The

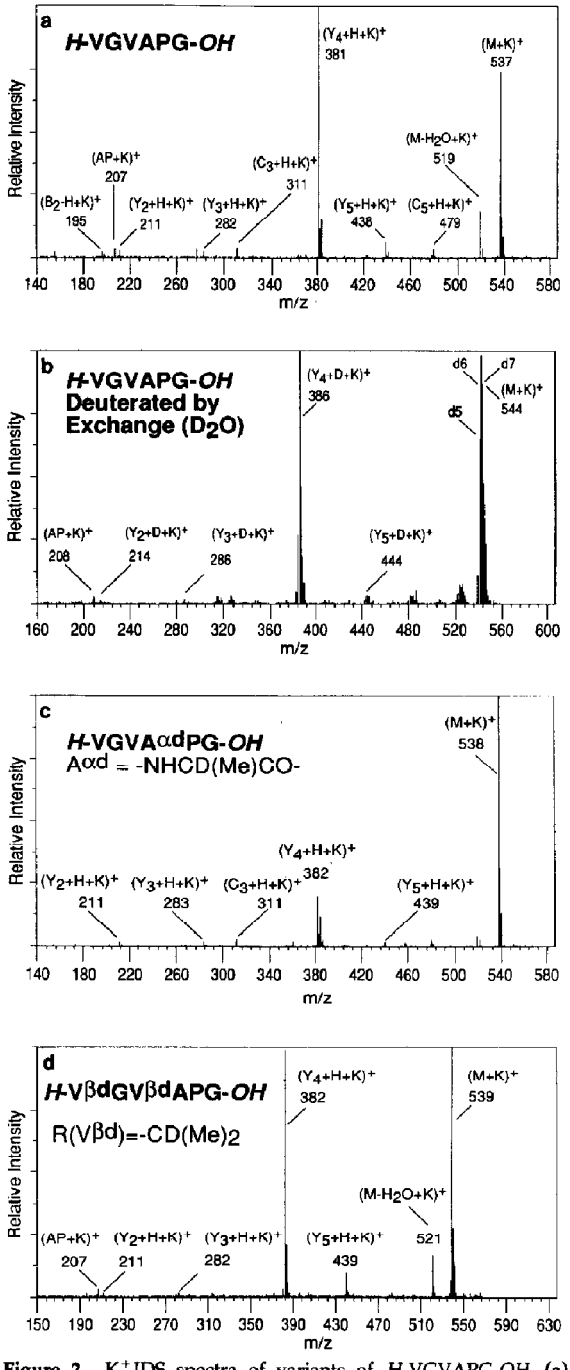
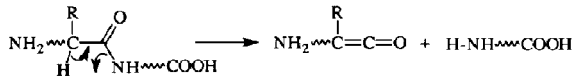


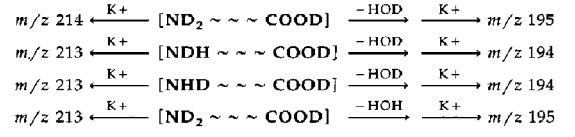
Figure 2. K^+IDS spectra of variants of *H*-VGVAPG-*OH*. **(a)** unlabeled; **(b)** peptide after exposure to D_2O; **(c)** *H*-VGVA$^{\alpha d}$PG-*OH*; **(d)** *H*-V$^{\beta d}$GV$^{\beta d}$APG-*OH*.



Scheme II. 1,2-elimination mechanism.

elimination of water from a peptide poses the same questions as the processes in which skeletal bonds cleave. To eliminate H_2O, a C-OH bond cleaves with an accompanying H-shift. What is the source of the shifting hydrogen atom? Water elimination is analogous to the process that forms B- and Y-type products. With small dipeptides, skeletal bond fragmentation is usually a minor process relative to water elimination; thus, they provide an opportunity for characterizing this particular process. Also, small peptides contain a relatively small number of exchangeable H atoms, and it is easier to approach 90 + % H/D exchange. If *H*-GP-*OH* is dissolved in D_2O, placed on the K^+IDS probe, and the experiment performed, the spectrum shown in Figure 3 is obtained. There are three exchangeable H atoms in this dipeptide. Complete exchange of these three H atoms yields a K^+ adduct of the intact peptide at *m/z* 214, with those molecules undergoing incomplete exchange represented by the peaks at lower mass-to-charge ratio values. The distribution of the $[M + K]^+$ peaks indicates that 90% of the exchangeable Hs have been replaced by deuterium atoms.

The dipeptide *H*-GP-*OH* has the elemental composition $C_7H_{12}N_2O_3$. The deuterated form that can be obtained via H/D exchange is $C_7H_9D_3N_2O_3$. In the spectrum of the deuterated dipeptide, the mass-to-charge ratio value of the dominant peak representing the dehydration product is 194, which corresponds to $[C_7H_9D_1N_2O_2 + K]^+$. The relationship of the isotopic variants for this species, relative to that for the $[M + K]^+$ ions, is as follows. If the loss of H_2O, in the form of DHO or H_2O, goes through a 1,2-elimination process, the degradation will produce the following ions:



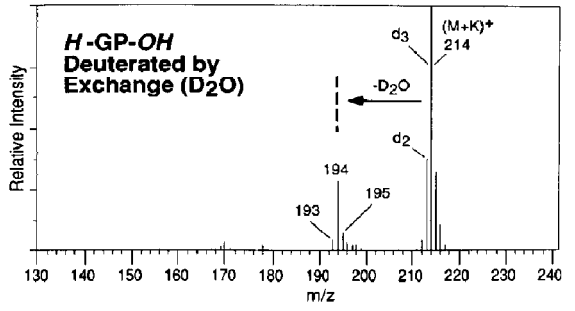
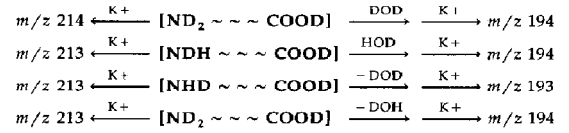


Figure 3. K^+IDS spectrum of *H*-GP-*OH* that had been exposed to D_2O.

Each expression above indicates the two termini of the deuterated peptide and the mass-to-charge ratio value of the corresponding K^+ adduct. The intensity of the K^+ adduct of each deuterated variant reflects its relative abundance. When a water molecule is lost from the peptide molecule, the adduct formed will have the mass-to-charge ratio value shown on the right side. If exchange of each candidate H atom occurs to approximately the same extent, and the water elimination occurs via a 1,2-elimination (involving an H atom close to the C-terminus) the expected ratio of *m/z* 194 to 195 would be 1:5, which is NOT in agreement with the experimental value of 4:1. However, if the loss of $H_2O/HDO/D_2O$ is through a 1,6-elimination in which the shifting H atom is from the N-terminus (allowing for a D-shift from the opposite end of the molecule), the elimination would allow for the loss of D_2O), and would give the following ions:



The expected ratio of *m/z* 193 to 194 will be $(40 \times 1/3):(100 + 40 \times 2/3) = 1:9$, which is in very good agreement with the experimental data (1:6). Simply, the spectrum suggests that most of the dipeptide molecules contain three deuterium atoms, and the water elimination that follows involves the loss of D_2O. This can only happen via a cyclic intermediate. Therefore, the loss of H_2O in the thermal degradation of this dipeptide occurs via a 1,6-elimination process.

The H/D exchange in D_2O was also undertaken with larger peptides such as the hexapeptide *H*-VGVAPG-*OH*, which has seven exchangeable H atoms. The resulting K^+IDS spectrum is shown in Figure 2b. The various isotopomers of the $[M + K]^+$ ion suggest extensive deuterium incorporation, with more than 93% of the exchangeable sites deuterated. In this hexapeptide, the dominant ion is the $[Y_4 + H + K]^+$ species, resulting from cleavage of the amide bond with an H shift onto the C-terminal fragment. An analysis of the isotopic species show that an exchangeable H shifts in the process; thus, a D-shift is observed in this case as well. It cannot be unambiguously determined whether the source of this shifting D is the N-terminal amine group, or an amide nitrogen in the N-terminal fragment. However, it is important to note that the bond cleavage/H shift is *not* a 1,2-elimination reaction, and the peptide backbone does not allow for a 1,3-elimination process, so Hs that are four or more skeletal atoms away must be involved in the chemistry.

The second way to prepare partially deuterated peptides is to use partially deuterated amino acids in the peptide synthesis. In this way, deuterium atoms can be placed on carbon atoms in the peptide skeleton

as well as in side chains. The following partially deuterated peptides were synthesized: *H*-VGVA$^{\alpha d}$PG-*OH*, *H*-VGVA$^{\beta 3d}$PG-*OH*, *H*-V$^{\alpha d}$GV$^{\alpha d}$APG-*OH*, *H*-V$^{\beta d}$GV$^{\beta d}$APG-*OH*, and *H*-AF$^{\beta d2}$LA-*OH*, with α and β designating the deuteron position(s). In these peptides, deuteriums are present on the backbone of the peptide chain such as -$CDCH_3$- (in $A^{\alpha d}$), and others on the side chains of the molecules such as -CD_3 (in $A^{\beta 3d}$) and -$CD(CH_3)_2$ (in the $V^{\beta d}$ residue).

The K^+IDS spectra of unlabeled *H*-VGVAPG-*OH*, as well as *H*-VGVA$^{\alpha d}$PG-*OH* and *H*-V$^{\beta d}$GV$^{\beta d}$APG-*OH* are shown in Figure 2a,c,d. The spectra show that the deuterium incorporation by synthetic methods is complete, and no reverse-exchange occurs. The results consistently show that, when Y-type ions are formed, H shifts only occur from nitrogen sites—amide groups and/or the N-terminus. The source of the shifting H atoms is not those Hs that are bound to carbon atoms in the amino acid residues.

Such deuteration experiments alone do not allow for unequivocal determinations of the sources of the shifting H that accompanies skeletal bond cleavage to form the dominant degradation product, (Y_n + H). To complete the investigation, other variants of the peptides were characterized.

Peptide Derivatization—Evidence for Cyclization

Insights were gained in the thermal degradation chemistry of small peptides via the N-acyl derivatives. When a peptide is N-acetylated, and there is no longer an amino group at the N-terminus, changes in the K^+IDS mass spectra are observed. For the underivatized hexapeptide *H*-VGVAPG-*OH*, as shown in Figure 2a, the dominant ions observed in the K^+IDS spectrum are the $[Y_n + H + K]^+$ ions, as has been previously discussed. When the N-terminus of *H*-VGVAPG-*OH* is blocked with an acetyl group forming CH_3CO-VGVAPG-*OH*, the dominant ions observed are *no longer* $[Y_n + H + K]^+$ ions. Instead, an unexpected series of ions of the type $[B_n + OH + K]^+$ and $[C_n + H + K]^+$ ions become dominant (see Figure 4). These observations suggest a scenario which is summarized in Scheme III. First, since there is such a dramatic change in the chemistry upon acylation, when the N-terminal amino group is removed, then the thermal degradation chemistry for underivatized peptides that yields molecules of the type (Y_n + H) must involve an H-shift from the N-terminus. If this group is removed, no Y-type products are formed. Second, if the Y-type products are all formed via an H-shift from the N-terminus, then the secondary structure of the decomposing peptide is critical, and must involve a cyclic intermediate. When the N-terminal amine group that can participate in H-bonding is removed, products that appear to involve cyclic intermediates through H-bonding to the C-terminus are observed. (It is readily apparent that *-OH shifts* accompanying the cleavage of skeletal bonds many atoms away occur through cyclic intermediates.) Thus, the peptides appear to react through cyclic conformations. The H-bonds involving the N-terminus usually dominate. However, if the N-terminus is blocked, the C-terminus participates in important secondary interactions. Third, if peptides undergo skeletal bond cleavages and H-shifts (or OH shifts) through cyclic intermediates, then one linear product and one *cyclic* product should be produced in each case. Note that the *dominant* (Y_n + H) product is formed by elimination of a cyclic, six-membered ring dipeptide. Thus, the dominant cyclic intermediate involves an H-bond via an eight-membered ring. Also, when the N-terminus is blocked and cyclic intermediates involving the C-terminus form, the dominant (B_n + OH) product is formed by, again, elimination of a cyclic dipeptide. Thus, cyclic interactions that involve six- and eight-membered rings simultaneously, as shown in Scheme III, form the dominant products.

To further investigate the possibility of remote H-shifts via cyclic intermediates, the synthesis of peptides with other types of N-terminal modifications, for subsequent analysis by K^+IDS, was pursued. Two forms of the amino acid alanine are available. In addition to the standard α-amino acid, NH_2-$CH(CH_3)$

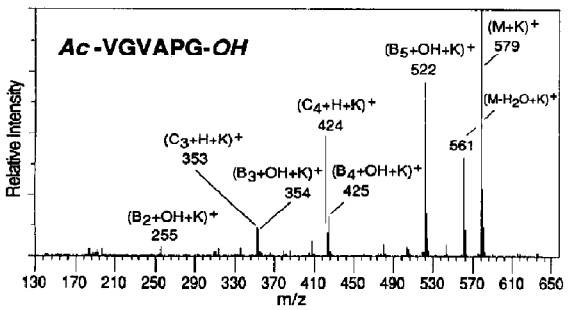
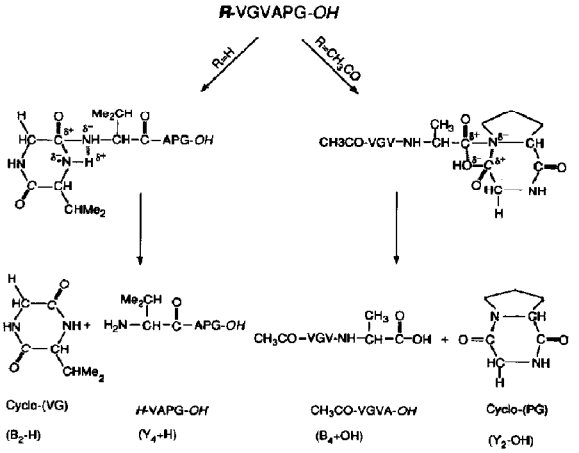


Figure 4. K^+IDS spectrum of CH_3CO-VGVAPG-*OH*.



Scheme III. Thermal degradation of peptides.

COOH, β-alanine is available, NH_2-CH_2-CH_2-COOH. The presence of β-alanine in a peptide will disrupt the normal sequence of the skeletal backbone. To investigate the influence of modifications of the N-terminus on thermal degradation, two peptides were synthesized, *H*-AGGFL-*OH* and *H*-(β-A)GGFL-*OH*, and their thermal degradation chemistries were compared. The resulting K^+IDS spectra are shown in Figure 5. For *H*-AGGFL-*OH* (Figure 5a), formation of $(Y_n + H)$ products dominate, with the $[Y_3 + H + K]^+$ ion indicating that elimination of a cyclic dipeptide from the N-terminus is the dominating thermal degradation pathway. When the N-terminal residue is changed to β-alanine (Figure 5b), the spectrum changes substantially. Amide bond cleavages with accompanying H-shifts still occur, although they no longer dominate the desorbed products. Note that if amide bond cleavages still involve H-shifts from the N-terminus, then the formation of $(Y_n + H)$ products would involve the formation of four-, seven-, ten-membered cyclic products. Apparently, none of these is as favored as the process that forms a six-membered cyclic dipeptide, which occurs for *H*-AGGFL-*OH* only. Also note that the ratio of thermal degradation to desorption of the intact molecule upon heating is smaller when β-A is at the N-terminus, leading to the $[M + K]^+$ ion as the base peak in Figure 5b. These changes in the spectrum when the N-terminal residue is modified in this way is consistent with the proposed importance of *remote* H-shifts from the N-terminus, accompanying skeletal bond cleavages throughout the peptide, and the importance of the formation of a cyclic product.

Termini, Side Chains, and Steric Effects

It is clear from the above data that the primary, low-energy, thermal degradation chemistry of peptides involves cyclic intermediates and cyclic products. When the N-terminus of a peptide is not blocked, cyclization involves the shift of an H atom from the N-terminus; when the N-terminus is blocked, cyclization involves the C-terminal OH group. The N-bound H atoms at the termini of a peptide are, in part, most active in their participation in cyclic interactions because fewer steric obstacles are present, relative to those encountered by interior amide N-H groups. If steric effects are important in the thermal degradation of peptides, the sizes of the side chain of the amino acid residue at the termini may influence the distribution of thermal degradation products. Insights into one aspect of steric effects can be realized through the K^+IDS spectra of the following series of peptides with various N-terminal residues: *H*-YGGFL-*OH*, *H*-VGGFL-*OH*, and *H*-AGGFL-*OH*. These are shown in Figures 6a,b and 5a, respectively. When the N-terminal residue is Y, V, or A, the spectra are essentially the same. The mechanism shown in Scheme III for the free peptide dominates. Note, in particular, the ratio of the intensities of the ions representing $[Y_3 + H + K]^+$ and $[Y_4 + H + K]^+$, which are formed by eliminating neutrals contain-

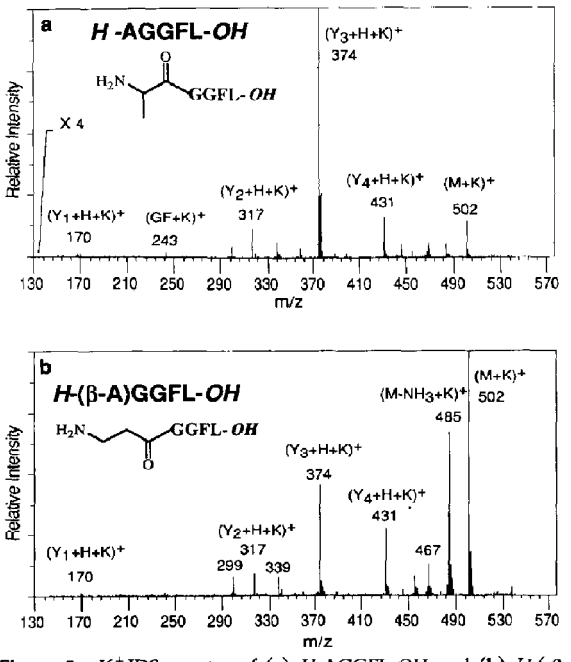


Figure 5. K^+IDS spectra of (a) *H*-AGGFL-*OH* and (b) *H*-(β-A)GGFL-*OH*.

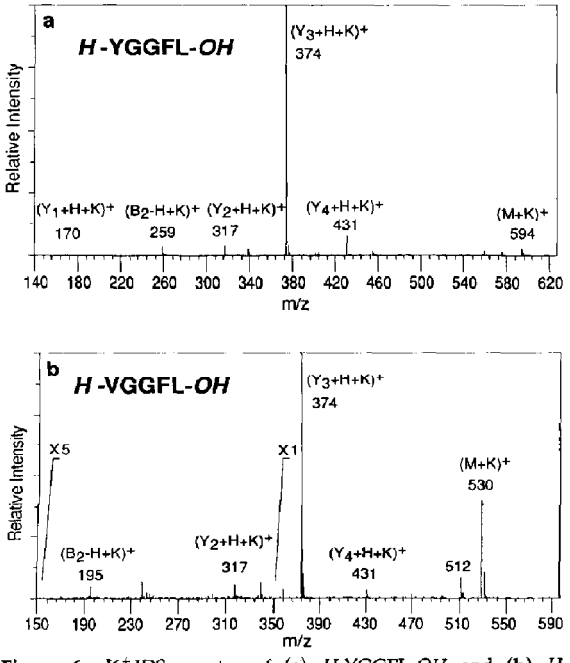


Figure 6. K^+IDS spectra of (a) *H*-YGGFL-*OH* and (b) *H*-VGGFL-*OH*.

ing six- and three-membered rings, respectively. Obviously, the former dominates. However, when the side chain of the N-terminal residue is smaller, changes are observed. Figure 7a shows the K^+IDS spectrum of another pentapeptide, *H*-GGVFL-*OH*, in which the N-terminal glycine contains the smallest side chain (least steric effects); the spectrum is very different. The relative probabilities of cleavages at various amide bonds change completely. In this case, the cleavage at the first amide bond is almost competitive with that at the second amide bond. Apparently, the size of the side chain in the N-terminal residue does have an influence on the distribution of the various cyclic intermediates, with the smallest cyclic intermediates being uncommon, unless the N-terminal residue is glycine. The same is observed in the spectrum of the tetrapeptide H-GGFL-OH, where the N-terminal residue is again G, Figure 7b. More peptide spectra will be useful to determine whether this effect is always observed for the N-terminal residues, and the precise secondary interactions that are involved in this steric effect.

Thermodynamic Aspects of the Thermal Degradation of Small Peptides

To better understand the mechanisms for the thermal degradation of peptides, thermodynamic aspects of the chemistry were considered. The experimental data presented here suggest that skeletal bond cleavages do not involve 1,2-elimination processes, but 1,*n*-elimination processes, where $n = 6$, 9, etc. Is one energetically favored over the other?

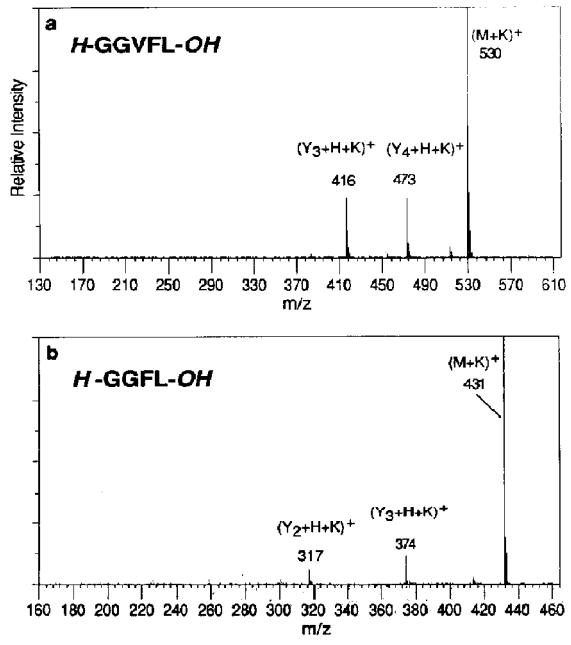
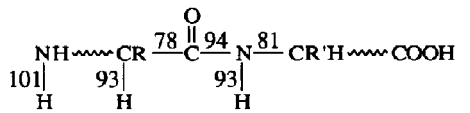


Figure 7. K^+IDS spectra of (a) *H*-GGVFL-*OH* and (b) *H*-GGFL-*OH*.

In all of the thermal degradation processes of peptide molecules that we have observed, two bonds are formed and two bonds are broken. The estimated reaction enthalpies for several possible processes are shown in Scheme IV. To approximate the reaction enthalpies for some of the degradation processes shown, the dissociation energies of the bonds formed ($BDE(B_{f1})$ and $BDE(B_{f2})$) and bonds broken ($BDE(B_{b1})$ and $BDE(B_{b2})$) are considered, and the reaction enthalpies are estimated as follows:

$$\Delta H^\circ_{r\times n} = BDE(B_{b1}) + BDE(B_{b2}) - BDE(B_{f1}) - BDE(B_{f2})$$

The bond dissociation energies (BDEs) that were used are shown below:



The bond dissociation energies for the three skeletal bonds of peptides were obtained from small molecules, for which thermodynamic data are available [16]. For example, BDE(Y-X bond) is actually that for the molecule $CH_3CO—NHCH_3$. Values used for BDE (H-X), where X = NHR, NR_2, CHR_2, etc., were also extracted from available data on small molecules. The BDE for an N-H bond in the terminal amine group is that for a small primary amine.

Reaction enthalpies for 1,2-elimination reactions that are presented again represent those for smaller analogous systems, where sufficient thermochemical information is available. For example, the estimated reaction enthalpy for the highest energy 1,2-elimination, to form {(B + H) and (Y − H)}, represents the enthalpy for the proces:

$$CH_3CONHCH_3 \rightarrow CH_3COH + NH{=}CH_2$$

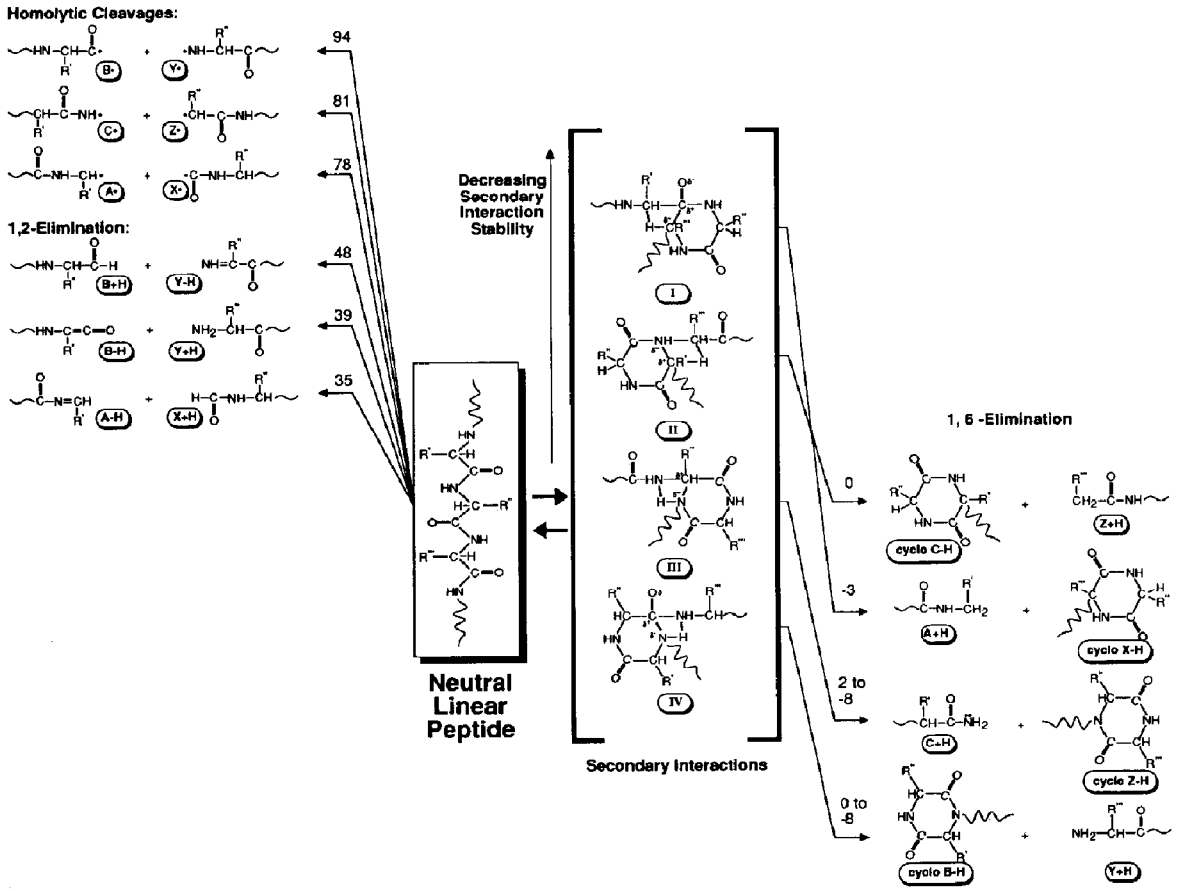
Scheme IV first points out that homolytic cleavage, involving the formation of two radicals, should not be expected since such reactions require much more energy than reactions in which the number of bonds broken is equal to the number of bonds formed, such as a 1,2-elimination. Products of simple homolytic cleavages are not observed under K^+IDS conditions. Lower energy 1,2-elimination reactions (requiring about 40 kcal/mol) also do not occur, as suggested by deuterium-labeling experiments. The lowest energy processes are those suggested on the right side of Scheme IV, in which a cyclic and a linear product are formed in each case. Like 1,2-elimination reactions, these 1,6-eliminations involve the cleavage of two bonds and the formation of two bonds. However, the latter have approximate reaction enthalpies that are less than or equal to 0 kcal/mol. The differences in the

two processes are fairly clear. In the 1,2-elimination, one of the bonds formed involves the conversion of a single bond to a double bond; double bonds have BDEs less than the sum of two single-bond BDEs. Also, in a 1,2-elimination, the environment from which the H moves is different from that to which it moves. In contrast, consider the 1,6-elimination process shown in Scheme IV that yields (cyclo-C-H) and (Z + H). One C-N bond is broken while one is formed. Also, a C-H bond is formed as a C-H bond is broken. Thus, of the mechanisms considered, the 1,6-elimination reactions represent the lowest energy pathways.

If the conversion of a peptide into two smaller peptides, one linear and one cyclic, is thermoneutral or exothermic, why are all not observed? Why does cleavage of one skeletal bond dominate? We suggest that the answer lies in a consideration of the intermediates through which the 1,6-elimination products are formed. These are also shown in Scheme IV, and are arranged to suggest their relative stabilities. In Scheme IV, intermediate IV is expected to be readily accessible. Dipole–dipole interactions will allow the molecule to align as shown, as a prelude to thermal degradation. In contrast, intermediate I would allow (cyclo-C-H) and (Z + H) species to be formed; however, it is an unfavored, high-energy transition state. Its formation requires unlikely secondary interactions according to considerations of the partial charges on the atoms of the peptide backbone. Thus, the B- and Y-type thermal degradation products are most likely formed as shown in Scheme IV—they evolve from the most stable secondary structure, and the overall reaction enthalpy is lowest. (In this case, the estimated enthalpy range reflects differences between N-H bond dissociation energies for Hs of either the N-terminal amino group or from an interior amide group.) The relative stabilities of the transition states determine, as usual, the reaction rates.

Response Discrimination

One question that was posed when Figure 1 was introduced remains. Obviously, whenever a B-type degra-



Scheme IV. Thermal degradation pathways for peptides (estimated energies in kilocalories/mole).

dation product is formed, a Y-type species is formed. However, the peaks representing the $[B_n - H + K]^+$ ions are less intense than those for the corresponding $[Y_n + H + K]^+$ ions. For example, the thermal degradation of the pentapeptide *H*-YGGFL-*OH* will produce $(Y_3 + H)$ and $(B_2 - H)$ in a 1:1 ratio. In the spectrum of *H*-YGGFL-*OH*, however, the abundance of $[Y_3 + H + K]^+$ is 30 times as great as that of $[B_2 - H + K]^+$. Why ?

For ion formation in K^+IDS, three processes are involved—degradation, desorption, and sampling (adduct formation with K^+ ions). We have investigated the latter as the most likely source, that is, that some discrimination exists in the adduct formation process. The nature of interactions between K^+ ions and neutral molecules is electrostatic, and should be sensitive to molecular size and structure, local dipole moments within the molecule, and polarizability. The binding energies are relatively small, 20–40 kcal/mol [17], and adduct ions are presumably stabilized by emission of an infrared photon or dissociate after some time period.

If small compounds such as amino acids are analyzed by K^+IDS, desorption can be made to dominate. Thus, equimolar amounts of two amino acids (A and B) will yield equal numbers of desorbed molecules for subsequent attachment to available K^+ ions. If the peaks representing $[A + K]^+$ and $[B + K]^+$ are of differing intensities, this would indicate a response discrimination. For amino acids, it was found that the responses *were* highly dependent on the size of individual amino acid and on the presence/absence of an aromatic side chain, as shown in Table 1. In the table, the response for Alanine is given a value of 1. Generally the greater the size (and polarizability), the greater the response. Dipeptides usually have greater responses than amino acids; tripeptides usually have greater responses than dipeptides. As the size continues to increase, however, the response differences gradually level off. The results for a series of small peptides are also listed in Table 1.

Since cyclic degradation products are formed in K^+IDS experiments, the relative responses for cyclic compounds were also tested. If equimolar amounts of *H*-GP-*OH* and cyclo-GP are analyzed by K^+IDS, the resulting spectra only contain the $[M + K]^+$ species of each. If the intensity of these two peaks is monitored, the results shown in Figure 8 are obtained. The time-dependent response reflects the desorption of the two species, and the relative responses clearly show that the linear form is sampled much more efficiently than the cyclic form by K^+ ions—the response is six times larger for the linear form. It is reasonable to believe that a linear molecule can form a more stable multidentate complex with K^+ than can a constrained cyclic molecule; thus, the results of these response studies are not unexpected.

Table 1. Relative Response Factors, R_{rel}

	Amino Acids								
	Ala	Gly	Ser	Val	Thr	Met	Leu	Ile	Phe
R_{rel}	1	1/6	2	7	8	8	18	22	32

	Small Peptides			
	Alaline	*H*-A-A-*OH*	*H*-A-A-A-*OH*	*H*-A-A-A-A-*OH*
R_{rel}	1	10	100	200

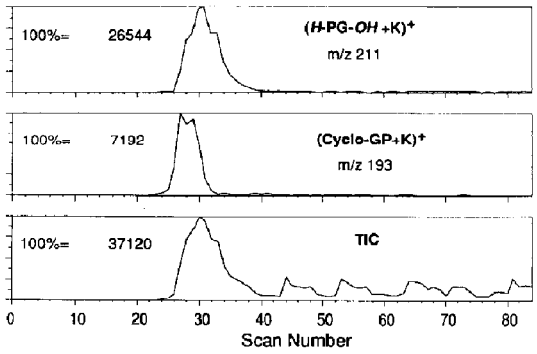


Figure 8. Mass chronograms showing the relative responses for the molecules *H*-GP-*OH* and cyclo-GP in the K^+IDS experiment.

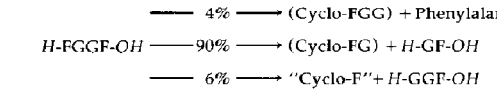
Based on the cyclization mechanism for thermal degradation and the subsequent discrimination in the K^+ adduct formation step against small molecules and against cyclic molecules, interpretation of K^+IDS spectra of peptides becomes straightforward. Since amide bonds cleave most frequently and there is a sampling discrimination against (cyclo-B_n-H), $[Y_n + H + K]^+$ ions dominate K^+IDS spectra while their corresponding $[B_n - H + K]^+$ ions are formed in smaller abundance.

Conclusions

K^+IDS is shown here to be a useful technique for the mass spectrometral characterization of the thermal degradation chemistry of small peptides. The spectra obtained provide both molecular weight and sequence information. In this work, two basic questions relevant to the technique have been answered, which allow spectra to be related to underlying thermal degradation processes.

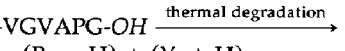
The dominant skeletal bond cleavages in the thermal degradation of peptides occur most frequently at amide bonds. Cyclo-degradation was found to be the major process for thermal degradation of peptides. The formation of a cyclic product is favored because it is the lowest energy process; the dominant cyclization process is the one with the most stable transition state. For underivatized peptides, cyclization involves the N-terminus; the shifting H that accompanies skeletal bond cleavage is from the N-terminus. For N-terminally blocked peptides, cyclization from the N-terminus is hindered, and cyclization involving the C-terminus can occur. In this case, the shifting H or OH is from the C-terminus.

The weak interactions between a K^+ ion and a neutral molecule leads to a finite lifetime for the nascent K^+ adducts. The limited lifetime is reflected in the response discriminations against smaller molecules and cyclic molecules, which give much weaker signals. The discrimination in K^+IDS results in simple, easy-to-interpret spectra of underivatized peptides, which are dominated by $[Y_n + H + K]^+$ ions. The determination of the relative responses of amino acids and small peptides makes it much easier to interpret K^+IDS spectra, and information regarding the probabilities of bond cleavages at different sites along a peptide backbone can be evaluated more accurately. For example, in Figure 1, the $[Y_2 + H + K]^+$ peak is about five times as intense as the $[Y_3 + H + K]^+$ peak, but this does not mean that the rate of bond cleavage at the second amide bond from the N-terminus is five times that for the third amide bond since there is a response discrimination against $(Y_2 + H)$. Since the response for $(Y_3 + H)$ is about five times that for $(Y_2 + H)$, the cleavage at the second amide bond is estimated to occur at a rate 25 times that for the third amide bond. For $[Y_1 + H + K]^+$, the peak is 80 times less intense than that for $[Y_2 + H + K]^+$, but cleavage of the third amide bond from the N-terminus is only 16 times that of the second amide bond since the response favors $(Y_2 + H)$. Thus, relative response studies suggest that the data in Figure 1 reflect the following distribution of primary, low-energy thermal degradation products, as an example:

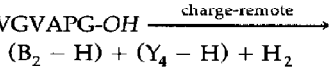


It should be stated that we certainly have not discovered here, for the first time, the importance of secondary structure in peptides! However, it is intriguing to realize that secondary interactions dominate the unimolecular thermal degradation chemistry of small peptides. There appears to be a total lack of chemistry that occurs via a linear form of these molecules. Secondary interactions not only determine the overall shape of the molecule, but create situations that lead to the lowest energy fragments.

Future publications will compare and contrast results presented here with those from FAB experiments, to discuss charge-induced versus charge-remote chemistry in peptides; however, a simple example will begin to provide some insights into the utility of the data presented here. It was suggested in the Introduction that the fragmentation chemistry that occurs in the K^+IDS experiment, since it does not involve a charge, could be a model for charge-remote chemistry. The use of triphenylphosphonium (TPP) derivatives of peptides has been demonstrated in the literature [18]. The charged TPP group can be placed at either the C- or N-terminus of peptides, and skeletal bonds throughout the resulting ion cleave in FAB–MS upon collisional activation—that is, by charge-remote mechanisms. Consider the hexapeptide *H*-VGVAPG-*OH*, and cleavage of a specific internal amide linkage. We have reported here that two products are formed by thermal processes:

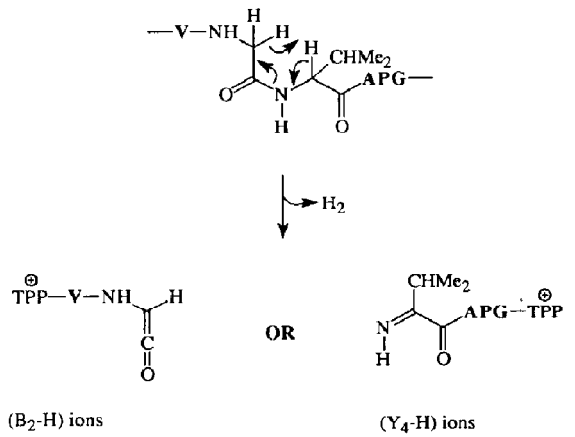


If an ethyl TPP group is attached to the C-terminus of *H*-VGVAPG-*OH* and the same bond cleaves, the N-terminal fragment is charged, and appears as a $(B_2$-H) product—that is, when the skeletal bond cleaves, an H shifts off the N-terminal fragment [19, 20]. What is the other product? To determine this, consider cleavage of the same bond when the TPP group (as the aminoethyl TPP derivative) is attached to the C-terminus. The product is not a $(Y_4 - H)$ product, but a $(Y_4 - H)$ species. Thus, data from two charge-remote cases suggest that the charge-remote chemistry involving cleavage of this skeletal bond yields three products as shown in Scheme V, not unlike previously reported mechanisms [7].



Thus, for cleavage of this amide bond in this particular hexapeptide, pure thermal reactions and charge-remote processes yield different products, and the H-shifts are the signature of each.

Two conclusions can be drawn from this example. First, based on the data presented here, one should not expect all charge-remote chemistry to parallel charge-free chemistry because the latter relies heavily on secondary interactions which would be dramatically altered in situations such as that given here, where a



Scheme V. The formation of $(B_n - H)$ and $(Y_n - H)$ ions from N-terminal or C-terminal TPP-derivatives.

fixed charge is appended to a peptide terminus. Thus, one would certainly not expect the TPP^+-VGVAPG-*OH* to yield similar products as *H*-VGVAPG-*OH* upon activation since the former does not have the N-terminal Hs which play such an important role in the degradation of the latter. If H-shifts were from sites local to skeletal bond cleavage in thermal processes, than more correlations would be expected.

Second, if secondary interactions occur in peptides when no charge is present, some must surely occur when a charge is present in protonated or charge-localized species. Thus, there may be some charge-stimulated processes occurring, which would seem to be charge-remote, depending on whether secondary structures are contributing factors.

Acknowledgments

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