Effect of Phenylalanine on the Fragmentation of Deprotonated Peptides

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The fragmentation reactions of a variety of deprotonated dipeptides and tripeptides containing phenylalanine have been studied using energy-resolved collision-induced dissociation, isotopic labeling and MS/MS/MS experiments. The benzyl α -group has a substantial effect on the fragmentation reactions observed. When the phenylalanine is in the C-terminal position of dipeptides or tripeptides a major fragmentation reaction is elimination of neutral cinnamic acid to form a deprotonated amino acid amide (c_1 ion) for dipeptides and a deprotonated dipeptide amide (c_2 ion) for tripeptides. Fragmentation of the $[M - H]^-$ ions of tripeptides with phenylalanine in the central position also results in substantial formation of the deprotonated amide of the N-terminal amino acid residue. When the phenylalanine residue is in the N-terminal position elimination of C_7H_8 from the $[M - H - CO_2]^-$ ion and formation of the benzyl anion become important fragmentation pathways. Sequence ions frequently observed are the y_1 ions, b_2 ions and a_3-N_t ions. (J Am Soc Mass Spectrom 2002, 13, 1242–1249) © 2002 American Society for Mass Spectrometry

Tandem mass spectrometry of protonated peptides is well-established as a practical method for the determination of the amino acid identity and sequence for the peptide [1–4].

As a result of many studies, the main features of the fragmentation of protonated peptides have been elucidated, at least at the phenomenological level [5, 6]. On the other hand much less is known concerning the fragmentation modes of deprotonated peptides. The majority of studies [7–18] have involved high-energy collision-induced dissociation (CID) and have been largely restricted to the $[M - H]^-$ ions of dipeptides with only a few tripeptides having been studied.

A recent paper from this laboratory [19] has reported the low-energy CID of the $[M - H]^-$ ions of a variety of dipeptides to pentapeptides containing H or alkyl α -groups. The fragmentation reactions observed are illustrated schematically in Scheme 1 using nomenclature adapted from that used [6, 7] for fragmentation of protonated peptides. A comparison of the low-energy CID results with the high-energy CID results reported by Eckersley et al. [12] showed that more extensive and sequence-specific fragmentation was observed in the low-energy CID experiments. This observation is in line with earlier work in this laboratory [20–23] which showed that low-energy collisional activation of negative ions imparted, on average, greater energy to the anion than did high-energy collisional activation

The early studies [7–18] of small deprotonated peptides suggested that the fragmentation modes of the $[M - H]^-$ ions depended significantly on the identity of the α -groups of the constituent amino acids. The present work reports a study of the low-energy CID of deprotonated peptides containing phenylalanine in order to evaluate the role of the benzyl α -group on the fragmentation of the deprotonated peptides. The fragmentation reactions of a number of deprotonated dipeptides and two tripeptides containing phenylalanine have been examined by high-energy CID by Waugh et al. [15]. A comparison will be made of the high-energy and low-energy CID results.

Experimental

Collision-induced dissociation (CID) studies were performed using an electrospray/quadrupole mass spectrometer (VG Platform, Micromass, Manchester, UK) with CID in the interface region between the atmospheric pressure source and the quadrupole mass analyzer. It is well known [24, 25] that CID can be achieved in this interface region, so-called cone voltage CID, and it has been clearly established [26–28] that the average energy imparted to the decomposing ions increases as the field in the interface region increases. Recent work [29–32] has shown that, by varying this field in steps, energy-resolved mass spectra [33–35] comparable to those obtained in variable, low-energy CID in quadrupole cells are obtained. The results of these cone-voltage CID studies are presented in the following either as CID mass spectra at a set cone voltage or as breakdown graphs expressing the percent of total ion abundance as

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Scheme 1

a function of the cone voltage, a measure of the field in the interface region. MS/MS/MS experiments were carried out using an electrospray/triple quadrupole mass spectrometer (Sciex API III, Concord, Canada). CID in the interface region produced the fragment ion of interest which was mass-selected by the first quadrupole analyzer and underwent collisional activation in the quadrupole collision cell with the fragmentation products being analyzed by the final quadrupole mass analyzer.

Ionization was by electrospray with the peptide sample, at micromolar concentration in 1:1 CH₃CN/1% aqueous NH₃, being introduced into the source at a flow rate of 30 μ L min⁻¹. The electrospray needle was held at -2.5–3.0 kV. Nitrogen, produced by a Whatman Model 75-72 N₂ generator (Whatman Inc., Haverhill, MA), was used as both nebulizing and drying gas. By using 1:1 CD₃CN/1% ND₃ in D₂O as the electrospray solvent the labile hydrogens of the peptide were exchanged for deuterium and the [M - D]⁻ ion formed in the ionization process. Under these conditions no evidence was seen for back-exchange of the labile hydrogens in the interface region, although significant back-exchange was observed when dry air was used as nebulizing and drying gas.

All peptide samples used were obtained from BACHEM Biosciences (King of Prussia, PA). CD₃CN (99.8 atom % D) and D₂O (99.9 atom % D) were obtained from Cambridge Isotope Laboratories (Andover, MA) while the ND₃OD (26% in D₂O, >99 atom % D) was obtained from CDN Isotopes (Pointe Claire, Quebec).

Results and Discussion

The low-energy collision-induced dissociation reactions of the [M - H]⁻ ions of a number of dipeptides, tripeptides, and one tetrapeptide containing phenylalanine were examined. The fragmentation modes observed depended significantly on the position of the phenylalanine in the peptide; as a consequence the results will be presented in these terms.

C-Terminal Phenylalanine

Figure 1 presents the breakdown graph for deprotonated glycylphenylalanine (GF). Two sequence ions, y₁ and c₁, are observed with the c₁ ion dominating the breakdown graph at higher collision energies. In addition, the benzyl anion (*m/z* 91) is observed, indicative of

Figure 1. Breakdown graph for deprotonated Gly-Phe. [M - H]⁻ (*m/z* 221) not shown on graph.

the presence of phenylalanine. Ion signals also are observed at *m/z* 147 and *m/z* 103. The former can be rationalized in terms of elimination of the N-terminal glycine as a neutral amide to form deprotonated cinnamic acid (*m/z* 147) which subsequently loses CO₂ to form deprotonated styrene (*m/z* 103). When the labile hydrogens were exchanged for deuterium the [M - D]⁻ ion was formed in the ionization process and all labile hydrogens were eliminated in forming *m/z* 147 and *m/z* 103. The y₁ ion showed incorporation of one labile hydrogen while the c₁ ion showed mainly incorporation of two labile hydrogens rather than the three that might have been expected. The mechanism of formation of the c₁ ion will be discussed below in light of the results obtained for tripeptides with a C-terminal phenylalanine. It should be noted that deprotonated dipeptides containing H or alkyl α -groups do not form c₁ ions [19]. The fragmentation of deprotonated leucylphenylalanine (LF) was very similar to that of deprotonated GF, including labile hydrogen retention data.

Waugh et al. [15] have recorded the high-energy CID mass spectrum of deprotonated GF produced by fast atom bombardment (FAB). They observed mainly formation of the y₁ and *m/z* 147 products along with the a₂ ion ([M - H - CO₂]⁻) and only minor formation of the c₁ and *m/z* 91 fragments. Their spectrum resembles that obtained at very low cone voltages (collision energy); clearly high-energy collisional activation must impart only a small amount of excitation energy to the fragmenting ions compared to low-energy collisional activation at typical cone voltages or collision energies. Similar conclusions were reached from the comparison

Figure 2. Comparison of the CID mass spectra of $[M - H]^+$ of Gly-Gly-Phe and $[M - D]^+$ of Gly-Gly-Phe- d_5 , 45 V cone voltage.

of the high- and low-energy collisional activation of deprotonated peptides containing H or alkyl α -groups [19]. By using a deuterated matrix in FAB, Waugh et al. also recorded the CID mass spectrum of the $[M - D]^+$ ion of the dipeptide in which all the labile hydrogens had been exchanged for deuterium. In agreement with the present results, they reported that the y_1 ion contained only one labile hydrogen and that the elimination of glycineamide involved loss of all labile hydrogens. However, in contrast to the present results, they reported that the (low abundance) c_1 ion contained three labile hydrogens. Similar results were reported for deprotonated alanylphenylalanine (AF).

Figure 2 compares the CID mass spectrum of the $[M - H]^+$ ion of GGF with that of the $[M - D]^+$ ion of the tripeptide in which all the labile hydrogens have been exchanged for deuterium. The c_2 ion is the dominant fragment ion with a_2 , b_2 and y_1 ions being observed in lesser yield; in addition, although the a_3 ion is barely observed, there is a significant ion signal for $a_3 - G$, i.e., loss of the N-terminal glycine residue from the a_3 ion. Waugh et al. [15] have reported the high-energy CID mass spectrum of deprotonated GGF produced by FAB. They observed significant yields of the c_2 and y_1 ions but, in addition, observed higher yields of the a_3 and y_2 ions which are not observed with significant yield in the low energy CID mass spectrum. The labeling results show that the c_2 ion incorporates mainly three labile hydrogens with only minor incorporation of four labile hydrogens. Simple bond cleavage in the $[M - H]^+$ ion to form c ions would lead to the incorporation of three labile hydrogens in the c_1 ions from dipeptides and four labile hydrogens in the c_2 ions derived from tripeptides. It is obvious that hydrogen migrations are occurring. A clue to the pathway to the c ions comes from MS³ experiments on the c_2 ion

Figure 3. Breakdown graph for deprotonated Gly-Ala-Phe. $[M - H]^+$ (m/z 292) not shown on graph.

derived from deprotonated GAF. At 3 eV centre-of-mass collision energy the c_2 ion (m/z 144) showed loss of H_2O (m/z 126, 9%), formation of deprotonated alaninamide (m/z 87, 55%), and formation of deprotonated glycineamide (m/z 73, 36%). The breakdown graph for deprotonated GAF (Figure 3), while complex, does show that the latter two ions are primarily, if not totally, products arising from fragmentation of the c_2 ion; the major primary ions are the c_2 ion and the y_1 ion with a more minor yield of $a_3 - G$ (m/z 191). Along the same line one notes the m/z 112 product ($c_2 - H_2O$) in the spectrum of Figure 2. Figure 4 presents the CID mass spectra of deprotonated GLF and deprotonated LGF. In both cases the c_2 ion is a major fragment. Note, in agreement with the results for GAF, the m/z 129 product (deprotonated leucinamide) in the spectrum of GLF and the m/z 73 product (deprotonated glycineamide) in the spectrum of LGF. These two products represent the deprotonated amide of the central amino acid residue. The loss of H_2O from the c_2 ions suggests the presence of a hydroxyl function in the c_2 ions derived from tripeptides with a C-terminal phenylalanine and

Figure 4. CID mass spectra for deprotonated Gly-Leu-Phe and deprotonated Leu-Gly-Phe. 60 V cone voltage. Ion signals from m/z 70 to m/z 300 multiplied by factor of two in top spectrum.

leads to the reaction mechanism proposed in Scheme 2 for deprotonated GF; a similar mechanism can be written for formation of the c_2 ions from the tripeptides. The mechanism is consistent with the deuterium labeling results of the present study. It is not clear whether the amide-N deprotonated species is formed directly in the ionization process or originates by proton migration in a carboxyl-deprotonated species. It should be noted that earlier studies [19] have shown that the methyl ester of trialanine was efficiently ionized by electrospray and that the ionization involved removal of an amidic hydrogen.

Bowie and co-workers [15] have proposed the mechanism outlined in Scheme 3 to rationalize formation of c_1 and the m/z 147 ion in dipeptides containing a C-terminal phenylalanine with the benzylic deprotonated species arising by proton transfer in the carboxyl-deprotonated species formed initially. While this reaction sequence is consistent with the present results for

Scheme 2

Scheme 3

formation of m/z 147, it is not consistent with the present labeling results for formation of the c_1 ion. Alternatively, it has been proposed [36] that elimination of the N-terminus residue (or residues) as an amide may occur by charge-remote fragmentation from the carboxyl-deprotonated species. The present study provides no data to choose between these two mechanisms.

For tripeptides containing H or alkyl α -groups the amino acid sequence can be derived from the observation of c_1 and y_1 ions with confirmatory evidence derived from the $a_3 - N_t$ fragment. For tripeptides containing C-terminal phenylalanine the dominant formation of c_2 ions as primary fragment ions and the further fragmentation of the c_2 ions to form both possible deprotonated amide ions makes the identification of the c_1 ion more problematic. This is illustrated by the CID spectra of Figure 4 where both deprotonated glycylamide (m/z 73) and deprotonated leucylamide are observed in both spectra. Similarly, the CID mass spectrum of deprotonated GVF (Figure 5) shows ion signals for both m/z 73 (deprotonated glycylamide) and m/z 115 (deprotonated valinamide). In these cases one has to rely on the formation of the $a_3 - N_t$ ion to identify the N-terminus amino acid residue. Thus, deprotonated LGF shows a signal for $a_3 - L$ while deprotonated GLF shows a signal for $a_3 - G$ and deprotonated GVF shows a signal for $a_3 - G$. The bottom panel of Figure 5 presents the CID mass spectrum of protonated GVF; it is clear that the order of G and V in the peptide cannot be established from CID of the protonated species while it can be from the negative ion CID mass spectrum. On the other hand, the CID spectra of protonated GLF and LGF do show substantial differences [37].

One tetrapeptide (VAAF) with a C-terminal phenylalanine was studied. Figure 6 shows the CID spectra obtained for the $[M - H]^-$ ion at three cone voltages illustrating the effect of collision energy on the fragmentation reactions observed. By analogy with the dipeptides and tripeptides discussed above, one might expect to see the c_3 ion (m/z 257) as a major product. Although

Figure 5. CID mass spectra of deprotonated (top) and protonated (bottom) Gly-Val-Phe. 60 V cone voltage (top), 30 V cone voltage (bottom).

a very minor ion signal is observed at m/z 257, there are much more prominent ion signals resulting from fragmentation of the c_3 ion, including $c_3 - H_2O$ and $c_3 - V$; in addition, the m/z 87 ion (deprotonated alaninamide) and the c_1 and c_2 ions probably arise largely from fragmentation of the c_3 ion. Some sequence information can be obtained from the y_1 and (low intensity) y_2 ions as well as from the $a_4 - V$ and $a_4 - V - A$ ion signals. However, in this case CID of the protonated peptide (Figure 7) gives a simpler spectrum with much sequence information in the y_1'' , y_2'' , and y_3'' ions as well as the b_2 and b_3 ions.

Central Phenylalanine

Figure 8 compares the CID mass spectrum of the $[M - H]^-$ ion of GFA with that of the $[M - D]^-$ ion of the

Figure 6. CID mass spectra of deprotonated Val-Ala-Ala-Phe at three cone voltages.

Figure 7. CID mass spectrum of protonated Val-Ala-Ala-Phe. 60 V cone voltage.

tripeptide in which the labile hydrogens have been exchanged for deuterium. Major fragment ions are the sequence-specific c_1 and y_1 ions along with a less abundant b_2 ion. The isotopic labeling shows that the y_1 ion incorporates to a significant extent two labile hydrogens as has been observed previously [19] in formation of y_1 ions from tripeptides. More interestingly, to a large extent the c_1 ion incorporates three labile hydrogens; this is in contrast to the formation of c_1 ions from dipeptides with a C-terminal phenylalanine (Figure 2) or formation of c_2 ions for tripeptides with a C-terminal phenylalanine where the number of labile hydrogens incorporated was one less than the number of labile hydrogens expected on the basis of simple bond cleavage. Clearly, in addition to the mechanism proposed in Scheme 2 there is another pathway to the c_1 ion in this case. Logically this pathway involves fragmentation of the benzylic-deprotonated species as outlined in Scheme 4. The labeling results show that the initial ionization process does not involve removal of a benzylic proton but it is not clear whether the formation of this species involves proton transfer in a carboxyl-deprotonated species or an amide-deprotonated species. In Scheme 4 we have proposed that the proton transfer occurs in an amide-deprotonated species

Figure 8. Comparison of the CID mass spectra of $[M - H]^-$ of Gly-Phe-Ala and $[M - D]^-$ of Gly-Phe-Ala- d_5 . 45 V cone voltage.

Scheme 4

largely because the analogous reaction involving proton transfer in a carboxyl-deprotonated species does not occur for dipeptides with a C-terminal phenylalanine.

Figure 9 presents the CID mass spectra of deprotonated GFG and deprotonated GFF. While the latter tripeptide has both a central and a C-terminal phenylalanine, the fragmentation reactions observed are more

Figure 9. CID mass spectra of deprotonated Gly-Phe-Gly (45 V cone voltage) and deprotonated Gly-Phe-Phe (60 V cone voltage).

Figure 10. CID mass spectra of deprotonated Phe-Gly (45 V cone voltage) and deprotonated Phe-Gly-Gly (60 V cone voltage).

characteristic of a central phenylalanine in that no c_2 ion (m/z 220) is observed. In agreement with the spectrum of Figure 8, both spectra show c_1 , y_1 , and b_2 ions indicative of the amino acid sequence. Significant ion signals are observed at m/z 160 for GFG, at m/z 174 for GFA, and at m/z 250 for GFF. In all three cases these ion signals correspond to loss of CO_2 along with the N-terminal residue as a neutral amide from the $[\text{M} - \text{H}]^-$ ion and probably involve remote loss of the neutral amide from the a_3 ion. Further fragmentation to deprotonated styrene (m/z 103) also appears to occur.

N-Terminal Phenylalanine

The top panel of Figure 10 presents the CID mass spectrum of deprotonated FG. The three major fragment ions observed are the y_1 ion (m/z 74), the benzyl anion (m/z 91), and a product of m/z 85. Exchange of the labile hydrogens for deuterium showed that the y_1 ion incorporated one deuterium, the benzyl anion one deuterium to some extent and the m/z 85 product mainly two deuteriums but with significant incorporation of only one deuterium. Bowie and co-workers [15] have observed these products in the high-energy CID of deprotonated FG but also observed a much more intense ion signal for $a_2 - \text{NH}_3$ as well as substantial ion signals for the a_2 ion and $[\text{M} - \text{H} - \text{H}_2\text{O}]^-$. The low-energy CID mass spectrum of deprotonated FL (not shown) was very similar to that of deprotonated FG, including formation of a product at m/z 141 analogous to the m/z 85 ion observed for FG. The m/z 85 ion signal corresponds to elimination of CO_2 plus toluene (C_7H_8) from the $[\text{M} - \text{H}]^-$ ion. The pathway to m/z 85 proposed by Bowie and co-workers [15] is presented in Scheme 5. An alternative pathway (Scheme 6) proceeds

from the a_2 ion ($[M - H - CO_2]^-$) and produces a more stable product ion. Styles and O'Hair [38] have observed proton migration from nitrogen to carbon (the first step in Scheme 6) in the fragmentation of the a_2 ion derived from glycylglycine. Some support for a mechanism involving a benzyl anion/neutral complex is the observation that the ratio (m/z 91)/(m/z 85) increased from 0.4 at low collision energy to ca. 1 at high collision energy. In effect, at high collision energy the complex dissociates before the internal proton transfer can occur. Both mechanisms would lead to incorporation of two labile hydrogens in the m/z 85 product and neither mechanism explains completely the isotopic labeling results.

The bottom panel of Figure 10 presents the CID mass spectrum of the $[M - H]^-$ ion of FGG. The major sequence ions observed are the y_1 , b_2 and $a_3 - F$ ions; interestingly, no c_1 ion corresponding to deprotonated phenylalaninamide is observed. Although the a_3 ion (m/z 234) is of very low abundance, $a_3 - NH_3$, $a_3 - C_7H_8$, and $a_3 - F$ ions are observed in substantial yield. Waugh et al. [15] have reported the high-energy CID mass spectrum of deprotonated FGG. The major fragment ions observed corresponded to the a_3 and b_2 ions with smaller yields of $a_3 - NH_3$, $a_3 - C_7H_8$, and y_1 ions.

Again it appears that much less energy is imparted to the $[M - H]^-$ ions in high-energy collisional activation. The $a_3 - C_7H_8$ ion is analogous to the m/z 85 ion observed in the fragmentation of deprotonated FG and a similar mechanism of formation probably is involved; the benzyl anion (m/z 91) also is observed. MS³ experiments on the m/z 142 ($a_3 - C_7H_8$) ion at 3 eV centre-of-mass collision energy showed that it fragmented to give m/z 87, m/z 85, and a product of m/z 58. Similar experiments on the b_2 (m/z 203) ion showed that it eliminated toluene to give m/z 111 and also formed the benzyl anion (m/z 91). Mechanistic speculation on these fragmentation reactions does not appear to be warranted.

Conclusions

The introduction of the benzyl α -group has a substantial effect on the fragmentation of deprotonated peptides when compared to peptides with only H or alkyl α -groups. The most pronounced effect is the formation of c ions by elimination of cinnamic acid or a cinnamic acid derivative when the phenylalanine is in the C-terminal position of di- or tri-peptides or in the central position of tripeptides. Deuterium labeling has shown that the detailed mechanism of this elimination reaction differs for C-terminal phenylalanine and central phenylalanine. When the phenylalanine is in the N-terminal position, significant elimination of C_7H_8 from the $[M - H - CO_2]^-$ ion as well as formation of the benzyl anion become important reactions.

The comparison of the CID mass spectrum of the $[M - H]^-$ ion of VAAF with that of the MH^+ ion indicates that, in this case at least, CID of the protonated species provides a simpler mass spectrum with equal sequence information. Protonated peptides often show facile cleavage at amide bonds forming y and b ions which provide sequence information. The present study and an earlier study [19] indicate that a single dominant cleavage mode may not occur for deprotonated peptides with the result that more complex CID mass spectra are observed although in most cases substantial sequence information is obtained. In this respect low-energy CID appears to be preferable to high-energy CID of negative ions.

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