Dehydration Versus Deamination of N-Terminal Glutamine in Collision-Induced Dissociation of Protonated Peptides

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Some of the most prominent "neutral losses" in peptide ion fragmentation are the loss of ammonia and water from N-terminal glutamine. These processes are studied by electrospray ionization mass spectrometry in singly- and doubly-protonated peptide ions undergoing collision-induced dissociation in a triple quadrupole and in an ion trap instrument. For this study, four sets of peptides were synthesized: (1) QLLLPLLLK and similar peptides with K replaced by R, H, or L, and Q replaced by a number of amino acids, (2) QL_nK (n = 0, 1, 3, 5, 7, 9, 11), (3) QL_nR (n = 0, 1, 3, 5, 7, 9), and (4) QL_n (n = 1, 2, 3, 4, 8). The results for QLLLPLLLK and QLLLPLLLR show that the singly protonated ions undergo loss of ammonia and to a smaller extent loss of water, whereas the doubly protonated ions undergo predominant loss of water. The fast fragmentation next to P (forming the y_5 ion) occurs to a larger extent than the neutral losses from the singly protonated ions but much less than the water loss from the doubly protonated ions. The results from these and other peptides show that, in general, when N-terminal glutamine peptides have no "mobile protons", that is, the number of charges on the peptide is no greater than the number of basic amino acids (K, R, H), deamination is the predominant neutral loss fragmentation, but when mobile protons are present the predominant process is the loss of water. Both of these processes are faster than backbone fragmentation at the proline. These results are rationalized on the basis of resonance stabilization of the two types of five-membered ring products that would be formed in the neutral loss processes; the singly protonated ion yields the more stable neutral pyrrolidinone ring whereas the doubly protonated ion yields the protonated aminopyrroline ring (see Schemes). The generality of these trends is confirmed by analyzing an MS/MS spectra library of peptides derived from tryptic digests of yeast. In the absence of mobile protons, glutamine deamination is the most rapid neutral loss process. For peptides with mobile protons, dehydration from glutamine is far more rapid than from any other amino acid. Most strikingly, end terminal glutamine is by far the most labile source of neutral loss in excess-proton peptides, but not highly exceptional when mobile protons are not available. In addition, rates of deamination are faster in lysine versus arginine C-terminus peptides and 20 times faster in positively charged than negatively charged peptides, demonstrating that these formal neutral loss reactions are not "neutral reactions" but depend on charge state and stability. (J Am Soc Mass Spectrom 2007, 18, 27–36) © 2007 American Society for Mass Spectrometry

The masses of fragment ions from the dissociation of peptide ions are commonly used to "sequence" the peptides. However, relative abundances of these ions and ions derived from them are not generally used for this process, despite the fact that they can carry additional sequence-specific information. The development of a sound basis for interpreting abundances can therefore be expected to aid peptide identification algorithms and has motivated the work of several laboratories [1–7]. It is also the motivation of the present work, which examines a highly specific fragmentation process, the "neutral loss" of ammonia and water from a glutamine residue at the amino terminus of a peptide. Neutral losses, commonplace in peptide fragmentation, can reveal or obscure structural features and have been less well studied than backbone cleavage.

Glutamine is one of the more labile naturally-occurring amino acids. Peptides with glutamine as the N-terminal amino acid are known to undergo partial deamination in solution, which converts the terminal glutamine residue into the cyclic pyroglutamyl residue (2-pyrrolidinone-5-carboxylic acid) [8]. Protonated peptide ions undergoing collision-induced dissociation also

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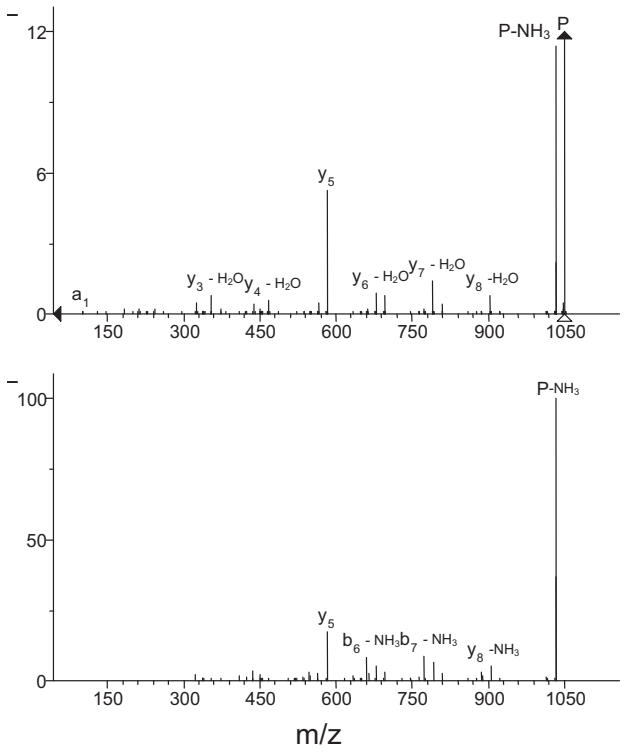


Figure 1. Comparison of the MS/MS spectrum of the singly protonated QLLLPLLLK ion measured in the ion-trap mass spectrometer (bottom) with a spectrum measured in the triple quadrupole instrument at an intermediate collision energy (top). While the precursor peak is completely depleted in the ion-trap spectrum, a large fraction of it still remains in the triple quadrupole spectrum, as is evident from the different intensity scales (y axis).

exhibit such a process [9]. Recently, several dipeptide and tripeptide ions have been found to undergo both deamination and dehydration at the N-terminal glutamine [10]. Harrison monitored the loss of ammonia and water as a function of in-source voltage [10]. We have repeated Harrison's experiment with glutamylglycine and have reproduced his results (in his Figure 1) very closely.

In the present study, we obtain the CID spectra at different energies, not by changing the in-source voltage as did Harrison [10], but rather by changing the collision energy in a triple-quadrupole mass spectrometer. This method permits us to isolate a single ion and follow its fragmentation without interference by other ions; this is particularly important when studying longer peptides, which produce numerous product ions. In this study, we vary the size of the peptides from 2 to 15 amino acids by using leucine residues as representative medium size amino acid with no reactive functional groups. We also examine cases where the ring expected to be formed by deamination is smaller or larger than that in the case of glutamine. The aim of the present study is to determine the relative extent of neutral losses of ammonia and water from protonated peptides as a function of peptide structure, peptide ion charge, and collision energy. For this purpose, we synthesized a series of peptides containing an N-terminal glutamine and several other peptides for comparison (see Tables). Each peptide was studied by electrospray ionization mass spectrometry as the singly protonated ion and, where possible, also as the doubly protonated ion.

Experimental

The peptides were synthesized in a Protein Technologies Inc. (Tuscon, Arizona) PS3 peptide synthesizer by using standard procedures and then dissolved in methanol/water (vol:vol = 1:1) containing 1% formic acid. For a few hydrophobic peptides with solubility too low to provide good quality spectra, methanol was replaced with acetonitrile, 2-propanol, or 1-propanol and/or the fraction of water in the solvent mixture was decreased to 20%.

Electrospray ionization mass spectrometry was carried out with a Micromass Quattro Micro triple quadrupole instrument (Waters Corp., Milford, MA). First the mass spectrum was measured at different cone voltages to determine the voltage at which the peptide ion peak is maximized. Then, selecting the precursor ion at that cone voltage into a collision cell (with 1.6 mTorr (0.21 Pa) Ar as collision gas), the MS/MS spectrum was measured at 20 different collision energies. The range of collision energies spanned from near zero up to a value where little precursor ion remained. The peak intensities of all the significant fragment ion peaks were calculated as a fraction from the total ion intensity and plotted as a function of collision energy.

Spectra were acquired in "centroid" mode, whereby signals within each individual time interval in a given spectrum were centered and integrated by the instrument data system. Typically, relative *m/z* values were within 0.2 of the theoretical *m/z* values throughout the *m/z* range of interest. Thus, it is easy to distinguish between loss of water ($MH^+ - 18$) and loss of ammonia ($MH^+ - 17$) in the singly-charged peptides. In the doubly-charged peptides, the peaks at $MH_2^{2+} - 9.0$ and $MH_2^{2+} - 8.5$ were clearly separated in most cases. To ensure correct identification, some spectra of doubly-charged peptides were also acquired in "profile" mode to confirm the identity of water and ammonia loss peaks.

To examine the influence of type of collisional excitation, MS/MS spectra of the same peptides were also measured with an ion trap instrument (LTQ ThermoElectron Corp., Waltham, MA). Samples were prepared in a manner similar to that described above. The sample was injected with a syringe pump at a flow rate of 10 μL/min into a flow splitter. An additional 190 μL/min of 70% acetonitrile/0.1% formic acid was directed into the splitter and the diluted sample flow was directed to the mass spectrometer. Tandem mass spectra were collected at collision energy setting of 35%. For selected peptides, MS^n (n = 3, 4) spectra were also measured.

Results and Discussion

Four sets of peptides were synthesized: (1) QLLLPLLLK and similar peptides with K replaced by R, H, or L, and Q replaced by one of several amino acids, (2) QL_nK, with $n = 0, 1, 3, 5, 7, 9, 11$, (3) QL_nR, with $n = 0, 1, 3, 5, 7, 9$, and (4) QL_n, with $n = 1, 2, 3, 4, 8$. The aim of the first set is to compare the rate of neutral losses with the rate of fragmentation at the proline and to compare glutamine with other amino acids. The other sets are aimed at studying the effect of peptide length and comparing tryptic (K or R at the C-terminus) with non-tryptic type peptides.

Comparison of the MS/MS Spectra Obtained with the Two Mass Spectrometers

The MS/MS spectra of the singly- and doubly-protonated ions of QLLLPLLLK are presented in Figures 1 and 2. As discussed in the Experimental section, one spectrum is obtained with the ion-trap instrument while in the triple-quadrupole instrument 20 spectra were recorded at varying collision energies. Of the 20 spectra, we chose the one that most closely resembles the spectrum obtained in the ion-trap instrument. As seen in Figures 1 and 2, the spectra obtained in the two mass spectrometers are fairly similar, they show the same predominant peaks with somewhat similar intensities (within a factor of two). The other spectra obtained with the triple-quadrupole instrument show gradual changes in peak intensities as discussed below. These, and other results described in this paper, show that findings developed here can be applied with equal confidence to either ion trap or low-energy collision cell instruments.

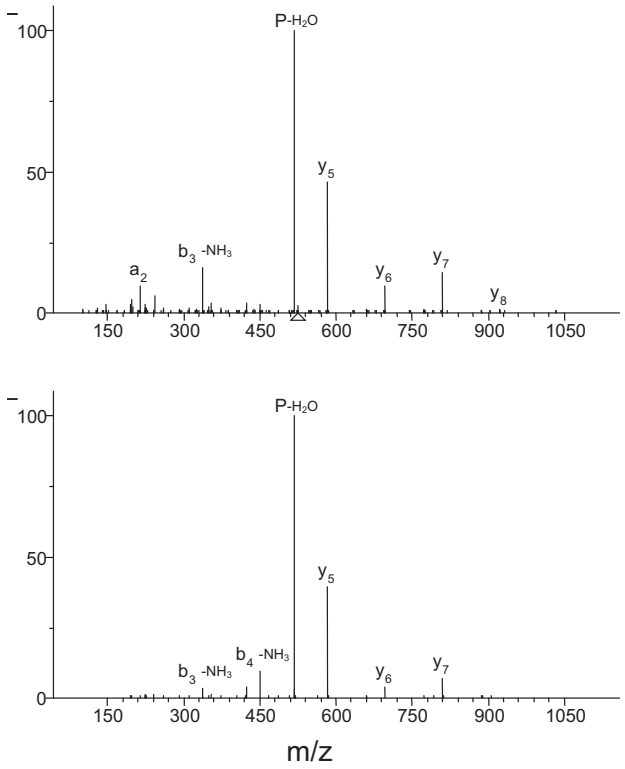


Figure 2. Comparison of the MS/MS spectrum of the doubly protonated QLLLPLLLK ion measured in the ion-trap mass spectrometer (bottom) with a spectrum measured in the triple quadrupole instrument at an intermediate collision energy (top).

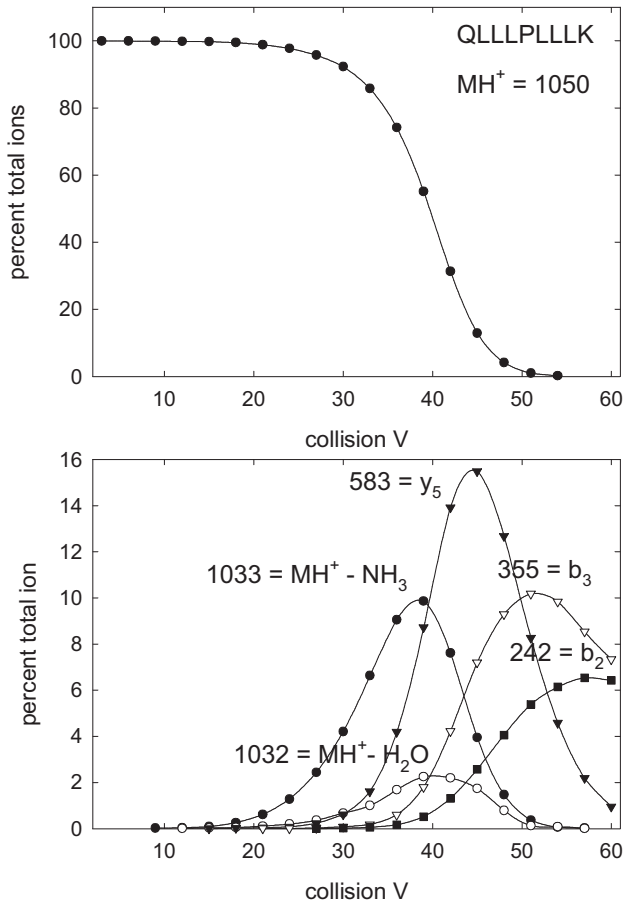


Figure 3. Decay of the precursor peak and formation of fragment peaks as a function of collision energy in the triple quadrupole MS/MS spectrum of singly protonated QLLLPLLLK.

Comparison of Neutral Losses from N-Terminal Glutamine to Fragmentation at Proline

In one set of experiments, we synthesized eleven peptides composed of nine amino acid residues with proline in the central position and leucine residues as space fillers. The aim of this set of measurements was to compare the extent of neutral losses of water and ammonia with the extent of fragmentation at the amino end of proline, which is well known as a rapid peptide bond cleavage. Results for QLLLPLLLK are presented in Figures 3 and 4. The MS/MS spectrum of the singly charged ion shows that as the collision energy increases and the precursor ion decomposes, the fastest fragmentation process is the neutral loss of NH_3. The product of this process ($MH^+ - NH_3$) reaches a maximum at a collision energy of 39 V, and at higher energies its level

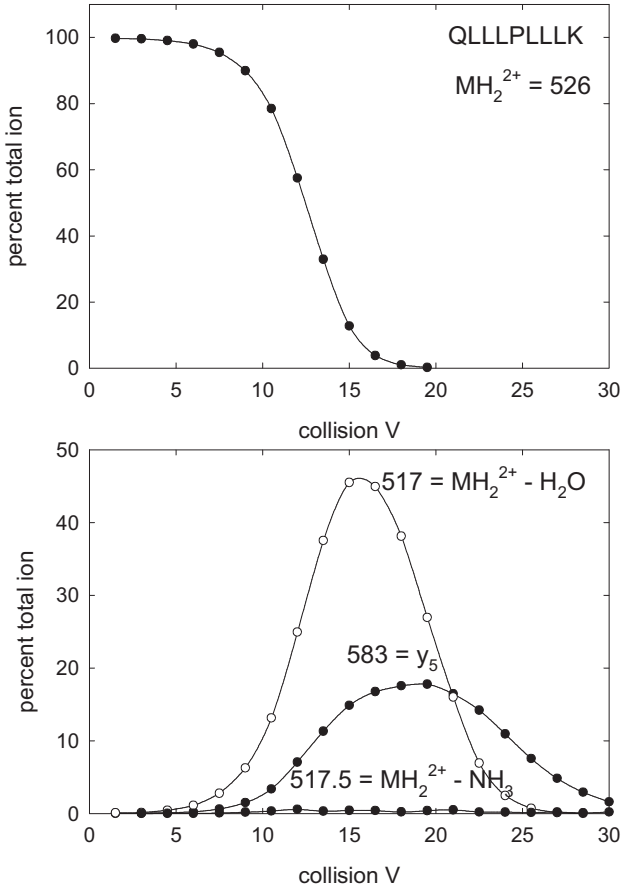


Figure 4. Decay of the precursor peak and formation of fragment peaks as a function of collision energy in the triple quadrupole MS/MS spectrum of doubly protonated QLLLPLLLK.

decreases due, presumably, to subsequent dissociation. The second most pronounced decomposition process is in the peptide bond L—P to produce the relatively stable y_5 ion. The concentration of this ion reaches a higher level than that observed for the ($MH^+ - NH_3$) ion and then decays as well. Neutral loss of water is also observed but it reaches a much lower level than the above products. The maximum concentration for each fragment ion (I_{max}) is a combined measure of the rate of production and the stability of that ion. From four repetitive experiments, we estimate the standard uncertainties in the I_{max} values to be ± 10%. Several b ions are formed in parallel with the y_5 ion; the predominant ones are b_2 and b_3 (Figure 3). The expected b_4 as well as ($b_4 - NH_3$) and ($b_4 - H_2O$) ions are also observed, but these ions apparently are less stable and reach only low levels (<3%, not shown in the Figure).

The results for the doubly protonated QLLLPLLLK ion are completely different (Figure 4). The most pronounced fragmentation process is the neutral loss of H_2O. Formation of the y_5 ion is slower and the loss of NH_3 is very slow. The levels of b ions are very small; apparently b_4 formed along with y_5 decomposes rapidly to ($b_4 - NH_3$), while ($b_4 - H_2O$) is formed at higher collision energies by fragmentation of the ($MH_2^{2+} - H_2O$) ion. MS^3 spectra collected for QLLLPLLLK in the singly- and doubly-protonated forms show formation of the expected $b_n - NH_3$ and $b_n - H_2O$ ions (along with $a_n - NH_3$ and a_n-H_2O), which confirm that the neutral losses of NH_3 and H_2O occur from the terminal glutamine.

Table 1 summarizes relative intensities (I_{max}) and energies of peak maxima (E_{coll}) for different peptides, from which one can visualize the shape of the abundance versus energy plots such as shown in Figures 3 and 4. To estimate the relative rates of precursor ion fragmentation into the different product ions, we calculated the ratios of intensities of the different product ion peaks at low extent of reaction (low energies), where relative intensities are constant and well before the curve approaches its maximum. Although the curves

Table 1. Maximum intensity (I_{max}, % total ions) and collision energy ($E_{coll.}$, V) for the ions formed by Loss of ammonia, loss of water, and fragmentation at proline (y_5). $E_{1/2}$ is the collision energy where precursor and products ion intensities are equal

	Singly protonated							Doubly protonated						
	$MH^+ - NH_3$		$MH^+ - H_2O$		y_5			$MH_2^{2+} - NH_3$		$MH_2^{2+} - H_2O$		y_5		
Peptide (M)	I_{max} (%)	$E_{(coll.)}$	I_{max} (%)	$E_{(coll.)}$	I_{max} (%)	$E_{(coll.)}$	$E_{1/2}$	I_{max} (%)	$E_{(coll.)}$	I_{max} (%)	$E_{(coll.)}$	I_{max} (%)	$E_{(coll.)}$	$E_{1/2}$
QLLLPLLLK	9	39	2	42	15	45	39.4	0.5		45	16	18	19	12.3
QLLLPLLLR	1.6	45	1.7	47	1.1	52	48.8	0.1		33	15	15	17	12.4
QLLLPLLLH	16	36	4	39	12	42	36.5			36	15	13	16	12.4
QLLLPLLLL	0.1		3	33	21	35	28.8			6	9	2	14	8.5
NLLLPLLLK	0.4	33			15	42	38.7			1.5	14	5	17	11.4
CcmLLLPLLLK	0.4	38			17	48	42.0	0.2		5	17	6	20	15.4
ELLLPLLLK			1.2	38	15	43	39.4			3	9	27	14	11.2
HLLLPLLLK			0.5	36	14	44	40.3			0.7	18	2.5	22	18.1
LLLLPLLLK			0.1		14	42	39.1			0.2	14	5.3	17	13.2
KLLLPLLLK	0.1		0.4	36	11.7	45	41.5	3	18			2.8	23	18.1
QLLLLLLLLLPLLLK	2	60			8	72	65.2			5	26	8.6	22	18.8
LQLLPLLLK			0.1		15.3	46	42.6	2.9	16	0.1		5.7	21	14.5

Table 2. Relative rates of loss of ammonia and water vs. fragmentation at proline ($k/k_{(y5)}$) derived from the results obtained with the triple quadrupole (QqQ) mass spectrometer at low collision energies and from the ion trap (IT) instrument

	Singly protonated				Doubly protonated			
	$MH^+ - NH_3$		$MH^+ - H_2O$		$MH_2^{2+} - NH_3$		$MH_2^{2+} - H_2O$	
Peptide (M)	QqQ	IT	QqQ	IT	QqQ	IT	QqQ	IT
QLLLPLLLK	7.1	6.0	1.2	2.3	0.3	0.1	4	2.5
QLLLPLLLR	19(12)[a]	19(7.3)[a]	13(5)[a]	21(7.9)[a]	0.2		4	1.6
QLLLPLLLH	25		2.7				2	
QLLLPLLLL		0.02	0.3	1.1			4	
NLLLPLLLK							0.6	
CcmLLLPLLLK							2	
ELLLPLLLK			0.8	1.2			0.4	
HLLLPLLLK			0.5	0.6			1.4	1.1
LLLLPLLLK			0.05				0.05	
KLLLPLLLK		0.03		0.5	1.7	0.8		
QLLLLLLLLLPLLLK	0.7	6.1(1.9)[a]		2.8(0.9)[a]			0.14	
LQLLPLLLK		0.02		0.05	1.2	0.2[b]		

[a]Numbers in parentheses are calculated using the total intensity of the y_5 and (y_5-17/18) peaks. They are inserted only when they are significantly different from the other numbers.
[b]This value may be too low since there is another peak (*m/z* 508.5) that correlates to another loss of water.

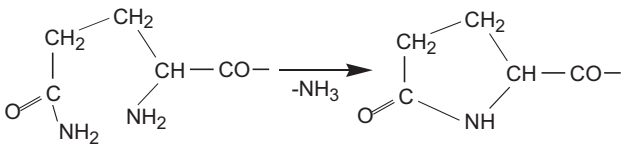
show product ion peak intensities as a function of collision energy, comparison of peak intensities at low extents of reaction is a form of "competition kinetics" for parallel processes. From these calculations, we estimate the rate of ammonia loss from the singly protonated ion of QLLLPLLLK, versus the rate of fragmentation at the proline residue, i.e., $k(MH^+ - NH_3)/k(y_5) = 7 \pm 1$. Similarly, we derive for the water loss a much lower ratio, $k(MH^+ - H_2O)/k(y_5) = 1.2 \pm 0.3$. For the doubly protonated ion we estimate in the same manner $k(MH_2^{2+} - NH_3)/k(y_5) = 0.3 \pm 0.1$ and $k(MH_2^{2+} - H_2O)/k(y_5) = 4 \pm 1$ (Table 2). From four repetitive experiments, we estimate the standard uncertainties in the relative rates to be approximately ± 25%.

Measurements were also done in an ion trap mass spectrometer, where collisional energy cannot be independently varied and excitation stops following dissociation, in contrast to the triple quadrupole instrument, where the product ions are subjected to the same collisional conditions as precursor ions. The ratios of product ion concentrations observed are $[MH^+ - NH_3]/[MH^+ - H_2O]/y_5 = 6.0/2.3/1$ for the singly protonated QLLLPLLLK and $[MH_2^{2+} - NH_3]/[MH_2^{2+} - H_2O]/y_5 = 0.1/2.5/1$ for the doubly protonated ion. These ratios are not too different from the ratios of the relative rates determined from the results obtained with the triple quadrupole instrument, i.e., 7/1.2/1, and 0.3/4/1, respectively. The small differences in ratios presumably originate from the different methods of ion activation in the collision cell and ion trap.

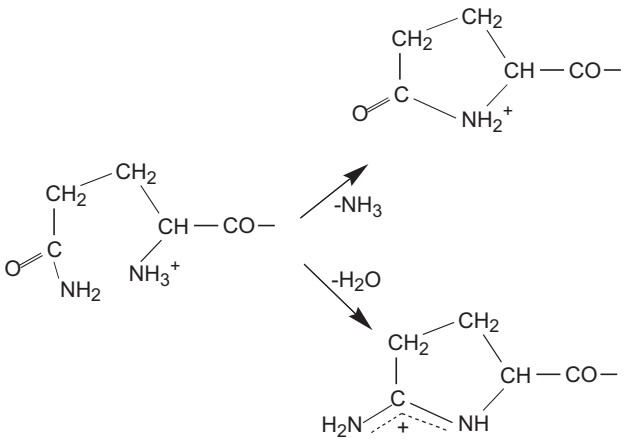
These results show that the fragmentation process in the singly-charged ion is neutral loss of NH_3 whereas in the doubly-charged ion it is predominantly the neutral loss of H_2O. In the singly-charged ion the proton is bound predominantly to the basic lysine residue at the C-terminus and has very small "residence time" at peptide bonds and N-terminus. The N-terminal glutamine then formally undergoes an NH_3 elimination reaction to form the cyclic pyroglutamyl residue (Scheme **1**), the same process that occurs, much more slowly, in unprotonated peptides in solution.

In the doubly-charged ion, however, the second proton is likely located on the second most basic site, the terminal glutamine. This protonated glutamine eliminates H_2O rather than NH_3. This is probably due to the stability of the expected product due to charge delocalization between the two nitrogens (Scheme **2**). We speculate that the mechanism involves an intermediate where the carbonyl of the side-chain amide group is protonated. This would not be surprising since it has been indicated that amides in the gas phase protonate on the carbonyl group [11].

The results for QLLLPLLLR are qualitatively similar to those described above. The doubly-charged ion undergoes predominantly loss of water while the singly-charged ion exhibits somewhat similar rates for loss of NH_3 and H_2O and slower formation of the y_5 ion (Table 1). The difference between the singly protonated QLLLPLLLR and QLLLPLLLK is expressed in their $E_{1/2}$ values, the collision energy at which the remaining intensity of the precursor peak is equal to the sum of the intensities of all the product peaks. It is apparent that decomposition of the singly-charged arginine peptide requires more energy than that of the lysine peptide



Scheme 1



Scheme 2

because of the higher basicity of R versus K [12]. The relative rates of fragmentation derived from results at low collision energies are again comparable to the ratios of the peaks observed in the ion trap instrument (Table 2). The values for QLLLPLLLR differ from those for QLLLPLLLK because they are determined relative to formation of the y_5 ion, which is different in the two peptides. Results for QLLLPLLLH are generally similar to those obtained with QLLLPLLLK, except that the value of $E_{1/2}$ for the singly-charged ion is slightly lower.

By replacing the basic amino acids K, R, and H with the neutral L, in QLLLPLLLL, we prevent localization of the first proton at the C-terminal residue. As a result, both the singly- and doubly-protonated ions show preference for water loss over ammonia loss. All of these effects were observed both in the triple quadrupole and the ion trap mass spectrometers under the respective conditions outlined above. The values of $E_{1/2}$ for both singly- and doubly-charged ions of QLLLPLLLL are lower than those for the peptides with basic amino acids (Table 1).

Note also that for the singly charged peptides in which the proton is expected to be localized on the C-terminal basic residue, $E_{1/2}$ values follow the same order as their gas-phase proton affinities, a trend consistent with the "mobile proton hypothesis" [2]. For H, K, and R the proton affinities are 988, 996, and 1051 kJ mol^{-1} [12], respectively, while the corresponding $E_{1/2}$ values are 36.5, 39.4, and 48.8 (Table 1).

Comparison of Glutamine with Other Amino Acids

Replacing glutamine by asparagine to form NLLLPLLLK strongly inhibits all neutral loss processes, presumably due to the requirement that a highly strained four-membered ring would be formed. To examine the situation for a six-membered ring, we synthesized CLLLPLLLK and alkylated the cysteine with iodoacetamide to form CcmLLLPLLLK. It is known that N-terminal S-carbamoylmethylcysteine in solution can cyclize by loss of NH_3 to form a product with a six-membered ring [13], the additional member being a sulfur atom. The results with this alkylated peptide show loss of NH_3 from the singly-charged ion and loss of H_2O from the doubly-charged ion, as observed with glutamine, but with lower efficiency; the maximum yields are an order of magnitude lower (Table 1) and the rate of water loss is a factor of two lower (Table 2). This suggests that the five-membered pyroglutamyl ring is the most stable structure and the most easily formed.

By replacing the terminal glutamine with glutamic acid, in ELLLPLLLK, cyclization can only lead to loss of water, and this process was found to require higher energy and reach lower levels than in the case of glutamine in both the singly- and doubly-charged ions. Upon replacing the terminal amino acid with H or L there is very little loss of water from both charge states; and upon replacing it with K there is only a little loss of ammonia from the doubly charged peptide. Extending the peptide with six additional leucine residues, QL_9PLLLK, increases the collision energy required to decompose these heavier peptide ions but the trend of ammonia loss from the singly-charged and water loss from the doubly-charged ions persists. And finally, when the glutamine is moved from the N-terminus to the adjacent position, cyclization is prevented and there are only minimal neutral losses (Table 1).

The results from the ion trap mass spectrometer are compared with those from the triple quadrupole instrument in Table 2 as discussed above. For the peptides mentioned above, the patterns of neutral losses are similar between the two instruments, with the ion trap generally having more intense neutral loss peaks from the precursor ion.

Further confirmation that the neutral losses of NH_3 and H_2O occur from the terminal glutamine was evident from the formation of the expected $b_n - NH_3$ and $b_n - H_2O$ ions (along with $a_n - NH_3$ and $a_n - H_2O$) in the MS^3 spectra collected for QLLLPLLLK and QLLLPLLLR in ion trap experiments. For the peptide LQLLPLLLK, MS^3 spectra show that the neutral loss of water from the singly charged peptide ion and the neutral loss of NH_3 from the doubly charged peptide ion occur at the glutamine in the second position. MS^3 spectra collected for the other peptides show losses of water or ammonia from the b ions as would be expected.

Effect of Peptide Length

Other peptides were synthesized to study the effect of peptide length on the neutral losses from the terminal glutamine (Table 3). To avoid possible effects attributable to the relative position of proline, these peptides do not contain proline. Two sets are based on QK and QR with a number of leucines (1, 3, 5, 7, 9, 11) between them. In this case, both the singly- and the doubly-protonated ions were studied. The third set is QL_n (n = 1, 2, 3, 4, 8). These peptides were observed as the

Table 3. Maximum intensity (I_{max}, % total ions) and collision energy ($E_{coll.}$, V) for the ions formed by loss of ammonia and water

	Singly protonated						Doubly protonated				
	$MH^+ - NH_3$		$MH^+ - H_2O$		$k_{(-NH3)}$		$MH_2^{2+} - NH_3$		$MH_2^{2+} - H_2O$		
Peptide (M)	I_{max} (%)	$E_{(coll.)}$	I_{max} (%)	$E_{(coll.)}$	$k_{(-H2O)}$	$E_{1/2}$	I_{max} (%)	$E_{(coll.)}$	I_{max} (%)	$E_{(coll.)}$	$E_{1/2}$
QK	33	12	22	12	1.6	9.5					
QLK	28	17	27	16	0.7	13.7	5	8	61	8	5.9
QLLLK	13.7	24	8.3	24	1.8	22.9			35	9	8.0
QLLLLLK	9.7	32	3.2	32	4.0	31.8	1.7	11	47	13	10.9
QLLLLLLLK	5.6	39	1.5	39	4.5	40.6	1.4		40	16	13.4
QLLLLLLLLLK	≈1	50				50.3			30	20	16.0
QLLLLLLLLLLLK						58.0			32	23	19.9
QR	6.9	15	3.5	15	1.3	14.8					
QLR	2.9	20	6.5	20	0.3	19.9	3.3	7	63	7	5.4
QLLLR	3.9	29	5.4	29	0.8	30.1			41	10	7.4
QLLLLLR	2.9	36	2.6	36	1.5	41.1	3.3	15	43	14	10.6
QLLLLLLLR	1.6	48	1.5	44	≈2	52.5	4.8	16	33	17	13.8
QLLLLLLLLLR	≈1	55	≈1	55		62.2	5	20	25	18	15.7
QG	36	9	44	9	0.7	5.9					
QL	31	10	44	10	0.8	7.2					
QLL	12	10	30	12	0.8	9.8					
QLLL	2.8	12	14	16	0.5	12.7					
QLLLL	1.3	15	9.3	20	0.6	17.4					
QLLLLLLLL			1.7	30		34.2	2.4	15			14.1
NLLL	0.8	12	2	12	0.4	10.1					

singly-charged ions, except for the longest one, which was also observed in the doubly-protonated state.

The doubly-charged ions of the QL_nK and QL_nR series show neutral loss of H_2O as the predominant process. The maximum level of $[MH_2^{2+} - H_2O]$ varies between I_{max} = 25 to 60% and appears to increase with decreasing peptide length (Table 3). The results for the nine-amino acid peptides, QL_7K and QL_7R, are similar to those found in Table 1 for the peptides of the same length that contain proline. The results are also similar in the two types of mass spectrometers. The loss of ammonia was relatively minor. It should be pointed out, however, that some of these doubly protonated ions underwent neutral loss of NH_3 as well as loss of an NH_4^+ ion to yield the singly protonated ion ($MH^+ - NH_3$). [Loss of NH_3 and loss of NH_4^+ were equally important in QLK, loss of NH_4^+ ion was more important in QL_3K and less important in the other peptides. Small peaks corresponding to loss of NH_4^+ were also observed in the ion trap from the doubly charged QL_5K, QL_7K, QLLLPLLLK, and QLLLPLLLR to form the singly charged ($MH^+ - NH_3$) ions].

The singly-charged ions of this series exhibit significant loss of both H_2O and NH_3 in the short peptides but the contribution of these neutral losses diminishes significantly as the peptide length increases and it becomes negligible in the longest peptide examined (Table 3). The relative rates of the neutral losses, $k(-NH_3)/k(-H_2O)$, derived from the peak ratios at low collision energies, are also listed in Table 3. It is noticed that the relative rate decreases from QK or QR when the first L is inserted but then increases with increasing number of L residues in the peptide chain, i.e., in the long peptides, loss of ammonia is faster than loss of water. This finding suggests that increased distance between the glutamine and the protonated amino acid residue decreases the likelihood of proton transfer to glutamine, or decreases the "residence time" of the proton on the terminal glutamine, resulting in decreased probability of water loss.

Plots of the $E_{1/2}$ values (the collision energy at which the total intensity of the fragment peaks equals the remaining intensity of the precursor peak) as a function of the precursor *m/z* values give very good linear correlations (Figure 5). For the singly charged ions, the slopes are 30, 38, and 47 V/kDa for the QL_n, QL_nK, and QL_nR series, respectively. The increase in $E_{1/2}$ value is in line with increasing basicity of the C-terminus. Since doubly charged ions possess twice the collisional energy as singly charged ions at the same collision voltage, to plot the results for the doubly charged ions on the same mass scale we multiplied their $E_{1/2}$ values by 2. The slopes for these peptides are very close for the QL_nK and QL_nR series, 24.4 and 25.6 V/kDa, respectively. Since the second proton in these doubly charged ions is not strongly bound, the $E_{1/2}$ values are expected to be the same for the QL_nK and QL_nR series. An equivalent relationship, where the collision energy needed to reach a degree of dissociation depended linearly on the molecular mass was reported by Haller et al. [14]. This reflects the idea that the rate of reaction depends on the energy per vibrational mode, which will tend to increase with increasing collision energy and decrease with numbers of oscillators (or, more coarsely, molecular mass). Stated differently, the energy requirement for a given reaction to occur at a given rate

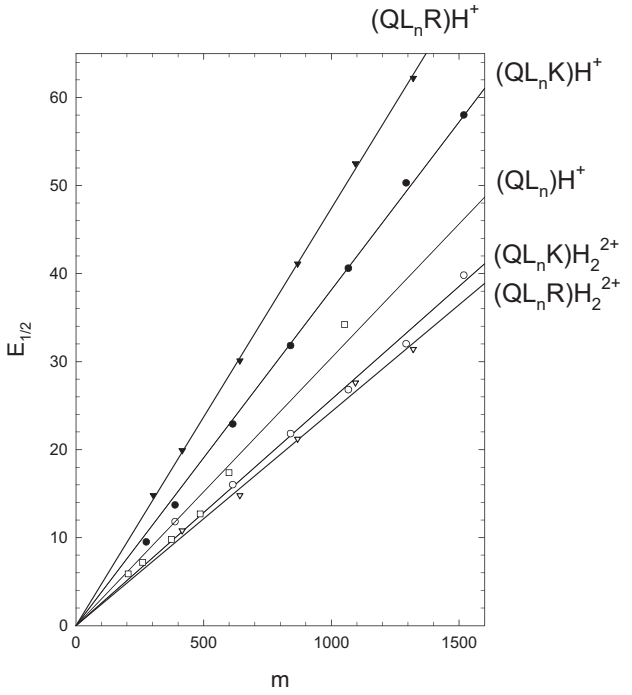


Figure 5. Dependence of $E_{1/2}$ (the collision energy at which the sum of the intensities of the fragment peaks equals the remaining intensity of the precursor peak) on peptide mass for various groups of peptide ions. To plot the results for the doubly charged ions on the same mass scale, we multiplied their $E_{1/2}$ values by 2.

depends on the heat capacity of the reactant, which in turn depends on its size (number of oscillators).

The singly-protonated ions of the QL_n series undergo neutral loss of water more than that of ammonia (Table 3), a trend that is reversed from that in the QL_nK series discussed above. The loss of water is favored in the QL_n series because the extent of protonation on the glutamine is higher (due to the absence of lysine or arginine), in line with the above mechanism.

Effect of Charge on Deamination

While the deamination process in N-terminal glutamine of singly charged tryptic peptides occurs at a location remote from the site where the charge is presumed to be localized, it was of interest to find if this reaction might be considered to occur independently of the charge, as it does in unprotonated peptides, though much more slowly. In Figure 6 we compare the extent of deamination of QLLLPLLLK (open circles) and QLLLPLLLR (solid circles) as a function of collision energy in the range where the loss of precursor ions is low (as shown by the corresponding triangles). The initial rate of deamination of the arginine terminal peptide is found to be slower than that of the lysine terminal peptides by about a factor of 2 (at the same collision energy, for the same peptide length). Similar comparisons for the shorter peptides of the QL_nK versus the QL_nR series show the same effect with even higher ratios. These results indicate that the higher stability of the proton in arginine (being a stronger base) somehow reduces the rate of deamination, suggesting the involvement of charge in the reaction. The observation that deamination becomes vanishingly small with increasing peptide size (distance from the charge site) also suggests that charge may be involved.

To further examine the effect of charge, we compared the rate of deamination of positively and negatively charged ions of the same peptides. In QG, QLLLK, QLLLLLK, and QLLLPLLLK the rate of deamination was (21 ± 5) times slower in the negatively charged versus the positively charged peptide ions. In both types of ions deamination took place along with backbone fragmentation and reached maximum levels at about the same collision energy (or slightly higher energy for the negative ions). Thus, negatively charged peptides show very low rates of deamination compared with the protonated species, confirming the involvement of charge in the latter cases. Since other studies described here suggest that the direct transfer of a proton to glutamine leads to loss of water, not ammonia, the mechanism of ammonia loss would appear to be quite different, perhaps involving some stabilization of the transition-state for ammonia loss without formal charge-transfer. Studies are underway to clarify the mechanism and kinetics of this process.

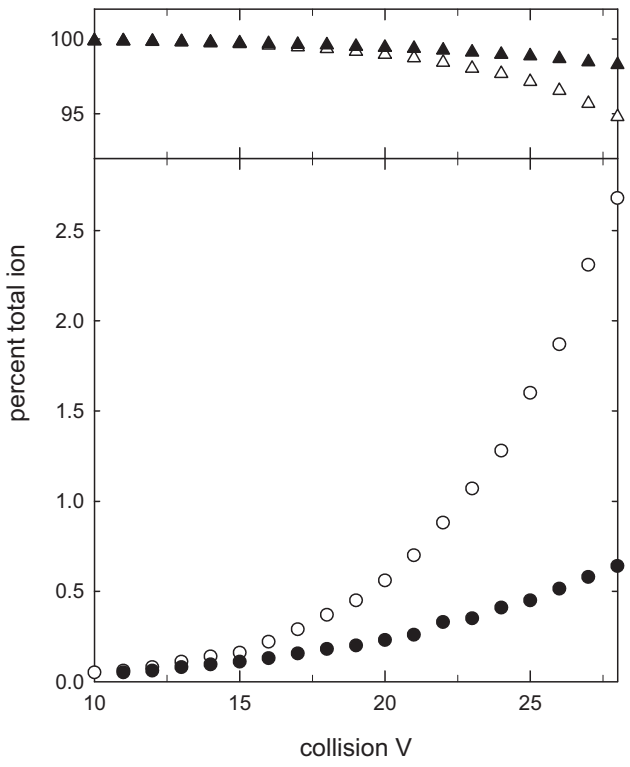


Figure 6. Decay of the precursor peaks (top) and formation of the ($MH^+ - NH_3$) peaks (bottom) as a function of collision energy in the triple quadrupole MS/MS spectrum of singly protonated QLLLPLLLK (open symbols) and QLLLPLLLR (filled symbols).

Table 4. Percent of products of water and ammonia loss peaks from peptide ions with and without excess (partially mobile [17]) protons for different N-terminal amino acids [15]

	n(protons) ≤ n(basic AA)			n(protons) > n(basic AA)		
N-terminal AA	-NH_3 (%)	-H_2O (%)	n(peptides)	-NH_3 (%)	-H_2O (%)	n(peptides)
A	1.97	2.58	937	0.20	0.48	739
C	8.05	2.8	40	0.50	1.0	38
D	2.40	1.80	711	0.17	0.27	511
E	4.22	11.5	734	0.08	0.14	619
F	2.35	2.87	468	0.22	0.38	449
G	2.05	2.31	687	0.26	0.74	527
H	2.12	3.13	590	—	—	5[a]
I	1.80	2.44	726	0.22	0.50	758
K	2.02	1.91	652	—	—	14[a]
L	2.02	2.84	1056	0.23	0.56	1030
M	1.03	1.26	137	0.19	0.48	196
N	6.36	1.77	665	0.33	0.54	528
P[b]	3.26	4.49	546	0.26	0.37	303
Q	15.24	5.75	170	<2.0[c]	4.8	292
R	3.09	1.06	241	—	—	5[a]
S	1.81	2.21	869	0.22	0.42	730
T	1.41	2.16	735	0.22	0.55	622
V	1.85	2.45	797	0.18	0.44	735
W	3.68	3.11	108	2.5	0.24	102
Y	2.27	2.53	436	0.21	0.37	393

[a]Since these are basic residues, and tryptic cleavage does not generally occur at the amino end of R and K, very few peptides were observed with sufficiently high charge states to fit in this category.
[b]Derived primarily from in-source dissociation of tryptic peptides since proline is not cleaved at its amino terminus by trypsin.
[c]Includes contribution from C^{13} isotope of the -H_2O product. Also, owing to ion-trap resolution, misassignment may occur from doubly and triply charged ions.

Comparison to Fragmentation of Tryptic Peptides

In the course of building an MS/MS library for yeast [15], we have created a collection of peptides derived from the tryptic digestion, all of which have a computed reliability >99%. These spectra were derived from publicly available sources of spectra, largely available from the Peptide Atlas [16] repository. Table 4 shows the average fraction of all product ions for neutral losses of ammonia and water from the precursor ion for each possible N-terminal amino acid. These are divided into two classes, those where the number of protons is less than or equal to the number of bases (R, K, H) and those with an excess of protons over bases. In the latter, a proton is expected to reside at the N-terminus, which is not the case for the former. Protons in this class have been referred to as partially-mobile protons [17].

Results are consistent with findings reported here. In the absence of excess protons, glutamine deamination is the most rapid of all loss processes. Even more prominently, when excess protons are available, dehydration of N-terminal glutamine is the most rapid of all neutral loss processes. We also note that for peptides containing excess protons, N-terminal carbamoylmethylcysteine is dehydrated more rapidly than any residue except glutamine, consistent with the present findings. Furthermore, glutamic acid, which is the most rapidly dehydrated N-terminal residue in the absence of excess protons, becomes the most resistant to dehydration when excess protons are available. While not the topic of the present study, we note that deamination of N-terminal tryptophan is an especially facile reaction for peptides with excess protons.

Conclusions

These studies report on the unique reactions of N-terminal glutamine in protonated peptides, where, if a mobile protons is available, water loss dominates the fragmentation process, while the unavailability of these protons leads primarily to ammonia loss. This has been traced to a rapid cyclization reaction of the glutamine, where water loss is encouraged by the associated formation of a highly stabilized, protonated basic residue. A similar residue, with a larger ring, can also be formed in the cyclization of N-terminal carbamidomethylated cysteine. Studies of the deamination process, which occurs when free protons are not available, show that this process too depends on the involvement of charge, though by a different, not clearly identified mechanism. Results from large numbers of tryptic peptides from yeast digests confirm these findings and show that these sequence-specific fragmentation processes have diagnostic value for peptide sequencing.

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