ELSEVIER

International Journal of Mass Spectrometry 210/211 (2001) 361–370

International Journal of Mass Spectrometry

www.elsevier.com/locate/ijms

Ion chemistry of protonated glutamic acid derivatives

Alex. G. Harrison

Department of Chemistry, University of Toronto, 80 St. George Street, Toronto, Ontario M5S 3H6, Canada

Received 2 January 2001; accepted 24 January 2001

Abstract

The fragmentation reactions of protonated glutamic acid derivatives of general formula H–Glu(X)–Y, specifically H–Gln–OH, H–Glu–NH_2, H–Glu–OMe, and H–Glu(OMe)–OH, have been studied as a function of internal energy and by tandem mass spectrometry (MS/MS/MS) experiments. The results show that loss of HX from the γ-carboxyl group leads not only to a protonated pyroglutamic acid derivative but also to a cyclic cationated anhydride or imide ion. Loss of HY from the α-carboxyl position is accompanied by loss of CO in agreement with the known instability of α-aminoacylium ions. The secondary fragmentation product $C_4H_6NO^+$ (*m/z* 84) is shown to arise by loss of HY + CO from the $[MH - HX]^+$ ions and by loss of HX from the $[MH - HY - CO]^+$ ions. Deuterium labeling experiments show that, although the primary loss of HX and HY+CO involve only labile hydrogens, both labile hydrogens and C-bonded hydrogens are lost in the further fragmentation of the $[MH - HX]^+$ and $[MH - HY - CO]^+$ primary ions. It is concluded that there are several pathways to the *m/z* 84 product ion and several structures for this fragment ion. (Int J Mass Spectrom 210/211 (2001) 361–370) © 2001 Elsevier Science B.V.

Keywords: Fragmentation mechanisms; Isotopic labelling; Glutamic acid derivatives

1. Introduction

Collision-induced dissociation (CID) of protonated peptides is a much-used approach in the determination of the constituent amino acid identity and sequence [1–3]. Both low energy and high-energy CID studies have been used in this respect [4]. With the increasing use of electrospray and matrix-assisted laser desorption ionization sources coupled to multiple quadrupole instruments, quadrupole ion traps, and Fourier-transform mass spectrometers, there is a need for a better understanding of the low-energy fragmentation processes of protonated peptides. Since the fragmentation reactions of peptides are determined in part by the identity of the constituent amino acids, there is considerable interest in establishing, in detail, the fragmentation reactions of protonated amino acids. The major low-energy fragmentation pathways of simple protonated amino acids have been proposed from observation of the fragment ions observed in Brønsted acid chemical ionization studies [5,6]. More recently, Kulik and Heerma [7] have surveyed the low-energy metastable ion fragmentation reactions and high-energy CID fragmentation reactions of protonated amino acids produced by fast-atom bombardment (FAB), whereas Dookeran et al. [8] have reported on the metastable ion fragmentation reactions and low-energy CID fragmentation reactions of the

E-mail: aharriso@chem.utoronto.ca

Dedicated to Nico Nibbering in appreciation of his science and his friendship over many years

PII S1387-3806(01)00405-5

MH^+ ions produced by FAB. There have been more detailed studies, both experimental and theoretical, of the fragmentation of the MH^+ of glycine [9–12], leucine and isoleucine [13], methionine [14–16], cysteine [17], threonine [18], lysine [19], aspartic acid [20], and arginine [21].

The present work reports a study of the fragmentation reactions of simple protonated glutamic acid derivatives, H–Glu(X)–Y using energy-resolved mass spectrometry [22–24] and isotopic labeling to provide more detailed information concerning the fragmentation reactions of protonated glutamic acid. A further communication will report on the fragmentation reactions of peptides containing glutamic acid and glutamine

2. Experimental

Collision-induced dissociation studies were performed using an electrospray ionization/quadrupole mass spectrometer (VG Platform, Micromass, Manchester, UK). It is clearly established [25] that CID can be achieved in the interface region between the atmospheric pressure ion source and the quadrupole mass analyzer, so-called cone-voltage CID. Further, it is known [26–28] that the average energy imparted to the decomposing ions increases as the field in this interface region is increased. Recent work in this laboratory [29–31] has shown that, by varying this field in steps, energy-resolved mass spectra can be obtained which are comparable to those obtained by variable, low-energy CID in quadrupole cells. The results of such energy-variation data are presented in the following as breakdown graphs expressing the percent of total ion abundance as a function of the cone voltage, a measure of the field in the interface region.

Ionization was by electrospray with the sample, at micromolar concentration in 1:1 CH_3CN/1% aqueous HCOOH, being introduced into the source at a flow rate of 30 $\mu L\ m^{-1}$. The electrospray capillary was held at 2.5–3.0 kV. N_2 was used as a nebulizing gas and as drying gas. The use of 1:1 CD_3CN/1% DCOOD in D_2O resulted in exchange of all labile hydrogens by deuterium and formation of the MD^+ ion in the ionization process.

MS/MS/MS experiments were carried out using an electrospray/triple quadrupole instrument (Sciex API III, MDS Sciex, Toronto). Fragment ions formed by CID in the interface region were mass selected by the first quadrupole stage and underwent collisional activation in the radio frequency-only quadrupole collision cell with analysis of the fragmentation products by the final mass-analyzing quadrupole.

All unlabeled amino acids and derivatives were obtained from BACHEM Biosciences (King of Prussia, PA) and were used as received. CD_3CN (99.8 at. % D) and D_2O (99.9 at. % D) were obtained from Cambridge Isotope Laboratories (Andover, MA) whereas DCOOD (95% in D_2O, 99.1 at. % D) and glutamic-2,4,4-d_3 acid were obtained from CDN Isotopes (Pointe Claire, Quebec).

3. Results and discussion

The two major primary fragmentation reactions of protonated glutamic acid involve loss of H_2O and combined loss of H_2O + CO [7,8]. It has been proposed [8] that H_2O loss occurs from the γ-carboxyl group with cyclization to form protonated pyroglutamic acid, analogous to the known dehydration of glutamic acid to pyroglutamic acid in solution [32]. The combined loss of H_2O + CO was believed [8] to involve the α-carboxyl group in line with the known instability of α-aminoacylium ions [6–13,33]. To confirm these proposals the fragmentation reactions of protonated H–Gln–OH, H–Glu-NH_2, H–Glu–OMe and H–Glu(OMe)–OH have been examined in detail in the present work.

Figs. 1–4 present the breakdown graphs for these four protonated species. Protonated H–Gln–OH shows loss of NH_3 and protonated H-Glu-NH_2 shows loss of H_2O as the dominant low-energy fragmentation reactions (reaction 1 in Scheme 1) with loss of HY + CO (reaction 2 in Scheme 1) increasing in importance as the collision energy is increased, although this fragmentation channel (producing *m/z* 101) is relatively minor for protonated glutamine.

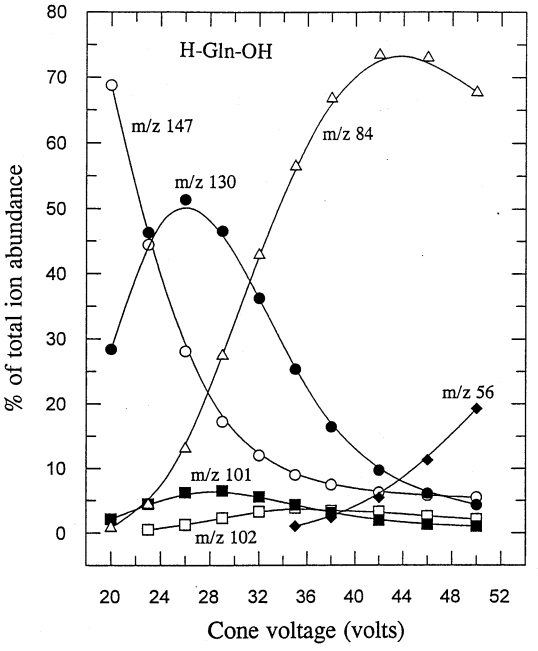


Fig. 1. Breakdown graph for protonated H–Gln–OH.

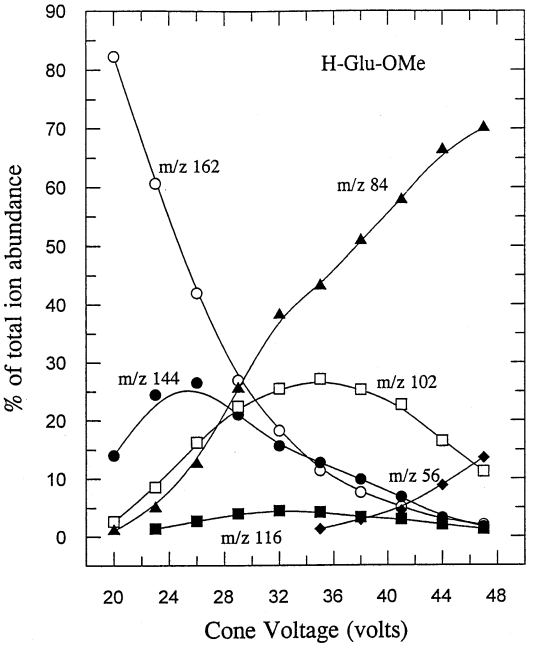


Fig. 3. Breakdown graph for protonated H–Glu–Ome.

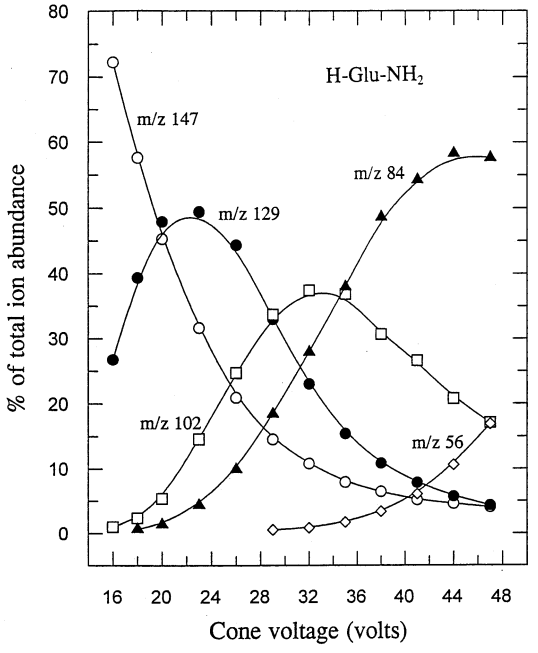


Fig. 2. Breakdown graph for protonated H–Glu–NH_2.

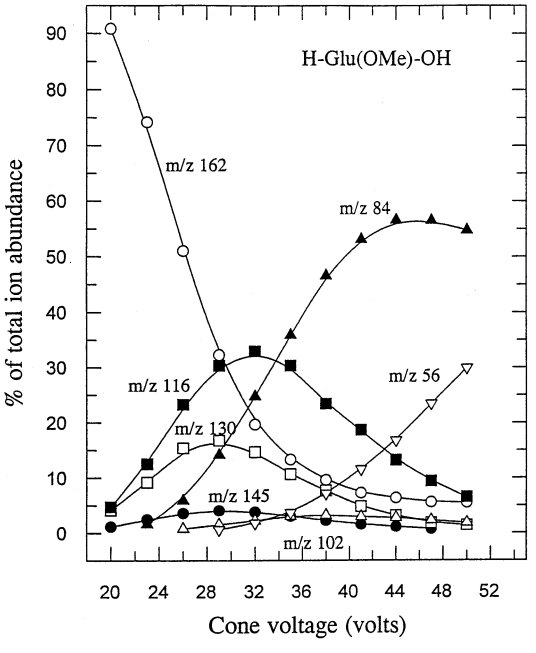
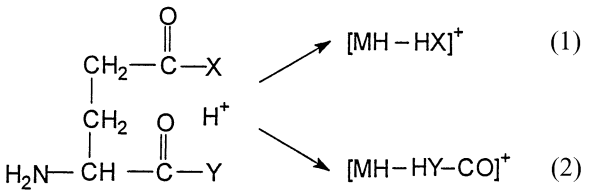


Fig. 4. Breakdown graph for protonated H–Glu(OMe)–OH.

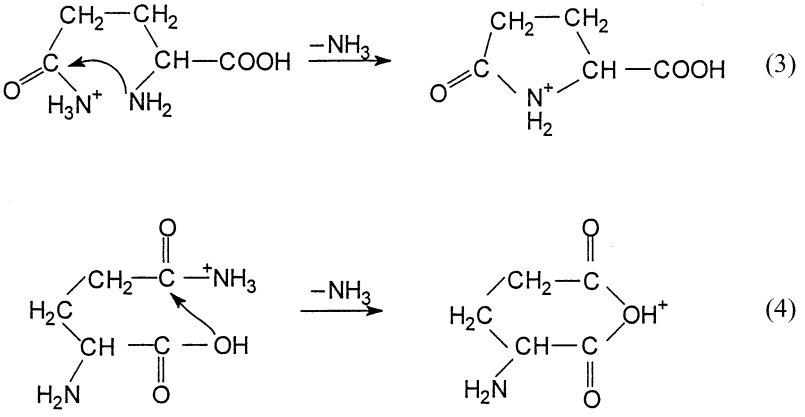


Scheme 1.

These results confirm the proposals made earlier [8] for protonated glutamic acid. The energy evolution of the two reaction channels is less clear for the two protonated methyl esters, although the results clearly support the contention that cleavage at the γ-carboxyl group results in HX loss only whereas loss of HY from the α-carboxyl group is accompanied by loss of CO. Clearly, the loss of HX from the γ-carboxyl group is favoured energetically but disfavoured entropically with the result that loss of HY + CO, which appears to be entropically favoured, increases in importance with increasing energy deposition in the fragmenting ions. The structure(s) of the $[MH - HX]^+$ ions and the pathways to and structure(s) of the ultimate fragmentation product at *m/z* 84 were the subject of further investigation.

Simple loss of HX from the γ-carboxyl group would not be expected to be energetically favoured or entropically disfavoured if the reaction was a simple bond cleavage to produce an acylium ion. It appears likely that interaction with other functional groups accompanies the fragmentation reaction. In our initial study [8] we assumed that cyclization accompanied HX loss to give the appropriate protonated pyroglutamic acid derivative as illustrated by reaction (3) in Scheme 2 using protonated glutamine as an example. An alternative route, illustrated by reaction (4) in Scheme 2, involves cyclization to a protonated or cationated cyclic anhydride (or imide) structure. Some support for this pathway is derived from the observation of low intensity ion signals at *m/z* 102 for H–Gln–OH, at *m/z* 102 for H–Glu(OMe)OH and at *m/z* 116 for H–Glu–OMe. In each case these ion signals correspond to loss of HX followed by loss of CO. It is unlikely that CO will be lost from the γ position since this would lead to formation of an unstable primary carbonium ion. A much more likely interpretation is that loss of HX is followed by migration of Y, as illustrated in Scheme 3, with loss of CO occurring from the α position to form a stable immonium ion. Such a migration also was found to occur for protonated aspartic acid derivatives [20].

It was further assumed in the earlier work [8] that the protonated pyroglutamic acid formed in reaction (3) in Scheme 2 fragmented by loss of H_2O + CO to give the fragment ion at *m/z* 84. In support of this assumption it was observed that low-energy CID of protonated pyroglutamic acid resulted in formation of *m/z* 84 as the only fragment. In the present study we observed that the MD^+ ion of pyroglutamic acid-d_2 resulted in specific loss of D_2O + CO, i.e. the *m/z* 84 ion shifted quantitatively to *m/z* 85 indicating incor-



Scheme 2.

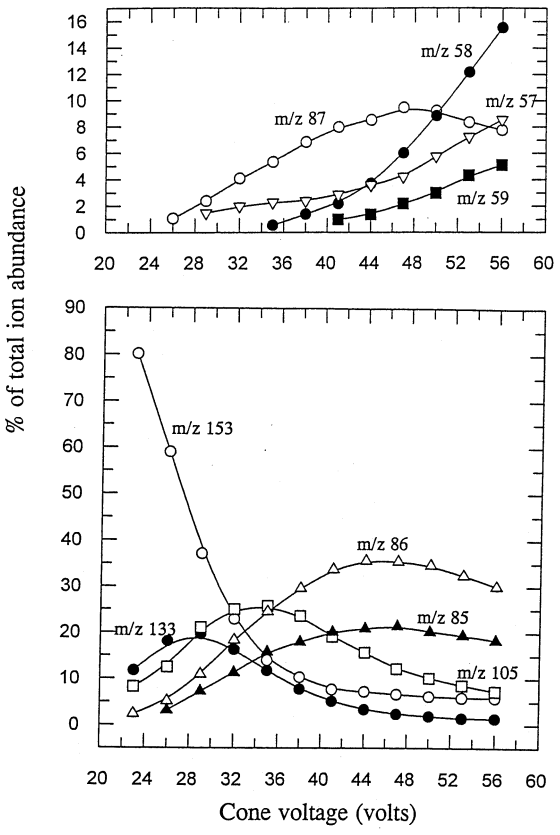


Fig. 5. Breakdown graph for MD^+ ion of glutamic acid-d_4.

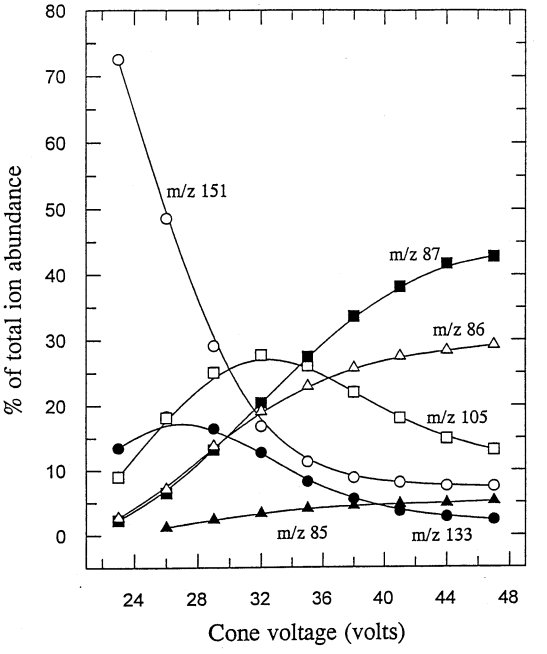
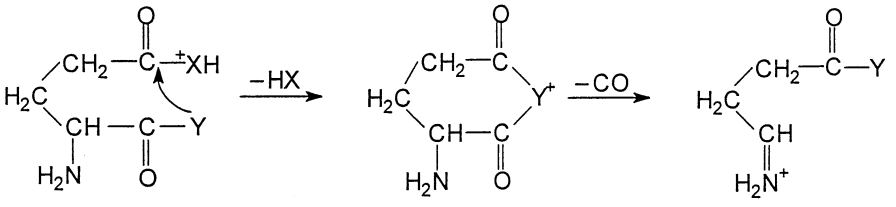


Fig. 6. Breakdown graph for MH^+ ion of glutamic-2,4,4,-d_3 acid.

poration of one labile hydrogen in the fragment ion. However, the situation is much more complex for the systems presently under study. Fig. 5 records the breakdown graph for the MD^+ ion of glutamic acid-d_4 (i.e. where all the labile hydrogens have been exchanged for deuterium) whereas Fig. 6 shows the breakdown graph for the MH^+ ion of glutamic-2,4,4-d_3 acid. The formation of the *m/z* 84 fragment ion clearly involves overall loss of two molecules of water and one carbon monoxide in some sequence. If the water loss involved only labile hydrogens (as is the case for pyroglutamic acid), the ion signal should be observed entirely at *m/z* 85 for the MD^+ ion of glutamic acid-d_4 and entirely at *m/z* 87 for the MH^+ ion of glutamic-2,4,4-d_3 acid. This obviously is not the case indicating that the water molecules lost incorporate to some extent hydrogens originally bonded to carbon. The primary fragmentation reactions, illustrated by reactions (1) and (2) of Scheme 1 do involve primarily the labile hydrogens. Figures 7–10 compare the CID spectra of the MH^+ ions of unlabeled H–Gln–OH, H–Glu–NH_2, H–Glu–Ome, and H–Glu(OMe)–OH with the CID spectra of the



Scheme 3.

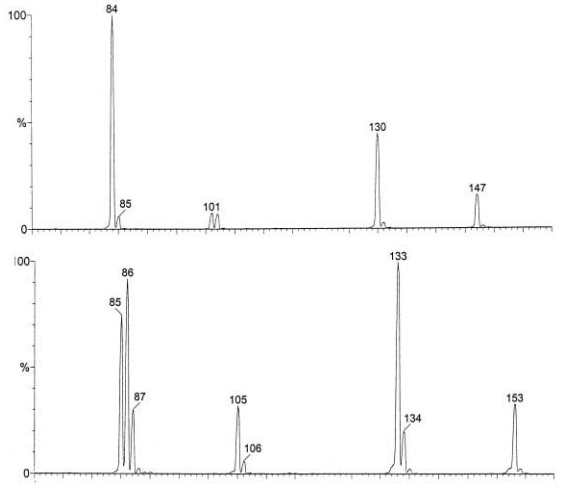


Fig. 7. Comparison of CID spectra of MH^+ ion of glutamine (top) and MD^+ ion of glutamine-d_5 (bottom). Cone voltage 35 V.

MD^+ ions of the species in which all the labile hydrogens have been exchanged for deuterium. Thus, the initial loss of H_2O from H–Glu–NH_2 (Fig. 8) becomes loss of D_2O (*m/z* 129 → *m/z* 133) whereas loss of NH_3 + CO (*m/z* 102) becomes loss of ND_3 + CO (*m/z* 105). Similar specificity is shown, on the whole, for the other systems, although there appears to be minor loss of ND_2H from the MD^+ ion of

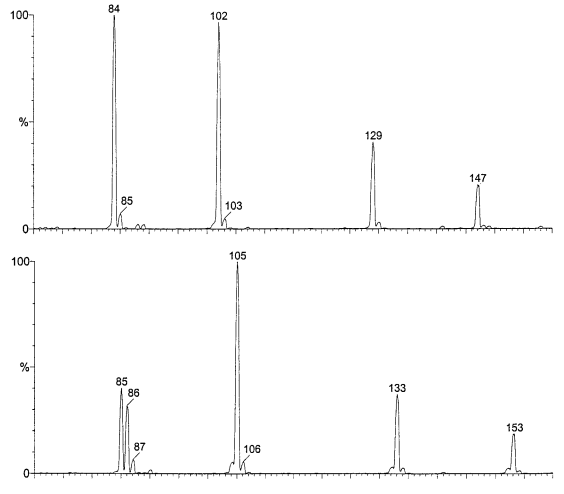


Fig. 8. Comparison of CID spectra of MH^+ ion of glutamic acid amide (top) and MD^+ ion of glutamic acid amide-d_5 (bottom). Cone voltage 35 V.

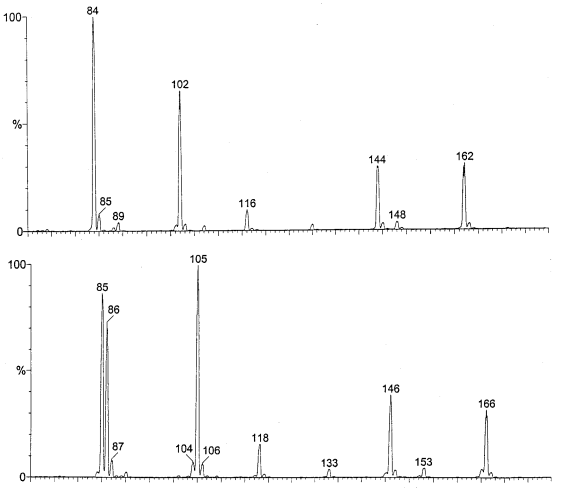


Fig. 9. Comparison of CID spectra of MH^+ ion of α-methylglutamate (top) and MD^+ ion of α-methylglutamate-d_3 (bottom). Cone voltage 35 V. Part of the *m/z* 146 signal and that at *m/z* 153 represent small glutamic acid impurity.

glutamine-d_5 and minor loss of HDO + CO from the MD^+ ion of γ-methylglutamate-d_3.

However, in all cases the ion signal at *m/z* 84 for the unlabeled species is observed at more than one *m/z* for the labeled species indicating involvement of both labile hydrogens and carbon-bonded hydrogens in the neutral losses forming the *m/z* 84 product. Clearly this

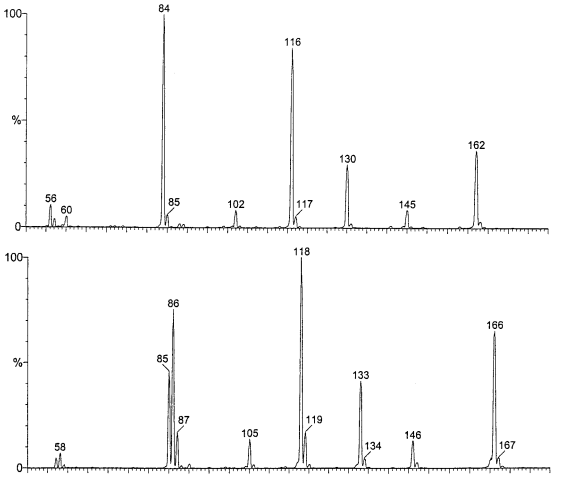


Fig. 10. Comparison of CID spectra of MH^+ ion of γ-methylglutamate (top) and MD^+ ion of γ-methylglutamate-d_3 (bottom). Cone voltage 35 V.

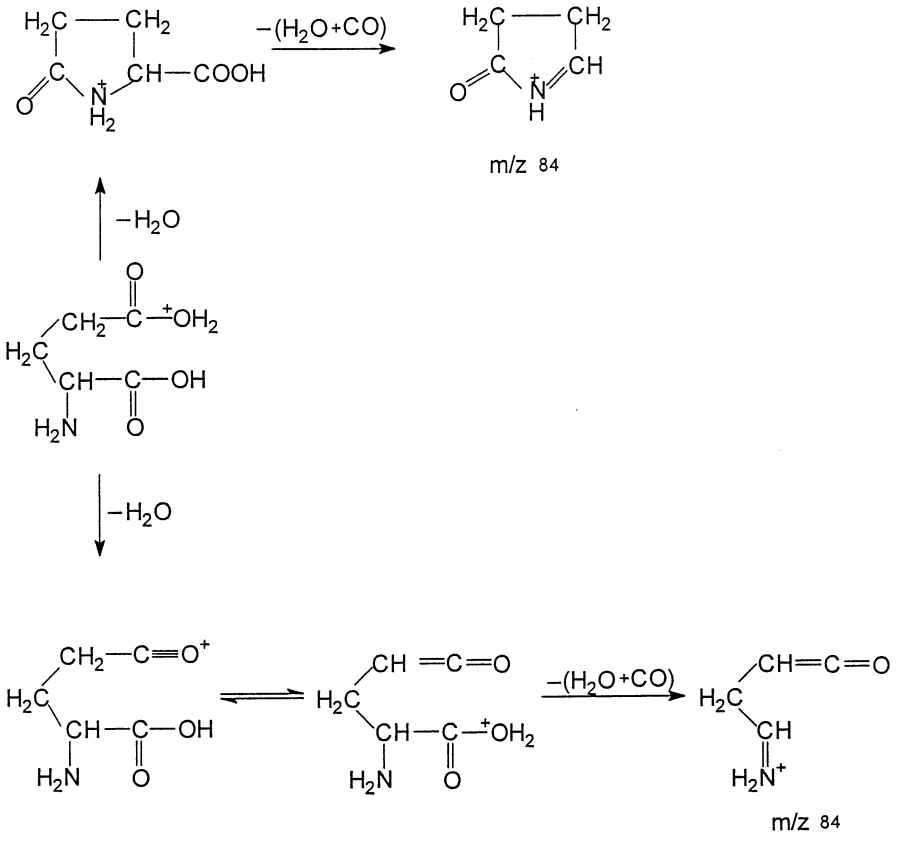
Table 1
MS^3 CID spectra of selected ions

Source	Ion studied	Intensity [neutral(s) lost]		
		m/z 85	*m/z* 86	*m/z* 87
Glutamine-d_5	$[MD - ND_3]^+$, *m/z* 133	75.8 $-(D_2O + CO)$	21.5 $-(DHO + CO)$	2.6 $-(H_2O + CO)$
Glutamic acid amide-d_5	$[MD - D_2O]^+$, *m/z* 133	73.9 $-(ND_3 + CO)$	22.0 $-(ND_2H + CO)$	3.5 $-(NDH_2 + CO)$
α-Methylglutamate-d_4	$[MD - D_2O]^+$, *m/z* 146	79.9 $-(CH_3OD + CO)$	20.1 $-(CH_3OH + CO)$	
Glutamic acid amide-d_5	$[MD - ND_3 - CO]^+$, *m/z* 105	29.1 $-D_2O$	57.4 $-DHO$	13.5 $-H_2O$
γ-Methylglutamate-d_4	$[MD - D_2O - CO]^+$, *m/z* 116	44.2 $-CH_3OD$	55.8 $-CH_3OH$	
Glutamic-2,4,4,-d_3 acid	$[MH - H_2O]^+$, *m/z* 133	6.2 $-(D_2O + CO)$	38.0 $-(DHO + CO)$	55.8 $-(H_2O + CO)$
Glutamic-2,4,4-d_3 acid	$[MH - H_2O - CO]^+$, *m/z* 105		11.6 $(-DHO)$	88.4 $(-H_2O)$

mixing does not occur in the primary fragmentation processes but must occur in the final fragmentation process(es) leading to *m/z* 84. To obtain confirmation of this proposal a number of MS^3 experiments were carried out with the results presented in Table 1. In all cases the relevant fragment ion, produced by CID in the interface region, was mass selected by the first quadrupole analyzer and underwent CID in the quadrupole collision cell with analysis of the fragments by the final quadrupole mass analyzer. The first observation of note is that not only does the $[MH - HX]^+$ ion fragment further by loss of HY + CO to give *m/z* 84 but also the $[MH - HY - CO]^+$ ions fragment further by loss of HX to give the *m/z* 84 fragment ion. Thus, there are a minimum of two routes to the *m/z* 84 fragment ion. In each of these routes there is involvement of both hydrogens bonded to oxygen or nitrogen and those bonded to carbon. Thus, the $[MD - ND_3]^+$ ion from glutamine-d_5 shows loss of D_2O + CO (75.8%), loss of HDO + CO (21.5%) and even loss of H_2O + CO (2.6%). Similar results are obtained for the loss of ammonia plus CO from the $[MD - D_2O]^+$ ion derived from glutamic acid amide-d_5 where there is significant loss of ND_2H + CO (*m/z* 86) and some loss of NDH_2 + CO (*m/z* 87). Fragmentation of the $[MD - ND_3 - CO]^+$ ion derived from glutamic acid amide-d_5 shows considerable H/D mixing with loss of D_2O (*m/z* 85), DHO (*m/z* 86), and H_2O (*m/z* 87) all being observed.

Further confirmatory evidence is provided by MS^3 experiments in the glutamic-2,4,4-d_3 system. Thus, the $[MH - H_2O]^+$ ion shows loss of H_2O + CO (55.8%), loss of HDO + CO (38.0%) and even loss of D_2O + CO (6.2%); clearly the hydrogens in the 2 or 4 position are involved significantly in the fragmentation process. In contrast to the $[MD - ND_3 - CO]^+$ ion derived from the labeled glutamic acid amide, which shows major loss of DHO and H_2O, the $[MH - H_2O - CO]^+$ ion derived from glutamic-2,4,4-d_3 acid shows mainly loss of H_2O with only minor loss of HDO. These results indicate that, in the HDO and H_2O losses from the $[MD - ND_3 - CO]^+$ ion of the labeled amide, the hydrogens do not originate to a significant extent from the 2 or 4position of the carbon backbone but must derive to a significant extent from the 3 position.

The isotopic labeling results are not consistent with scrambling of all H/D prior to fragmentation but rather point, with reference to Scheme 1, to more than one mechanism for loss of HY + CO from the $[MH - HX]^+$ ion and more than one mechanism for loss of HX from the $[MH - HY - CO]^+$ fragment ion. Two plausible pathways for further fragmentation of the $[MH - HX]^+$ ion, which are at least qualitatively

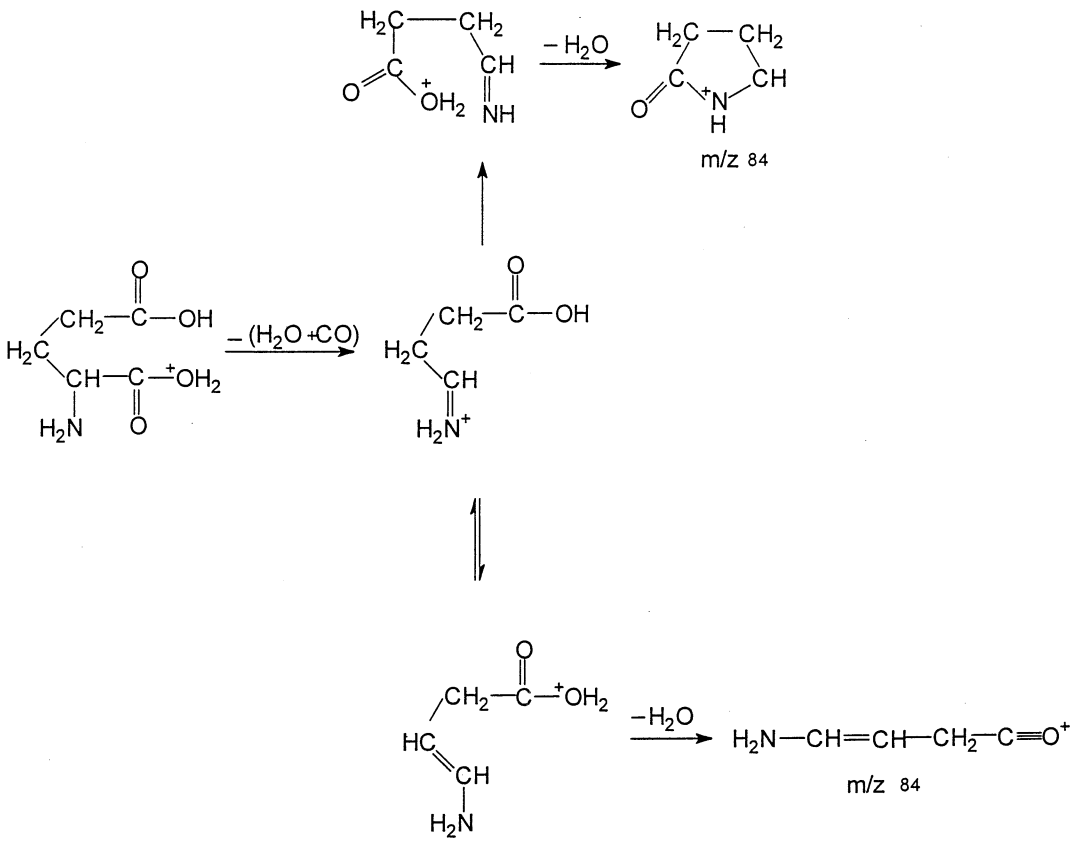


Scheme 4.

consistent with the isotopic labeling results, are illustrated in Scheme 4 using protonated glutamic acid as the example. There undoubtedly is some cyclization to protonated pyroglutamic acid on loss of HX and, by analogy with pyroglutamic acid itself further loss of H_2O + CO should involve only labile hydrogens. Loss of HX also can lead to an acylium ion (formed at low internal energies as a protonated anhydride structure). Within this ion reversible transfer of a proton from the 4 position to the carboxylic hydroxyl group can trigger elimination of H_2O + CO incorporating one or more hydrogens from the 4 position and formation of an acyclic *m/z* 84 ion. Two plausible pathways also can be postulated for further fragmentation of the $[MH - HY - CO]^+$ ion as illustrated in Scheme 5 again using protonated glutamic acid as the example. We cannot rule out the possibility that loss of H_2O may be accompanied by cyclization to form the immonium ion derived from pyroglutamic acid; such a process would most likely involve only loss of labile hydrogens in the water lost. In addition, the labeling results indicate participation of hydrogen(s) from the 3 position in the water-loss process and a pathway leading to an acylium ion structure is shown in Scheme 5. The results thus point to at least four pathways to formation of the *m/z* 84 fragment ion and, most likely, three distinct structures for the ions of *m/z* 84. These ions all fragment further by loss of CO and it is possible that they could be distinguished on the basis of kinetic energy releases in the loss of CO but we are unable to carry out such experiments.

4. Conclusions

The present results show that loss of HX (X = OH, NH_2, OCH_3) only from protonated glutamic



Scheme 5.

acid derivatives occurs from the γ-carboxyl function while loss of HY + CO (Y = OH, NH_2, OCH_3) together occurs from the α-carboxyl function. Exchange of the labile hydrogens for deuterium shows that these primary fragmentation reactions involve almost exclusively loss of labile hydrogens. The $[MH - HX]^+$ and $[MH - HY - CO]^+$ primary fragment ions undergo further fragmentation by loss of HY + CO for the former and loss of HX for the latter, in all cases forming *m/z* 84 ($C_4H_6NO^+$). The isotopic labeling results indicate that carbon-bonded hydrogens (particularly from C3 and C4 of the backbone) are involved to a significant extent in these secondary fragmentation processes. Plausible mechanisms leading to three distinct structures for $C_4H_6NO^+$ have been presented. The results show that loss of HX from the γ-carboxyl position leads not only to cyclization to a protonated pyroglutamic derivative but also to cyclization to a cationated anhydride or imide structure. The present work also shows that earlier proposals [8] that the *m/z* 84 ion observed in the CID spectra of protonated glutamic acid and protonated glutamine had the structure of the pyroglutamic acid immonium ion are only partly correct; other structures clearly are involved.

Acknowledgements

The continuing financial support of the Natural Sciences and Engineering Research Council (Canada) is gratefully acknowledged. The author is particularly indebted to Professor Michael Siu (Centre for Research in Mass Spectrometry, York University) for

providing access to the API III instrument and to Houssain El Arabi for his assistance in carrying out the MS^3 experiments.

References

[1] K. Biemann, Methods Enzymol. 193 (1990) 455.
[2] Mass Spectrometry of Peptides, D.M. Desiderio (Ed.), CRC Press, Boca Raton, FL 1991.
[3] K. Biemann, in Biological Mass Spectrometry, T. Matsuo, R.M. Caprioli, M.L. Gross, Y. Seyama (Eds.), Wiley, New York, 1993, p. 276.
[4] A.L. Burlingame, R.K. Boyd, S.J. Gaskell, Anal. Chem. 66 (1993) 634R.
[5] G.W.A. Milne, T. Axenrod, H.M. Fales, J. Am. Chem. Soc. 92 (1970) 5170.
[6] C.W. Tsang, A.G. Harrison, J. Am. Chem. Soc. 98 (1976) 1301.
[7] W. Kulik, W. Heerma, Biomed. Env. Mass Spectrom. 15 (1988) 419.
[8] N.N. Dookeran, T. Yalcin, A.G. Harrison, J. Mass Spectrom. 31 (1996) 500.
[9] S. Beranova, J. Cai, C. Wesdemiotis, J. Am. Chem. Soc. 117 (1995) 9492.
[10] R.A.J. O'Hair, P.S. Broughton, M.L. Styles, B.T. Frink, C.M. Hadad, J. Am. Soc. Mass Spectrom. 11 (2000) 687.
[11] F. Rogalewicz, Y. Hoppilliard, Int. J. Mass Spectrom. 199 (2000) 235.
[12] B. Balta, M. Basma, V. Aviyente, C. Zhu, C. Lifshitz, Int. J. Mass Spectrom. 201 (2000) 69.
[13] G. Bouchoux, S. Bourcier, Y. Hoppilliard, C. Mauriac, Org. Mass Spectrom. 28 (1993) 1064.
[14] J. van der Greef, M.C. ten Noever de Brauw, J.J. Zwinselman, N.M.M. Nibbering, Org. Mass Spectrom. 17 (1982) 274.
[15] Y.-P. Tu, A.G. Harrison, Rapid Commun. Mass Spectrom. 12 (1998) 849.
[16] R.A.J. O'Hair, G.E. Reid, Eur. Mass Spectrom. 5 (1998) 325.
[17] R.A.J. O'Hair, M.L. Styles, G.E. Reid, J. Am. Soc. Mass Spectrom. 9 (1998) 1275.
[18] R.A.J. O'Hair, G.E. Reid, Rapid Commun. Mass Spectrom. 12 (1998) 999.
[19] T. Yalcin, A.G. Harrison, J. Mass Spectrom. 31 (1996) 1237.
[20] A.G. Harrison, Y.-P. Tu, J. Mass Spectrom. 33 (1998) 532.
[21] J.J. Zwinselman, N.M.M. Nibbering, J. van der Greef, M.C. ten Noever de Brauw, Org. Mass Spectrom. 18 (1983) 525.
[22] S.A. McLuckey, G.L. Glish, R.G. Cooks, Int. J. Mass Spectrom. Ion Phys. 39 (1981) 219.
[23] D.D. Fetterolf, R.A. Yost, Int. J. Mass Spectrom. Ion Phys. 44 (1982) 37.
[24] S.A. McLuckey, R.G. Cooks, in Tandem Mass Spectrometry, F.W. McLafferty (Ed.), Wiley, New York, 1983, p. 303.
[25] A.P. Bruins, in Electrospray Mass Spectrometry: Fundamentals, Instrumentation and Applications, R.B. Coles (Ed.), Wiley, New York, 1997, Chap. 3.
[26] A. Donò, C. Paradisi, G. Scorrano, Rapid Commun. Mass Spectrom. 11 (1997) 1687.
[27] C. Collette, E. DePauw, Rapid Commun. Mass Spectrom. 12 (1998) 165.
[28] C. Collette, L. Drahos, E. DePauw, K. Vékey, Rapid Commun. Mass Spectrom. 12 (1998) 1673.
[29] A.G. Harrison, Rapid Commun. Mass Spectrom. 13 (1999) 1663.
[30] A.G. Harrison, J. Mass Spectrom. 34 (2000) 1253.
[31] A.G. Harrison, I.G. Csizmadia, T.-H. Tang, J. Am. Soc. Mass Spectrom. 11 (2000) 427.
[32] M. Orlowski, A. Meister, in The Enzymes, Vol. 4, P.D. Boyer (Ed.), Academic, New York, 1971, p. 129, and references cited therein.
[33] T. Yalcin, C. Khouw, I.G. Csizmadia, M.R. Peterson, A.G. Harrison, J. Am. Soc. Mass Spectrom. 6 (1995) 1165.