An Investigation of the Energetics of Peptide Ion Dissociation by Laser Desorption Chemical Ionization Fourier Transform Mass Spectrometry

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The energy dependence of competing fragmentation pathways of protonated peptide molecules is studied via laser desorption-chemical ionization in a Fourier transform ion cyclotron resonance spectrometer. Neutral peptide molecules are desorbed by the technique of substrate-assisted laser desorption, followed by post-ionization with a proton transfer reagent ion species. The chemical ionization reaction activates the protonated peptide molecules, which then fragment in accordance with the amount of excess energy that is deposited. Chemical ionization forms a protonated molecule with a narrower distribution of activation energy than can be formed by activation methods such as collision activated dissociation. Furthermore, the upper limit of the activation energy is well defined and is approximately given by the enthalpy of the chemical ionization reaction. Control over the fragmentation of peptide ions is demonstrated through reactions between desorbed peptide molecules with different reagent ion species. The fragmentation behavior of peptide ions with different internal energies is established by generation of a breakdown curve for the peptide under investigation. Breakdown curves are reported for the peptides Val-Pro, Val-Pro-Leu, Phe-Phe-Gly-Leu-Met-NH₂, and Arg-Lys-Asp-Val-Tyr. The experimentally derived breakdown curve of Val-Pro has been fitted by using quasi-equilibrium Rice-Ramsperger-Kassel-Marcus theory to model the unimolecular dissociation of the protonated peptide to provide a better understanding of the mechanisms for the formation of fragment ions that originate from protonated peptides. (*J Am Soc Mass Spectrom* 1995, 6, 1069-1078)

In the last two decades, mass spectrometry has played an increasingly important role in the analysis of biomolecules. Much of the success associated with the analysis of peptides and proteins has resulted from the development of new desorption-ionization methods, such as fast-atom bombardment [1], electrospray ionization [2, 3], and laser desorption [4, 5], that provide the ability to measure the molecular weights of large biomolecules directly. These methods have been used together with tandem mass spectrometry to obtain data that can be used to determine the sequence of amino acids in a peptide. The tandem mass spectrometry methods that have been used to examine peptide structures include collision activated dissociation (CAD) [6-13], surface-induced dissociation (SID) [14-20], and photodissociation (PD) [21-28]. Although ion activation plays an important role in peptide mass

spectrometry, few studies have examined the details of the energetics and kinetics of peptide ion decomposition. We report here the results of a quantitative study of the energy dependence of common peptide fragmentation reactions.

CAD, the most widely used method of ion activation, has well established capabilities to sequence and identify post-translationally modified peptides [29, 30] and peptide fragments produced by the enzymatic digestion of proteins [9]. Although CAD has been highly successful to fragment peptide ions smaller than m/z 3000 and readily produces structurally significant ions, its utility to study the controlled energetics of fragmentation is limited due to the wide distribution of energies imparted to the precursor ions [31, 32]. The more recent technique of surface-induced dissociation (SID) also has been applied to the analysis of peptides. In contrast to CAD, SID deposits a relatively narrow distribution of energies into a precursor ion [33]. However, this energy is difficult to determine quantitatively [34]. SID studies of ions for which the

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energetics of fragmentation have been well characterized show that only a fraction of an ion's laboratory energy is converted into internal energy. For example, for $W(\text{CO}_6^+)$, 13% of the kinetic energy of the ion is converted into internal energy by SID [35]. More recent work, which used self-assembled monolayers to coat the collision surface, allows a different fraction of lab energy to be converted into internal energy during SID [36, 37]. Wysocki and co-workers [37] have demonstrated the formation of a strong series of sequence-specific ions formed by SID of singly and doubly charged peptide ions formed by electrospray ionization. In addition to these collision methods, the use of photodissociation (PD) has been investigated as a means to obtain structural information from peptide ions. In particular, infrared multiphoton dissociation has been found to provide efficient fragmentation of peptide ions. Studies of energy dependence of ion fragmentation are best carried out by single photon dissociation, so that the energy deposited into the precursor ion can be well characterized. Photodissociation is of limited utility for such fundamental studies because peptides are transparent throughout much of the visible and ultraviolet spectrum. Strong absorption occurs only in fairly limited regions, for example, below 220 nm.

As an alternative to the above-mentioned ion activation methods to dissociate biomolecules, we have investigated the use of chemical ionization as a means to deposit a well defined amount of energy into laser-desorbed peptide molecules [laser desorption-chemical ionization (LD-CI)] [38-41]. In these studies, laser-desorbed peptide *neutral* molecules P , of proton affinity $\text{PA}(P)$, are allowed to interact with protonated reagent ion species RH^+ of proton affinity $\text{PA}(\text{R})$, as in reaction 1. The internal energy of PH^+ is approximately equal to minus the enthalpy of proton transfer, a number that can be obtained by calculating the difference between the proton affinity of the reactant (R) and the peptide molecule (P), as in eq 2 [42]. Therefore, the internal energy of PH^+ can be varied in a controlled manner by the choice of reagent ion RH^+ . It is the selective use of reagent ions that allows control over the fragmentation of the peptide.

$$\Delta H = \text{PA}(\text{R}) - \text{PA}(P) \quad (2)$$

In an effort to better understand the energetics of peptide ion dissociation, we have used LD-CI to generate energy-dependent mass spectra of peptides. The data can be plotted as a breakdown curve, that is, a plot of the fractional intensities of the ions in a mass spectrum versus the internal energy of the molecular ion, to provide detailed information about the fragmentation channels of a peptide ion and about the energy dependence of the rates of dissociation into these channels. Previous breakdown curves that were generated for simple compounds aimed to compare

experimentally derived data with those calculated by theory. These types of experiments were applied to problems such as to distinguish isomeric molecules for which the electron impact spectra are nearly identical [43, 44], to establish bond dissociation energies or isomerization barriers [45], and to control the extent of fragmentation [46]. Here, we report breakdown curves for Val-Pro (VP), Val-Pro-Leu (VPL), and the pentapeptides Phe-Phe-Gly-Leu-Met- NH_2 (FFGLM- NH_2) and Arg-Lys-Asp-Val-Tyr (RKDVY). Some of the details of the fragmentation can be extracted from a comparison of such data to calculated breakdown curves obtained via Rice-Ramsperger-Kassel-Marcus (RRKM) theory.

Experimental

All experiments were performed in a Fourier transform mass spectrometer (FTMS) designed and constructed at the University of Georgia specifically for LD-CI studies. The details of this instrument have been published [40]. Briefly, the mass spectrometer consists of two vacuum chambers, separated by a gate valve, which accommodates the introduction and transfer of solid sample probes from an antechamber to the main vacuum chamber that houses the analyzer cell. The main vacuum chamber is pumped by a 330 L/s turbomolecular pump to a base pressure of 2×10^{-10} torr. The magnetic field is induced by an electromagnet operated at 1 T. Reagent ions for the chemical ionization of peptides are prepared by electron bombardment of a volatile gas or gas mixture admitted through one or more pulsed valves. The reagent ions are trapped in a cubic analyzer cell and allowed to react with peptide neutral molecules desorbed from the surface of a sample stub that is supported 5 mm outside an opening in one of the analyzer cell detection plates. The execution of all the experimental events, as well as data collection and manipulations, are controlled by the data system (IonSpec, Irvine, CA).

The desorption of peptide neutral molecules is performed by using substrate-assisted laser desorption (SALD) [47]. With this technique, an amount of sinapinic acid (Aldrich Chemical Co., St. Louis, MO) that corresponds to 500 monolayers (100 ng/mm^2) is applied to a stainless steel probe by electrospraying a methanol solution of the organic substrate. The equivalent of 500 monolayers of a peptide sample ($100\text{--}500 \text{ ng/mm}^2$) is applied on top of the sinapinic acid film, also by electrospraying a methanol solution. An excimer laser provides the pulsed ultraviolet radiation for desorption of the peptide samples. The 248 (KrF) and 337-nm (N_2) lines of the excimer laser are used to desorb peptide samples from the organic substrate. The irradiance of the laser light incident upon the sample is in the range of $1\text{--}10 \text{ MW/cm}^2$. All mass spectra are recorded from a single laser shot, with a spot size of 0.25 mm^2 , which corresponds to approxi-

mately 100 pmol of sample desorbed per mass spectrum.

The RRKM calculations used to determine the theoretical rate constants for the unimolecular dissociations of protonated VP employ the method of steepest descent to evaluate the reduced energy level densities and sum of states [48]. The computer program that carried out this algorithm was written in-house by using Turbo Pascal™ (copies of the program are available from the authors upon request). The accuracy of the RRKM program has been evaluated by modeling the unimolecular decomposition of dioxane, for which the kinetic behavior is known (Dunbar, R. C., private communication). The accuracy of the program for the calculation of energy level densities for reactants that contain free rotors has been verified by the investigation of the “small” and “large” models described by Forst and Prasil [48]. The vibrational frequencies of the protonated molecule of VP used in the RRKM calculations are obtained by semiempirical (AM1) calculations. The appearance energies of the fragment ions are taken from the mass spectral data. The thermal energy for a room temperature reactant ion is added to the activation energy. The reaction path degeneracy in modeling the kinetic behavior of both fragments is assumed to be unity. The intercepts of the abscissa on the calculated breakdown curve reflect the slowest reaction rate that can be observed with our experimental sequence [300-ms delay between LD and detect event; $k(E) = 3.3 \text{ s}^{-1}$].

Results and Discussion

Proton Transfer Thermodynamics

For quantitative studies of the energetics of ion decomposition, the use of chemical ionization (CI) as a means of ionization–activation offers distinct advantages over other methods that induce fragmentation. One advantage is that CI produces ions with a well defined upper limit to the energy distribution [42]. The proton transfer reaction that leads to chemical ionization is a type of chemical activation. Chemical activation long has been used to produce an activated complex with a narrow internal energy distribution [49]. In high pressure ion sources that are used for CI experiments in most mass spectrometers, collisional damping of the activated ion broadens its initial internal energy distribution and reduces the average internal energy. However, in the FTMS CI experiment, reagent gases are introduced in a pulse and the proton transfer to the peptide occurs at low pressure ($< 10^{-8}$ torr), which prevents collisional damping of the activation energy. In contrast to electron impact ionization or collisional activation, which produce broad energy distributions with a long tail at high energy, those generated by CI will have a much narrower energy distribution, with a maximum internal energy determined by eq 2 [42], as shown schematically in Figure 1. For exothermic reac-

Figure 1. Hypothetical curves that illustrate the probability of forming an ion with a given internal energy [$P(E)$] versus energy (E) for chemical ionization (CI) and electron impact (EI). Each CI curve represents ionization with a different reagent ion. The CI curves represent the energy distribution imparted by the ionization reaction. Further collisions with reagent or sample molecules could lead to broadening of the distribution on the low energy side.

tions, the CI process also occurs at or near 100% efficiency, that is, 100% of the ion–molecule collisions result in proton transfer. This provides a very sensitive method of ionization, and as well as makes it a good method for coupling with pulsed vaporization techniques such as LD.

The maximum energy deposited into an analyte molecule by proton transfer CI is equal to minus the enthalpy of the proton transfer reaction, when we assume the formation of an ion–molecule complex that is sufficiently long lived for the energy to be distributed among all vibrational modes. Because the vibrational modes of the peptide greatly outnumber those of the reagent, all excess energy is assumed to be distributed within the peptide. For example, protonation of the peptide RKDVY by CH_5^+ yields a collision complex with 105 atoms and 309 vibrational modes. The methane molecule that results from proton transfer has five atoms and nine vibrational modes. If we assume that energy is divided evenly among all vibrational modes, the methane neutral would carry off 3% ($9/309$) of the available energy. Similarly, it could be argued that protonation of the same dipeptide by N_2H^+ would result in less than 1% of the energy in the neutral product N_2 . Although the upper limit of energy transferred by CI is given by the enthalpy of the proton transfer reaction, the lower bound is less well defined in these experiments. For LD-CI, the reagent gas is introduced in a pulse and the neutrals are pumped away before the laser desorption of the peptide ($P < 10^{-8}$ torr). Thus there are no collisions of the initially formed peptide ions with reagent neutral molecules, in contrast to chemical ionization in a high pressure ion source. However, some of the protonated molecule of the peptides that are formed by CI may have a collision with a second peptide neutral molecule. This collision could result in reduction of the internal energy of the protonated molecule. Another factor that can lead to broadening of the internal energy distribution is incomplete equilibration of the energy of proton

transfer between the reagent molecule and the analyte. This may occur if the ion-molecule complex formed by the reaction of the reagent ion and the sample molecule is short lived. We assume here that these complexes are long lived and that complete equilibration of energy occurs among all vibrational modes of the complex. However, for the most highly exothermic reactions, incomplete equilibration of energy may reduce the amount of energy that is present in the protonated peptide.

The proton affinities of the peptide and of the reagent species must be known to determine the enthalpy of the proton transfer. The proton affinities of the reagents used in this study are well established [50], but the proton affinities of peptides are not. Therefore we have measured the proton affinity of the peptides studied here by using a bracketing method that we have reported previously [51, 52]. Briefly, the measurements involve observation of the occurrence or nonoccurrence of a proton transfer reaction between a peptide and a reference base, as in reaction 3, or its reverse reaction, in which P represents a peptide, B a reference base, and PH^+ and BH^+ their respective protonated forms. Proton transfer is expected to occur at the collision rate if the reaction is exoergic:

In the forward reaction, SALD is used to generate the intact neutral peptide species that reacts with a protonated reference base. Peptide molecules are allowed to react with reagents of increasing basicity until the proton transfer reaction is no longer observed; thereby an upper limit for the gas-phase basicity of the neutral species is established. The technique of matrix-assisted laser desorption (MALDI) is used for the reverse reaction to generate the protonated form of the peptide, which reacts with neutral reference bases of decreasing basicity until no reaction is observed; thereby a lower limit of gas-phase basicity is established. Consistent measurements between both the SALD and MALDI experiments confirm the gas-phase basicity, from which we derive the proton affinity by estimation of the entropy of protonation [51]. The mass spectral data used to determine the proton affinity of the dipeptide VP are shown in Figure 2. Figure 2a and b shows the results of the bracketing experiment that established the lower limit of the gas-phase basicity (GB) for VP. Here, SALD was employed to desorb intact neutral molecules of VP that were allowed to react with the reference bases trimethylamine and diethylamine. When reacted with trimethylamine ($\text{GB} = 223.3 \pm 1.5$ kcal/mol), VP was found to abstract a proton from the protonated reference base. The absence of a protonation reaction with diethylamine ($\text{GB} = 224.1 \pm 1.5$ kcal/mol), however, indicates that the lower limit of the GB of VP was 223.3 ± 1.5 kcal/mol. In Figure 2c and d, the upper limit of the GB of VP is determined by using MALDI to generate the protonated form of

Figure 2. Mass spectral data used for bracketing the proton affinity of VP. Reactions of the protonated reference bases (a) (trimethylamine + H^+) and (b) (diethylamine + H^+) with neutral VP molecules desorbed via SALD at 337 nm. Reactions of VPH^+ , desorbed via MALDI, with the reference bases (c) trimethylamine ($\text{GB} = 223 \pm 1.5$ kcal/mol) and (d) diethylamine ($\text{GB} = 224.1 \pm 1.5$ kcal/mol).

the peptide. When trimethylamine was allowed to interact with VPH^+ , no proton transfer reaction was observed. Diethylamine was found to undergo proton transfer from VPH^+ which established the upper limit of the GB of VP to be 224.1 ± 1.5 kcal/mol. Together, these mass spectra were used to bracket the GB of VP to a value of 223.7 ± 1.9 kcal/mol (9.70 eV). From this value, and with the assumption that the entropy of protonation is due solely to the translational entropy of the proton ($T\Delta S = 9.4$ kcal/mol at 350 K, the temperature of the ion cyclotron resonance (ICR) cell in these experiments), we obtain a proton affinity (PA) of 233.1 ± 1.9 kcal/mol (10.1 eV).

Effect of Laser Desorption on Energetics of Fragmentation

When LD is used in conjunction with proton transfer CI, it may be expected that under certain conditions, the LD step will contribute to the amount of energy deposited into the peptide molecule. For instance, we have shown that laser desorption at wavelengths that are absorbed by the peptide (193-nm LD), the product ion distribution reflects not only the energetics of the CI process, but also the energy deposited into the neutral during the LD step as well [47]. The role of the laser desorption wavelength can be examined by comparison of the product ions observed in the LD-CI mass spectra of the tripeptide Val-Pro-Leu (VPL) obtained at different wavelengths. The breakdown curves obtained with three laser wavelengths are shown in Figure 3. Figure 3a displays the results of LD-CI of VPL (Au-Pd substrate; 193-nm laser desorption) with a series of increasingly acidic reagent ions, NH_4^+ , C_2H_5^+ , and N_2OH^+ , that comprise an energy spread to 1.2–4.2

Figure 3. Breakdown curve for VPL obtained via LD-CI with differing experimental conditions. (a) 193-nm laser desorption of a neat sample, CI by using NH_4^+ , C_2H_5^+ , and N_2OH^+ ($-\Delta H = 1.2\text{--}4.2$ eV). (b) 248-nm laser desorption coupled with SALD, CI by using NH_4^+ , C_2H_5^+ , N_2OH^+ , and N_2H^+ ($-\Delta H = 1.2\text{--}5.0$ eV). (c) 337-nm laser desorption coupled with SALD, CI by using NH_4^+ , C_2H_5^+ , N_2OH^+ , and N_2H^+ ($-\Delta H = 1.2\text{--}5.0$ eV).

eV for the activation of the protonated tripeptide ion. (The estimates of the energy of protonation are based on a PA of 10.2 eV for the tripeptide, which has been determined by bracketing measurements [51]. Data from the LD-CI of VPL that employed the technique of substrate-assisted laser desorption (sinapinic acid substrate; 248-nm laser desorption) with the same series of CI reagents and for N_2H^+ ($-\Delta H_{\text{reagent}} = 5.0$ eV) are presented in Figure 3b. It is apparent that the b_2 and y_2 ions of VPL are diagnostic of the amount of internal energy present in the protonated parent ion, with the y_2 favored at higher energies and the b_2 favored at lower energies. The "crossover" region of the curves, in which the y_2 and b_2 ions have the same relative abundance, is characteristic of the internal energy of the parent ion. In Figure 2a, this occurs at ~ 3.3 eV;

however, in Figure 3b this crossover appears at 4.2 eV, which is 0.9 eV higher. Based on this comparison, the data suggest that laser desorption at 193 nm (Au-Pd substrate) forms gas-phase peptide molecules with approximately 1 eV more internal energy than those formed by substrate-assisted laser desorption [47]. We also have investigated the use of 337-nm laser desorption in conjunction with the substrate-assisted approach for laser desorption of "cool" peptide molecules (Figure 3c). Here, the curves cross at 4.4 eV, which suggests that the peptides have 0.2-eV less internal energy than those formed by using 248 nm LD. The results of this study can be understood by consideration of the electronic spectra of peptides. Peptides such as those studied here have much higher molar absorptivity at 193 nm ($10,000 \text{ M}^{-1}/\text{cm}$) than at 248 nm, ($< 100 \text{ M}^{-1}/\text{cm}$) whereas the organic substrate—sinapinic acid—absorbs strongly at both wavelengths ($> 10,000 \text{ M}^{-1}/\text{cm}$). The data suggest that more energy is imparted to a peptide when LD occurs at a wavelength that is strongly absorbed by the sample. The desorption of even cooler peptide molecules at 337 nm is likely a result of the large difference in molar absorptivities between the substrate ($\epsilon_{337} = 15,000 \text{ M}^{-1}/\text{cm}$) and the peptide ($\epsilon_{337} = < 100 \text{ M}^{-1}/\text{cm}$).

For the experiments reported here, we used 337-nm laser desorption to minimize the contribution of the LD step to the activation of the parent ion. The reproducibility of LD-CI mass spectra obtained with these conditions is striking. The laser energy varies $\pm 10\%$ shot-to-shot, and other experimental parameters associated with laser desorption, such as the laser spot size and the thickness of the sample and the substrate, have similar or larger variations on a sample-to-sample basis. In Figure 4, we see LD-CI mass spectra of the pentapeptide RKDVY that were acquired on three separate days with NH_4^+ as the reagent ion ($-\Delta H = 1.6$ eV). Figures 4a and b (desorbed from a stainless steel sample probe) show data acquired from separately prepared samples on consecutive days, from which variations in ion abundance are approximately $\pm 10\%$. In contrast, Figure 4c shows the mass spectrum acquired under conditions in which the peptide acquired thermal energy during LD. The sample was desorbed from a Macor sample stub coated with a 75-nm Au-Pd film. The relatively thick stainless steel allows rapid diffusion of heat away from the probe surface, while the Macor acts to localize heat near the surface of the probe, which causes the desorption of internally "hot" peptide molecules or neutral peptide fragments. The presence of the c_2 and c_3 fragment ions in Figure 4c, which are absent in Figures 4a and b, is believed to be the result of localized heating of the ceramic sample probe. The significance of these results is that variations of the laser desorption parameters (substrate coverage and irradiance) that almost certainly are present do not appreciably affect the mass spectra. The fragment ions formed in the mass spectra obtained with the metal probe tip and by using 337-nm SALD appear

Figure 4. LD-CI mass spectra of the peptide RKDVY. Neutral peptide molecules desorbed via SALD at 337 nm and ionized with NH_4^+ . (a) Acquired September 1, 1992, peptide desorbed from stainless steel probe. (b) Acquired September 2, 1992, peptide desorbed from stainless steel probe. (c) Acquired April 30, 1992, peptide desorbed from Au-Pd coated Macor probe.

to be determined by the chemistry of the protonation reaction.

Breakdown Curves of Peptides

We have used the LD-CI technique to produce breakdown curves for peptides, from which the energy dependence of ion fragmentation can be observed. LD-CI mass spectra of the peptide FFGLM- NH_2 are shown in Figure 5. The trends in the mass spectra are condensed into the breakdown curve, shown in Figure 6. The most apparent trend in the breakdown curve is the characteristic decrease in the abundance of the protonated molecule with increasing internal energy. The protonated molecule disappears from the mass spectrum when it is ionized with N_2H^+ ($-\Delta H = 5.2$ eV). The fragment ion $[\text{M} + \text{H} - 44]^+$ occurs from the loss of the CONH_2 group from the C-terminus of the protonated molecule. The appearance energy of this ion is between 1.3 and 3.2 eV. $[\text{M} + \text{H} - 44]^+$ dominates the mass spectra when the enthalpy of proton transfer is between -3.2 and -4.3 eV. Other significant ions that emerge at higher energies ($-\Delta H > 4.3$ eV) are the ions a_1 , x_1 , and the z_2 . An interesting observation in these spectra is the lack of y-type ions.

Figure 5. LD-CI mass spectra of FFGLM- NH_2 via SALD at 337 nm. Proton transfer CI with $(\text{CH}_3)_3\text{NH}^+$, NH_4^+ , C_2H_5^+ , COSH^+ , COH^+ , N_2OH^+ , CO_2H^+ , and N_2H^+ represents an energy range between 0.2 and 5.2 eV.

CAD and PD mass spectra of peptides typically contain y-type ions for almost all peptides except for those that contain a highly basic N-terminus amino acid residue, such as arginine or lysine [52-56].

A similar study has been carried out for the peptide RKDVY. The PA of this pentapeptide has not been determined yet by bracketing measurements because it is more basic than any of the bracketing compounds available for this study. The exclusive presence of the protonated molecule as a result of ionization by $(\text{CH}_3)_3\text{NH}^+$ (PA = 10.1 eV) implies a near thermoneutral reaction. For this peptide, a reasonable estimate of the PA is that it is approximately equal to that of the amino acid arginine—its most basic amino acid residue. The PA of arginine is known to be in excess of 10.5 eV [51]. Energies of proton transfer reported here therefore represent lower limits. The mass spectra shown in Figure 7 represent energies deposited into the molecule that range from 0.4 [CI with $((\text{CH}_3)_3\text{NH}^+)$

Figure 6. Breakdown curve for FFGLM- NH_2 produced from LD-CI spectra. The breakdown curve represents an energy range between 0.2 and 5.2 eV.

Figure 7. LD-CI mass spectra of RKDVY via SALD at 337 nm. Proton transfer CI with $(\text{CH}_3)_3\text{NH}^+$, CH_3NH_3^+ , NH_4^+ , H_3O^+ , C_2H_5^+ , and COH^+ represents an energy range between > 0.4 and > 4.3 eV.

to 4.3 eV (CI with COH^+). The breakdown curve obtained from the resulting mass spectra is shown in Figure 8. The very basic arginine and lysine residues that reside at the N-terminus of the peptide result in the dominance of the N-terminal fragments ions (i.e., b- and c-type ions) throughout the LD-CI spectra. The most abundant ion in the series is b_1 , with an appearance energy between 0.4 and 1.0 eV, and which is the most abundant ion at energies greater than 1.0 eV. Another interesting trend observed is the increase of the b_2 and b_3 intensities with increasing energy. The dominance of the b-type ions in these LD-CI spectra stands in contrast to previous CAD spectra in which the b-type series is prominent only for peptides that lack a basic amino acid residue in the molecule [57]. The CAD spectra of peptides that contain the basic arginine residue at the N-terminus were shown to be dominated by a-type ions [52, 53]. However, Buehler et al. [57] have shown that CI of thermally desorbed peptides that contain N-terminal arginine produces

Figure 8. Breakdown curve for RKDVY produced from LD-CI spectra. The breakdown curve represents an energy range between > 0.4 and > 4.3 eV.

significant abundances of b-type ions. This may suggest a fundamental difference in fragment ion formation between those produced by CI and CAD. Finally, for internal energies greater than 3.0 eV, the intensity of the c_1 ion grows rapidly with increasing exothermicity, and shows a fivefold increase in the ion's abundance at 3.4 eV compared to that at 3.0 eV.

Application of Quasi-Equilibrium · Rice-Ramsperger-Kassel-Marcus Theory

Peptide ions are expected to exhibit decomposition behavior that is consistent with quasi-equilibrium theory (QET), and so their various unimolecular decomposition pathways should depend only on their structure and internal energy [58]. Here we fit experimental data obtained by LD-CI with breakdown curves generated by RRKM theory to investigate the mechanisms associated with the unimolecular dissociations of protonated peptides. In this study, the theoretically derived breakdown curve for the dissociations of protonated VP was compared to that obtained experimentally by LD-CI. The internal energy deposited into the protonated molecule spans an energy range of 0.7–4.5 eV, shown in a series of mass spectra in Figure 9. The complete breakdown curve for protonated VP via LD-CI is shown in Figure 10. The predominant fragment ions observed in this energy range are a_1 and y_1 . The abundance of these ions was modeled with RRKM theory. The two adjustable parameters in fitting the theoretical data to the experimental data are the critical energies of the two fragmentation reactions and the number of free rotors lost or gained by the precursor ion when it passes through the transition state to product ions. With two adjustable parameters, there are many combinations that will allow a reasonable fit of the data. We have made the assumption that the experimentally observed appearance energies closely approximate the critical energies for the two pathways.

Figure 9. LD-CI mass spectra of VP via SALD at 337 nm. Proton transfer CI with CH_3NH_3^+ , NH_4^+ , $\text{C}_2\text{H}_5\text{OH}_2^+$, CH_3OH_2^+ , H_3O^+ , C_2H_5^+ , COH^+ , N_2OH^+ , and CH_3^+ represents an energy range between 0.6 and 4.4 eV.

Figure 10. Breakdown curve for VP produced from LD-CI spectra. The breakdown curve represents an energy range between 0.6 and 4.4 eV.

This assumption could be in error if there are substantial kinetic shifts for the fragmentation reactions. Although the long time scale of the ICR experiment (300 ms allowed for fragmentation to occur) allows the possibility to observe slow reactions, infrared cooling can introduce an "intrinsic kinetic shift," as has been described in detail by Dunbar [59]. Thus our approximation of the critical energies, although necessary for fitting the data, introduces some uncertainty into the fit, and so the results should not be construed as quantitatively precise, but rather, as producing a qualitative picture of the ion fragmentation mechanism. A plot of the unimolecular rate constant ($k(E)$) versus internal energy (E) for the formation of the a_1 and y_1 fragments that best model the experimental data is shown in Figure 11. The experimentally and theoretically derived breakdown curves for these competing pathways are overlaid in Figure 12. The fractional ion abundance of the y_1 ion (m/z 116) in the experimental breakdown curve was corrected to compensate for the

Figure 11. The calculated plot of the unimolecular rate constant [$k(E)$] versus internal energy for the formation of the a_1 and y_1 fragments that originate from the protonated molecule of VP.

Figure 12. Experimental and calculated (RRKM) breakdown curves of the a_1 and y_1 fragments of protonated VP. The solid lines represent experimental results whereas the broken lines represent calculated kinetic behavior.

formation of a secondary fragmentation product. As depicted in Scheme I, this product is a result of the loss of formic acid (46 u) from the y_1 , which forms the immonium ion at m/z 70 (appearance energy = 1.9 eV). Therefore, the contribution of the immonium ion to the total fractional ion abundance was added to that of the y_1 ion. To model the behavior of both fragments a_1 and y_1 , a vibrational frequency of 1640 cm^{-1} , which corresponds to the *amide I* band, is assumed to be lost to the reaction coordinate. The *amide I* band is a normal mode that arises in part from the carbonyl stretch that is vibrationally coupled to the C bond N stretch and to some extent with the NH band [60]. The choice of this frequency is not critical because calculations of the rate constants for both fragments were found to be only weakly sensitive to this parameter. The calculated breakdown curve was fitted to that obtained experimentally by selecting the appropriate values of critical energy and by making some assumptions about the transition states of the competing reactions. To model the behavior of the y_1 ion, the best agreement with experimental results was obtained by assuming that the fragment ion is formed via a rearrangement reaction, with one rotational degree of freedom being frozen out in the transition state. This assumption is supported by other work that has established that formation of y -type ions occurs with cleavage of the amide bond, accompanied by the transfer of hydrogen atoms to the C-terminal fragment [61, 62]. Results obtained by Orlando and co-workers [63] have

Scheme I

extended this investigation and determined that a hydrogen atom attached to nitrogen and not carbon migrates during cleavage of the amide bond. This suggests a "tight" transition state for the formation of the y_1 , consistent with the assumption of the loss of one free rotor that provides the best fit of the RRKM data.

The theoretically derived behavior of the a_1 fragment was shown to fit experimental results best when it was assumed to be formed by a direct cleavage of the amide bond. Here, one rotational degree of freedom was increased in the transition state at the expense of vibrational states. This suggests that the a_1 is formed through a loose transition state prior to dissociation. To date, a detailed study of the mechanism of a -type ion formation has not been published. However, a generally accepted mechanism for the formation of the a -type fragments is that it is initiated by the formation of a b -type fragment, followed by loss of CO to form the immonium ion shown in Scheme II [64, 65]. Our experimental findings do not support this mechanism, due to the absence of a b -type fragment in any of the mass spectra obtained at lower internal energies. On the contrary, we observe the presence of b -type fragments within a narrow energy threshold at approximately 1.1 eV higher in energy than the appearance energy of the a_1 . Our data suggest that the formation of the a_1 excludes a rearrangement prior to dissociation and is consistent with a direct cleavage reaction. Studies of other peptides should allow greater insight into the mechanism of the formation of a -type fragments.

It is interesting to note that the protonated molecule of VP is observed as a minor peak in the mass spectra collected for reaction energies greater than 1.5 eV, even though the RRKM calculation suggests a dissociation rate ($> 100 \text{ s}^{-1}$) for complete fragmentation should have occurred on the time scale of our experiment (300 ms). As described in the foregoing text, the lower limit of the internal energy distribution of the protonated molecule produced by chemical ionization is not well defined because collisions of some of the initially formed protonated ions with other laser-desorbed molecules can lead to loss of some of the energy of activation. The results suggest that this process occurs to a small extent in this experiment. Although this reduces the quantitative precision of these measurements, the results give a good qualitative picture of the general trends in reactivity.

Scheme II

Conclusion

We have demonstrated the use of LD-CI to study the unimolecular fragmentation of protonated peptides. The LD-CI technique allows the formation of protonated peptide molecules with well defined, discrete amounts of internal energy. This allows breakdown curves for peptides to be generated directly from the mass spectra. We also have shown that energy deposited into analyte molecules by the LD process can be minimized when the SALD technique is used in conjunction with 337-nm radiation.

The energetics of competing fragmentation patterns for peptides can be investigated by using QET-RRKM theory. Theoretical breakdown curves, produced from calculated unimolecular rates of reaction versus internal energy can be compared to those obtained experimentally. However, it is important to recognize some of the uncertainties in these experiments that make these results more qualitative than quantitative. First is that the internal energy distribution of ions formed by chemical ionization is not well characterized. As previously discussed, collisions of the protonated molecule formed by CI can lead to a broadening of the low side of the energy distribution. Second is that the internal energy of laser-desorbed molecules has not been established quantitatively, although data here suggest that this energy is small. Despite these limitations, studies such as these provide qualitative information about critical energies and transition states in the formation of important peptide fragment ions, and will lead to a better understanding of the mechanism of the formation of such ions.

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References

1. Barber, M.; Bordoli, R. S.; Elliot, G. J.; Sedgwick, R. D.; Tyler, A. N. *J. Chem. Soc. Chem. Commun.* **1981**, 325.
2. Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F. *Mass Spectrom. Rev.* **1990**, 9, 37.
3. Tanaka, K.; Waki, H.; Idao, Y.; Akita, S.; Yoshida, Y. Y.; Yoshida, T. *Rapid Commun. Mass Spectrom.* **1988**, 2, 151.
4. Karas, M.; Hillenkamp, F. *Adv. Mass Spectrom.* **1989**, 11A, 416.
5. Smith, R. D.; Loo, J. A.; Edmonds, C. G.; Barinaga, C. J.; Udseth, H. R. *Anal. Chem.* **1990**, 62, 882.
6. Hunt, D. F.; Bone, W. M.; Shabanowitz, J.; Rhodes, J.; Ballard, J. M. *Anal. Chem.* **1981**, 54, 1704.

7. Amster, I. J.; Baldwin, M. A.; Cheng, M. T.; Proctor, C. J.; McLafferty, F. W. *J. Am. Chem. Soc.* **1983**, *105*, 1654.
8. Cody, R. B.; Amster, I. J.; McLafferty, F. W. *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 6367.
9. Hunt, D. F.; Shabanowitz, J.; Yates, J. R.; Griffin, P. R.; Zhu, N. Z. In *Mass Spectrometry of Biological Materials*; McEwen, C. N.; Larsen, B. S., Eds.; New York: Marcel Dekker, 1990; chap. 5.
10. Morris, H. R.; Dell, A.; Panico, M.; Thomas-Oates, J.; Rogers, M.; McDowell, R.; Chatterjee, A. In *Mass Spectrometry of Biological Materials*; McEwen, C. N.; Larsen, B. S., Eds.; New York: Marcel Dekker, 1990; Chap. 4.
11. Gross, M. L.; Tomer, K. B.; Cerny, R. L.; Giblin, D. E. In *Mass Spectrometry in the Analysis of Large Molecules*; McNeal, C. J., Ed.; Chichester: Wiley, 1986; p 171.
12. Raschdorf, F.; Dahinden, R.; Domon, C.; Muller, D.; Richter, W. J. In *Mass Spectrometry in the Analysis of Large Molecules*; McNeal, C. J., Ed.; Chichester: Wiley, 1986; p 49.
13. Cooks, R. G.; Kaiser, R. E., Jr. *Acc. Chem. Res.* **1990**, *23*, 213.
14. Cooks, R. G.; Amy, J. W.; Bier, M. E.; Schwartz, J. C.; Schey, K. L. *Adv. Mass Spectrom.* **1989**, *11*, 33.
15. Williams, E. R.; Henry, K. D.; McLafferty, F. W.; Shabanowitz, J.; Hunt, D. F. *J. Am. Chem. Soc. Mass Spectrom.* **1990**, 413.
16. Cooks, R. G.; Ast, T.; Mabud, A. *Int. J. Mass Spectrom. Ion Processes* **1990**, *100*, 209.
17. McCormick, A. L.; Shabanowitz, J.; Hunt, D. F.; Wysocki, V. H. *Proceedings of the 39th ASMS Conference on Mass Spectrometry and Allied Topics*; Nashville, TN, 1991; p 823.
18. McCormick, A. L.; Jones, J. L.; Wysocki, V. H. *J. Am. Soc. Mass Spectrom.* **1992**, *3*, 859.
19. Aberth, W. *Proceedings of the 36th ASMS Conference on Mass Spectrometry and Allied Topics*; San Francisco, CA, 1988; p 73.
20. Cole, R. B.; LeMeillour, S.; Tabet, J.-C. *Anal. Chem.* **1992**, *64*, 365.
21. Bowers, W. D.; Delbert, S. S.; McIver, R. T., Jr. *Anal. Chem.* **1986**, *58*, 969.
22. Lebrilla, C. B.; Wang, D. T. S.; Mizoguchi, T. J.; McIver, R. T., Jr. *J. Am. Chem. Soc.* **1989**, *111*, 8593.
23. Griffith, P. R.; Kumar, S.; Shabanowitz, J.; Charbonneau, J.; Pearl, C. N.; Walsh, K. A.; Hunt, D. F.; Petra, P. H. *J. Biol. Chem.* **1989**, *264*, 19066.
24. Tecklenburg, R. F., Jr.; Miller, M. N.; Russell, D. H. *J. Am. Chem. Soc.* **1989**, *111*, 1161.
25. Martin, S. A.; Hill, J. A.; Kitrell, C.; Biemann, K. *J. Am. Soc. Mass Spectrom.* **1990**, *1*, 107.
26. Williams, E. R.; Furlong, J. J. P.; McLafferty, F. W. *J. Am. Soc. Mass Spectrom.* **1990**, *1*, 288.
27. Zimmerman, J. A.; Watson, C. H.; Eyler, J. *Anal. Chem.* **1991**, *63*, 361.
28. Gorman, G. S.; Amster, I. J. *Org. Mass Spectrom.* **1993**, *28*, 1602.
29. McLafferty, F. W., Ed. *Tandem Mass Spectrometry*; New York: Wiley, 1983.
30. Busch, K. L.; Glish, G. L.; McLuckey, S. A. In *Mass Spectrometry/Mass spectrometry: Techniques and Applications of Tandem Mass Spectrometry*; New York: CH Publishers, 1988.
31. Wysocki, V. H.; Kenttamaa, H. I.; Cooks, R. G. *Int. J. Mass Spectrom. Ion Processes* **1987**, *75*, 181.
32. McLafferty, F. W.; Bente, P. F., III; Kornfeld, R.; Tsai, S.-C.; Howe, I. J. *Am. Chem. Soc.* **1973**, *95*, 2120.
33. Wysocki, V. H. In *Mass Spectrometry in the Biological Sciences: A Tutorial*; Gross, M. L., Ed.; Dordrecht: Kluwer Academic Publishers, 1992; Chap. 3.
34. Bier, M. E.; Amy, J. W.; Cooks, R. G.; Syka, J. E. P.; Ceja, P.; Stafford, G. *Int. J. Mass Spectrom. Ion Processes* **1987**, *77*, 31.
35. Wysocki, V. H.; Ding, J.-M.; Jones, J. L.; Callahan, J. H.; King, F. L. *J. Am. Soc. Mass Spectrom.* **1992**, *3*, 27.
36. (a) Winger, B. E.; Julian, R. K., Jr.; Cooks, R. G.; Chidsey, C. E. D. *J. Am. Chem. Soc.* **1991**, *113*, 8967; (b) Wysocki, V. H.; Jones, J. L.; Ding, J. M. *J. Am. Chem. Soc.* **1991**, *113*, 8970.
37. Jones, J. L.; Dongre, A. R.; Somogyi, A.; Wysocki, V. H. *J. Am. Chem. Soc.* **1994**, *116*, 8368.
38. Amster, I. J.; Land, D. P.; Hemminger, J. C.; McIver, R. T., Jr. *Anal. Chem.* **1989**, *61*, 184.
39. Amster, I. J.; Land, D. P.; Hemminger, J. C.; McIver, R. T., Jr. *Adv. Mass Spectrom.* **1989**, *11*, 680.
40. Speir, J. P.; Gorman, G. S.; Cornett, D. S.; Amster, I. J. *Anal. Chem.* **1991**, *63*, 65.
41. Speir, J. P.; Gorman, G. S.; Amster, I. J. In *Mass Spectrometry in the Biological Sciences: A Tutorial*; Gross, M. L., Ed.; Dordrecht: Kluwer Academic Publishers, 1992; Chap. 11.
42. Harrison, A. G. *Chemical Ionization Mass Spectrometry*; Boca Raton, FL: CRC Press, 1984.
43. Li, Y. H.; Herman, J. A.; Harrison, A. G. *Can. J. Chem.* **1981**, *59*, 1753.
44. Verma, S.; Ciupek, J. D.; Cooks, R. G. *Int. J. Mass Spectrom. Ion Processes* **1984**, *62*, 219.
45. Malinovich, Y.; Arakawa, Y.; Haase, G.; Lifshitz, C. *J. Phys. Chem.* **1985**, *89*, 2253.
46. Baer, T.; Dutuit, O.; Mestagh, H.; Rolando, C. *J. Phys. Chem.* **1988**, *92*, 5674.
47. Speir, J. P.; Amster, I. J. *Anal. Chem.* **1992**, *64*, 1041.
48. Forst, W.; Prasil, Z. *J. Chem. Phys.* **1969**, *51*, 3006.
49. Robinson, P. J.; Holbrook, K. A. *Unimolecular Reactions*; London: Wiley-Interscience, 1972; Chap. 8.
50. Lias, S. G.; Liebman, J. F.; Levin, R. D. *J. Phys. Chem. Ref. Data* **1984**, *13*, 695.
51. Gorman, G. S.; Speir, J. P.; Turner, C. A.; Amster, I. J. *J. Am. Chem. Soc.* **1992**, *114*, 3986.
52. Gorman, G. S.; Amster, I. J. *J. Am. Chem. Soc.* **1993**, *115*, 5729.
53. Martin, S. A.; Biemann, K. *Int. J. Mass Spectrom. Ion Processes* **1987**, *78*, 213.
54. Biemann, K.; Scoble, H. A. *Science* **1987**, *238*, 992.
55. Johnson, R. S. Ph.D. Thesis, Massachusetts Institute of Technology, 1988.
56. Biemann, K. *Biomed. Environ. Mass Spectrom.* **1988**, *16*, 99.
57. Buehler, R. J.; Flanagan, E.; Greenwood, L. J.; Friedman, L. *J. Am. Chem. Soc.* **1974**, *96*, 3990.
58. Rosenstock, H. M.; Wallenstein, M. B.; Wahrhaftig, A. L.; Eyring, H. *Proc. Natl. Acad. Sci. USA* **1952**, *38*, 667.
59. Dunbar, R. C. *Int. J. Mass Spectrom. Ion Processes* **1983**, *54*, 109.
60. Bellamy, L. J. *The Infra-red Spectra of Complex Molecules*; New York: Wiley, 1975; Chap. 12.
61. Hunt, D. F.; Yates, J. R., III; Shabanowitz, J.; Winston, S.; Hauer, C. R. *Proc. Natl. Acad. Sci. USA* **1986**, *83*, 6233.
62. Mueller, D. R.; Eckersley, M.; Richter, W. J. *Org. Mass Spectrom.* **1988**, *23*, 217.
63. Kenny, P. T.; Nomoto, K.; Orlando, R. *Rapid Commun. Mass Spectrom.* **1992**, *6*, 95.
64. Hunt, D. F.; Shabanowitz, J.; Yates, J. R.; Griffin, P. R.; Zhu, N. Z. In *Mass Spectrometry of Biological Materials*; McEwen, C. N.; Larsen, B. S., Eds.; New York: Marcel Dekker, 1990; Chap. 5.
65. Johnson, R. S.; Martin, S. A.; Biemann, K. *Int. J. Mass Spectrom. Ion Processes* **1988**, *86*, 137.