Fragmentation of Protonated Tripeptides: The Proline Effect Revisited

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The fragmentation of protonated tripeptides under metastable ion conditions and collision-induced conditions are reported. The majority of protonated tripeptides cleave at the C-terminal amide bond to form b_2 and y_1 ions, with the relative amounts depending on the proton affinities of the amino acids derived from the C-terminal amino acid residue. Protonated tripeptides that have a proline residue at the C-terminal position fragment almost exclusively by cleavage of the amide bond adjacent to the proline and give mainly y_1 ions; where there is a proline residue in the central position, fragmentation of the protonated tripeptide occurs at both the N-terminal and the C-terminal amide bonds to form y_2 and b_2 ions; finally, two of the tripeptides with proline at the N-terminal position, Pro-Pro-Pro and Pro-Gly-Gly, fragment largely by cleavage at the N-terminal, the former by cleavage of the amide bond and the latter by direct formation of the a_1 ion. Protonated Pro-Val-Gly fragments to give predominantly b_2 and a_2 ions at low energy, but the a_1 ion becomes the dominant product at higher energies. In the fragmentation of protonated peptides, the lack of b ions formed by cleavage at the amide bond C-terminal to proline (i.e., with the proline residue in the oxazolone ring) has conventionally been attributed to ring strain in a bicyclic oxazolone. Here we show, however, that the bicyclic oxazolone structure containing proline derived from protonated Gly-Pro-Gly is only 2.7 kcal/mol higher in free energy, at the B3LYP/6-31++G(d,p) level of theory, than the isomeric nonbicyclic oxazolone derived from protonated Pro–Gly–Gly; that is, strain appears to be a small or negligible factor. Formation of the y_2 ion in protonated Gly-Pro-Gly has a barrier of 3 kcal/mol higher free energy than that of the b_2 ion. In comparison, the difference increases to 10 kcal/mol in protonated Gly-Phe-Gly. The neutral products that are formed along with the y_2 ion are CO and methanimine, not aziridinone as once proposed.

Introduction

Despite the importance of mass spectrometry in protein analysis, fragmentation of protonated peptides is understood only at the empirical level,[1–3] and the fine details of many of the fragmentation pathways are still not known. Protonated peptides fragment primarily along the backbone at the amide bonds and produce mainly b, y, and a ions. These fragmentation pathways
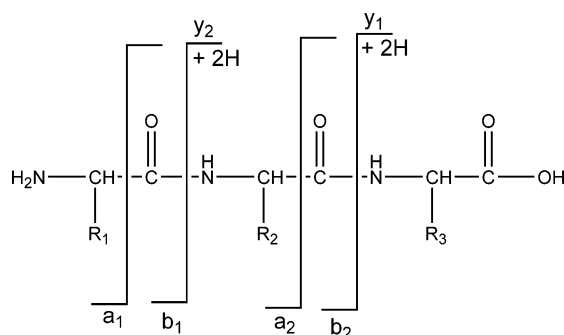
have been studied by a wide variety of methods including collision-induced dissociation (CID)[4–7] and isotopic labeling.[8–10] The use of CID continues to be of importance in peptide

sequencing and protein identification. A low (<100 eV) energy collision-induced dissociation spectrum of a protonated peptide typically consists of series of b, a, and y fragment ions which reflect the amino acid sequence.[3,11,12] In solution, the most basic site on a peptide devoid of histidine, lysine, and arginine residues is the nitrogen atom of the N-terminal amino group.[13,14] However, once the protonated peptide ion has been desorbed into the gas phase, e.g., via electrospray,[15] transfer of the "ionizing" proton to amidic functional groups, the carbonyl oxygen and the amidic nitrogen atoms, on the backbone becomes competitive.[16–31] The peptide then fragments at the protonated peptide linkage.[3,11,12] A nonterminal residue has two adjacent peptide linkages, one N-terminal and the other C-terminal to it. The relative propensities of fragmentation of the two peptide linkages in different amino acid residues have been systematically examined in 1465 ion trap spectra of doubly protonated tryptic peptides.[32] It was found that proline and to a smaller extent glycine and serine residues have biases toward fragmentation on their N-terminal peptide bonds, whereas residues such as valine and isoleucine have biases toward their C-terminal peptide bonds. Proline is unique in that it is the only amino acid found in mammalian proteins that contains a secondary amine.

Doubly protonated tryptic peptides tend to fragment to yield two ionic products. For singly protonated peptides, dissociation can yield only one charged product. If the charge is retained on the N-terminal fragment, a b ion is produced; this ion may

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subsequently lose CO to form an a ion. Alternatively, if the charge is retained on the C-terminal fragment, a y ion is produced. The b ion was long believed to be an acylium ion;[12-14] however, seminal work by Harrison's,[33-35] Wesdemiotis',[36,37] and Hunt's[38] groups has established that the b_2 ion, at least that formed from a peptide that comprises residues containing only alkyl side chains, has an oxazolone structure. The b ion can further dissociate to produce the a ion, an iminium ion. The y ion has been proposed to be a protonated truncated peptide or amino acid;[16-19] earlier MS/MS[8] and MS/MS/MS[10] experiments had shown the y_n ions to be protonated peptides or amino acids.

It is apparent from the diverse CID patterns observed that the fragmentation routes of protonated peptides depend significantly on the identity and positions of the amino acids constituting the peptides. Of particular interest is the pronounced effect of the proline residue in directing fragmentation of the peptide bond N-terminal to it. In addition to the systematic study pointed out earlier[32] other published accounts on tandem MS of peptides, e.g., ubiquitin, have often noted unusually abundant product ions resulting from cleavage of peptide linkages on the N-terminal side of proline residues.[39] This propensity for selective fragmentation has also been noted for singly protonated peptides[40-45] and multiply charged protein ions.[46] This phenomenon is sometimes known as the proline effect and was first attributed to the relatively high proton affinity of proline.[47] Vaisar and Urban investigated a number of peptides that contained a modified amino acid residue of relatively high proton affinity, most notably one that contained a six-membered piperidine ring as opposed to proline's five-membered pyrolidine ring, and concluded that proton affinity is not the reason.[48] They attributed the preferred cleavage N-terminal (as opposed to C-terminal) to proline in terms of increased ring strain in forming a bicyclic b ion when the C-terminal peptide bond is cleaved. The issue of ring strain will be addressed in this present study.

In addition, we will report a systematic study on the fragmentation of tripeptides that contain proline in different residue positions, with a view to sorting out differences in fragmentation propensities; both mass spectrometry and computational chemistry are used in attempts to elucidate mechanisms. The use of the tripeptide system eliminates possible competition from and participation of distant residues in dissociation.

Experimental Methods

Fragmentation reactions of the protonated tripeptides occurring on the metastable ion time scale were explored using B/E linked scans[49,50] on a BE double-focusing mass spectrometer (VG 70-250S, Micromass, Manchester, U.K.). Ionization of the peptide of interest was by fast atom bombardment (FAB) using Xe atoms with the peptide in either a glycerol or thioglycerol matrix.

Collision-induced dissociation (CID) studies were performed on two SCIEX triple-quadrupole mass spectrometers, API III and API 3000. Samples were typically 10 μM peptides in 50/50 water/methanol containing 1% acetic acid. These were continuously infused by means of a syringe pump at a typical flow rate of 3 μL/min into the pneumatically assisted electrospray probe with air being the nebulizing gas. The optimum probe position was established from time to time but was typically with the tip about 2 cm from the interface plate and with the spray off-axis from the orifice. Mass spectra were acquired in the positive ion detection mode with unit mass

TABLE 1: Metastable Ion Fragmentation of Protonated Tripeptides

peptide	% of total ion signal				
	$b_3{}^a$	y_2	b_2	a_2	y_1
Gly-Pro-Gly	19.1	15.8	60.6	4.6	
Gly-Pro-Ala	27.1	11.7	61.3		
Ala-Pro-Gly	13.4	39.0	42.4	5.2	
Ala-Pro-Leu	14.7	50.3	31.5	1.4	2.1
Ala-Pro-Phe	9.8	57.4	27.3	1.2	4.3
Gly-Pro-Pro	12.0	4.9	80.9		2.2
Pro-Pro-Pro		67.6	29.7		2.7

^{a} H$_2$O loss from MH$^+$.

resolution at a step size of 0.1 *m/z* unit and at a dwell time of 10 ms/step. Typically, 10 scans were summed to produce a mass spectrum. Collision-induced dissociation was performed with argon or nitrogen as the neutral gas. In the API III, the gas pressure in q2 was continuously monitored with an upstream baritron gauge, the read out of which was converted into collision gas thickness (CGT)[51] by the mass spectrometer software. (CGT is the product of the neutral gas number density and the length of q2.) For single collision CID, CGT was kept at a value of 50 atoms cm^{-2}. The results of many CID studies are presented in this article as breakdown graphs expressing the percent of total ion abundance as a function of the collision energy. All peptide samples were obtained from BACHEM Biosciences (King of Prussia, PA).

Computational Method

DFT calculations employing the hybrid B3LYP method (using Becke's three-parameter exchange functional[52] and the correlational functional from Lee, Yang, and Parr[53]) with the 6-31++G(d,p) basis set[54] in Gaussian 98[55] have been used to calculate the optimized geometries, energetics, and vibrational frequencies. Initially, structure optimizations were performed at the Hartree-Fock level using a 3-21G basis set,[56] but as the energetics of the overall reaction profile at both levels of theory are very similar, we only report the results of the DFT study here. Zero-point energies and thermal corrections obtained from the DFT calculations were used to determine relative enthalpies at 298 K. Finally, entropy terms were included to obtain relative free energies. Details of the energies from the computations are given in the Supporting Information in Table 1S and structural details are given in Figure 1S.

Results and Discussion

The fragmentation pathways followed by a number of protonated tripeptides, the majority containing a proline residue, were examined in both metastable ion studies and by single-collision CID. The products of metastable ion fragmentations are summarized in Table 1, and the major ions observed from fragmentation of protonated tripeptides under single-collision CID (at E_{cm}= 3 eV) are summarized in Table 2. Breakdown graphs expressing the percent of total ion abundance as a function of collision energy are shown in Figures 1-4 for a selected number of tripeptides. Under single-collision CID, fragmentation yield is typically small in the E_{cm} range examined. To facilitate viewing, the primary axis refers to the product ion abundances, whereas the secondary axis refers to the precursor ion abundance. The relative abundances are dependent on E_{cm}.
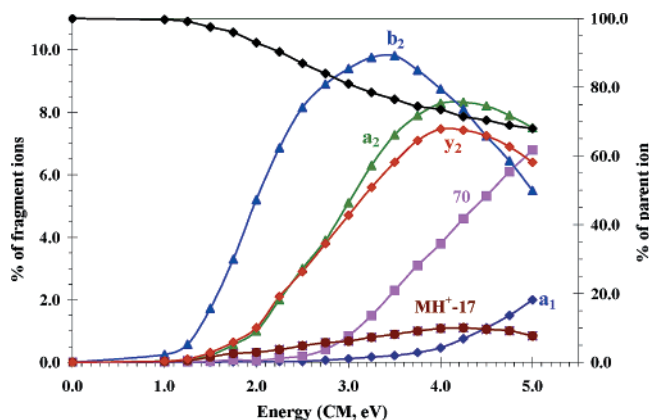
Figure 1. Breakdown curves for collision-induced dissociation of protonated Gly-Pro-Gly under single-collision conditions.
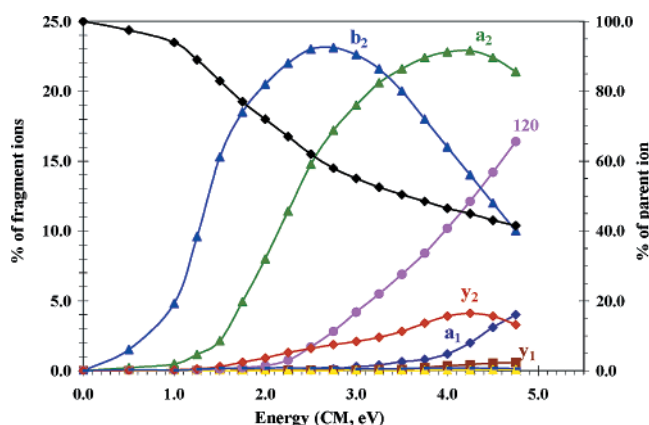

Figure 3. Breakdown curves for single-collision CID of protonated Pro-Gly-Gly.

Figure 2. Breakdown curves for collision-induced dissociation of protonated Gly-Phe-Gly under single-collision conditions.
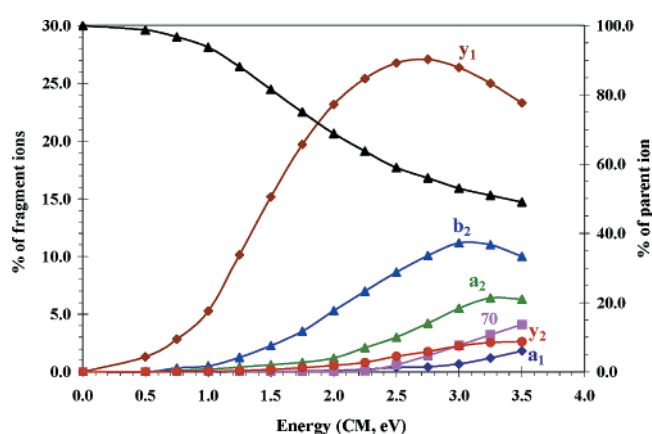
Figure 4. Breakdown curves for single-collision CID of protonated Ala-Ala-Pro.

TABLE 2: Major Ions Formed at $E_{cm} = 3.0$ eV from Fragmentation of Protonated Tripeptides under Single Collision CID (Reported Anything >10% of Total Ions for Products)

peptide	major ions
Gly-Gly-Gly	b_2 (67%), y_2 (23%)
Ala-Ala-Ala	b_2 (75%), y_2 (12%)
Leu-Leu-Leu	b_2 (52%), a_2 (24%), a_1 (12%)
Pro-Pro-Pro	y_2 (53%), b_2 (14%), a_1 (17%)
Gly-Pro-Gly	b_2 (45%), a_2 (24%), y_2 (22%)
Gly-Pro-Ala	b_2 (54%), a_2 (22%), y_2 (16%)
Ala-Pro-Ala	y_2 (53%), b_2 (26%), a_2 (10%)
Lys-Pro-Val	y_2 (50%), a_1−17 (15%)
Ala-Pro-Leu	y_2 (45%), b_2 (34%), a_2 (11%)
Gly-Phe-Gly	b_2 (47%), a_2 (39%)
Gly-Phe-Ala	b_2 (59%), a_2 (33%)
Ala-Leu-Ala	b_2 (56%), a_2 (26%)
Pro-Gly-Gly	a_1 (80%), b_2 (15%)
Pro-Val-Gly	b_2 (40%), a_2 (30%), a_1 (21%)
Leu-Gly-Gly	a_1 (66%), b_2 (24%)
Val-Gly-Gly	a_1 (50%), b_2 (22%)
Leu-Ala-Pro	y_1 (60%), b_2 (23%)
Ala-Ala-Pro	y_1 (55%), b_2 (23%), a_2 (11%)
Ala-Phe-Pro	y_1 (47%), b_2 (33%), a_2 (15%)
Gly-Gly-Phe	y_1 (52%), b_2 (14%), y_2 (12%), Phe (20%)
Gly-Gly-Leu	y_1 (44%), b_2 (21%), y_2 (12%), Leu (17%)
Gly-Gly-Ala	b_2 (47%), y_1 (22%), y_2 (22%)

Under single-collision conditions at 3 eV, much of the protonated tripeptide remains undissociated; the numbers given in Table 2 are for product ions only. In metastable ion fragmentation, significant loss of H_2O is observed to form, nominally, the b_3 ion; this product, however, is not significant under CID conditions.
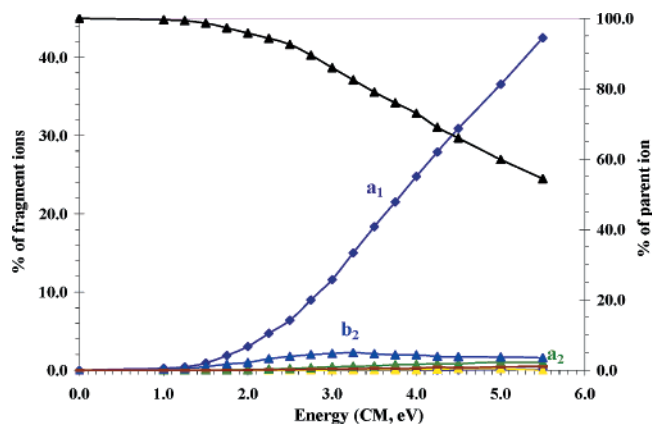
In interpreting the fragmentation patterns of protonated tripeptides, when the predominant products are the b_2 and a_2 ions, the major pathway is thought to be cleavage of the C-terminal amide bond; when the y_2 ion is the major product, then it is thought to be cleavage of the N-terminal amide bond. When the a_1 or y_1 ions are the major products, determination of where cleavage first occurred is less certain because the a_1 ion can be formed either via the b_2 ion, the a_2 ion, or directly from the protonated tripeptide, $[M + H]^+$; similarly, the y_1 ion can be formed either from the y_2 ion or by direct cleavage of the C-terminal amide bond of $[M + H]^+$.

Of the 25 protonated tripeptides examined here, the majority, except those derived from Ala-Pro-Leu and Ala-Pro-Phe (studied under metastable fragmentation conditions, Table 1) and Ala-Pro-Ala, Pro-Pro-Pro, Val-Gly-Gly, Leu-Gly-Gly, Pro-Gly-Gly, and Lys-Pro-Val (studied under CID conditions, Table 2), cleave predominantly at the C-terminal amide bond. For the first four of the tripeptides in this minority group, which all have a central proline residue, the propensities for cleavage at the N- and C-terminal amide bonds are approximately equal.

For the next three tripeptides, of the general formula X-Gly-Gly, the major products are a_1 ions, with minor amounts of b_2 ions, but no significant amount of a_2, throughout the collision-energy range examined (0−6 eV); this suggests that the majority of the a_1 ion produced may not be formed via the b_2 ion but directly from $[M + H]^+$. A precursor ion scan of the a_1 ion derived from protonated Leu-Gly-Gly (Figure 5) is in accordance with the above interpretation that the majority of this a_1 ion, the leucine iminium ion, arises directly by fragmentation of the $[M + H]^+$ ion; smaller amounts come directly from the b_2 and
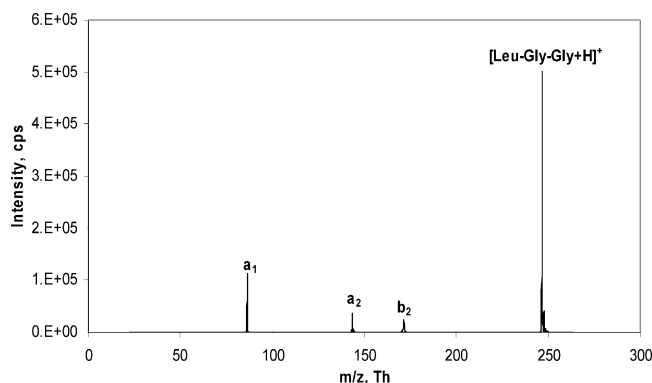
Figure 5. Precursor ion scan of the a_1 ion of protonated Leu-Gly-Gly at E_{cm}= 3 eV.

also from the a_2 ion. Harrison et al. have also observed the direct formation of the a_1 ion in the metastable ion fragmentation of protonated Pro-Gly-Gly (97% b_2 and 100% a_1).[35] The a_1 ion is also formed directly from the b_2 ion derived from many protonated tripeptides in which the central residue is glycyl.[57,58]

Fragmentation of protonated Lys-Pro-Val under single-collision conditions gave approximately 50% of y_2. The other major product, the a_1 −17 ion, results from a pathway unique to this tripeptide, and we attribute this to the preferred site of protonation being on the side chain of the lysine residue.

In addition to the protonated tripeptides listed above, there are other tripeptides from which *minor* amounts of y_2 ions are produced. These are protonated Gly-Gly-X (where X = Phe, Leu, Ala, and Gly), protonated Gly-Pro-X (where X is Gly and Ala), and protonated Ala-Ala-Ala. In summary then, formation of y_2 ions, implying cleavage at the N-terminal amide bond, occurs in all tripeptides with a proline residue in the central position and also in small amounts from Gly-Gly-X and Ala-Ala-Ala. From *all* of the protonated tripeptides, with the exception of Lys-Pro-Val, there are significant if not predominant products (b_2 and y_1 ions) from cleavage at the C-terminal amide bond.

In the absence of any strongly basic group in the side chain of an amino acid residue, the preferred equilibrium location for the "ionizing" proton on the tripeptide is either on the amino group of the N-terminal residue and attached via a hydrogen bond to one of the oxygen atoms of an amide group on the peptide backbone, or attached to one amide oxygen and hydrogen bonded to the other.[59] The preference for cleavage at the C-terminal amide bond has been examined using a combination of quantum chemical and Rice-Ramsperger-Kassel-Marcus (RRKM) calculations on protonated glycylglycylglycine.[60,61] The barrier to formation of the b_2 ion was found to 26.4 kcal mol^{-1}, considerably lower than that for the formation of the y_1 via a mechanism involving formation of diketopiperazine as the neutral N-terminal product (barrier 37.1 kcal mol^{-1}). Formation of the y_2 ion along with CO and $H_2C{=}NH$ was calculated to have a barrier of 39.8 kcal mol^{-1}, but the RRKM calculations showed this reaction to be quite slow relative to that for the formation of the b_2 ion. Nevertheless, a minor but significant amount of y_2 ion was observed in our CID experiment on protonated glycylglycylglycine.

We will now carry out a more systematic analysis of the types of fragmentation pathways followed as a function of the structure of the tripeptide.

(a) Tripeptides Containing Only One Type of Amino Acid Residue. In the CID spectrum of protonated Gly-Gly-Gly, under single-collision conditions at an E_{cm} of 3 eV, fragmentation occurred mainly at the C-terminal amide bond, although there

is a significant amount (slightly >20%) of the y_2 ion formed. Similarly, fragmentations of protonated Ala-Ala-Ala and of protonated Leu-Leu-Leu occurs mainly at the C-terminal amide bond with formation of mainly b_2 ions.

(b) Tripeptides Containing Two Types of Amino Acid Residues. Replacement of the C-terminal residue of protonated Gly-Gly-Gly by a different amino acid residue makes little difference; fragmentations of protonated Gly-Gly-Ala, protonated Gly-Gly-Leu, and protonated Gly-Gly-Phe again occur mainly at the C-terminal amide bond. The major difference is that y_1 ions are produced, with the ratio of y_1 to b_2 increasing with the basicity of the C-terminal amino acid. The proton affinities of the relevant amino acids (in kcal mol^{-1}) are glycine 211.9, alanine 215.5, leucine 218.6, and phenylalanine 220.6.[62]

Replacement of the N-terminal residue of Gly-Gly-Gly has a more profound effect on the type of fragmentation observed. Under single-collision conditions protonated X-Gly-Gly ions, where X is Leu, Val, and Pro, fragment predominantly to a_1 ions, with minor amounts of b_2 ions. This is illustrated for protonated Pro-Gly-Gly under single-collision CID in Figure 3. Previously, one of us has observed that protonated PGG produces substantial amounts of a1 ion under metastable ion conditions.[35] By contrast, under CID conditions, protonated Gly-Gly-Gly fragments to yield the b_2 ion as the major product with no a_1 ion.

The two protonated tripeptides in which the terminal residues are Gly and the central residues are Phe or Pro (i.e., Gly-Phe-Gly and Gly-Pro-Gly) fragment differently. The dissociation of protonated Gly-Phe-Gly (Figure 2) is simpler, with products of cleavage only at the C-terminal amide bond (to give the b_2 and a_2 ions); that is, unlike in the CID spectrum of protonated Gly-Gly-Gly, there is no evidence of cleavage at the N-terminal amide bond. By contrast, protonated Gly-Pro-Gly (Figure 1) requires slightly higher energy for fragmentation but also gives predominately the b_2 ion with 15−20% y_2, an amount similar to that observed for protonated Gly-Gly-Gly. Further, the CID spectrum of the b_2 ion formed from protonated Gly-Pro-Gly was found to be distinctly different from that of protonated cyclo-Gly-Pro, the ion expected if the b_2 ion had a diketopiperazine structure.[36,63] Metastable ion fragmentation of protonated Gly-Phe-Gly gives exclusively the b_2 (97%) ion,[64] whereas for protonated Gly-Pro-Gly, it gives b_2 (60.6%) and y_2 (15.8%) ions. From these observations, it is difficult to support the claim that cleavage at the C-terminal of proline is made unfavorable by the formation of a strained bicyclic oxazolone.[48,65]

The general pattern that has emerged from studying the fragmentations of protonated tripeptides is that, when all of the residues bear relatively simple side chains (e.g., a H or a methyl group), similar types of breakdowns occur, namely cleavage at the C-terminal amide bond and formation of the b_2 and a_2 ions. Introduction of a residue with a relatively complex side chain at either the C- or N-terminal results in cleavage adjacent to that amino acid residue; this is attributed to the greater basicities of residues with large side chains.

We will now carry out a systematic study of the effect of the location of a proline residue on the fragmentation pathway of protonated tripeptides.

(c) Tripeptides Containing Proline. *(i) Pro-Pro-Pro.* Under metastable ion conditions, protonated Pro-Pro-Pro forms mainly the y_2 ion (67.6%) with the b_2 ion (29.7%) being the only other significant product. The CID spectrum of protonated Pro-Pro-Pro also has the y_2 ion as the major product at low energy, with smaller amounts of b_2 and a_1 being formed. At higher energies, an ion of $m/z = 126$ is formed. A precursor ion scan

shows that this ion is formed from both the b_2 and MH^+ ions and a likely structure has two five-membered rings fused together, as shown.
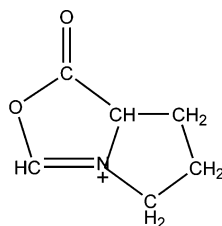
Possible structure of ion of m/z = 126

In forming the ion at 126 Th, the cleavage occurs at the C-terminal amide bond (thereby producing the b_2 ion) and the ring from the N-terminal residue, c-C_4NH_8, is lost as a cyclic imine.

(ii) Tripeptides with a Proline Residue at the C-Terminus. Protonated tripeptides (Ala-Ala-Pro, Leu-Ala-Pro, and Ala-Phe-Pro) with a proline residue at the C-terminus cleave at the C-terminal amide bond. In protonated Ala-Ala-Pro and Leu-Ala-Pro, the major product is the y_1 ion, rather than the b_2 ion (see Figure 4 for the breakdown curve of protonated Ala-Ala-Pro). The preferential formation of the y_1 ion in these peptides is attributed to the high proton affinity of proline. Protonated Ala-Phe-Pro gives a mixture of y_1 and b_2 ions. The presence of Phe in the N-terminal fragment increases the proton affinity of the neutral N-terminal fragment (an oxazolone or a diketopiperazine) relative to that of proline. This result is in essential agreement with those obtained by Harrison.[64] He observed formation of both b_2 (51%) and y_1 (46%) ions with some loss of water, in the metastable ion fragmentation of protonated Ala-Phe-Pro. As noted previously, most protonated tripeptides cleave at the C-terminal amide bond and, in this respect, fragmentations of the three peptides in this X−Y−Pro group follow this general trend.

(iii) Tripeptides with a Proline Residue at the Central Position. When proline is the central residue of the tripeptide and the two terminal residues are those derived from any combination of the two *smallest* amino acids (glycine and alanine), then, except for in the fragmentation of protonated Ala-Pro-Ala under single-collision conditions, fragmentation occurs predominantly at the C-terminal side of the proline to give the b_2 and a_2 ions (see Figure 1 for protonated Gly-Pro-Gly). This again indicates that formation of bicyclic oxazolones is not energetically prohibitive.

The major product from protonated Ala-Pro-Leu under metastable ion and CID conditions given in Tables 1 and 2 is the y_2 ion, but, under single-collision conditions, the *sums* of the amounts of a_2 and b_2 ions are the same as that of the y_2 ion (45%). The amount of b_2 plus a_2 is also appreciable (32.9%) in the metastable fragmentation; that is, in protonated Ala-Pro-Leu, cleavage occurs at both the C-terminal and N-terminal sides of the proline residue.

Fragmentation of protonated Ala-Pro-Phe under metastable ion conditions also gives predominantly the y_2 ion (57%), whereas protonated Gly-Pro-Pro gives mainly b_2 (81%).

In the fragmentation of protonated Lys-Pro-Val, cleavage at the N-terminal side of the proline is dominant under single-collision conditions. The y_2 ion is the major product, but there are also significant amounts of the a_1-17 ion, probably produced by loss of the side chain amino group as ammonia from the a_1 ion. The most basic site in Lys-Pro-Val is on the side chain of the lysine residue, and protonation at that site is probably the
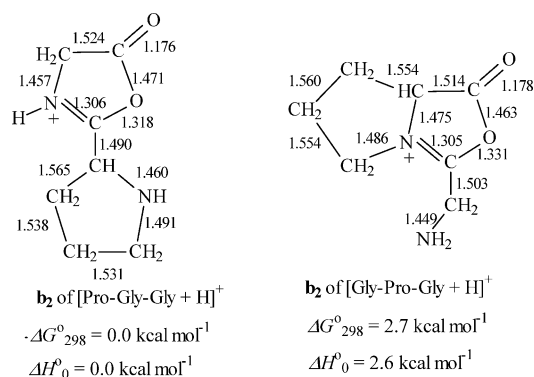

b₂ of $[Pro-Gly-Gly + H]^+$

$\Delta G^o_{298} = 0.0$ kcal mol^{-1}

$\Delta H^o_0 = 0.0$ kcal mol^{-1}

b₂ of $[Gly-Pro-Gly + H]^+$

$\Delta G^o_{298} = 2.7$ kcal mol^{-1}

$\Delta H^o_0 = 2.6$ kcal mol^{-1}

Figure 6. Structures of b_2 ions derived from protonated Gly-Pro-Gly and protonated Pro-Gly-Gly as calculated at B3LYP/6-31++G(d,p).

reason that this peptide displays a different type of fragmentation than the other ions with the proline in the central position.

(iv) Tripeptides with a Proline Residue at the N-Terminus. We have examined only three tripeptides with a proline residue at the N-terminus, and from this limited sample, it is less possible to generalize. With protonated Pro-Pro-Pro, the major product is the y_2 ion, i.e., the N-terminal amide bond cleaves; with protonated Pro-Gly-Gly, the major product is the a_1 ion, involving either cleavage of the N-terminal amide bond followed by loss of CO, or direct cleavage of the C_α−CO bond. These products are atypical for fragmentation of protonated tripeptides. However, protonated Pro-Val-Gly mainly undergoes cleavage at the Val-Gly bond, yielding a_2 and b_2 ions; that is, fragmentation of this tripeptide follows the more common pathway observed for other protonated tripeptides. Even here, the a_1 ion becomes the dominant product at higher collision energies. These observations are consistent with metastable ion studies results. Under metastable ion fragmentation protonated Pro-Gly-Gly shows formation of a_1 (22.3%) and b_2 (46.5%) with $b_3 = 17.9\%$ and $b_2 - H_2O = 13.2\%$ while protonated Pro-Val-Gly shows reduced formation of a_1 (9.5%0 and more pronounced formation of b_2 (77.4%) with some some formation of a_2 (13.2%).

Vaiser and Urban,[48] and subsequently Wysocki et al.,[65] have suggested that the strain introduced by forming a bicyclic oxazolone in the cleavage of the amide bond C-terminal to proline disfavors fragmentation at this bond and, coupled with the high proton affinity of the secondary amide that the N of proline generates, leads to enhancement of the cleavage at the amide bond N-terminal to the proline. The relatively large proton affinity of the prolyl residue then results in the charge being retained on the C-terminal fragment to give a y_n ion.

In an attempt to assess whether the strained bicyclic oxazolone derived from a proline-containing peptide is energetically unfavorable, we used B3LYP/6-31++G(d,p) calculations to examine the b_2 ions that would be produced from peptides Gly-Pro-X and from Pro-Gly-X. The resulting structures (Figure 6) have similar geometric parameters in the oxazolone rings, with the largest difference being the C−N and C−O bonds that are both longer (by 0.018 and 0.013Å, respectively) in the bicyclic ion. The bicyclic oxazolone derived from Gly-Pro-Gly is only 2.7 kcal/mol higher in free energy (at 298 K) than the nonbicyclic oxazolone derived from Pro-Gly-Gly, thereby indicating that the strain in the bicyclic system is not a large destabilizing factor.

Density Functional Calculations. *(a) Structures of Protonated Tripeptides.* The lowest energy structures for all protonated tripeptides have the "mobile" proton attached to one of the three basic sites, the oxygens of the two amide groups and
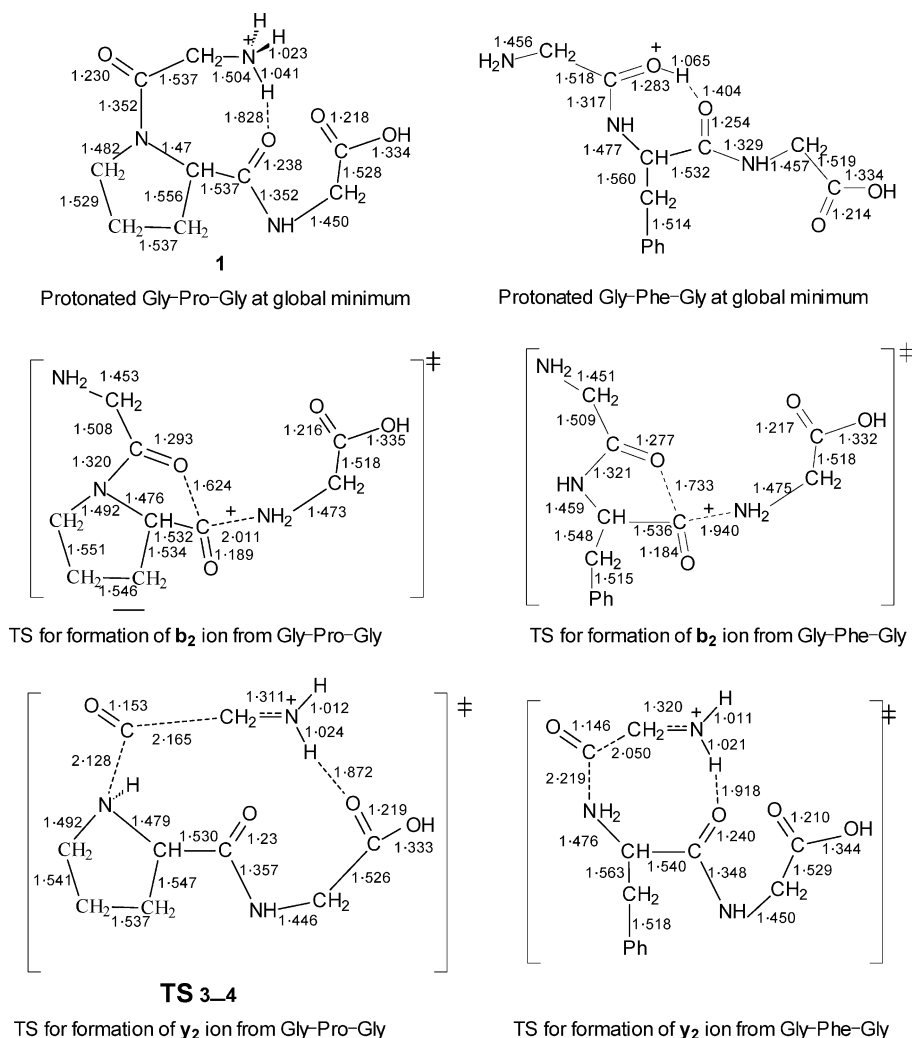
Protonated Gly-Pro-Gly at global minimum   Protonated Gly-Phe-Gly at global minimum

TS for formation of **b₂** ion from Gly-Pro-Gly   TS for formation of **b₂** ion from Gly-Phe-Gly

TS 3—4

TS for formation of **y₂** ion from Gly-Pro-Gly   TS for formation of **y₂** ion from Gly-Phe-Gly

Figure 7. Structures of protonated Gly-Pro-Gly and protonated Gly-Phe-Gly and the highest transition states on the profiles to formation of the b_2 and y_2 ions as calculated at B3LYP/6-31++G(d,p).

the terminal amino group and hydrogen bonded to one of the others. For each protonated tripeptide, many tautomers have similar energies, and in some instances, the structure with the lowest energy differs depending on whether only electronic energies are considered, or whether thermal corrections and entropies are included in order to bring the structure up to 298 K. Using free energies at 298 K as the criterion, for the three tripeptides that we have examined at B3LYP/6-31++G(d,p), the lowest energy tautomers have different structures. For protonated Gly-Gly-Gly, the lowest energy structure has the proton attached to the oxygen of the N-terminal amide group and it is hydrogen bonded to the terminal amino group.[59] We have optimized more than 20 structures for protonated Gly-Pro-Gly, and the one at the global minimum, ion **1**, (Figure 7 and Scheme 1) has the "mobile" proton on the terminal amino group with a hydrogen bond to the oxygen of the C-terminal amide group. This creates a larger ring (eight-membered) than in the best structures for protonated Gly-Gly-Gly (a five-membered ring) and for protonated Gly-Phe-Gly (a seven-membered ring, shown in Figure 7). In ion **1**, there is an electrostatic attraction between the terminal carboxy group and the NH_3^+ group, resulting in a compact structure.

In the best structure for protonated Gly-Phe-Gly, the "mobile" proton lies between the two oxygens of the amide groups and, from the structural parameters, appears to be strongly attached to both (Figure 7). Formally, it is attached to the oxygen of the

N-terminal amide group, and this results in a lengthening of the C=O bond and shortening of both the C−N and C−C bonds of the N-terminal amide group. The O−H distance is long (1.065Å compared with 0.965 Å in H_2O), the OH···H hydrogen bond is very short (1.404 Å), and the C=O of the C-terminal amide group, the recipient of the hydrogen bond, is slightly longer than that in an average amide (1.225 Å in acetamide).

(b) Fragmentation of Protonated Tripeptides. Cleavage of a protonated peptide is charge proximal, and to initiate cleavage of an amide bond, the "mobile" proton must be transferred to the nitrogen atom of one of the amide groups. When the proton moves to the nitrogen of the N-terminal amide group, then cleavage of the adjacent N−C bond, after a subsequent proton transfer, yields the y_2 ion. Alternatively, if the proton is transferred to the nitrogen of the C-terminal amide group, then the C-terminal amino acid residue leaves as a glycine and the b_2 ion is formed.

(i) Formation of b_2 Ions. We have previously reported a detailed study of the reaction profile for formation of the b_2 ion from protonated Gly-Gly-Gly.[59] As there are many similarities in the profiles regardless of which amino acid residue is in the central position of protonated Gly-X-Gly, here we give only the structures of the transition states for the rate-determining step for the protonated ions derived from Gly-Pro-Gly and Gly-Phe-Gly (Figure 7). In this step, the "mobile" proton is attached to the nitrogen of the C-terminal amide, and the oxygen of the
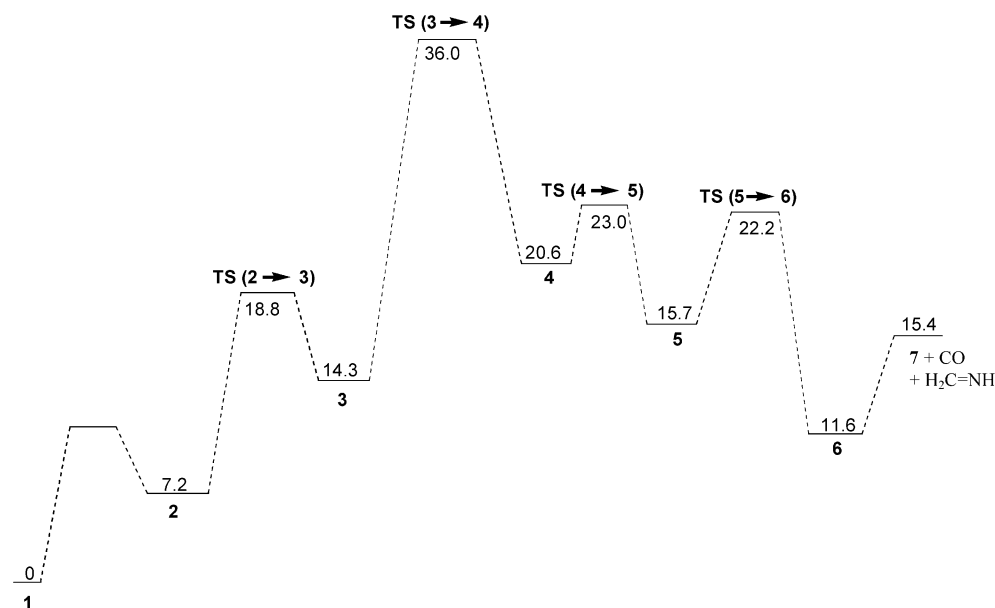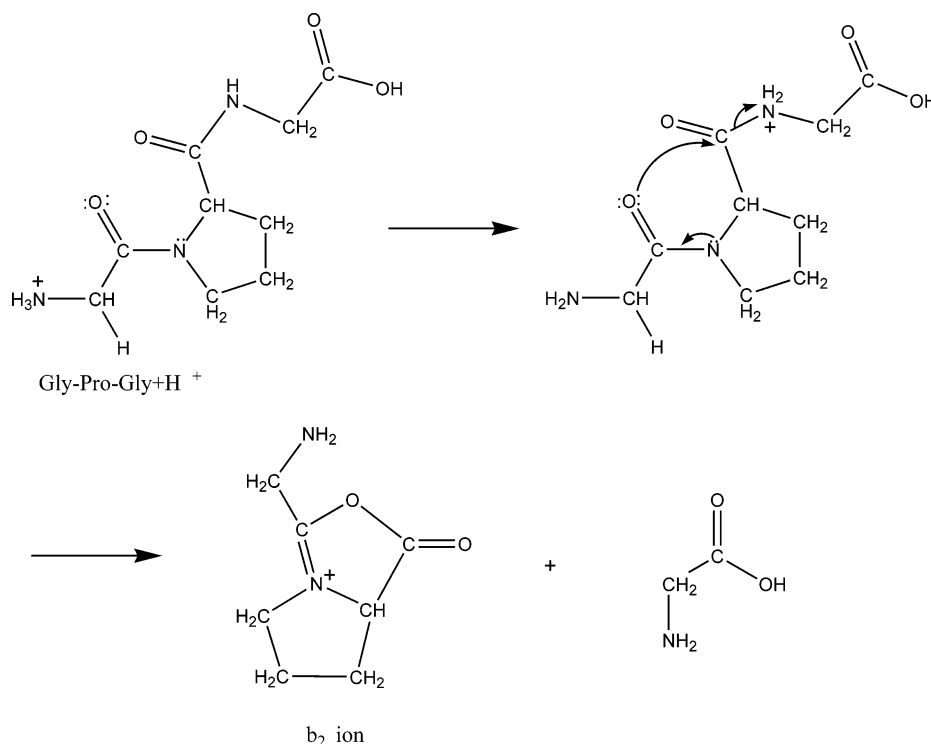
Figure 8. Reaction profile for fragmentation of protonated Gly-Pro-Gly into the y_2 ion at B3LYP/6-31++G(d,p). Free energies (in kcal mol^{-1}) at 298 K are relative to structure 1.

SCHEME 1

other amide group is attacking the carbon of the C-terminal amide to form an oxazolone, with concomitant cleavage of the C−N bond (Scheme 1).

In all of the transition states leading to the formation of b_2 ions, the C···N bond that is breaking is long (~2Å) and the O···C bond that is forming is considerably shorter (~1.7Å); that is, the transition state is more like the product than the reactant. In comparing the three transition states, that for protonated Gly-Phe-Gly has the longest O···C distance (1.733Å) and shortest C···N distance (1.940Å); that is, this ion has the earliest transition state. The calculated free energy barriers (all in kcal mol^{-1}) for formation of b_2 ions from protonated Gly-X-Gly ions are all similar, 31.3 for X = Gly, 32.7 for X = Pro, and 28.9 for X = Phe. Here it is interesting to note that

formation of the b_2 ion from protonated Gly-Phe-Gly has the *lowest* barrier, which agrees with the experimental observation (Figures 1 and 2). As we can see from Figures 1 and 2, the b_2 ion from protonated Gly-Phe-Gly has lower threshold of formation than the b_2 ion from protonated Gly-Pro-Gly.

(ii) Formation of y_2 Ions. Here we present the reaction profile in terms of free energies for formation of the y_2 ion from protonated Gly-Pro-Gly (Figure 8) as obtained at the B3LYP/6-31++G(d,p) level of theory. The overall mechanism involves transfer of the "mobile" proton from the terminal amino group, as in structures **1** and **2**, to the nitrogen of the proline and subsequent cleavage to form the y_2 ion (Scheme 2). In the structures in Scheme 2, we have arbitrarily drawn dotted lines and reported distances only when X···H is less than 2.0 Å.

SCHEME 2
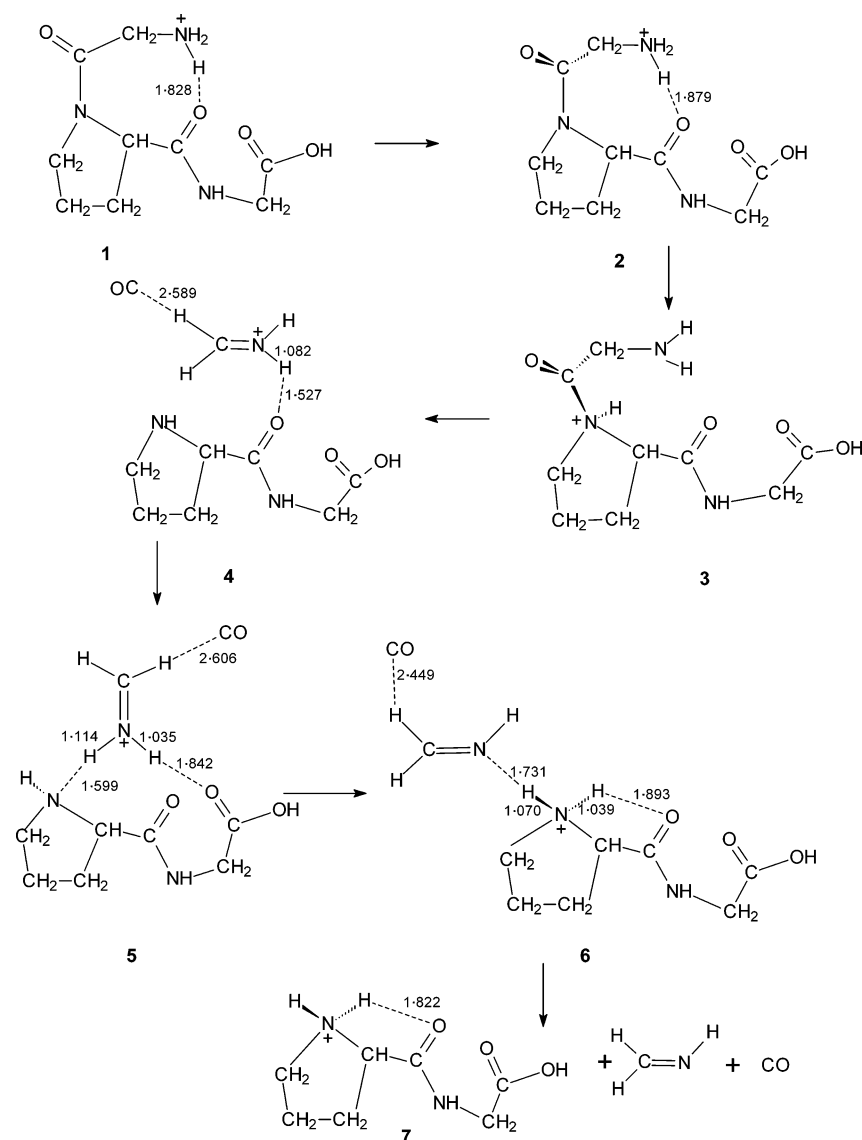
The structure at the global minimum, **1**, is protonated at the terminal amino group, and one of the hydrogens from the NH_3^+ forms a hydrogen bond with the carbonyl oxygen of the C-terminal amide group. Migration of the "mobile" proton to the nitrogen of the proline residue requires initially loosening the hydrogen bond; this is achieved by rotation about the N-terminal amide bond to give structure **2**. The same hydrogen then migrates to the nitrogen atom of the prolyl residue, forming ion **3**; the overall barrier to this process is provided by **TS2 → 3** and is about 20 kcal mol^{-1} above structure **1**. The rate-determining step then occurs via transition state **TS(3→4)**, as given in Figure 7. This is a complicated step in which the ion essentially fragments into three molecules in a concerted loss of CO and dissociation of the amide bond. The products are a methyliminium ion solvated by a dipeptide Pro-Gly and CO. At B3LYP/6-31++G(d,p), the barrier to this process is 36.0 kcal mol^{-1}. In a subsequent rearrangement, the proton migrates from the iminium ion to the nitrogen of the proline residue, and the ultimate products are protonated Pro-Gly (the y_2 ion), methanimine, and carbon monoxide.

The barrier to formation of the y_2 ion from protonated Gly-Phe-Gly is 39.1 kcal mol^{-1} at B3LYP/6-31++G(d,p), 3.1 kcal mol^{-1} higher than that for the proline-containing tripeptide. For protonated Gly-Pro-Gly, formation of the y_2 ion requires

approximately 3 kcal mol^{-1} more energy than formation of the b_2 ion, whereas for protonated Gly-Phe-Gly, formation of the y_2 ion requires approximately 9 kcal mol^{-1} more energy than formation of the b_2 ion. The observation of only b_2 and a_2 ions in the CID spectrum of protonated Gly-Phe-Gly is consistent with the calculated large energy difference between the rate-determining transition states for formation of b_2 and y_2 ions. For protonated Gly-Pro-Gly both b_2 and y_2 ions are observed experimentally, with the former being in larger amounts at low collision energies. This is consistent with the calculated *small* difference in barrier heights favoring the formation of the b_2 ion. The preference for cleavage to form the b_2 ion rather than the y_2 ion in protonated Gly-Pro-Gly may appear to be an anomaly in view of the proline effect. However, both proline and glycine have been found to show strong biases for cleavages at their N-terminal amide bonds,[65] which mitigates the proline effect, and b_2 ions typically have unusually high abundances among *b* product ions. These factors may have contributed to the propensity for b_2 ion formation.

From neutral fragment reionization (N_rR) studies, Wesdemiotis and co-workers have concluded that in the fragmentation of peptides the N-terminal amino acid residue is lost as an aziridinone when y_n ions are formed.[10,66,67] However, based on density functional calculations examining the fragmentation of

protonated glycylglycine and subsequently on protonated gly-clglycylglycine, Paizs and Suhai[60,61] have concluded that formation of the aziridinone along with the y_1 ion is a high energy pathway, but one that likely became accessible in the N_fR experiments. A lower energy pathway, involving loss of CO from protonated glycylglycine to form a proton-bound complex of $H_2C=NH$ and glycine, has a transition state that is structurally very similar to those found in this work for formation of the y_2 ion. On the protonated glycylglycine surface, the key transition state in the fragmentation pathway is 38.4 kcal mol^{-1} above the reactant at 0 K; this compares with ΔE (kcal mol^{-1}) at 0 K values of 38.5 for protonated Gly-Pro-Gly and 40.4 for protonated Gly-Phe-Gly on the energy hypersurfaces present herein. One major difference between the profiles for the tripeptides and that for protonated glycylglycine is that for the latter the final products, CO, $H_2C=NH$, and protonated glycine, are *higher* in energy than the key transition state, whereas on protonated tripeptide surfaces, the products are *lower*. Consequently, unlike for protonated glycylglycine, the barrier to formation of the y_2 ion from protonated tripeptides is determined by the highest transition state (**TS 3 → 4**).

Conclusions

Fragmentation of a peptide bond involves protonation at the nitrogen of an amide bond (the less preferred site for protonation). In the absence of major interactions with the side chain, the nitrogen of the C-terminal amide bond is probably the more basic (due to stabilization through hydrogen bonding with the other amide group or with the terminal amino group) of the two amidic nitrogens, and hence, cleavage at the C-terminal amide bond is the most common reaction. Of the 25 protonated tripeptides examined here, 17 undergo cleavage predominantly at the C-terminal amide bond. The position of a proline residue in a tripeptide has a major effect on the type of fragmentation that occurs. When a prolyl residue is at the C-terminal, then *only* products of cleavage at the C-terminal are observed. This is attributed to the high basicity of the secondary amide formed at the nitrogen of the prolyl residue. When the prolyl residue is at the N-terminal, fragmentation again occurs at the C-terminal bond but is accompanied by formation of large amounts of a_1 ion, possibly directly from the $[M + H]^+$ ion. When the prolyl residue is at the central position, then usually both b_2 and y_2 ions are formed; that is, cleavage of the two amide bonds is competitive. The lower barrier to formation of the y_2 ion here can be explained in terms of the higher basicity of the secondary amide containing the prolyl nitrogen allowing protonation at the N-terminal amide bond to become competitive. The C$-$N$^+$ bond produced by this protonation is more sterically crowded than those involving primary amides and might therefore be expected to be weaker and break more easily. Our computational study showed that, unlike proposed in the literature, there is only a small destabilization attributable to strain in the bicyclic b_2 ion. In summary, then, comparing fragmentation pathways for the protonated tripeptides Gly-Pro-Gly and Gly-Phe-Gly the prolyl residue has the effect of slightly increasing the barrier to b_2 formation and slightly decreasing that to y_2 formation relative to that in protonated Gly-Phe-Gly. The net effect is that protonated Gly-Pro-Gly produces both y_2 and b_2 ions, whereas Gly-Phe-Gly gives only the b_2 ion.

Supporting Information Available: Details of the energies from the computations and structural details. This material is available free of charge via the Internet at http://pubs.acs.org.

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