Low-Energy Dissociation Pathways of Small Deprotonated Peptides in the Gas Phase

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Abstract: The unimolecular dissociation dynamics of small deprotonated peptides generated with an external fast atom bombardment source have been investigated using Fourier transform ion cyclotron resonance mass spectrometry. Because the charge site is well defined in peptides lacking strongly acidic side chains, deprotonated peptides present a good model system for investigating the unimolecular dissociation dynamics of "large" molecules. Off-resonance collisional activation was used to determine the low-energy fragmentation pathways available to the peptides, which greatly contrast those of higher-energy dissociation techniques. Dissociation is governed by the site of deprotonation and yields partial sequence information in favorable cases. Almost all observed pathways were brought about by charge-induced mechanisms. The lowest energy dissociation pathway for all peptides without acidic side chains is elimination of the conjugate base of the C-terminus amino acid as the ionic fragment. This generally occurs in up to 100% yield with no competition. For peptides with acidic side chains alternate pathways are also observed. However, in most cases through competing or sequential dissociation processes the C-terminus amino acid could be determined. Calculations were carried out at the AM1 level to determine the minimum energy configurations of these species. Intramolecular hydrogen bonding to solvate and stabilize the charge is observed to be prevalent. The calculations provide further support for the dissociation mechanisms presented. Application of statistical RRKM calculations to these systems allows a qualitative understanding of the energetic changes associated with the observed dissociation processes, distinguishing in particular processes arising from competitive as opposed to sequential dissociations. The bimolecular reactivity of deprotonated peptides was also investigated. Several reactions taking advantage of the nucleophilicity of the deprotonated carboxylic group were observed.

Introduction

Relatively little is known about the *low-energy* dissociation pathways of biopolymers in the gas phase. Yet, an understanding of these processes is critical to developing methods for obtaining sequence-specific information of peptides by collisional activation techniques. The majority of previous investigations have utilized high-energy collision-induced dissociation of quasimolecular ions to obtain sequence information.[1] The dissociation pathways exhibited by many peptides appear to be controlled by the site of charge, but for many protonated and cationized species the charge site is not well-known or necessarily localized. Calculations on amino acids and simple peptides to determine the most likely site of protonation or cationization have been reported.[2] The most stable form does not necessarily represent a reactive configuration, which could be energetically less favorable yet easily accessed when the molecule is activated. Solvation of the charge site by other basic functional groups in the molecule results in further complications. The major conclusion from these studies is that for many protonated or cationized peptides there tend to be a variety of sites close in energy where the charge could reside. Even though the experimental methodology often yields useful sequence information, this makes obtaining a systematic understanding of the actual high- or low-energy dissociation dynamics for these species difficult.

Deprotonated peptides, which can be formed easily by the same methods employed for positive ions (fast atom bombardment or laser desorption), circumvent this charge localization problem. The site of charge in the absence of acidic side chains will be the carboxyl terminus, which can be solvated by internal hydrogen bonding. A consistent pattern of charge localization is expected to result in specific and perhaps predictable dissociation pathways. Of particular interest then is determination of the most energetically favorable pathway(s) for dissociation of deprotonated peptides. Studies of deprotonated peptides have been somewhat more limited. Bowie and co-workers have published a series of papers on the high-energy fragmentation patterns of dipeptides, in which they observe that some sequence information can be obtained from these patterns.[3a-e] More recently, Adams and co-workers have carried out studies on anionic complexes of serine- and threonine-containing peptides, both deprotonated and complexed with calcium. They looked at both the high-energy CID, and the metastable decay processes of these peptides. For most peptides both groups observe multiple fragmentation pathways.[3f]

Fourier transform ion cyclotron resonance (FT-ICR) spectroscopy is a powerful tool both for studies of negative ions and for studies of low-energy collision dynamics. Low-energy pathways for unimolecular dissociation can be investigated using off-resonance excitation.[4] Application of radio frequency excitation slightly off resonance from the frequency of a selected ion causes the ion to oscillate with low (<5 eV center of mass), bounded translational energies. As a result excitation can be carried out for long time periods (>1 s). In the presence of a collision gas the ion undergoes multiple collisions, each one resulting in some of the translational energy being transferred into internal excitation. The internal energy of the ion will slowly increase

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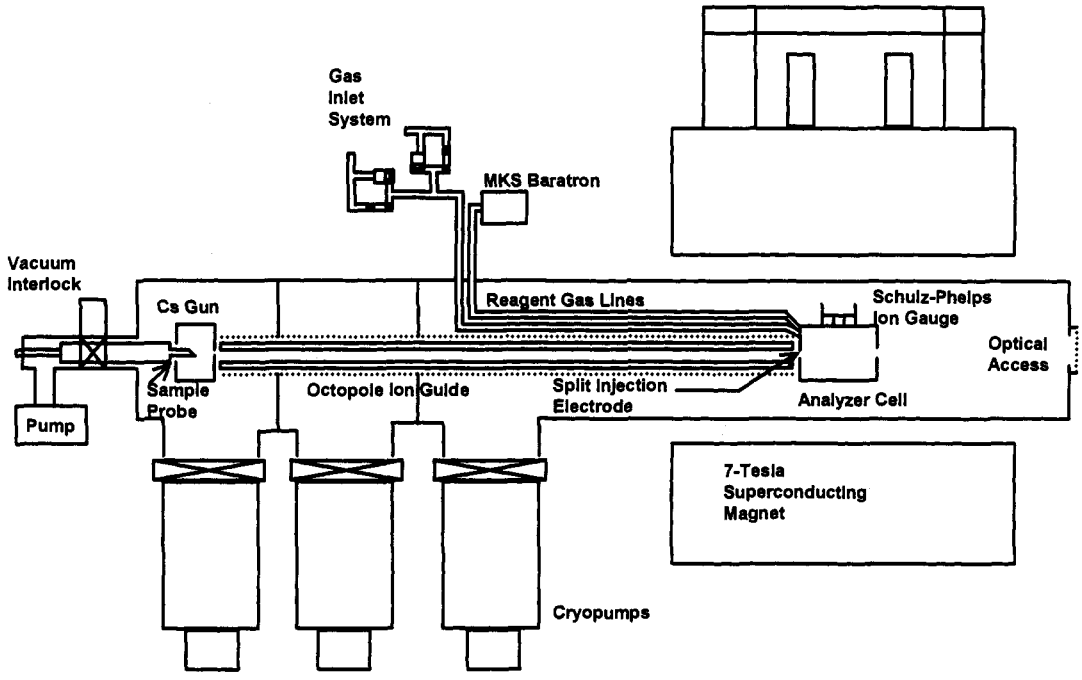

Figure 1. Schematic diagram of external ion source FT-ICR.

until the ion undergoes unimolecular dissociation. Generally the products are further off resonance and so are not excited by the radio frequency field. Instead they collisionally relax. Thus off-resonance excitation allows determination of the low-energy fragmentation pathways. Energetic requirements for the systems studied can be specified by an RRKM analysis, which provides a better understanding of observed sequential and competitive dissociation processes.

Very few bimolecular reactions of peptides other than proton transfer reactions have been reported.[5] In the gas phase a peptide is a fairly inert species, at least while in its protonated or cationized form. However, a peptide deprotonated on the carboxyl terminus possesses a reasonably nucleophilic site. As a result bimolecular reactions with reagents subject to nucleophilic attack are likely to be observed.

In the present work we have investigated the low-energy dissociation pathways of a range of deprotonated peptides of varying complexity. The effect of adding acidic side chains, and hence varying the site of charge, was probed. AM1 geometry calculations and RRKM statistical calculations were carried out to further understand the mechanisms of unimolecular dissociation. In addition, the bimolecular reactivity of simple deprotonated peptides was studied using a variety of traditional reagents which are susceptible to nucleophilic attack.

Experimental Section

A modified IonSpec Corp. external source FT-ICR equipped with a fast atom bombardment (FAB) source and specifically designed for quantitative studies of biomolecule reaction dynamics was used to carry out these studies. The instrument, schematically shown in Figure 1, has three differentially pumped regions containing an ion source, octopole ion guide, and analyzer cell. APD cryopumps (APD-6, 1000 L/s each) provide pumping in each of the three regions of the vacuum chamber with operating pressures of 5×10^{-10} Torr as measured by an internal Granville-Phillips ionization gauge located above the pump adjacent to the magnet. Due to the proximity of the magnetic field the cryopump motors are shielded with cold rolled steel. The ion source region is equipped with an Antek cesium ion gun (Model 160-250B) with a PS4 power supply. Samples are transferred into vacuum through an interlock using an MDC high vacuum motion feed through assembly. The octopole ion guide, designed and constructed in our laboratory, allows ions to be transported efficiently from the source region to the analyzer cell 47 in. away in the high magnetic field. The octopole with $^1/_8$ in. rods is powered by an ENI power amplifier (Model 2100L) driven by a Wavetek function generator (Model 190). The higher voltages required to driven the octopole are derived from an air coil transformer with the secondary coil forming a tuned LC circuit with the octopole rods which resonates at 1.2 MHz. The stainless steel trapped ion analyzer cell with dimensions $2 \times 2 \times 3$ in. is centered in the homogeneous region of an Oxford Instruments MarkII, 7-Tesla superconducting magnet. An inlet manifold equipped with two Varian (Model 951-5106) leak valves and two General Valve (series 9) pulsed valves is configured to deliver gas directly to the analyzer cell. The General Valve pulsed valves do not perform well in the high fringing fields generated by our magnet and this necessitates locating them several feet from the cell. Thus, obtaining a short burst of gas over a controlled period of time is not possible with our current setup. Pressures in the source region are measured by a home-built ionization gauge of the Schulz–Phelps geometry[6] mounted directly on the cell and calibrated against a MKS (Model 390) pressure transducer, configured to work in a high magnetic field. A static gas tap from the pressure transducer to the cell allows accurate measurement of the gas pressures to the cell. The cell has been modified to incorporate a split injection electrode directly opposite the octopole ion guide exit.[7] Control pulses for the ion source, radio frequency octopole ion guide, and FTMS analyzer cell are generated by an OMEGA data system manufactured by IonSpec Corp. which digitizes and processes the transients collected. The data system allows for transients up to 256 kilobytes in length to be recorded. All control is through a 486 computer which allows storage and manipulation of the data.

In a typical experiment the peptide is dissolved in a thin layer of liquid matrix (usually 2:1 glycerol and ammonium hydroxide) coated on a copper tip. The sample is then desorbed by a 100 ms pulse of 6–8 keV cesium ions. Concurrently a 50–150 V, 1.2 MHz radio frequency electric field is applied to the octopole rods and a 9–18 V differential is applied to the split electrode. Ions are typically trapped with 1.0–2.5 V trapping potential and the transmitter and receiver plates are left at ground. Spectra are

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(7) In initial experiments we found that a differential bias voltage on the transmitter plates of the ICR analyzer cell was required to maximize ion trapping efficiency. This acts to slow down the ions as they traverse the cell but skews the ion orbits in the analyzer cell. To avoid distortion of the electrostatic potentials in the cell, a split injection electrode consisting of two plates at the ion entrance to the cell that can be independently biased during ion injection was incorporated.

Table 1. Product Distributions[a] observed for the Low-Energy Collision Activated Dissociation of a Variety of Simple Peptides

peptide	*m/z* deprotonated peptide	C-terminus product	other products
	(a) Non-Acidic and Slightly-Acidic Side Chains		
gly-gly	131.0451	100	
gly-leu	187.1083	100	
gly-ala	145.0613	100	
gly-gly-ala	202.0828	100	
gly-gly-ileu	244.1298	100	
gly-gly-gly	188.0672	100	
gly-gly-val	230.1141	100	
ala-leu-ala	272.1610	100	
gly-gly-phe	278.1141	100	
met-leu-phe	408.1957	100	
ala-leu-ala-leu	385.2451	100	
val-ala-ala-phe	405.2137	100	
tyr-gly-gly	294.1090	75	25 *m/z* 219[b]
thr-val-leu	330.2029	20	80 *m/z* 286[b]
leu-ser-phe	364.1872	25	75 *m/z* 334[b]
leu-ser	217.1188	0	82 *m/z* 187[b]
			18 *m/z* 74 gly-H$^-$
	(b) Very-Acidic Side Chains		
gly-trp	260.1035	78	22 *m/z* 131
gly-his	211.0831	30	27 *m/z* 149 $-(CO_2 + H_2O)$
			27 *m/z* 74 gly-H$^-$
			7.5 *m/z* 193 $-H_2O$
			7.5 *m/z* 110[b]
glu-leu	259.1294		90 *m/z* 128[a]
			10 *m/z* 241 $-H_2O$
gly-glu	203.0668		54 *m/z* 185 $-H_2O$
			17 *m/z* 128[b]
			14 *m/z* 74 gly-H$^-$
			10 *m/z* 141 $(-(CO_2 + H_2O)$
			5 *m/z* 159 $-CO_2$
val-gly-ser-glu	389.1672		44 *m/z* 260[b]
			31 *m/z* 128[b]
			25 *m/z* 173[b]

[a] Reported as percent of total products. [b] See text for details.

derived from averaging 5–10 transients. For systems giving weaker signal intensity as many as 50 transients were averaged. All peptides and other reagents were obtained from Sigma and used as received. The time allowed before detection was dependent on the experiment being performed.

In all of the collision-activated dissociation studies reported here nitrogen was used as the collision gas. The species of interest was isolated through a series of ejection pulses and could be stored for several minutes with no loss of signal. Detection delays up to several seconds often resulted in better signal intensity and resolution, probably the result of relaxation of any excess translational energy the ions gain during formation and injection into the analyzer cell. Species were activated 1–4 kHz off resonance at lower frequency (higher mass) for 1000 ms with a radio frequency pulse of 1.0–5.0 V zero-to-peak. Dissociation was carried out against both static ($0.5–1.0 \times 10^{-7}$ Torr) and pulsed ($1.0–5.0 \times 10^{-6}$ Torr) gases. For static experiments the total delay time was 5 s, whereas for pulsed experiments a 3 min detect delay was necessary to allow the gas to be fully pumped away. In general the energies for excitation were higher for static experiments (compared to pulsed) since lower pressures resulted in fewer collisions during the reaction period. It is noteworthy that none of the energies employed excite the ions to radii of more than a few millimeters, so resolution should not be greatly affected. The average ion energy was varied by changing the frequency of excitation and the amplitude of the radio frequency field.

Geometry optimization calculations were carried out on using the Hyperchem program suite (Autodesk, Inc.) on an IBM 486 DX33 computer. Structures were initially optimized using molecular mechanics, typically with AMBER force fields. Final further optimization was carried out using semiempirical methods at the AM1 level. The AM1 model has been found to be a fast and accurate calculation method for ions as well as neutrals. It has also been demonstrated to be able to accurately handle intramolecular hydrogen bond interactions.[8]

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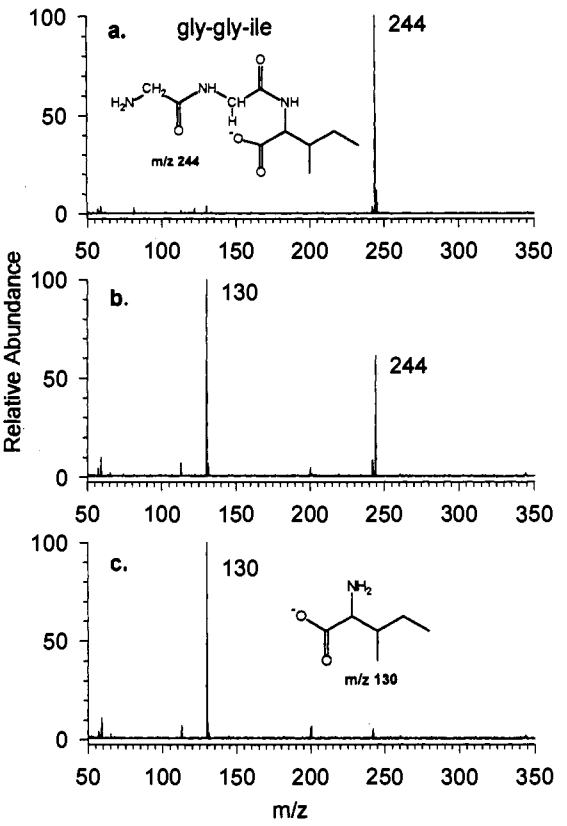
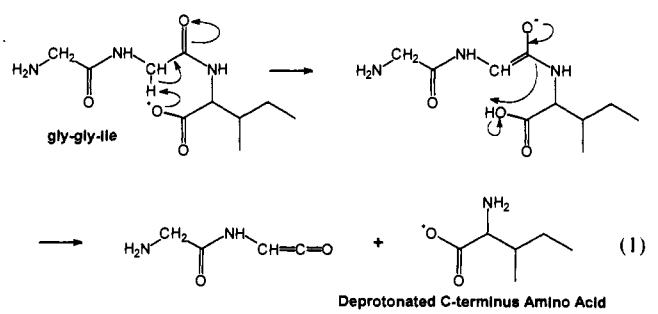


Figure 2. Low-energy collision-induced dissociation of the deprotonated peptide gly-gly-ile (m/z = 244.1): (a) isolated spectrum of the parent peptide; (b) irradiation with $\Delta\omega/2\pi$ = 4400 Hz (m/z = 246.5) and $\langle E_{com} \rangle$ = 0.24 eV at a pressure of 1×10^{-6} Torr for 1 s (approximately 100 collisions) yields partial dissociation of the parent to give the deprotonated C-terminal amino acid (m/z = 130.1); (c) irradiation with $\langle E_{com} \rangle$ = 0.42 eV at a pressure of 1.5×10^{-6} Torr for 1 s (approximately 150 collisions) yields 100% dissociation. The small peaks in the spectrum are not products but can be assigned either to noise or to imcomplete isolation of the selected ion.

Results

Unimolecular Reactions: Non-Acidic Side Chains. Table 1 summarizes the low-energy dissociation patterns observed for all peptides studied. Collisional activation of all peptides with non-acidic side chains results in exclusive elimination of the conjugate base of the C-terminus amino acid as the observed product ion. The type of data obtained for these experiments are illustrated for the case of the deprotonated peptide gly-gly-ile in Figure 2. The reactant ion is isolated with a series of radio frequency ejection pulses. Collision gas is pulsed into the system and off-resonance excitation of the isolated ion results in controlled fragmentation with only one product. The proposed mechanism for this dissociation is shown in reaction 1. While the secondary structure

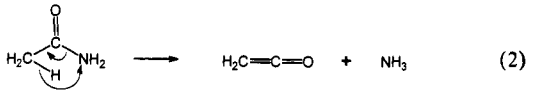


(1)

of deprotonated peptides in the gas phase is not known, the only reasonable site of deprotonation for molecules with simple side

chains is the carboxyl terminus, as depicted here. We propose a mechanism in which dissociation is initiated by proton abstraction from the adjacent amino acid through a favorable seven-member-ring intermediate. Simple analysis based on the approximate relative acidities of the sites predicts this step to be endothermic by about 30 kcal mol^{-1} (1.3 eV).[9] This initial abstraction is followed by rapid rearrangement to yield the C-terminus amino acid as the charged product. The overall endothermicity of the process is estimated from bond strengths to be approximately 40 kcal mol^{-1} (1.7 eV). Labeling studies by Bowie et al.[3a] on dipeptides confirm that the proton we propose is initially abstracted is present in the final product.

It should be noted that the observed products in reaction 1 could also result from a simple 4-center elimination reaction, as has been proposed for the related compound acetamide as shown in reaction 2.[10] This reaction has measured activation parameters



(2)

of log A = 14.7 and E_a = 73.4 kcal mol^{-1} (3.2 eV).[10a] However, as discussed by Benson et al.,[10b] these numbers are not consistent with a four-center elimination reaction, which should have a tight transition state and a lower A-factor than observed. They suggest that for the 4-center elimination reaction log A = 13.0 and E_a = 68 kcal mol^{-1} (2.9 eV) are more appropriate values.

In the stuides of deprotonated peptides without acidic side chains, cleavage occurs only at the C-terminus amino acid. This means that the charge localization at the C-terminus necessarily plays a key role in determining the site specificity of the reaction. If a charge-remote four-center elimination reaction was possible in this low-energy dissociation regime then dissociation at every amide bond should be equally possible, but for all the peptides without acidic side chains investigated dissociation at the C-terminus amino acid is exclusively observed. In the proposed dissociation mechanism in reaction 1, the carboxylate anion can be considered a catalyst for the four-center elimination reaction. The estimated E_a of 68 kcal mol^{-1} for the 4-center process can be considered a reasonable upper limit for the activation energy of the observed catalyzed process. AM1 calculations reveal the lowest-energy geometry of peptides without acidic side chains containing at least three amino acids to be a structure with hydrogen bonding interactions between the deprotonated C-terminus and the N-terminus and/or other amide hydrogens. However, the amide and N-terminus hydrogens are not particularly acidic and dissociation pathways involving deprotonation of these sites would be necessarily a much higher energy process than hydrogen abstraction from the adjacent amino acid. In the collisional activation process enough energy is added to the molecule so that all of the lower energy structures will be easily accessible.

The energetics of the off-resonance excitation process can be quantified. The amount of translational energy that the ion gains is given by eq 3 where ω_c is the cyclotron frequency and ω and E_0 are the frequency and electric field used for excitation.[11]

$$E_{tr} = \frac{E_0^2 q^2 \sin^2\left(\frac{(\omega - \omega_c)t}{2}\right)}{2m(\omega - \omega_c)^2} \quad (3)$$

Experimentally, exciting at a frequency below the resonance frequency (higher mass) gave better results, though in principle the amount of energy absorbed should be the same. The translational energy of the ion oscillates with time with an average value given by eq 4. The total number of oscillations in a time t (seconds) is given by eq 5.

$$\langle E_{tr} \rangle = \frac{E_0^2 q^2}{4m(\omega - \omega_c)^2} \quad (4)$$

$$n = t\left(\frac{\omega}{2\pi} - \frac{\omega_c}{2\pi}\right) \quad (5)$$

The translational energy in the center of mass frame is given by eq 6.

$$\langle E_{com} \rangle = \langle E_{tr} \rangle \left[\frac{m_{gas}}{m_{gas} + m_{ion}}\right] \quad (6)$$

Typical operating conditions are those used to obtain the data shown in Figure 2. Irradiation of gly-gly-ile with $\Delta\omega/2\pi$ = 4400 Hz at $\langle E_{com} \rangle$ = 0.24 eV at a pressure of 1 × 10^{-6} Torr for 1 s yields partial dissociation (Figure 2b). These conditions correspond to approximately 100 collisions with 4400 oscillations. This large number of oscillations compared with the number of collisions justifies the use of an average translational energy as given by eq 4. Increasing $\langle E_{com} \rangle$ to 0.42 eV and the pressure to 1.5 × 10^{-6} Torr results in 100% dissociation of the peptide with no other product channels observed (Figure 2c). For most experiments the amount of fragmentation was varied by changing e_0 and/or by varying the pressure. The extent of fragmentation can also be finely controlled by varying the frequency of excitation (ω) and the time of excitation (t). Figure 3 demonstrates this for gly-gly-ile. In Figure 3a dissociation was examined at three different frequencies. The energy was varied by changing the applied radio frequency pulse amplitude. Figure 3a shows that the extent of dissociation is a function of the average ion energy and independent of the frequency chosen for excitation over a frequency range of 1.5–3.5 kHz of the frequency chosen for excitation. Figure 3b shows that, as expected, the extent of dissociation increases as the time of excitation increases. A small fraction of the ions always dissociate under very low energy conditions. This is attributed to a fraction of the ion population being formed initially in the FAB desorption process with high internal energies. A more complete analysis of the kinetic data will be the subject of a separate paper.[12]

Unimolecular Reactions: Weakly-Acidic Side Chains. For reference, Table 2 contains estimated acidities (ΔH_{acid}) of the various amino acid side chains. These values were estimated from comparison of similar compounds with known acidities available in the literature.[13] Also noteworthy is that ΔH_{acid} has been experimentally determined by bracketing techniques for glycine and alanine to be 342.4 and 340.7 kcal mol^{-1}, respectively.[14] Recently the acidities of all the amino acids have been determined by kinetic methods and are in good agreement with these values.[15]

(9) The endothermicity can be estimated from the difference in acidities between a carboxylic acid site and a model ketone (CH_3CONMe_2 has an acidity of 375 kcal mol^{-1} from ref 12b). Likewise the total exothermicity for the reaction was estimated by a thermodynamic analysis of the bonds broken and formed during reaction.

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(11) For our cell geometry E_0 is 0.949V/0.0508 where V is the applied rf voltage and 0.0508 is the cell electrode spacing in meters.

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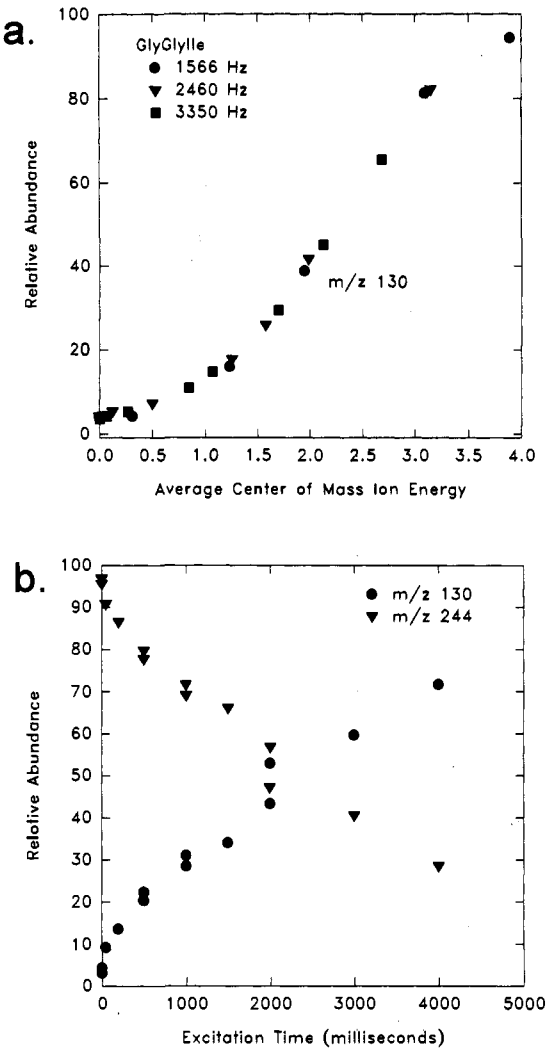
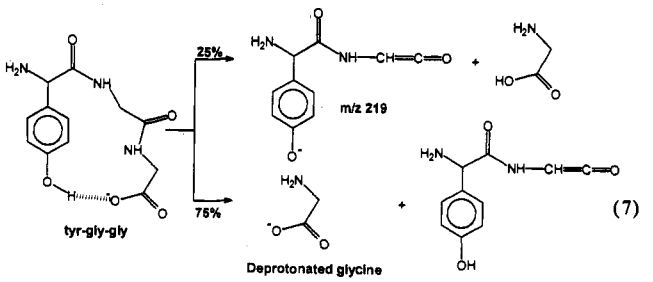


Figure 3. (a) Dissociation of deprotonated gly-gly-ile at various excitation energies. Three different frequencies for excitation were examined. For each frequency the ion translational energy was varied by varying the amplitude of the applied radio frequency electric field. Excitation was for 1 s at a pressure of 9×10^{-8} Torr. (b) Extent of fragmentation of deprotonated gly-gly-ile as a function of excitation time. Irradiation at $\Delta\omega/2\pi$ = 4400 Hz (m/z = 246.5) and $\langle E_{com} \rangle$ = 1.57 eV at a pressure of 8×10^{-8} Torr results in increasing dissociation with time.

These measured values are also included in Table 2. From these a value of about 342 kcal mol^{-1} for the acidity of a generic peptide with no acidic side chains which deprotonates on the C-terminus is estimated. Further stabilization from hydrogen bonding with the side chain is possible for some of the larger amino acids, which would be reflected by an increased acidity.

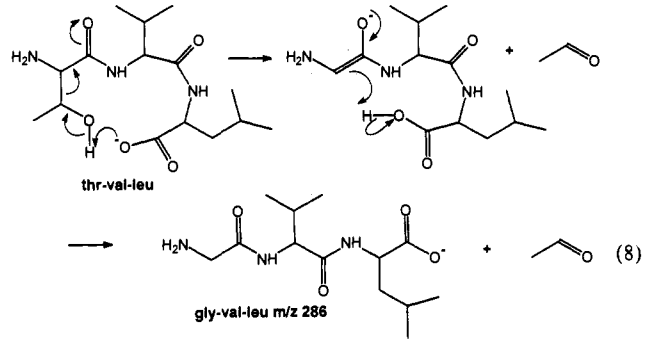
Peptides containing tyrosine, serine, and threonine, all of which are moderately acidic, exhibit slightly different behavior compared to the compounds with non-acidic side chains studied. Peptides containing tyrosine, which is comparable in acidity to the carboxyl terminus (347 kcal mol^{-1} vs 342 kcal mol^{-1}), yield two products upon collisional activation, as shown in reaction 7 for the deprotonated peptide tyr-gly-gly. The lowest energy structure is one which adopts a conformation allowing intramolecular hydrogen bonding between the tyrosine side chain and C-terminus amino acid, as shown. AM1 calculations show this structure to be more stable by 9.3 kcal mol^{-1} than the open form of the peptide and more stable by 2.4 kcal mol^{-1} than a structure with hydrogen bonding between a N-terminus hydrogen and the deprotonated C-terminus. Dissociation occurs by the same mechanism as shown for gly-gly-ile and the two products could then arise from a competition of charge retention between the two acidic sites of



(7)

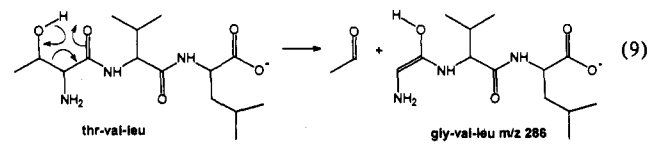
the molecule. When the C-terminus is deprotonated typical dissociation behavior yielding deprotonated glycine as the product ion is observed. When the tyrosine side chain retains the charge, the product at m/z 219 is observed. The observation of these two products arising form a competitive proton transfer reaction implies the possibility of formation of a long-lived dissociation complex.

Peptides containing smaller alcoholic groups like serine and threonine also yield two products. An example with the peptide thr-val-leu is shown in Figures 4 and 5. The reason for the differences in these spectra is discussed later. One product corresponds to the loss of the side chain yielding an ion at m/z 286 and the other is the expected C-terminus amino acid, in this case deprotonated leucine at m/z 130. Because the resulting



(8)

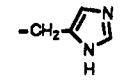
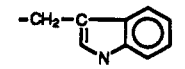
fragment at m/z 286 corresponds to peptide with glycine in the place of threonine, further dissociation should yield the deprotonated C-terminus amino acid. Off-resonance collisional activation of the m/z 286 fragment from thr-val-leu yielded deprotonated leucine as expected. This is shown in Figure 6. If the alcoholic amino acid is at the C-terminus (i.e. leu-ser) the peptide should then first lose the aldehyde from the side chain and then sequentially eliminate deprotonated glycine. This process was also confirmed by initial product isolation and further collisional activation of deprotonated leu-ser. With leu-ser no deprotonated serine was observed, confirming that loss of the side chain is the lowest energy process. This implies that the pathway to yield the C-terminus amino acid occurs exclusively through a sequential process, and not through two competing processes. The initial loss of the side chain is thought to arise through either a charge-induced mechanism (shown in reaction 8) or charge-remote rearrangement (shown in reaction 9). As



(9)

discussed by Adams[3f] either of these mechanisms can account for the observed products. We have no additional experimental evidence to support one or the other of the mechanisms, but Adams showed that for peptides with threonine where the deprotonated C-terminus was bound to a Ca^{2+} ion the loss of the side chain was

Table 2. Estimated Side Chain Acidities for the Amino Acids and Measured Acidity Values of the Amino Acids.[c] Accepted Abbreviations and Monoisotopic Molecular Weights Given for Reference

peptide	abbreviation		$NH_2CHRCOOH$ structure of R	MW	acidities ΔH_a(kcal mol^{-1}) side chain	acidities ΔH_a(kcal mol^{-1}) amino acid[a]
glycine	Gly	G	–H	75.0321	>390[b]	342.0
alanine	Ala	A	$-CH_3$	89.0477	>390[b]	341.2
valine	Val	V	$-CH(CH_3)_2$	117.0790	>390[b]	339.4
leucine	Leu	L	$-CH_2CH(CH_3)_2$	131.0947	>390[b]	339.1
isoleucine	Ile	K	$-CH(CH_3)CH_2CH_3$	131.0946	>390[b]	338.8
methionine	Met	M	$-CH_2CH_2SCH_3$	149.0511	>390[b]	335.8
proline	Pro	P	$-CH_2CH_2CH_2-$	115.0634	>390[b]	340.3
lysine	Lys	L	$-CH_2CH_2CH_2CH_2NH_2$	146.1056	>390[b]	337.5
arginine	Arg	R	$-CH_2CH_2CH_2NHC(NH)NH_2$	174.1117	>390[b]	331.9
phenylalanine	Phe	F	$-CH_2Ph$	165.0790	380[b]	336.5
serine	Ser	S	$-CH_2OH$	105.0426	375[b]	332.7
threonine	Thr	T	$-CH(OH)CH_3$	116.0583	373[b]	332.1
asparagine	Asn	N	$-CH_2CONH_2$	132.0535	362[b]	331.7
glutamine	Gln	Q	$-CH_2CH_2CONH_2$	146.0692	362[b]	331.7
cysteine	Cys	C	CH_2SH	121.0198	355[b]	332.9
histidine	His	H		155.0695	353[c]	331.0
tryptophan	Trp	W		204.0899	352[c]	336.9
tyrosine	Tyr	Y	$-CH_2PhOH$	181.0739	347[b]	356.4
aspartic Acid	Asp	D	$-CH_2COOH$	133.0375	345[b]	
glutamic Acid	Glu	E	$-CH_2CH_2COOH$	147.0532	344[b]	

[a] From ref 14. [b] From ref 12b. [c] From ref 12a.

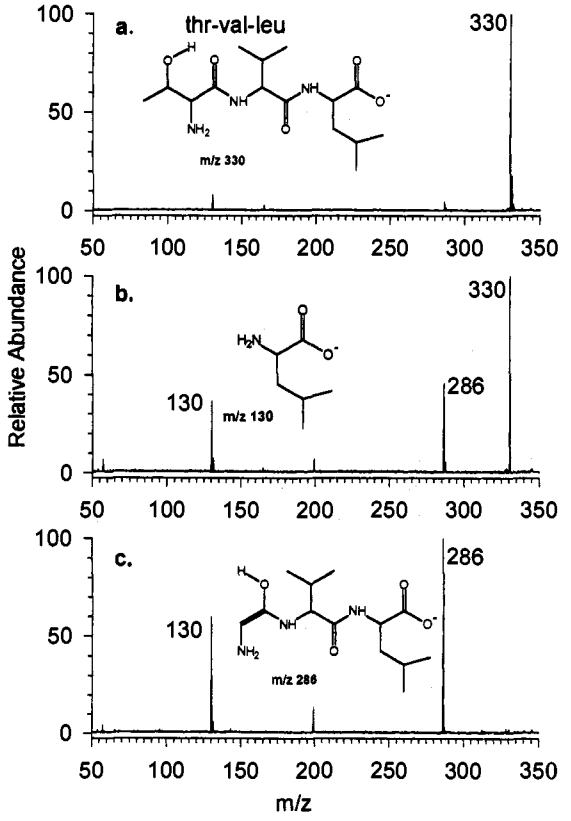


Figure 4. Low-energy dissociation of the deprotonated peptide thr-val-leu (m/z = 330.2) against a pulsed gas pressure: (a) isolated parent ion; (b) irradiation with $\Delta\omega/2\pi$ = 1866 Hz (m/z = 332) and $\langle E_{com}\rangle$ = 0.29 eV at a pressure of 10^{-6} Torr for 1 s (approximately 100 collisions) yields partial dissociation and two products; (c) increasing irradiation at $\langle E_{com}\rangle$ = 0.41 eV yields 100% dissociation.

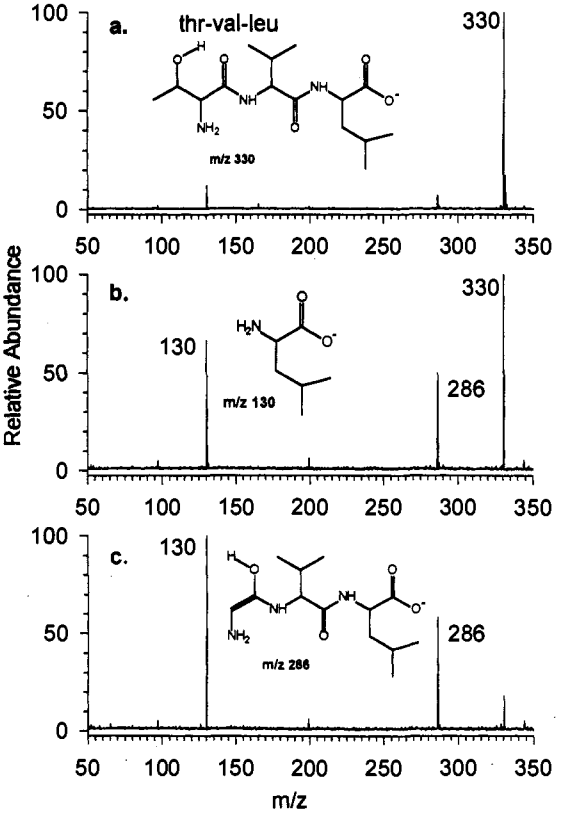


Figure 5. Low-energy dissociation of the deprotonated peptide thr-val-leu (m/z = 330.2) against a static gas pressure: (a) isolated parent ion; (b) irradiation with $\Delta\omega/2\pi$ = 1866 Hz (m/z = 332) and $\langle E_{com}\rangle$ = 1.50 eV at a pressure of 10^{-7} Torr for 1 s (approximately 10 collisions) yields partial dissociation and two products; (c) increasing irradiation to $\langle E_{com}\rangle$ = 2.16 eV yields 100% dissociation.

still observed, supporting the charge-remote mechanism of reaction 9. Molecular mechanics calculations by Adams on deprotonated thr-val-leu showed that the lowest energy structure mimics that shown in reaction 8 with hydrogen bonding between the deprotonated C-terminus and the side chain. However, we have done further calculations at the AM1 level and observe that

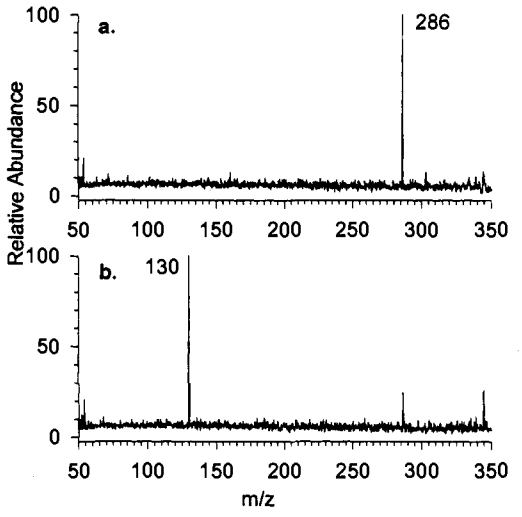


Figure 6. (a) Isolation of the daughter ion of m/z 286 from the dissociation of thr-val-leu; (b) off-resonance excitation with $\Delta\omega/2\pi$ = 2370 Hz (m/z = 288) and $\langle E_{com}\rangle$ = 0.43 eV at a pressure of 10^{-6} Torr for 1 s (approximately 100 collisions) yields complete fragmentation to give the C-terminus amino acid (m/z = 130).

a structure with hydrogen bonding between the deprotonated C-terminus and the N-terminus and terminal amide bond and a hydrogen bond between the hydroxyl group of the side chain and the adjacent carbonyl is lower in energy than a structure with ahydrogen bond between the deprotonated C-terminus and the hydroxyl group by 2.0 kcal mol^{-1}. Though all the structures will be accessible with the energy available from collisional activation, this is further support for the charge-remote mechanism. As will be further mentioned in the discussion a charge-remote mechanism fits the general trend of dissociation mechanisms observed. In addition the thermal rearrangement of 4-hydroxy-4-methyl-2-pentanone, which occurs by a mechanism analogous to the mechanism in reaction 9, has known activation parameters of E_a = 32.3 kcal mol^{-1} (1.4 eV) and $A = 10^{12}$ s^{-1}.[10c]

Unimolecular Reactions: Strongly-Acidic Side Chains. Preliminary studies were carried out on peptides containing amino acids with acidities comparable to that of the C-terminus carboxyl group. For even the simple dipeptides containing such functionalities determination of the C-terminus amino acid was often not possible, and multiple fragmentation patterns were usually observed. Peptides containing histidine and tryptophan side chains, which are estimated to be slightly less acidic than the carboxyl terminus, still yield the deprotonated C-terminus amino acid as one of their dissociation pathways. Peptides containing tryptophan in addition eliminate the tryptophan side chain, while peptides with histidine also lose CO_2 and/or H_2O.

Peptides containing glutamic acid yield dissociation patterns similar to the high energy spectra.[3e] Two types of products observed for a more complicated peptide, in this case val-gly-ser-glu, are indicated in reaction 10. Two products probably arising from a competitive dissociation process are observed. This again suggests the existence of a long-lived dissociation complex. With peptides having glutamic acid at the C-terminus position the major product of chemical interest observed is an intact peptide less the C-terminus amino acid as shown for val-gly-ser-glu in reaction 10. In this case an ion of m/z 260 proposed to correspond to the simpler deprotonated peptide val-gly-ser is observed. Further dissociation of this product confirms its identity and yields an ion of m/z 230 corresponding to loss of formaldehyde from the serine side chain. An ion corresponding to m/z 173 was also observed (both by us and by Bowie et al.[3]) but we were unable to assign a reasonable structure. Likewise, in the case of gly-glu deprotonated glycine is observed. In all glutamic acid containing peptides an ion of m/z 128 was observed. When glutamic acid is at the C-terminus this ion is proposed to correspond to the product shown in reaction 10. This structure was confirmed by Bowie et al. by MS/MS experiments.[3e] When glutamic acid is at the N-terminus the ion at m/z 128 was also observed and is proposed to arise from deprotonated glu-leu as shown in reaction 11. This mechanism is analogous to the reactions exhibited by peptides without acidic side chains except the ion observed corresponds to the fragment containing the glutamic acid side chain, which is deprotonated. In addition, the smaller peptides with acidic side chains often showed water and/or carbon dioxide loss, a process never observed for peptides without acidic side chains.

Table 3. Reagents Tested To Probe Bimolecular Reactivity of Amino Acids and Peptides

reagent	observed reactions
o-$CH_3PhN{=}C{=}O$	none
$CH_3N{=}C{=}S$	none
acetone (CH_3COCH_3)	none
CH_3COCl	none
ethylene oxide	none
formaldehyde	none
acetic anhydride ($CH_3CO{-}O{-}COCH_3$)	m/z 119
trifluoroacetic anhydride ($CF_3CO{-}O{-}COCF_3$)	m/z 113
phenylsilane ($SiPhH_3$)	proton transfer
acetic acid	proton transfer
$Fe(CO)_5$	adduct formation and $Fe(CO)_4^-$ formation
trifluoroacetic acid	proton transfer

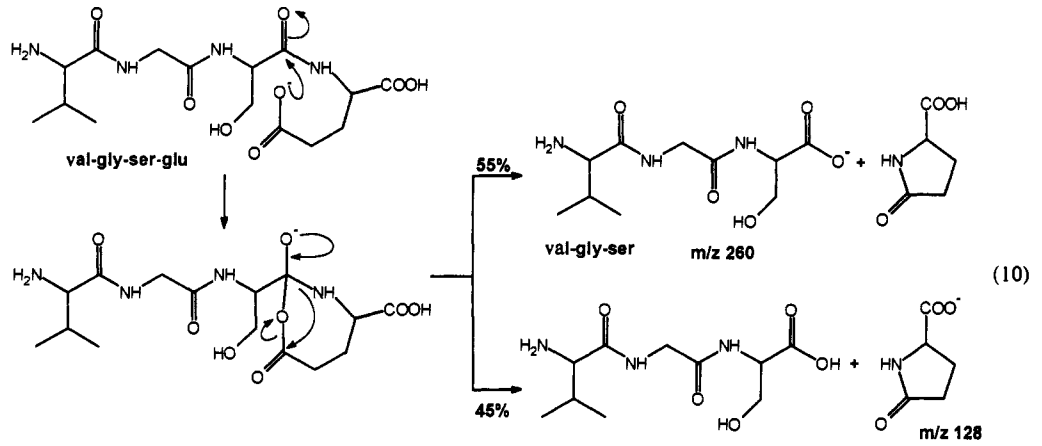
Bimolecular Reactions. Simple deprotonated peptides were exposed to various reagents susceptible to nucleophilic attack to see if any bimolecular reactivity could be observed. Table 3 provides a summary of reagents tried and reactivity observed. Most bimolecular reactions of deprotonated peptides with other reagents are expected to be endothermic and off-resonance excitation was employed to promote product formation. A variety of reactive electrophiles exhibited no reactivity, including formaldehyde, ethylene oxide, and *p*-tolyl isocyanate. Species with labile protons like acetic acid, trifluoroacetic acid, and phenylsilane tended to undergo fast proton transfer as shown in reaction 12. Transition metal carbonyl complexes, like iron pentacarbonyl, known to be reactive with carboxylic acids[16] formed dimer complexes with the peptides as shown in reaction 13. In ten seconds at an iron pentacarbonyl pressure of 5×10^{-8} Torr the reaction goes to completion with the resulting product distribution shown in reaction 13.

Bimolecular reactivity of anyhydrides at a reasonable rate without any excitation was observed, specifically with acetic and trifluoroacetic anhydride. Reactions 14 and 15 exemplify reaction pathways observed for a typical peptide reacting with trifluoroacetic anhydride and acetic anhydride, respectively. These reactions are in good agreement with known gas-phase reactivity of anhydrides with simple carboxylic acids.[17] Though none of the observed processes yielded any useful sequence information, these results are noteworthy since other than isotopic hydrogen exchange and proton transfer processes there are few previously reported instances of bimolecular reactions of biomolecular ions with small molecules in the gas phase.

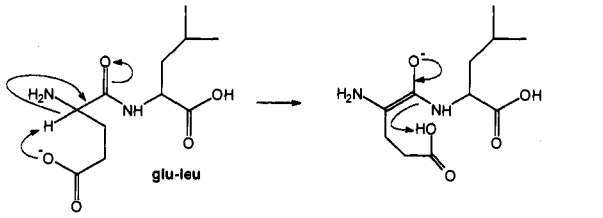
Discussion

Off-resonance excitation has been previously demonstrated to be a simple method with which to effect low-energy dissociations of ions in an ICR cell.[4] Here we have shown its applicability to studying simple deprotonated peptides. The example of the

(16) Lame, K. R.; Sallans, L.; Squires, R. R. *J. Am. Chem. Soc.* **1986**, *108*, 4368–4378.
(17) (a) Bowie, J. H. *Aust. J. Chem.* **1975**, *28*, 559–562. (b) Bowie, J. H.; Williams, B. D. *Org. Mass Spectrum.* **1975**, *10*, 141–145.



(10)



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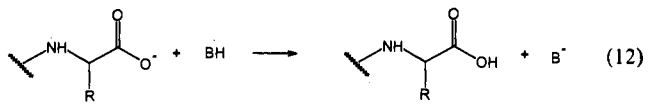

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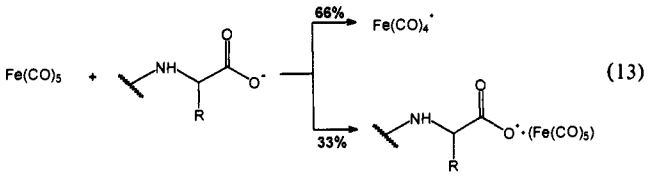
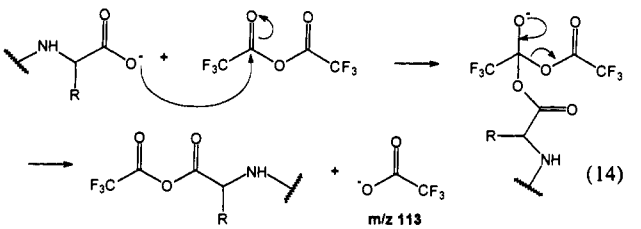


(13)



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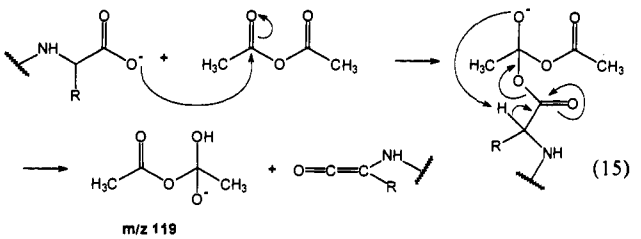

(15)

dissociation of gly-gly-ile given in Figures 2 and 3 and reaction 1 shows many of the key features of the technique being used. The amount of fragmentation can be finely controlled by varying the average ion translational energy, the length of the excitation period, and the collision gas pressure. Complete fragmentation can be obtained with efficient collection and detection of the nascent fragment ions. This is in contrast to high-energy collision-induced dissociation techniques where to obtain fragmentation significant attenuation of the ion beam, accompanied by a loss of fragment ion signal intensity, is necessary. Off-resonance excitation is highly selective and only ions with resonance frequencies close to the applied excitation frequency are excited. The products generally are far enough off resonance that they are not excited and instead collisionally relax. High-energy collision-induced dissociation (CID) is usually conducted under multiple collision conditions, and fragments may be further excited in energetic encounters with the collision gas. This aspect of the experiment also provides advantages over infrared multiphoton dissociation (IRMPD)[18] and other photoexcitation methods[19] where everything trapped in the cell (products and reactants) is subject to excitation if the wavelengths employed are absorbed by the ions.

RRKM calculations have been carried out for several of the systems studied and are shown in Figure 7 for the simple peptides gly-gly-ile, thr-val-leu, and gly-val-leu. Appendix 1 details the assumptions made and equations used in carrying out the RRKM analysis on these large molecules. Though it is difficult to obtain quantitative information a more thorough qualitative understanding of the energetics of dissociation of these molecules can be obtained. Because the activation energies are not known the calculations were initially carried out for gly-gly-ile at a variety of activation energies ranging typically from 2 to 3 eV (46–69 kcal mol^{-1}). These numbers are about one and a half to two times the estimated reaction endothermicity and, based on the parameters for acetamide,[10] are probably reasonable guesses. One of the most important things that immediately becomes apparent in these calculations is that internal energies significantly in excess of the activation energy are required to obtain dissociation rates of 10 s^{-1} (an appropriate rate for the time scales associated with our experiments) even for the small peptides being studied here. Despite this requirement for high internal energy, reasonably selective fragmentation patterns are observed. For gly-gly-ile, Figure 7a shows that to obtain a dissociation rate of 10 s^{-1} with an assumed E_0 (threshold or activation energy) of 2.5 eV will require a total of 5 eV of internal excitation, or 2.5 eV excess energy over the threshold energy. This is in comparison to high-energy CID experiments, where to observe dissociation with the same activation parameters requires a dissociation rate of at least 10^6 which would require at least 9 eV of internal excitation, or 6.5 eV of excess energy over threshold. Figure 3 shows that to obtain almost complete dissociation requires an $\langle E_{com} \rangle$ of 4.0 eV for 3 collisions. This implies an average efficiency for conversion

(18) (a) Moini, M.; Eyler, J. R. *Int. J. Mass Spec. Ion Proc.* **1987**, *76*, 47–54. (b) Moini, M.; Eyler, J. R. *Int. J. Mass Spec. Ion Proc.* **1989**, *87*, 29–40.

(19) (a) Bowers, W. D.; Delbert, S.; Hunter, R. L.; McIver, R. T., Jr. *J. Am. Chem. Soc.* **1984**, *106*, 7288–7289. (b) Bowers, W. D.; Delbert, S.; McIver, R. T., Jr. *Anal. Chem.* **1986**, *58*, 969–972.

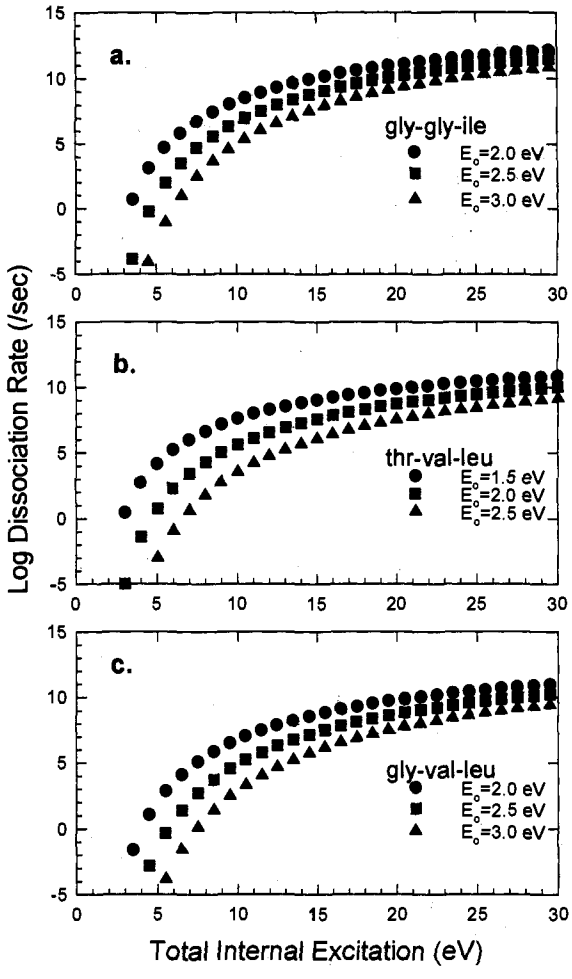

Figure 7. RRKM calculations for the dissociation of small deprotonated peptides as a function of internal energy for various activation energies: (a) rates of dissociation of gly-gly-ile to form deprotonated isoleucine; (b) rates of dissociation of thr-val-leu to form deprotonated gly-val-leu (m/z 286); (c) rates of dissociation of deprotonated gly-val-leu to yield deprotonated leucine (m/z 130). These results indicate that internal energies significantly in excess of threshold are required for large molecules to obtain an appreciable dissociation rate.

of translational to internal vibrational energy of at least 42% for each collision [about 39 kcal mol^{-1} (1.7 eV) collision^{-1}] to accumulate the required internal energy.

Only in the reactions of peptides with acidic side chains were multiple products observed. In the case of tyr-gly-gly competing processes were observed. Competition was posulated to arise by one dissociation pathway with the products resulting from placing the proton on two sites comparable in acidity. This proton transfer competition probably results in the multiple products observed for peptides involving strongly acidic side chains as well. However, it is observed and expected from an RRKM analysis that if multiple major products from two different dissociation pathways are observed they tend to arise due to sequential rather than competitive pathways. This is the case with peptides containing threonine and serine (i.e. thr-val-leu, leu-ser-phe, and leu-ser). The initial fragmentation pathway involves elimination of the alcoholic side chain. As we have shown, the resulting fragment ion can then be further dissociated to yield the deprotonated C-terminus amino acid. Since no deprotonated serine is observed in the dissociation of leu-ser we propose that the process to yield the deprotonated C-terminus amino acid occurs exclusively by sequential fragmentation after loss of the hydroxyl side chain. The fragment ion intensities observed for thr-val-leu at two different pressures are shown in Figures 4 and 5. Figure 4 shows that at higher pressures (in this case 10^{-6} Torr) more of the fragment arising from side chain elimination is observed. In contrast, at the lower pressure used to obtain the spectra in Figure 5, the deprotonated C-terminus amino acid is the more abundant ion observed. RRKM analysis can also be used to explain the different branching ratios observed at different pressures. RRKM calculations for a variety of activation energies are shown for the two processes in Figure 7, parts b and c. When dissociation first occurs the fragments are still vibrationally excited. The charged fragment (m/z 286 in this case) retains a substantial fraction of the initial internal energy deposited in excess of the threshold energy and can further dissociate. In this case, the second dissociation process, leading to the deprotonated C-terminus amino acid, can occur on a time scale comparable to collisional or radiative relaxation, provided it has a similar (or slightly higher) activation energy. Since it is not efficiently excited by the off-resonance pulse it will also slowly lose energy (at pressures of 10^{-6} Torr collisional relaxation can become significant on a 10 ms time scale). The product distributions observed for unimolecular dissociation at 10^{-6} Torr (Figure 4) compared with those observed at 5×10^{-8} Torr (Figure 5) further support this postulated sequential process. Collisional relaxation of the product(s) occurs faster at the higher pressures of the former experiment and so significantly less of the deprotonated C-terminus amino acid is observed. Hence, to determine low-energy dissociation pathways, a pulsed gas experiment which allows higher collision pressures to be obtained is more appropriate. An idea of the necessary energetics can be determined by considering the RRKM calculations shown in parts b and c of Figure 7 for thr-val-leu and gly-val-leu. For example, if E_0 for the loss of the threonine side chain is 1.5 eV and the internal excitation is 3.5 eV the fragment ion can retain an average internal vibrational energy of almost 2.0 eV, meaning no extensive further dissociation would be observed. This means that to observe dissociation the loss of the C-terminus amino acid must have an activation energy of less than 2.0 eV. Collisional energy transfer processes of large molecules are not well understood. To rationalize the observed product distributions with the energetic data available, it seems likely that a fraction of the molecules are dissociating with even higher internal energies than those postulated here. These highly excited molecules would then more readily undergo sequential dissociation on the time scales of our experiments and yield the observed product distributions.

There are salient features which characterize almost all of the dissociation processes considered in this study. The dissociations are controlled by the site of charge, whether it is on the C-terminus of the peptide or on the side chain of an amino acid in the peptide, except perhaps for the threonine- and serine-containing peptides. The importance of the size of the internal ring formed to bring about the dissociation is also apparent. The general mechanism for dissociation of peptides without acidic side chains (reaction 1) involves formation of a seven-member ring. This ring size selectivity is also evident when considering the dissociation of the deprotonated peptide val-gly-ser-glu (reaction 9) and comparing it to the observed dissociations of other peptides containing glutamic acid (reaction 10). The fragment resulting from the dissociation pathway of reaction 1 is never observed. If the charge is on the glutamic acid side chain for this peptide an eight-member ring would be required to abstract the appropriate proton. The major products observed result from the initial formation of a more favorable seven-member ring. Likewise, the major product observed for the deprotonated peptide glu-leu is proposed to result from a six-member-ring transition state (reaction 10). Water loss is a process observed for many of the smaller peptides with acidic side chains and it seems likely this corresponds to cyclization of the peptide. In the case of dipeptides this would result in formation of rings with six to eight members. It is evident water loss is not exclusively due to the presence of acidic side chains as it is not an observed product from deprotonated val-gly-ser-

glu, which would form a much larger ring upon cyclization. The importance of the ring size formed in the transition state provides further evidence that the decomposition of the serine- and threonine-containing peptides occurs by a charge-remote mechanism, which would have a six-member-ring transition state. Further work probing the fragmentations of model compounds and peptides of various composition is needed to understand more thoroughly these dissociation mechanisms.

Off-resonance collisional activation of deprotonated peptides is a useful technique for simple systems because it allows identification of the C-terminus amino acid in many instances. However, most of the molecule is lost as a neutral, making the determination of further sequence information impossible. A more ideal experiment would leave the neutral fragment as an ion with an intact C-terminus mimicking the parent ion. Then sequential dissociations could allow the sequence of the peptide to be rapidly determined. The simplest way to do this would be by a bimolecular reaction with some reagent that would add to the C-terminus prior to collisional activation to yield the desired product. Something that would add to mimic the chemistry of glutamic acid on the C-terminus would be ideal, for as was shown in reaction 10 these peptides undergo a potentially useful dissociation pathway to yield a peptide with an intact C-terminus. Though none of the reactions investigated afforded any sequence information they are noteworthy because there are few previously reported studies of bimolecular reactions of peptides in the gas phase. Understanding the bimolecular reactivity of quasimolecular ions formed from peptides can aid in the development of methods to obtain sequence-specific information for these species.

Conclusions

It has been demonstrated that collisional activation of deprotonated peptides can yield useful but limited sequence information. In general the low-energy pathway for dissociation of peptides without acidic side chains is elimination of the deprotonated C-terminus amino acid. This reaction pathway is controlled and catalyzed by the deprotonated carboxyl group. Peptides with acidic side chains exhibit more complex behavior but still yield useful sequence information. Statistical analysis using RRKM theory of these systems aids in the qualitative understanding of the energetics of the various processes. Further work to understand the mechanisms of collisional activation in the off-resonance excitation process and obtain more quantitative energetic information is in progress. In particular it is possible to use a series of deprotonated peptides to form a homologous set of molecules that dissociate with similar activation parameters. This can then be used to probe the effect of size on the collisional activation process.

Appendix

RRKM Calculations. RRKM calculations to model the unimolecular dissociation dynamics were carried out using the Whitten–Rabinovitch approximation.[20] External rotations were assumed to be inactive. In this case the functional form of the sum of states, $G(E^\ddagger)$, as a function of the excess energy in the activation complex, $E^\ddagger$, is given by eq 16. The density of states, $N(E)$, as a function of total internal energy, E, for the reactant species is given by eq 17. $E_z^\ddagger$ is the zero point energy (ZPE) of the activated complex, E_z is the ZPE of the reactant species, s is the number of degrees of freedom of the reactant species, and $\nu_i^\ddagger$ and ν_i are the vibrational frequencies of the activated and reactant species, respectively. The activated complex is assumed to have one less degree of freedom than the reactant molecule.

$$G(E^\ddagger) = \frac{(E^\ddagger + a^\ddagger E_z^\ddagger)^{s-1}}{(s-1)!\prod_{i=1}^{s-1} h\nu_i^\ddagger} \tag{16}$$

$$N(E) = \frac{(E + aE_z)^{s-1}}{(s-1)!\prod_{i=1}^{s} h\nu_i} \tag{17}$$

The rate expression can then be simplified to the form shown in eq 18.

$$k = \frac{G(E^\ddagger)}{hN(E)} = \left(\frac{E^\ddagger + a^\ddagger E_z^\ddagger}{E + aE_z}\right)^{s-1} \frac{\prod_{i=1}^{s} \nu_i}{\prod_{i-1}^{s-1} \nu_i^\ddagger} \tag{18}$$

For "large" molecules the ZPE and sum and density of states are very large. For example, the ZPE of a 5-unit glycine polymer is about 10 eV. To carry out the actual calculation a reasonable mechanism and activated complex is first proposed. Vibrational frequencies are estimated from known frequencies for groups functionally similar to those found in peptides. Any additional frequencies not directly assignable were assumed to be low-energy modes, and internal rotations were constrained in the cyclic transition states. Typical frequency factors were 10^{13} s^{-1}. To test the accuracy of the Whitten–Rabinovitch approximation we also performed calculations using a direct count algorithm in the case of gly-gly-ile for comparison. For rate constants of less than 10^8 s^{-1} very good agreement was observed. This justifies using the simpler (and faster) approach outlined here to obtain unimolecular dissociation rate constants in the energy regime in which we are interested.

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