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Phenyl thiocarbamoyl and related derivatives of peptides: Edman chemistry in the gas phase

Scott G. Summerfield[1], Hanno Steen[2], Mathew O'Malley, Simon J. Gaskell*

Michael Barber Centre for Mass Spectrometry, Department of Chemistry, UMIST, Manchester M60 1QD, UK

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Abstract

Formation of the b_1 ion during the low energy collision-activated dissociation of the N-terminal phenyl thiocarbamoyl (PTC) derivatives of protonated peptides is analogous to the condensed-phase cleavage step of the Edman degradation. Previous studies confined to the analysis of tryptic peptides are here extended to probe the influence of peptide structure and extent of protonation on the prevalence of this fragmentation. The data are consistent with a requirement for protonation of the peptide backbone at the N-terminal amide linkage. Generally, PTC derivatives of peptides that incorporate as many or more basic amino acid residues than charges fail to undergo favored cleavage of the N-terminal amide bond, reflecting the absence of a proton resident on the peptide backbone to promote such fragmentation. Exceptions to this rule may be explained in terms of proton bridging between basic sites to release an ionizing proton for residence on the peptide backbone. Replacement of the PTC derivative by the pentafluoro-PTC analog results in similar fragmentation chemistry but with preferential loss of the derivatized N-terminal residue as a neutral fragment. Thus, judicious choice of derivatization procedure enables not only the direction of fragmentation but also of charge retention. (Int J Mass Spectrom 188 (1999) 95–103) © 1999 Elsevier Science B.V.

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1. Introduction

Electrospray and tandem mass spectrometry (MS/MS) are now very widely employed for the characterization of peptides and proteins. For protein analysis, a common strategy involves initial enzymatic digestion followed by MS/MS sequencing of the components of the resulting peptide mixture. Partial or total sequence information for individual peptides may be used to refine a search of protein databases whereby a comparison is made between the experimental data and those predicted for each database protein [1]. Substantial research effort in a number of centers has been devoted to the elucidation of the nature and mechanism of peptide ion decomposition. The techniques employed have included the analysis of structural analogues, isotopic labeling [2–4], sequential MS (MS^n) [5–8], reionization of neutral fragments [9,10], and molecular modeling [11,12].

* Corresponding author. E-mail: simon.gaskell@umist.ac.uk

[1] Present address: SmithKline Beecham, Department of Drug Metabolism and Pharmacokinetics, The Frythe, Welwyn, Herts. AL6 9AR, UK.

[2] Present address: Center for Experimental Bioinformatics, Odense University, 5230 Odense M, Denmark.

Dedicated to Brian Green for his many innovative contributions to mass spectrometry instrument development and for his tireless help in teaching us how to take advantage of them.

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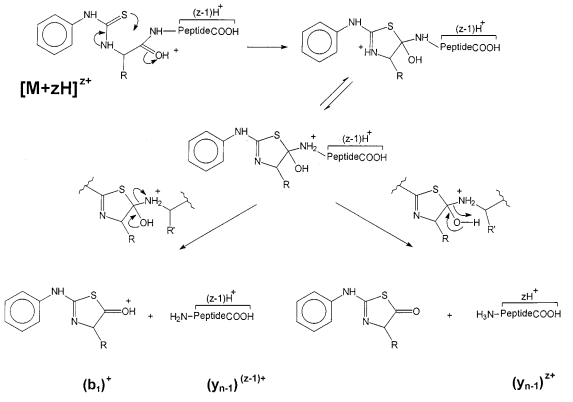

Fig. 1. Proposed mechanism for the fragmentation of PTC derivatives of protonated peptides (of *n* amino acid residues) to yield b_1 and y_{n-1} ions (adapted from [15]).

Thus, for example, the mechanism of formation of b_n ions (formed by cleavage of an amide bond with charge retention on the N-terminal fragment) has been elucidated by Harrison and co-workers and reported in a series of papers [10,13,14]. It is now understood that the genesis of b-type ions involves nucleophilic attack of a carbonyl oxygen on the carbonyl carbon of the adjacent (C-terminal side) amide linkage to yield an oxazolone structure. The mechanism readily explains the general absence of b_1 ions in the product ion spectra of underivatized peptides (and equally their presence in N-acetylated analogs) [14].

We recently reported [15] the electrospray-tandem mass spectrometric analysis of N-terminal phenylthiocarbamoyl (PTC) derivatives of tryptic peptides with low energy collision-activated dissociation (CAD) of $[M + 2H]^{2+}$ ions. There was a marked propensity to cleavage of the N-terminal amide bond, yielding complementary b_1 and y_{n-1} singly charged fragments from the *n*-residue peptide. A significant process was also observed which resulted in formation of the doubly charged y_{n-1} ion. The fragmentation chemistry was rationalized as shown in Fig. 1, drawing a close analogy with the accepted mechanism of formation of b-type ions discussed above [13,14]. The predominance of b_1 over other sites of b-type cleavage for the PTC derivatives may be explained by considering the higher nucleophilicity of the sulfur of the thiocarbonyl group, in comparison with carbonyl oxygens. A close correspondence also exists between the proposed gas-phase chemistry and condensed-phase Edman chemistry. In the latter case, initial derivative formation is followed by a cleavage reaction promoted by anhydrous acid. In our earlier report [15] we suggested that the further fragmentation of b_1 ions derived from peptide PTC derivatives was consistent with isomerization of the initially formed thiazolone to a phenyl thiohydantoin structure (providing a further parallel with Edman chemistry). A subsequent more detailed examination of this latter aspect by Yalcin et al. [16], however, provided no evidence for such isomerization.

Here we report an extension of our earlier studies on the CAD of ions derived from peptide PTC derivatives and describe an evaluation of the influence of peptide structure and charge state on the importance of cleavage of the N-terminal amide bond. The data are discussed in terms of the existing model of charge directed fragmentation. In addition, we describe the influence on this ion fragmentation chemistry of the use of the pentaflurophenyl-thiocarbamoyl derivative.

2. Experimental

2.1. Sources of peptides and reagents

All peptides and reagents were obtained from Sigma (Poole, Dorset, UK).

2.1. Preparation of PTC derivatives

Derivatization was performed using a modification of the procedure described previously [15]. The dry peptide (1 μg) was dissolved in 10 μL of ethanol-water-triethylamine-phenyl isothiocyanate (77:11:11:1, by volume). The reaction was allowed to proceed for 10 min at 50 °C; solvents and excess reagent were then removed by vacuum centrifugation. Reaction byproducts and impurities were then removed by solvent partition. Heptane–ethyl acetate (10/1, v/v; 50–200 μL) was added to the dried, derivatized digest, followed by an equivalent volume of water.

This mixture was shaken vigorously and then centrifuged to break the dispersion. The organic (top) layer was removed and replaced by a second aliquot of the organic solvent mixture. Extraction was repeated to remove all of the yellow coloration in the solution or solid remaining. The mixture was then dried under vacuum. It was observed that if the water was added first during the extraction then a yellowish solid formed at the bottom of the aqueous layer and was difficult to extract into the organic top layer. Incomplete removal of byproducts and impurities led to increased background interference during electrospray mass spectrometry and a lower analyte signal. Derivatized peptides were stored dry at −30 °C; the acidified electrospray solvent was added immediately prior to analysis.

2.2. Preparation of para-bromophenyl thiocarbamoyl (pBr-PTC) derivatives

The peptide was dissolved in ethanol-water-triethylamine (2/1/3, v/v/v; 6 μL per 1 μg of peptide). para-bromophenyl isothiocyanate reagent was then added as a 0.2 M ethanolic solution (5 μL per 1 μg of peptide) and the reaction was allowed to proceed for 35 min at 50 °C. After drying in a vacuum centrifuge, the sample was redissolved in water and washed with chloroform-heptane-ethyl acetate (5/5/1, v/v/v); the washing steps were analogous to those used for the PTC derivative.

2.3. Preparation of the pentafluorophenyl thiocarbamoyl (pentafluoroPTC) derivatives

The peptide was dissolved in 19:1 trifluoroethanol-triethylamine (19/1, v/v; 10 μL per 1 μg of peptide). (No water was used to avoid destruction of the reagent.) Pentafluorophenyl isothiocyanate (2 μL) was added and reaction was allowed to proceed for 10 min at 50 °C. Following drying in a vacuum centrifuge, the product was dissolved in water and washed with 5:5:1 chloroform-heptane-ethyl acetate (5/5/1, v/v/v); the washing steps were analogous to those used for the PTC derivative.

2.4. Mass spectrometry

Electrospray MS and MS/MS experiments were performed using a Quattro tandem quadrupole instrument upgraded to Quattro II specifications (Micromass, Manchester, UK). The electrospray solvent was acetonitrile-water (1/1, v/v) containing 0.1% formic acid, delivered at 1 to 3 μL/min using a syringe driver (Harvard Apparatus, South Natick, MA, USA). External calibration of the mass-to-charge ratio scale was performed with a mixture of NaI (2 g/L) and RbI (0.05 g/L) in isopropanol-water (1/1, v/v). For MS/MS experiments, argon was used as the collision gas at an indicated manifold pressure of $\sim 1 \times 10^{-6}$ bar. Collision offset potentials are given for each analysis in Sec. 3. Product ion scanning experiments used a quadrupole scan rate of 100 Th/s. Data acquisition and processing were performed via the MassLynx data system. For product ion scanning, the resolution of the precursor ion was reduced to achieve transmission of the full isotopic envelope; product ion resolution was set to unit for singly charged products. Product ions are designated according to the Biemann variant [17] of the Roepstorff and Fohlman [18] nomenclature. Thus, b- and y-type ions are both derived by cleavage of a peptide bond, with charge retention on N- and C-terminal fragments, respectively; a subscript indicates the number of amino acid residues in the ion. The original nomenclature for singly charged ions is supplemented for multiply charged ions by an indication of the charge state. Multiply charged ions imply the incorporation of additional protons.

3. Results and discussion

The first stage of this work concentrated on the analysis of peptides with sequences such as those expected to result from hydrolysis of proteins using the enzyme, trypsin, which cleaves C-terminal to Arg and Lys residues. Electrospray mass spectrometry of such peptides commonly yields prominent $[M + 2H]^{2+}$ ions where one of the ionizing protons is presumed to be sequestered by the basic sidechain of

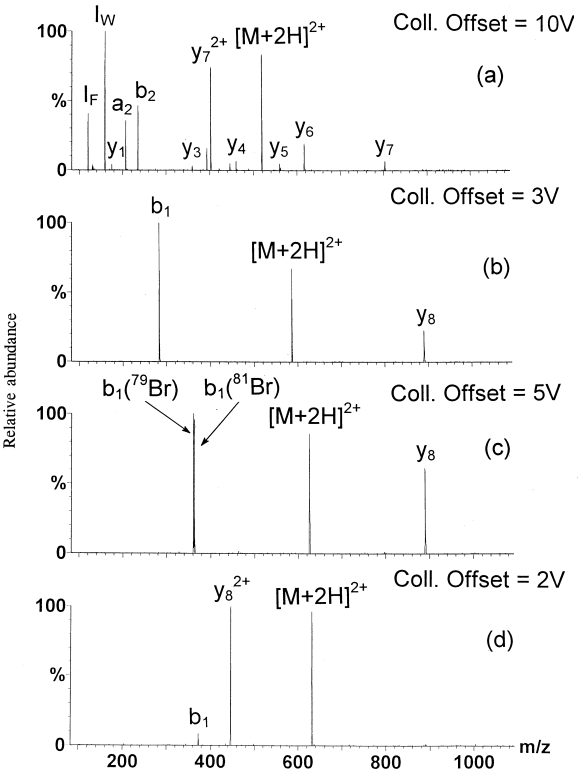

Fig. 2. Product ion spectra recorded following low energy CAD of $[M + 2H]^{2+}$ ions of the peptide, FSWGAEGQR, and derivatives. (a) the underivatized peptide; (b) PTC derivative; (c) 4-bromo-PTC derivative; (d) the pentafluoro-PTC derivative. Collision offset potentials are shown for each analysis. Ions labeled as I_F and I_W correspond to immonium ions derived from the phenylalanine and tryptophan residues, respectively.

the C-terminal residue [12]. Fig. 2(a) shows the product ion spectrum of the $[M + 2H]^{2+}$ ion derived from the peptide, FSWGAEGQR. The spectrum, which is generally typical of those observed for doubly protonated tryptic peptides, incorporates an almost complete series of y ions but with a_2 and b_2 as the single prominent N-terminal fragments. As argued previously [12], the fragmentation pattern may be rationalized in terms of the promotion of fragmentation by the "mobile" proton not associated with the C-terminal residue. Of the complementary y- and b-series ions formed in this manner, the y ions will be the more stable by virtue of incorporating the basic site which retains the proton and therefore suppresses charge-proximal second-generation cleavage of the peptide backbone. Initially formed b ions incorporate no strongly basic site so that the proton conferring charge is mobile and can promote subsequent generations of fragment ions, leading to a concentration of N-terminal fragment ion current in the b_2 and a_2 ions. The genesis of b-type ions via oxazolone formation [13,14] explains the lack of observation of the b_1 ion. The particular propensity to form, and the evident stability of, the y_7^{2+} ion [Fig. 2(a)] remains unexplained.

The simplicity of the product ion spectrum of the $[M + 2H]^{2+}$ ion of the PTC derivative of FSWGAEGQR [Fig. 2(b)] is in marked contrast to that of the underivatized peptide. Cleavage at a single site in the peptide backbone is observed to yield the complementary b_1 and y_8 ions. The finding is in keeping with earlier observations of Edman-like chemistry arising from nucleophilic attack by the sulfur of the derivative group on the carbonyl carbon of the first peptide bond (Fig. 1) [15]. The product ion spectrum recorded for the $[M + 2H]^{2+}$ ion of the p-bromophenyl thiocarbamoyl derivative is closely similar to that of the PTC derivative; the spectrum shown in Fig. 2(c) was obtained by selection for CAD of both isotopomers of the precursor ion so that both $[^{79}Br]b_1$ and $[^{81}Br]b_1$ ions are observed. The spectrum of product ions derived from $[M + 2H]^{2+}$ of pentafluorophenyl thiocarbamoyl (pentafluoro-PTC) shows an interesting difference from those of the other derivatives examined [Fig. 2(d)]. Cleavage at the N-terminal peptide bond is again the sole process evident but the y_8^{2+} ion is observed almost exclusively (and using a collision energy, in the laboratory frame-of-reference, of only 4 eV). Evidently the strongly electron withdrawing effect of the pentafluorophenyl group sufficiently destabilizes incipient charge retention on the N-terminal fragment that it is lost almost exclusively as the neutral species.

Electrospray mass spectrometry analyses of small peptides not incorporating a basic residue yield predominantly singly protonated species which are expected to be heterogeneous with respect to the site of charge. Analysis of the PTC derivative of Leu-enkephalin (YGGFL) yields the expected $[M + H]^+$

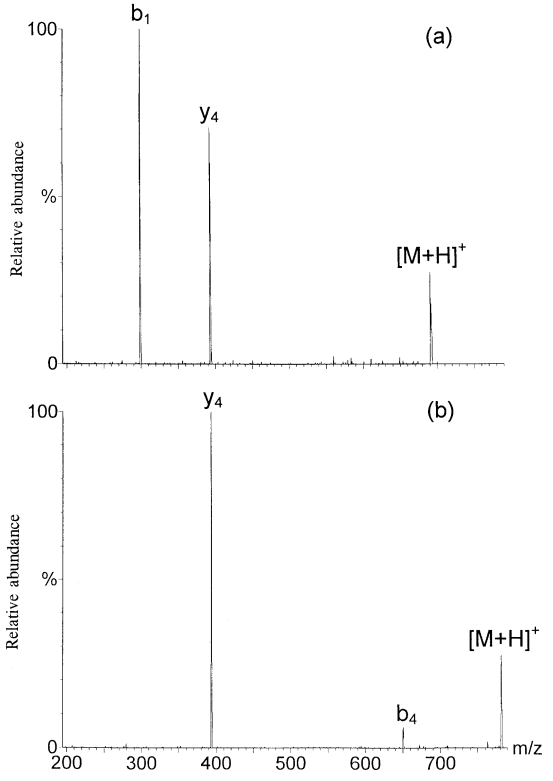

Fig. 3. Product ion spectra recorded following low energy CAD of the $[M + H]^+$ ions of derivatives of YGGFL (Leu-enkephalin). (a) PTC derivative, collision offset = 9 V; (b) pentafluoro-PTC derivative; collision offset = 5 V.

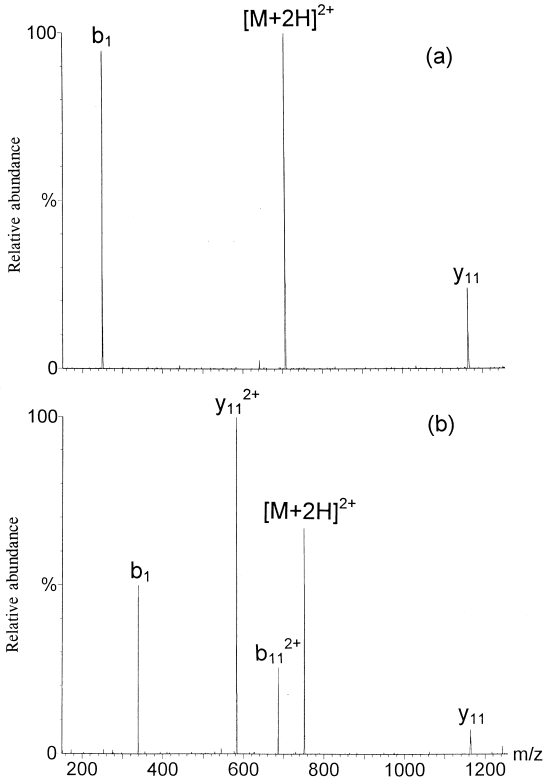

Fig. 4. Product ion spectra recorded following low energy CAD of the $[M + 2H]^{2+}$ ions of derivatives of IYLGGPFSPNVL. (a) PTC derivative, collision offset = 7 V; (b) pentafluoro-PTC, collision offset = 16 V.

ion (spectrum not shown); upon CAD, the product ion spectrum [Fig. 3(a)] is dominated by the b_1 and y_4 ions arising from the now-familiar gas-phase Edman chemistry. The finding is in keeping with the required mobility of the ionizing proton to yield a proportion of the precursor ion population incorporating protonation of the carbonyl oxygen of the N-terminal peptide bond (Fig. 1). Collisional activation of $[M + H]^+$ ions of the PTC derivatives of tryptic peptides (incorporating a strongly preferred site of charge on the C-terminal residue) failed to yield significant fragments at low collision energies (data not shown); elevation of the collision offset to values in excess of 50 eV promoted fragmentation of the PTC moiety but provided no evidence of preferential cleavage of the N-terminal peptide bond. These findings are again consistent with the requirement for protonation at the N-terminal peptide bond to promote Edman-type chemistry. CAD of the $[M + H]^+$ ion of the pentafluoro-PTC derivative of Leu-enkephalin [Fig. 3(b)] yields predominantly the y_4 fragment, confirming the propensity for loss of the fluorinated derivative of the N-terminal residue as a neutral fragment. The b_4 fragment, corresponding to cleavage of the C-terminal peptide bond, is of minor importance relative to y_4 but nevertheless is formed in much higher yield than any other N-terminal fragment; this observation is currently unexplained.

Electrospray mass spectrometry of the PTC derivative of IYLGGPFSPNVL yields predominantly the $[M + 2H]^{2+}$ ion despite the lack of a strongly basic amino acid residue. CAD of this ion gave the product ion spectrum shown in Fig. 4(a); b_1 and y_{11} ions predominate. The equivalent result for the pen-

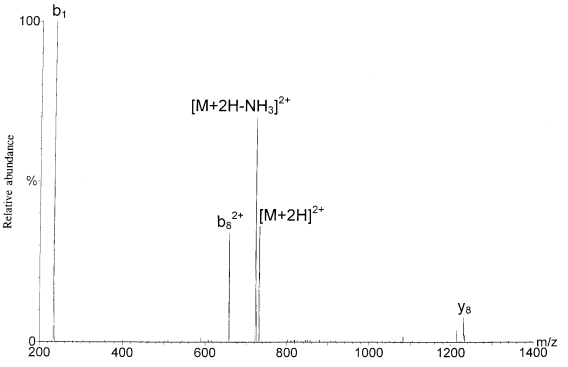

Fig. 5. Product ion spectrum recorded following low energy CAD of the $[M + 2H]^{2+}$ ions of the PTC derivative of PKPQQFFGLM-amide (substance P); collision offset = 5 V.

tafluoro-PTC derivative [Fig. 4(b)] shows the expected enhancement of the doubly charged y ion derived from scission of the N-terminal peptide bond, though the singly charged complementary fragments from the same cleavage remain prominent. Interestingly [in the light of the observations with Leu-enkephalin, Fig. 3(b)], the doubly charged b ion arising from cleavage at the C-terminus is of significant abundance; again, further study is required to explain this finding.

Several peptides have also been investigated which incorporate basic amino acid residues at or near the N-terminus. $[M + 2H]^{2+}$ and $[M + 3H]^{3+}$ ions derived from the PTC derivatives of tryptic peptides incorporating a His residue at the N-terminus fragment predominantly to yield the b_1 and complementary fragments (data not shown). Fig. 5 shows the product ion spectrum for the $[M + 2H]^{2+}$ ion of the PTC derivative of PKPQQFFGLM-amide (substance P). Aside from the loss of ammonia, the only prominent product ions are those that arise from cleavage of the N-terminal amide bond, with or without charge retention on the N-terminal fragment. Evidently the presence of the basic lysine residue at position 2 does not preclude charge-directed cleavage of the N-terminal amide bond. Similarly, analyses of PTC derivatives of tryptic peptides incorporating N-terminal histidine residues also fragment to yield b_1 and y_{n-1} ions (data not shown).

The product ion spectrum of the $[M + 2H]^{2+}$ ion

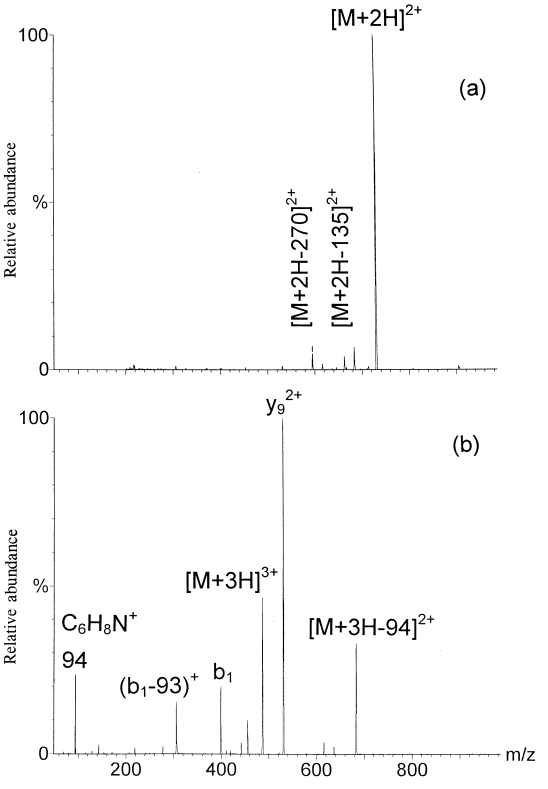

Fig. 6. Product ion spectra recorded following low energy CAD of the PTC derivative of KRPPGFSPFR. (a) $[M + 2H]^{2+}$ precursor ion, collision offset = 9 V; (b) $[M + 3H]^{3+}$ precursor ion, collision offset = 6 V.

of KRPPGFSPFR (Lys^1-bradykinin) is shown in Fig. 6(a); the poor fragmentation efficiency is consistent with the notion that the ionizing protons are sequestered on the strongly basic guanidino groups of the arginine residues. Minor fragments are observed corresponding to partial or complete loss of the derivative group; analogous losses are observed for the pentafluoro-PTC derivative but to a greater extent at a given collision potential (data not shown). Addition of a further proton has the expected effect of opening the fragmentation pathway involving cleavage of the N-terminal amide bond; the complementary y_9^{2+} and b_1 ions are produced [Fig. 6(b)]. In addition, cleavage of the derivative group is observed, either from the $[M + 3H]^{3+}$ ion or the b_1 fragment. In contrast to these observations, the product ion spectra of the $[M + 2H]^{2+}$ ions of the PTC and pentafluoro-PTC

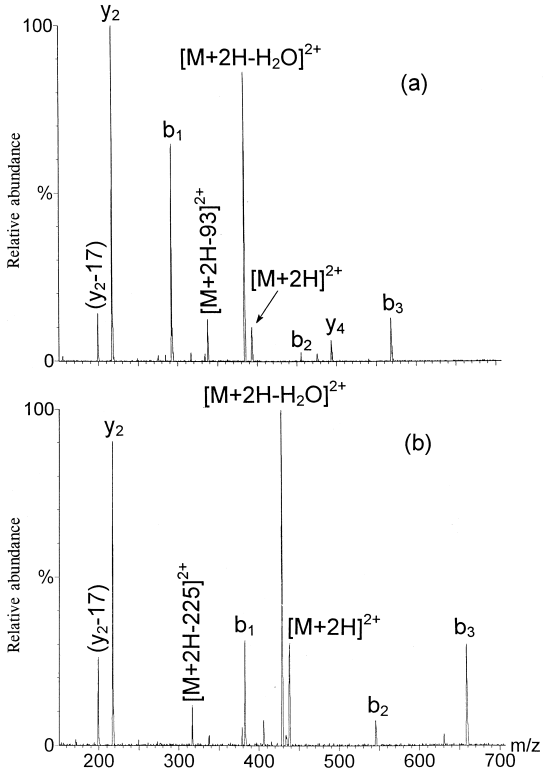

Fig. 7. Product ion spectra recorded following low energy CAD of the $[M + 2H]^{2+}$ ions of the PTC derivative of RYLPT (proctolin). (a) PTC derivative, collision offset = 5 V; (b) pentafluoro-PTC, collision offset = 2 V.

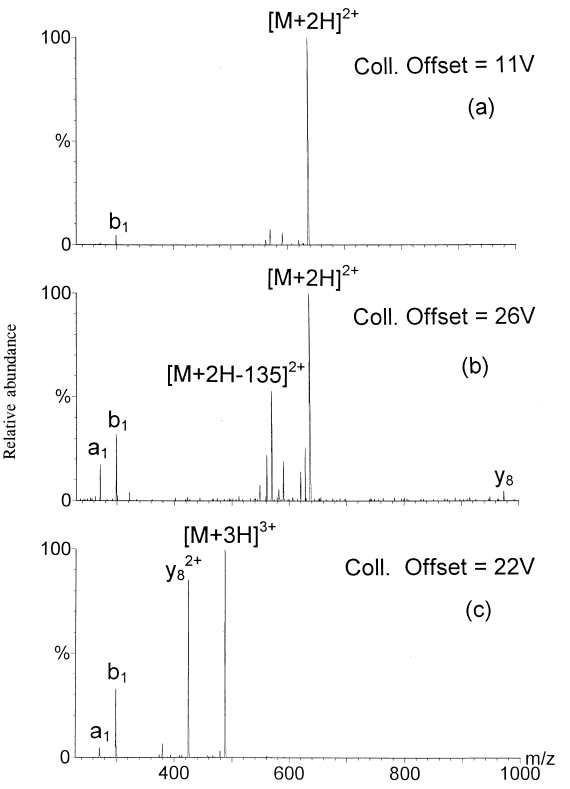

Fig. 8. Product ion spectra recorded following low energy CAD of the PTC derivative of YGGFLRRIR. (a) $[M + 2H]^{2+}$ precursor ion; (b) $[M + 2H]^{2+}$ precursor ion; (c) $[M + 3H]^{3+}$ precursor ion. Collision offset potentials are shown for each analysis.

derivatives of RYLPT (proctolin) show significantly more complex fragmentation patterns despite the incorporation of one more proton than basic amino acid residues (Fig. 7). For both derivatives, b_1 ions are observed but other cleavages, including water loss, peptide chain cleavages and fragmentation of the derivative group [15] are prominent.

CAD of ions derived from PTC derivatives of peptides that incorporate as many or more basic amino acid residues than charges commonly fail to undergo favored cleavage of the N-terminal amide bond, consistent with the absence of a proton resident on the peptide backbone to promote this fragmentation. Thus, for example, such analysis of the $[M + 3H]^{3+}$ ion of the PTC derivative of DRVYIHPFHLLVYS (renin substrate factor) does not yield a prominent b_1 ion (data not shown). The peptide YGGFLRRIR (dynorphin) provides an exception to this generalization. Fig. 8 shows the product ion spectra of the $[M + 2H]^{2+}$ and $[M + 3H]^{3+}$ ions of the PTC derivative of YGGFLRRIR. As expected, the fragmentation efficiency of the doubly charged ion is poor, though elevation of the collision offset potential to 26 V (corresponding to a collision energy in the laboratory frame-of-reference of 52 eV for the doubly charged ion) increases the yield of b_1 and y_8 ions, in addition to promoting fragmentation of the derivative group [Fig. 8(b)]. The observation of the a_1 ion at significant abundance is presumably a reflection of the further collisional activation of the b_1 first-generation product ion. CAD of the $[M + 3H]^{3+}$ analog [Fig. 8(c)] using a collision energy of 66 eV gives almost exclusive fragmentation of the N-terminal amide bond to yield complementary b_1 and y_8^{2+} ions, despite the

fact that the three ionizing protons might be accommodated on the three arginine residue sidechains. Evidently, the relatively high collision energy is sufficient to mobilize a proton to the peptide backbone to promote the observed cleavage. The comparative facility with which this occurs may be associated with the proximity of the three arginine residues, perhaps facilitating proton bridging between guanidino groups and therefore releasing a proton to the peptide backbone. This suggestion is consistent with recent findings in this laboratory on the relative decomposition efficiencies of melittin (GIGAVLKVLTTGLPALISWIKRKRQQ-amide) $[M + 4H]^{4+}$ ions and of an analog incorporating modified arginine residues; the data were interpreted in terms of proton bridging within the –KRKR– sequence. Bowers and co-workers [19] have argued that proton bridging between the N- and C-terminal arginine residues of bradykinin establishes an energetically preferred macrocyclic conformation for the $[M + H]^{+}$ ion.

4. Conclusions

The discussion in Sec. 3 has emphasized the correspondence between the gas-phase processes described here and condensed-phase Edman chemistry. In solution, cleavage of the derivatized N-terminal amino acid residue is achieved by treatment with anhydrous acid. A proton serves the same purpose in the gas phase but an important difference is that the condensed-phase chemistry involves acid in vast molar excess so that differences in the basicities of the various sites on the peptide structure are of little consequence. Condensed-phase cleavage proceeds because of the attack of the relatively strongly nucleophilic thiocarbonyl sulfur on a proximal electrophilic site, namely the carbon of the protonated carbonyl group of the first amide linkage. In the gas phase, the extent to which this occurs is determined also by the extent of protonation of the N-terminal carbonyl oxygen and this will be influenced by the favorability of sequestration of ionizing protons at alternative sites and also by coulombic repulsion which may affect the distribution of protons in multiply charged species. A particularly favorable situation exists with peptides derived from tryptic digestion of proteins and therefore incorporating basic arginine or lysine residues at the C-terminus. Electrospray formation of $[M + 2H]^{2+}$ ions provides species which include a mobile proton in addition to that expected to be sequestered by the C-terminal residue. A proportion of the precursor ion population will therefore incorporate a proton on the N-terminal amide oxygen, promoting nucleophilic attack by the thiocarbonyl sulfur. (In the case of histidine-containing tryptic peptides, the commonly formed $[M + 3H]^{3+}$ ions will show similar properties.) A consequence of these observations is that complex mixtures of tryptic peptides may be screened for the presence of specific N-terminal residues by conversion to PTC derivatives and tandem mass spectrometry analyses with scanning of precursors of the appropriate b_1 ion [20]. The observation of preferential retention of charge on the C-terminal fragment when the pentafluoro-PTC derivatives are subjected to CAD provides a further example of the control of peptide ion fragmentation which may be used to analytical advantage. Thus, MS/MS analysis provides (via observation of neutral loss) a sensitive indication of the identity of the N-terminal residue while MS/MS/MS analysis (e.g. using an ion trapping instrument) would enable additional sequence information to be obtained via CAD of the y_{n-1} fragment.

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