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RESEARCH ARTICLE

Towards Understanding the Tandem Mass Spectra of Protonated Oligopeptides. 2: The Proline Effect in Collision-Induced Dissociation of Protonated Ala-Ala-Xxx-Pro-Ala (Xxx = Ala, Ser, Leu, Val, Phe, and Trp)

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Abstract

The product ion spectra of proline-containing peptides are commonly dominated by y_n ions generated by cleavage at the N-terminal side of proline residues. This proline effect is investigated in the current work by collision-induced dissociation (CID) of protonated Ala-Ala-Xxx-Pro-Ala (Xxx includes Ala, Ser, Leu, Val, Phe, and Trp) in an electrospray/quadrupole/time-of-flight (QqTOF) mass spectrometer and by quantum chemical calculations on protonated Ala-Ala-Ala-Pro-Ala. The CID spectra of all investigated peptides show a dominant y_2 ion (Pro-Ala sequence). Our computational results show that the proline effect mainly arises from the particularly low threshold energy for the amide bond cleavage N-terminal to the proline residue, and from the high proton affinity of the proline-containing C-terminal fragment produced by this cleavage. These theoretical results are qualitatively supported by the experimentally observed y_2/b_3 abundance ratios for protonated Ala-Ala-Xxx-Pro-Ala (Xxx = Ala, Ser, Leu, Val, Phe, and Trp). In the post-cleavage phase of fragmentation the N-terminal oxazolone fragment with the Ala-Ala-Xxx sequence and Pro-Ala compete for the ionizing proton for these peptides. As the proton affinity of the oxazolone fragment increases, the y_2/b_3 abundance ratio decreases.

Key words: Peptides, CID, Fragmentation pathways, Proline effect

Introduction

Protein identification in proteomics is mainly carried out by tandem mass spectrometry (MS/MS) of protonated or multiply-protonated peptides. Under the typical low-energy collision conditions these peptide ions usually fragment at amide bonds to give series of *b* and/or *y* ions [1, 2], which contain the N- and C-terminus, respectively. Ideally, these series of *b* and *y* ions and their satellite fragments (generated by loss of small neutrals like water, ammonia, and CO) provide sufficient information to sequence the investigated peptides, mostly by using bioinformatics tools. Therefore, it is of great importance to understand the mechanism, energetics, and kinetics of the related primary backbone cleavages [3].

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Two major classes of peptide fragmentation chemistries need to be considered in this respect, one occurring in peptide ions with and the other in peptide ions without mobile protons. In the case of peptide ions with *mobile protons* [4], excitation leads to amide nitrogen protonated species with weakened amide bonds, which are then cleaved to form primary fragments. Two types of such amide bond cleavage mechanisms exist, with 'loose' or 'tight' transition states, respectively [3]. The former involves simultaneous cleavages of the C_α–CO and CO–NH_2^+ bonds and forms a_n and y_m ions by elimination of CO (a_1-y_1 or a_n-y_m pathways [5, 6]). The latter always involves rearrangement type reactions, and one of the products contains a newly formed ring. The majority of b_n ions is thought to be formed on the b_n-y_m pathways [7–12] where the N-terminal adjacent amide oxygen initiates cleavage of the protonated amide bond by nucleophilic attack on its carbon center. This reaction leads to formation of *b* fragments with a C-terminal five-membered oxazolone ring [13–21]. Alternatively, the N-terminal amino group can initiate the amide bond cleavage forming cyclic peptide isomers of b_n ions [22]. The b_2-y_m_*cyclic_peptide* pathway (cleavage of the second amide bond from the N-terminus) requires *trans* to *cis* isomerization of the N-terminal amide bond [23] to form the diketopiperazine derivative b_2 ion. This reaction can be energetically and/or kinetically controlled. Cyclic peptide isomer b_n ions of intermediate size (like b_3 and to lesser extent b_4) are not likely to be formed on this pathway, because of the significant ring strain that arises from a medium-sized ring accommodating all but one amide bonds in the *trans* form [3, 9]. (Only the amide bond introduced by ring formation can be *cis* or *trans*). This constrain is not valid any longer for larger b_n ions ($n \geq 5$) [24–26]. However, entropic factors can hinder the corresponding b_n-y_m_*cyclic_peptide* pathways while such constraints do not exist for the corresponding 'oxazolone' b_n-y_m pathways [3, 9]. For these reasons, the b_n-y_m_*cyclic_peptide* pathways are usually less active than the corresponding 'oxazolone' b_n-y_m channels [3, 9]. It was, however, recently demonstrated [24–26] that the cyclic peptide isomers of the larger b_n ions ($n \geq 5$) can easily be formed from oxazolone-terminated b_n isomers by nucleophilic attack of the N-terminal amine on the protonated oxazolone ring. The third type of rearrangement pathways involves amide bond cleavage initiated by side chain nucleophiles [27–31].

A recent study indicates that significantly different mechanisms are active for amide bond cleavages of peptide ions lacking mobile protons [32]. This peptide ion class includes the practically important, Arg-terminated singly protonated tryptic peptides formed by MALDI and ESI-generated multiply charged peptide ions where the number of arginines equals the number of ionizing protons. It was demonstrated [32] that three distinct proton mobilization pathways can occur for these peptide ions which involve Arg side chain guanidinium, amidic, or carboxylic protons. For $[G_nR + H]^+$ (n=2–4) ions, the latter proton mobilization channel is the energetically most favored, which leads to salt-bridge stabilized intermediates and transition structures on the amide bond cleavage pathways. Mobilization of amidic protons leads to imine-enol (-COH=N-) isomers from which oxazolone terminated *b* ions can be formed. For $[G_nR + H]^+$ (n=2–4) ions the salt-bridge and imine-enol pathways were energetically more favored than the classical b_n-y_m pathways initiated by mobilization of a guanidinium proton.

The mechanism, energetics, and kinetics of amide bond cleavage have so far been studied for peptide ions with a limited set of amino acid residues [3, 33–36]. Relatively detailed information is available for small protonated oligoglycines [5, 7, 8, 10], oligoalanines [9, 33, 35], or Leu-enkephalin [17, 36], which produce CID spectra with numerous abundant *b* and *y* ions. Often, however, CID of certain peptides produces product ion spectra dominated by only a few peaks. One particularly intriguing such case is proline-containing peptides. Here, the product ion spectra are typically dominated by y_m ions formed by cleavages N-terminal to Pro (i.e., the related y_m ions contain proline residues at their N-termini). This so-called 'proline effect' was for example demonstrated by Schwartz and Bursey when studying [37] the fragmentation of protonated Ala-Ala-Ala-Ala-Ala, Ala-Ala-Pro-Ala-Ala, and Ala-Ala-Ala-Pro-Ala under CID conditions. They found that the product ion spectra were dominated by the y_3 and y_2 ions for Ala-Ala-Pro-Ala-Ala, and Ala-Ala-Ala-Pro-Ala, respectively; while numerous abundant fragments were generated in CID of protonated Ala-Ala-Ala-Ala-Ala. This behavior was explained by the high proton affinity (PA) of Pro [38, 39]. As a consequence of the high PA, protonation at the Pro amide nitrogen was considered energetically favored over protonation at other amide nitrogens, giving rise to preferred bond cleavage at the Pro residue.

In a subsequent study Vaisar and Urban investigated [40] the fragmentation patterns of protonated Val-Ala-Pro-Leu-Gly and Val-Ala-Pip-Leu-Gly. In the latter peptide, the proline residue of Val-Ala-Pro-Leu-Gly was substituted by pipecolic acid (Pip), which has a six-membered side chain ring instead of the 5-membered ring of proline. The PAs of Pro and Pip are most likely close to each other and the fragmentation patterns of protonated Val-Ala-Pro-Leu-Gly and Val-Ala-Pip-Leu-Gly were therefore both expected to feature dominant y_3 ions. However, while protonated Val-Ala-Pro-Leu-Gly did show prevalent y_3 ions (i.e., a 'proline effect'), the product ion spectrum of protonated Val-Ala-Pip-Leu-Gly did not display a similar effect. Instead, a dominant b_3 ion and only a weak y_3 ion were observed. This indicates that the proline effect cannot be explained by considering only the energetically favored protonation of the Pro residue caused by its high PA. Vaisar and Urban suggested [40] that cleavage N-terminal to Pro is enhanced, because cleavage C-terminal to Pro would lead to bicyclic transition structures as well as N-terminal products that are energetically not favorable. However, this proposal was

later refuted by Siu and co-workers who demonstrated that these bicyclic products are not energetically disfavored [41]. The proline effect was also investigated in statistical analysis of validated product ion spectra [42], and detailed fundamental studies on doubly protonated VPDPR and analogs [43], and protonated polyprolines [44]. Counterman and Clemmer demonstrated that proline containing peptides often show multiple resolved features in their ion mobility spectra [45]. This phenomenon was explained by coexisting isomers due to *cis* and/or *trans* forms of the Xxx-Pro amide bond. Poutsma and co-workers have determined the PAs of Pro and various derivatives [46]. Recently, the Wysocki and Poutsma groups studied the fragmentation chemistry of various oligopeptides containing proline [47].

In the present study, we have examined the proline effect using experimental and theoretical tools. CID of protonated Ala-Ala-Xxx-Pro-Ala (Xxx includes Ala, Ser, Leu, Val, Phe, and Trp) was studied in a quadrupole/time-of-flight (Qq-TOF) instrument. The potential energy surface of protonated Ala-Ala-Ala-Pro-Ala was characterized including backbone protonation sites and amide bond cleavage transition structures. Using these data the proline effect is analyzed for these peptides in the framework of the 'Pathways in Competition' fragmentation model [3] that considers detailed mechanistic, energetic, and kinetic aspects of the pre-cleavage, amide bond cleavage, and post-cleavage phases of peptide fragmentation.

Experimental

All experimental work was carried out using an electrospray/quadrupole/time-of-flight (QqTOF) mass spectrometer (QStar, SCIEX, Concord, Canada). MS^2 experiments were carried out in the usual fashion for $[M + H]^+$ ions by mass-selecting the ions of interest with the mass analyzer Q with fragmentation in the quadrupole collision cell q and mass analysis of the ionic products with the time-of-flight analyzer. Ionization was by electrospray with the sample, dissolved at micromolar levels in 1:1 CH_3OH:1% aqueous formic acid, introduced into the ion source at a flow rate of 80 μL min^{-1}. Nitrogen was used as nebulizing and drying gas and as collision gas in the quadrupole collision cell. The peptides used Ala-Ala-Xxx-Pro-Ala (Xxx includes Ala, Ser, Leu, Val, Phe, and Trp) were obtained from Celtek Peptides (Nashville, TN, USA); they showed no impurities in their mass spectra and were used as received.

Computational Details

A conformational search engine [3, 17, 26, 48, 49] devised specifically to deal with protonated peptides was used to scan the potential energy surface (PES) of protonated Ala-Ala-Ala-Pro-Ala. We have characterized the energetics of the N-terminal amino and amide nitrogen and oxygen protonated forms and the transition structures of the b_n–y_m amide bond cleavages. To obtain theoretical proton affinities for the N-terminal oxazolone fragments with the Ala-Ala-Xxx sequences (Xxx = Ser, Leu, Val, Phe, and Trp), both the neutral and N-terminal amine and C-terminal oxazolone ring nitrogen protonated forms were computed. These calculations began with molecular dynamics (MD) simulations using simulated annealing on the above isomers and protonation sites using the Discover program (Biosym Technologies, San Diego, CA, USA), in conjunction with the AMBER force field [50] modified by us to allow amide nitrogen and oxygen protonated species, oxazolone terminated structures, and b_n–y_m amide bond cleavage TSs. During the MD simulations, structures were regularly saved for further refinement by full geometry-optimization using the same force field. Special attention was paid to possible *cis/trans* isomers of the Ala(3)–Pro(4) amide bond. For each distinct protonation site (amide oxygen, amide nitrogen), *cis/trans* isomer, and TSs a large number (>5000) of candidate structures were generated in MD simulations. In the next stage of the process, these structures were analyzed by our own conformer-family search program. This program is able to group optimized structures into families based on the similarity of the most important characteristic torsion angles. The most stable species in these families were then fully optimized at the HF/3-21G, B3LYP/6-31G(d), and the B3LYP/6-31 + G(d,p) levels. Relative energies were calculated by using the B3LYP/6-31 + G(d,p) total energies and zero-point energy corrections (ZPE) determined at the B3LYP/6-31G(d) level. Entropies were calculated using the rigid-rotor harmonic oscillator (RRHO) approximation (Table S2 in the Supporting Information). In absolute terms these are likely to be exaggerated due to the presence of too many low-frequency modes; however relative entropies are more accurate due to cancellation of errors. Proton affinity values are approximated from zero-point corrected electronic energies. The Gaussian [51] suite of programs was used for all ab initio and DFT calculations.

Results and Discussion

Fragmentation Reactions of $[M + H]^+$ Ions of the Investigated Peptides

The product ion mass spectra of the six $[M + H]^+$ ions at 16 eV collision energy are shown as stick spectra in Figure 1 (Ala-Ala-Ala-Pro-Ala) and Figures S1–5 (Ala-Ala-Xxx-Pro-Ala with Xxx = Ser, Leu, Val, Phe, and Trp, see Supporting Material). There is a close resemblance between the fragment ion spectra of protonated Ala-Ala-Ala-Pro-Ala reported by Schwartz and Bursey [37] and that displayed in Figure. 1. The main backbone fragmentation channels of the peptides studied are summarized in Scheme 1. Each fragmentation spectrum shows a dominating y_2 product ion, evidence of the proline effect. Dominance of the y_2 signal is maintained in a wide collision energy range (8–20 eV) as investigated in the present work (data not shown). The y_2 ions are always accompanied by a less abundant b_3 peak

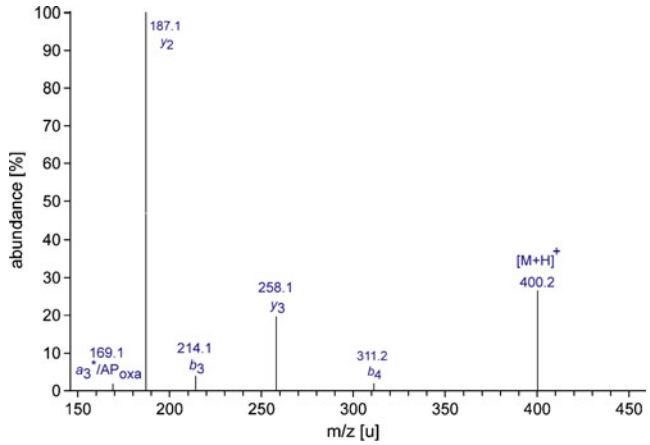
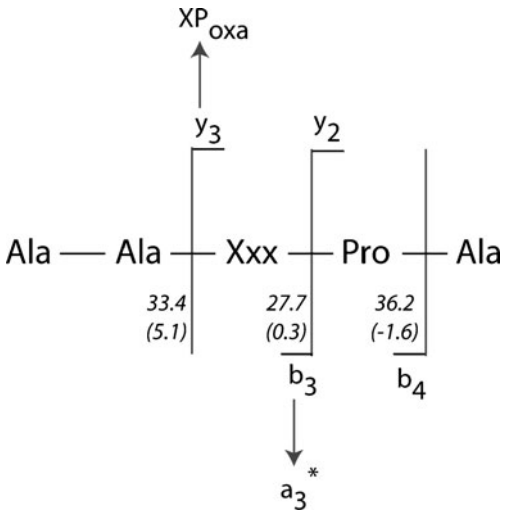


Figure 1. Product ion mass spectrum (recorded at 16 eV collision energy) of protonated Ala-Ala-Ala-Pro-Ala

(Scheme 1). Since the b_4 ions are always weak in these spectra, it is likely that b_3 arises as a primary fragment from cleavage of the Xxx-Pro amide bond and *not* from the $b_4 \rightarrow b_3$ pathway. Additionally, each spectrum displays a low or moderately abundant y_3 fragment generated by the cleavage of the Ala-Xxx amide bond. This y_3 fragment is protonated Xxx-Pro-Ala that can easily fragment by losing the C-terminal Ala residue [15]. This reaction can occur on both the 'oxazolone' b_2-y_1 and the b_2-y_1*_cyclic_peptide* (diketopiperazine) pathways; Figure 1 and Figures S1, S2, S3, S4 and S5 (see Supporting Information) display the corresponding product ion as 'XP_{oxa}', even though this might not be an oxazolone if formed on the b_2-y_1*_cyclic_peptide* pathway. Furthermore, b_3 (AAX_{oxa}) can fragment [52] by eliminating CO and ammonia and the elemental composition of the resulting a_3^* ion is the same as that of XP_{oxa} for each investigated peptide. Therefore, for example, the ion at *m/z* 169.1 for protonated Ala-Ala-Ala-Pro-Ala can be composed on three different pathways. The only case when y_2 does not fully dominate the product ion spectrum is protonated Ala-Ala-Ser-Pro-Ala (Figure S1 in the Supporting Information). Here one observes abundant $[M + H\text{-}H_2O]^+$ and y_3-H_2O peaks, both likely due to loss of water from the Ser side chain [3, 53].



Scheme **1**. Fragmentation pathways of protonated Ala-Ala-Xxx-Pro-Ala. Computed threshold energies (kcal mol^{-1}) and activation entropies (cal mol^{-1} K^{-1}) are given for the studied amide bond cleavage pathways of protonated Ala-Ala-Ala-Pro-Ala in italics

Protonation Energetics and Fragmentation Pathways of Protonated Ala-Ala-Ala-Pro-Ala

To gain a deeper understanding of the proline effect and the related mechanistic, energetic and kinetic phenomena, we carried out detailed quantum chemical calculations on protonated Ala-Ala-Ala-Pro-Ala. These studies aimed at elucidating the relative energies of the possible backbone protonation sites and transition structures of the amide bond cleavage pathways. In doing so we considered both the *cis* and *trans* isomerization states of the Ala(3)–Pro(4) amide bond [45].

Energies relative to the all-*trans* N-terminal amino protonated isomer are presented in Table 1. The energetically most favored protonation site of Ala-Ala-Ala-Pro-Ala is the N-terminal amino group (Table 1), with the *trans* Ala(3)–Pro(4) isomer slightly higher in energy than the *cis* form (relative energy at −1.4 kcal mol^{-1}). Protonation at the oxygens of the Ala(1)–Ala(2), Ala(2)–Ala(3), and Ala(3)–Pro(4) amide bonds is energetically more favored for the *cis* isomer than for the *trans* form. The most favored amide oxygen protonation site for the all-*trans* isomer is that of the Ala–-Pro amide bond (3.9 kcal mol^{-1}). Protonation at the Ala(2)–Ala(3) and Ala(3)–Pro(4) amide oxygens of the *cis*-isomer is essentially isoenergetic to protonation at the N-terminus of the all-*trans* isomer. Isomers protonated at the amide oxygen of the Pro(4)–Ala(5) amide bond with *cis* configuration of the Ala(3)–Pro(4) peptide bond turned out to be unstable in the DFT calculations. Here, proton transfers occurred to other basic sites in the molecule. No such species could therefore be located within ca. 16 kcal/mol of the global minimum structure of Ala-Ala-Ala-Pro-Ala.

The energetically most favored amide nitrogen protonation site is the one at the Ala–Pro amide bond, at 14.6 kcal mol^{-1}

Table 1. Relative Energies (kcal mol^{-1}) of Various Protonation Sites of Protonated Ala-Ala-Ala-Pro-Ala Calculated with Respect to the N-terminal Amine Protonated Species of the All-*trans* Isomer

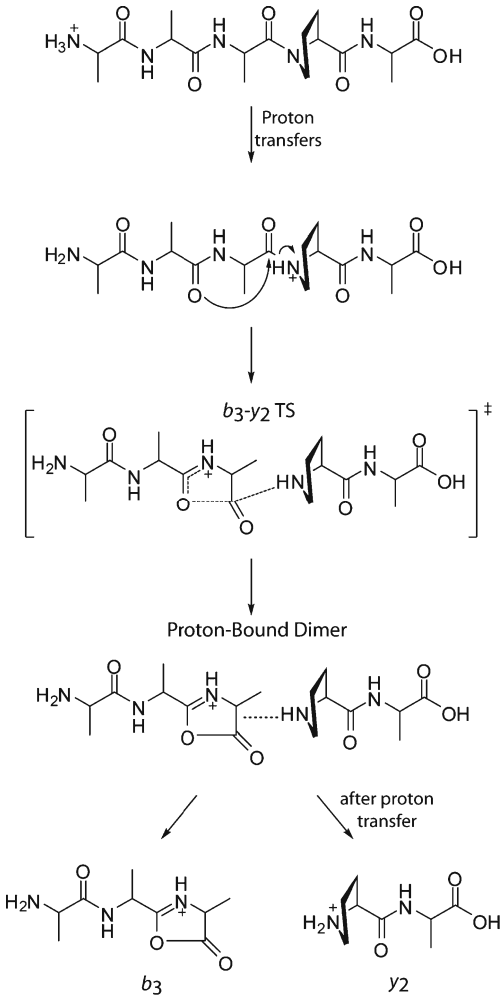
Protonation site	Relative energy	
	trans (Ala–Pro)	*cis* (Ala–Pro)
N-terminal NH_2	0.0	−1.4
Ala(1)–Ala(2) amide O	10.0	4.4
Ala(2) –Ala(3) amide O	6.4	−0.1
Ala(3) –Pro(4) amide O	3.9	0.2
Pro(4) –Ala(5) amide O	10.8	none located
Ala(1) –Ala(2) amide N	23.3	21.8
Ala(2) –Ala(3) amide N	18.9	18.6
Ala(3) –Pro(4) amide N	14.6(S)/15.2(R)	
Pro(4) –Ala(5) amide N	18.3	20.3

relative energy. It is worth noting here that due the proline side chain the amide nitrogen becomes a chiral center upon protonation. Both the *R* and the *S* configurations were computed, the latter is slightly more favored (0.8 kcal mol^{-1}) than the former, although this difference is clearly within the limits of our computational approach. The Ala(2)–Ala(3) amide nitrogen protonated structures with the *trans* and *cis* Ala(3)–Pro(4) bonds are nearly equienergetic at 18.9 and 18.6 kcal mol^{-1} relative energies, respectively. These structures are critical intermediates on the b_2–y_3 pathway [33].

These energetics clearly indicate that the Pro side chain stabilizes protonation at the nitrogen of the Ala-Pro amide bond. These results suggest that the b_3–y_2 pathway is preferred over the other b_n–y_m pathways in the pre-cleavage phase [33] of the fragmentation of protonated Ala-Ala-Ala-Pro-Ala.

In the next step, we determined the amide bond cleavage transition structures of protonated Ala-Ala-Ala-Pro-Ala. In these calculations (Table 2) we restricted ourselves to 'oxazolone' b_n–y_m pathways because of the following reasons. Using IR spectroscopy and theory, Stipdonk and co-workers recently demonstrated [19] that the b_2 ion of protonated Ala-Ala-Ala is an oxazolone and not a diketopiperazine. We assume the same mechanism applies for cleavage of the Ala(2)–Ala(3) amide bond of protonated Ala-Ala-Ala-Pro-Ala. The cyclic peptide isomer of b_3 with the Ala-Ala-Ala sequence is energetically clearly disfavored compared to the oxazolone isomer [54]. This suggests the b_3-y_2 *_cyclic_peptide* channel is not competitive with the 'oxazolone' b_3–y_2 pathway for protonated Ala-Ala-Ala-Pro-Ala.

Mechanistic details of the 'oxazolone' b_3–y_2 pathway are presented in Scheme 2; similar mechanisms can be drawn for the b_2–y_3 and b_4–y_1 channels. After mobilization of the ionizing proton to the Ala(3)–Pro(4) amide nitrogen the Ala (2) amide oxygen attacks the carbon center of the protonated amide bond. This reaction forms the oxazolone ring of b_3 cleaving the Ala-Pro amide bond. The critical energy of this reaction is 27.7 kcal mol^{-1} (Scheme 1) and the corresponding activation entropy (at 298.15 K) is 0.3 cal mol^{-1} K^{-1} (Table 2). The absolute configuration of the proline nitrogen



Scheme **2**. Formation of b_3 and y_2 ions on the b_3-y_2 pathway

atom plays an important role; fragmentation with *R* configuration (27.7 kcal mol^{-1}) is much preferred over *S* configuration (33.8 kcal mol^{-1}). The calculated critical energies for cleavage at other amide bonds are much higher (Scheme 1 and Table 2). Amide bond cleavage on the b_2–y_3 and b_4–y_1 channels requires 33.4 and 36.2 kcal mol^{-1}, respectively. The activation entropy of the b_2–y_3 pathway is more positive (5.1 cal mol^{-1} K^{-1}) than that of b_3–y_2, but this effect is not large enough to render b_2–y_3 competitive to b_3–y_2 even at 600 K. The activation entropy of the b_4–y_1 pathway is more negative (−1.6 cal mol^{-1} K^{-1}) than that of b_3-y_2. These considerations demonstrate that the b_3–y_2 pathway is clearly preferred over the other b_n–y_m pathways in the *amide bond cleavage phase* [3] of the fragmentation of protonated Ala-Ala-Ala-Pro-Ala.

Under low-energy collision conditions the two fragments generated by cleavage of the Ala-Pro amide bond do not separate immediately. Instead, they initially form a proton-bound dimer (PDB). During dissociation of this PBD the N-terminal oxazolone and C-terminal Pro-Ala fragments compete for the ionizing proton. Since the proton affinity of

Table 2. Threshold Energies (kcal mol^{-1}) and Activation Entropies (cal mol^{-1} K^{-1}) of Various Backbone Peptide Fragmentation Pathways of Protonated Ala-Ala-Ala-Pro-Ala Calculated Relative to the N-terminal Amine Protonated All-*trans* Species. *Cis*/*trans* Denote *Cis* and *Trans* Isomerization States of the Ala–Pro Amide Bond, and R/S Denote the Absolute Configuration of the Proline Nitrogen Atom

Fragmentation pathway	*cis*/*trans*; R/S	Threshold energy	Activation entropy
b_2–y_3	*trans*	33.4	5.1
	cis	34.3	4.9
b_3–y_2	R	27.7	0.3
	S	33.8	6.7
b_4–y_1	*trans*	36.2	−1.6

the latter (230.1 kcal mol^{-1} computed in this work) is 4.5 kcal mol^{-1} higher than that of the former (225.6 kcal mol^{-1} computed in this work, see Table S3 in the Supporting Information), one expects that y_2 will be much more abundant than b_3 in the experimental spectrum. This is indeed the case; the y_2/b_3 abundance ratio is 25.8 in Figure. 1. These considerations on the post-cleavage phase [3] of the fragmentation of protonated Ala-Ala-Ala-Pro-Ala explain why y_2 is dominantly formed on the b_3–y_2 pathway while b_3 is only a minor fragment. Taken together all the pre-cleavage, amide bond-cleavage, and post cleavage phases of the fragmentation of protonated Ala-Ala-Ala-Pro-Ala clearly favor cleavage of the Ala-Pro amide bond and formation of the y_2 fragment providing a reasonable explanation for the proline effect observed in CID of this peptide ion.

The Proline Effect in CID of Protonated Ala-Ala-Xxx-Pro-Ala

The proline effect was further probed by CID of protonated peptides in the Ala-Ala-Xxx-Pro-Ala (Xxx includes Ala, Ser, Leu, Val, Phe, and Trp) series. Here, the amino acid residue in the third position was systematically varied. Such peptide series were previously studied by MS/MS. For example, Morgan and Bursey investigated the Gly-Gly-Xxx series and N-benzoylated peptides [55, 56] and Harrison and co-workers studied the Val-Xxx and Gly-Xxx-Phe [57, 58] series. Analysis of the product ion spectra of the related peptides and rationalizing the observed fragmentation tendencies led to improved fragmentation mechanisms of peptides [3, 5, 8, 9]. Often, a linear free energy relationships [59] could be established from the observed fragmentation tendencies.

The product ion spectra of protonated Ala-Ala-Xxx-Pro-Ala (Xxx includes Ala, Ser, Leu, Val, Phe, and Trp) are presented in Figure. 1 and Figures S1, S2, S3, S4 and S5. All spectra display the proline effect by showing a clearly pronounced y_2 peak. In Table 2 we present fragment ion abundance data for the b_3–y_2 pathways along with proton affinities of the corresponding N-terminal fragments. For all peptides but Ala-Ala-Ser-Pro-Ala, the y_2 and b_3 ions account for more than 70% of the total fragment ion current of the investigated peptides (Table 2). In the PBDs formed by cleavage of the Xxx-Pro amide bond on the b_3–y_2 pathway the (N-terminal) oxazolone with sequence Ala-Ala-Xxx and the (C-terminal) Pro-Ala fragment compete for the ionizing proton. The PA of the C-terminal Pro-Ala fragment is computed to be 230.1 kcal mol^{-1} (see above). However, the PA of the N-terminal oxazolone fragment Ala-Ala-Xxx varies with the C-terminal residue Xxx from 225.1 to 230.8 kcal mol^{-1} (Table S3 in the Supporting Information). Following our arguments of the post-cleavage phase made above, the abundance ratio y_2/b_3 should therefore decrease with increasing proton affinity of the N-terminal oxazolone fragment. This tendency is clearly seen in Table 3. It must be noted here that the ln(y_2/b_3) versus PA(oxazolone) relationship cannot be approximated by a simple linear curve as a linear free energy relationship. This can be at least partially explained by the presence of competing fragmentation pathways and/or further fragmentation of the primary b_3 fragment to a_3* and additional formation of y_2 on the $y_3 \rightarrow y_2$ pathway. It is unfortunately impossible to quantify the real abundance of a_3* because of the isobaric XP_{oxa} that can easily be formed from y_3. Furthermore, the calculated PA of the oxazolone with the Ala-Ala-Trp sequence is 230.8 kcal mol^{-1}, which is higher than that of Pro-Ala at 230.1 kcal mol^{-1}. This indicates that more abundant b_3 ions should be observed for protonated Ala-Ala-Trp-Pro-Ala than y_2 fragments. This is clearly not the case, the y_2/b_3 ratio is around 4 for a wide range of collision energies. One can explain the mismatching theoretical and experimental data here by considering the uncertainties of our theoretical model, which is clearly more than 2 kcal mol^{-1}. Another cause for the disagreement between theory and experiment could be that the dissociation of the PBD is not governed by the relative proton affinities and rather gas-phase basicities need to be used to describe this process.

Table 3. Relative Weight (%) of the y_2 and b_3 Ions and the Logarithm of the Abundance Ratio of These Ions in the Product Ion Spectra of Ala-Ala-Xxx-Pro-Ala (Xxx = Ala, Ser, Leu, Val, Phe, and Trp) and Proton Affinities (kcal mol^{-1}, Computed in the Present Work) of the Corresponding Oxazolone Fragments

Peptide	y_2 and b_3	ln(y_2/b_3)	Oxazolone	PA
Ala-Ala-Ala-Pro-Ala	81.7	3.25	AAA	225.6
Ala-Ala-Ser-Pro-Ala	48.6	3.20	AAS	225.1
Ala-Ala-Leu-Pro-Ala	87.7	2.83	AAL	227.5
Ala-Ala-Val-Pro-Ala	91.0	2.79	AAV	227.6
Ala-Ala-Phe-Pro-Ala	74.0	2.62	AAF	228.6
Ala-Ala-Trp-Pro-Ala	78.7	1.38	AAW	230.8

Conclusions

The CID spectra of proline-containing peptides typically show abundant *y*-ions formed by cleavage of the amide bond N-terminal to Pro residues. We have analyzed this 'proline effect' by computational means for protonated Ala-Ala-Ala-Pro-Ala. It was demonstrated that the proline residue significantly stabilizes the Ala-Pro amide nitrogen protonation site and the corresponding b_n–y_m transition state. The high proton affinity of the proline residue results in a high proton affinity of the C-terminal fragment and therefore preferred formation of the y_2 ion during the post-cleavage phase of fragmentation. These theoretical results are qualitatively supported by experimentally observed y_2/b_3 fragment ion abundance ratios for the peptide series Ala-Ala-Xxx-Pro-Ala (Xxx includes Ala, Ser, Leu, Val, Phe, and Trp). The y_2/b_3 abundance ratio decreases as the PA of the N-terminal oxazolone-terminated fragment increases. We are currently performing similar studies on peptide series like AAP, AAAP, and AAAAP.

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