RESEARCH ARTICLE

Investigations of the Mechanism of the "Proline Effect" in Tandem Mass Spectrometry Experiments: The "Pipecolic Acid Effect"

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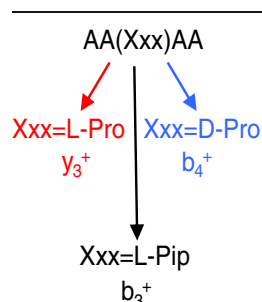
Abstract. The fragmentation behavior of a set of model peptides containing proline, its four-membered ring analog azetidine-2-carboxylic acid (Aze), its six-membered ring analog pipecolic acid (Pip), an acyclic secondary amine residue *N*-methyl-alanine (NMeA), and the D stereoisomers of Pro and Pip has been determined using collision-induced dissociation in ESI-tandem mass spectrometers. Experimental results for AAXAA, AVXLG, AAAXA, AGXGA, and AXPAA peptides are presented, where X represents Pro, Aze, Pip, or NMeA. Aze- and Pro-containing peptides fragment according to the well-established "proline effect" through selective cleavage of the amide bond N-terminal to the Aze/Pro residue to give y^n ions. In contrast, Pip- and NMA-fragment through a different mechanism, the "pipecolic acid effect," selectively at the amide bond C-terminal to the Pip/NMA residue to give b^n ions. Calculations of the relative basicities of various sites in model peptide molecules containing Aze, Pro, Pip, or NMeA indicate that whereas the "proline effect' can in part be rationalized by the increased basicity of the prolyl-amide site, the "pipecolic acid effect" cannot be justified through the basicity of the residue. Rather, the increased flexibility of the Pip and NMeA residues allow for conformations of the peptide for which transfer of the mobile proton to the amide site C-terminal to the Pip/NMeA becomes energetically favorable. This argument is supported by the differing results obtained for AAPAA versus AA(D-Pro)AA, a result that can best be explained by steric effects. Fragmentation of pentapeptides containing both Pro and Pip indicate that the "pipecolic acid effect" is stronger than the "proline effect."

Keywords: Peptide fragmentation, Proline effect, Non-protein amino acid, Collision-induced dissociation

Introduction

The discovery of the complete sequence for the human genome was a triumph for molecular biology and genetics research. Rather than an endpoint, this discovery launched the quest for the identification of all of the proteins that are coded by the genome. Research in proteomics has exploded in the past decade and tremendous strides are being taken toward a complete understanding of the proteomes of humans and other organisms. In part, the tremendous growth in the field has been made possible by sophisticated and highly automated mass spectrometry approaches to sequencing proteins and peptides [1–5]. So-called "bottom-up" proteomics involves enzymatic digests of the protein of interest followed by tandem mass spectrometric sequencing of the resulting peptides. Under low-energy collision-induced dissociation conditions, peptides fragment through several different mechanisms depending on the charge and number of basic residues in the peptide [6]. For peptides in which the number of ionizing protons is greater than the number of basic residues [7], the mobile proton model [8–10] is active and fragmentation is initiated by transfer of a proton to the amide residue to be cleaved. Most of the time, the

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site of the mobile proton is nonselectively determined and the peptide fragments to give a wide variety of fragment ions that can be used in computer-based sequencing algorithms [11–15].

Unfortunately, certain amino acid residues are known to promote selective cleavages that dominate the spectra and suppress other nonselective cleavages that can aid in peptide identification [16–32]. Examples of these selective cleavages include the "proline effect," a preference for cleavage N-terminal to proline residues [16, 17, 19, 20, 24–30] to give y_n^+ ions, where n represents the number of residues in the fragment counting from the C-terminus for y-type ions and from the N-terminus for b-type ions [33], the tendency for aspartic acid (D)- and glutamic acid (E)-containing peptides to fragment C-terminal to the acidic residues when no mobile proton is present [18, 20–23, 26, 29], and the recently discovered ornithine effect [32].

Numerous experimental and theoretical studies have been carried out in order to understand the origin and mechanism for the "proline effect" [16, 17, 19, 20, 24–30]. Early studies centered on the enhanced basicity of the proline residue as the main cause of the selective cleavage [16]. Vaisar and Urban investigated the fragmentation behavior of a single set of peptides AVXLG [X=Pro, Pip, and N-methylalanine, (NMeA)] in a triple quadrupole instrument [19]. In their analysis, they postulated that the instability of the b_3^+ oxazolone ion is the dominating factor in the "proline effect" rather than the enhanced proton affinity of proline. Schemes 1 and 2 show proposed mechanisms for y_3^+/b_2^+ and b_3^+ formation from AGPGA based on initial transfer of the mobile proton to the carbonyl group N-terminal to and C-terminal to the proline residue, respectively. In these schemes, the dissociation is broken into two steps: (1) Cleavage of the amide bond after transfer of the mobile proton to the amide N, and (2) decomposition of the b_n/y_m intermediate. If the b_n/y_m intermediate falls apart directly, b_n^+ ions will be formed [33]. Alternatively, the nascent protonated oxazolone b_n^+ ion can transfer a proton to the neutral fragment to give y_m^+ ions [6, 33]. The enhanced basicity of the secondary amine of proline will make this proton transfer energetically favorable and will thus lead to enhanced y-type ions.

While a bicyclic oxazolone containing a proline residue will certainly be strained, Grewal et al. determined that the bicyclic b_2^+ oxazolone ion derived from GPG is only 11.8 kJ/mol higher than the non-bicyclic b_2^+ ion derived from PGG [25]. Our recent IRMPD work showed that the b_2^+ ion GP formed from protonated tripeptides is formed with only an oxazolone structure [34]. In contrast, AP, IP, and VP b_2^+ ions are formed with both oxazolone and diketopiperazine structures, with the oxazolone dominating [34].

The preference for y_m^+ ions over b_n^+ ions in proline containing peptides is clearly related to the enhanced basicity of the proline-containing y-fragment in step 2 of the decomposition. What is less clear is why the mobile proton is initially directed to the amide carbonyl N-terminal to the Pro residue preferentially. Paizs and co-workers recently completed a combined experimental/theoretical study on AAXPA peptides (X=A, S, L, V, F) using a Qq-TOF instrument and density functional theory calculations [30]. All of the AAXPA peptides gave strong y_2^+ ions that dominated the mass spectrum, except AASPA, in which water loss peaks from the protonated peptide and y_3^+ could compete with y_2^+ formation. Computationally, they found that in AAAPA, the most favorable amide protonation site in the all trans form of the peptide is the amide oxygen between A_3 and P_4. In addition, they found that the nitrogen atom of that amide is more basic than the nitrogen atoms of alanine amides by about 16–24 kJ/mol. Their conclusion was that the "proline residue stabilizes protonation at the nitrogen of the Ala-Pro amide bond" [30]. Protonation at the amide between P_4 and A_5 was determined to be less favored by 29 (oxygen atom) and 16 kJ/mol (nitrogen atom). They concluded that in the early phase of the dissociation, the enhanced basicity of the proline residue favors transfer of the mobile proton to the amide N-terminal to that residue such that the amide bond N-terminal to the proline residue is cleaved. In the latter part of the dissociation, the decomposition of the b_n/y_m intermediate, a proton transfer occurs from the nascent b-type ion to the nitrogen atom in the proline ring resulting in a preference for y-type ions.

These results explain the observation that proline-containing peptides tend to fragment N-terminal to the proline
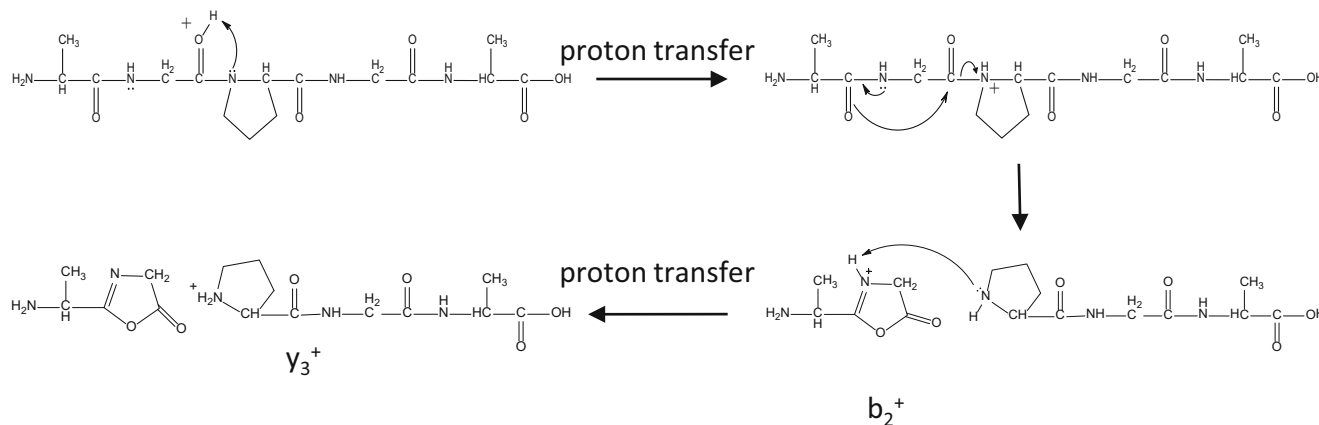
Scheme 1.
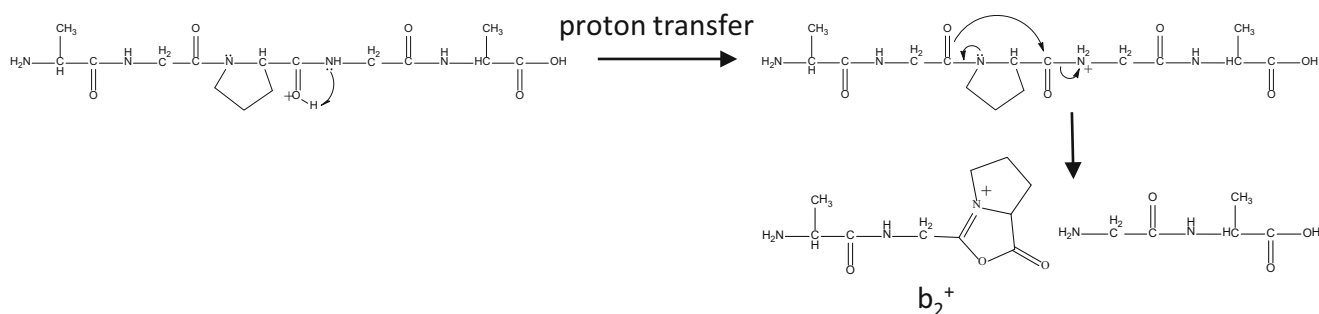
Scheme 2.

residue to give y-type ions. Unfortunately, they do not address Vaisar and Urban's finding that VAPipLG and VA(NMeA)LG peptides fragment selectively to form b_3^+ ions [19]. In an effort to reconcile the available data on the "proline effect," we have undertaken a systematic study of the fragmentation behavior of small peptides containing proline (**2**), its six-membered ring analog – pipecolic acid (Pip, **3**), its four-membered ring analog – azetidine-2-carboxylic acid (Aze, **1**), or N-methylalanine (NMeA, **4**), a residue with a secondary amine but no ring. The proton affinities of the four proline analog residues are 933, 937, 944, and 931 kJ/mol, for **1** to **4**, respectively and, therefore, the residues should behave similarly with respect to the energetics of proton transfer [35, 36].

We show here that Vaisar and Urban's results for VAXLG appear to be general in that proline-containing peptides give enhanced y_n^+ ions, whereas Pip- and NMeA-containing peptides give enhanced b_n^+ ions, giving rise to a "pipecolic acid effect." In addition, we show that peptides containing the highly strained Aze residue fragment similarly to proline-containing peptides to give enhanced y_n^+ ions. Finally, analysis of pentapeptides containing more than one proline analog indicates that the "pipecolic acid effect" is more selective than the "proline effect." These results are consistent with a mechanism that involves transfer of the mobile proton to different residues for Aze/Pro and Pip/NMeA peptides.

Experimental

Peptides were synthesized via standard solid-phase synthesis techniques starting with Fmoc-protected amino acids attached to Wang resin beads [37]. The C-terminal amino acid was deprotected and then coupled with an Fmoc-protected amino acid. The process was repeated until the desired peptide is complete. The peptide was then cleaved from the resin using trifluoroacetic acid and triisopropylsilane. The resulting peptides were dissolved in slightly acidified (1% HOAc) 50:50 (v:v) H_2O: CH_3OH and diluted to ca. 1×10^{-5} M. The following peptides were synthesized in this manner: AAXAA, AAAXA, AGXGA, AVXLG, and AXPAA, where X=(Aze, Pro, Pip, or NMeAla). In addition AAProPipA, AProPipAA, AA(D-Pro)AA, AV(D-Pip)LG, APipAProA, and AProAPipA were synthesized by this procedure.

Mass spectrometry experiments were performed in two different tandem mass spectrometers. Peptides AAXAA, AAAXA, AGXGA, AVXLG, AXPAA, and AAXXA and AXAXA were examined in an LCQ-Deca (Finnigan, San Jose, CA) quadrupole ion trap instrument. Peptides AA(D-Pro)AA and AV(D-Pip)LG were examined in a Velos Pro (ThermoScientific, San Jose, CA) dual-linear ion trap instrument. In both instruments, protonated peptide ions were generated from electrospray ionization of directly-infused peptide solutions. Electrospray and ion focusing conditions were adjusted to maximize the signal for the protonated peptides of interest. For LCQ experiments, peptide ions were isolated with q_z=0.250, and the isolation width was set to allow for maximum signal of the mono-isotopic parent ion, while still maintaining isolation. The activation amplitude was adjusted in order to reduce the intensity of the parent M+H$^+$ ion to less than 5% of its original intensity and was in the range of 25%–35%. For the Velos Pro experiments, an identical q_z value of 0.250 was used, compared with the LCQ Deca, with an isolation width of 1.0 m/z. The L-forms of each peptide were also run to determine whether results were comparable to those from the LCQ Deca. We saw no substantial differences between data obtained in the LCQ and Velos Pro for AAPAA and AVPLG.

Theoretical Methods

All quantum calculations were carried out using the Gaussian98 and Gaussian98W suites of programs [38]. Starting geometries for acetyl-azetidine-2-carboxylic acid (**5**), acetyl-proline (**6**), acetyl-pipecolic acid (**7**), acetyl-NMeA (**8**), N-acetyl-Pro-NHMe (**9**), N-acetyl-Pip-NHMe (**10**), and N,N-dimethylacetamide (NNDMA) were obtained using the GMMX conformational search algorithm in PCModel [39]. The GMMX routine generates random conformations through rotations about all single bonds of a molecule. The MMX force field is used to evaluate the energy of each random conformation. For this study, all conformations within 32 kJ/mol of the lowest energy conformer were used as starting structures for a series of ab initio and density functional theory calculations of increasing size. Ultimately, minimized geometries and harmonic vibrational frequencies were obtained at the B3LYP/6-31+G* level of theory [40, 41]. Single point energy calculations were carried out at the B3LYP/6-311++G(d,p) level at the B3LYP/6-31+G(d) geometries. Enthalpies at 298 K were

obtained using the B3LYP/6-311++G(d,p) energy, and ZPE and thermal corrections from unscaled harmonic vibrational frequency calculations.

The process was repeated for the above molecules protonated at the amide carbonyl oxygen and the amide nitrogen for **5**–**8** and NNDMA, and for the two carbonyl oxygen atoms for **9** and **10**. Relative proton affinities for the various sites were obtained directly from 298 K enthalpies for the neutral and protonated molecules according to Equation 1, where M and

$$PA = H_{298}(M) + H_{298}(H^+) - H_{298}(MH^+) \qquad (1)$$

MH^+ are the neutral and protonated molecule, respectively and $H_{298}(H^+)$ was taken as 5/2RT=12.3 kJ/mol. Absolute proton affinities for all species were also calculated via an isodesmic approach with NNDMA serving as the reference compound according to Equation 2.

$$NNDMAH^+ + M \rightarrow MH^+ + NNDMA \qquad (2)$$

The experimental proton affinity of NNDMA (carbonyl oxygen) was taken as 908.0 kJ/mol [42]. This level of theory performs well for amides, giving a raw calculated PA for the carbonyl oxygen in NNDMA of 907.9 kJ/mol.

Materials

All synthetic materials were purchased from commercial vendors and were used as supplied. Fmoc-Ala-Wang, Fmoc-Gly-Wang, and Fmoc-Ala were purchased from ChemPep, Inc. (Wellington, FL, USA). Fmoc-Gly, Fmoc-Val, Fmoc-Leu, and Fmoc-Pro, and Fmoc-NMA were purchased from Novabiochem (EMD, Millipore, Billerica, MA). Fmoc-Pip and Fmoc-Aze, and were purchased from Neosystems (Strasbourg, France). Solid phase synthesis reagents dimethylformamide, trifluoroacetic acid, piperidine, triisopropylsilane, and dichloromethane were purchased from SigmaAldrich (St. Louis, MO, USA) while 2-(6-chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU), and N,N,N'N'-tetramethyl-O-(1-H-benzoltriazol-1-yl)uronium hexafluorophosphate (HBTU) were purchased from Novabiochem (EMD, Millipore, Billerica, MA, USA).

Results and Discussion

Peptides with a Single Pro-Analog Residue

Our initial investigations centered on the fragmentation behavior of peptides AAXAA, where X=L-Pro, D-Pro, Pip, NMA, and Aze. Our goal was to extend the studies of Schwartz and Bursey [16] to include the four-membered ring analog Aze, the six-membered ring analog Pip, the non-cyclic amino acid NMeA, and D-Pro. Protonated peptides were generated using electrospray ionization and fragmented in one of the tandem mass spectrometers. Figure 1a and b show fragmentation

spectra for AA(Aze)AA and AA(Pro)AA at normalized collision energy of 30%.

As can be seen from these spectra, the dominant peaks are from cleavage N-terminal to the proline analog residue to give y_3^+ ions. Small peaks are also seen corresponding to b_2^+, b_3^+, and b_4^+ for AA(Aze)AA and b_4^+ for AAPAA. In contrast, Fig. 1c and d show that fragmentation of AA(Pip)AA and AA(NMeA)AA gives dominant peaks corresponding to b_3^+, with smaller y_3^+ and b_4^+ peaks. It is difficult to rationalize these results simply in terms of the proton affinities of the proline analogs or in terms of the proton affinities of the proline analog residues in the peptides.

The proton affinities of the four proline analog residues are 933, 937, 944, and 931 kJ/mol, for **1**–**4**, respectively [35, 36]. As all four residues have nearly the same proton affinity, they should behave similarly with respect to proton transfer to form y_n^+ type ions upon the decomposition of the b_n/y_n intermediate in the final step of Scheme 1. Therefore, the differences in the fragmentation pathways for the AAXAA peptides must be arising from geometric factors that allow for transfer of the mobile proton to different amide residues in the different peptides. In an effort to begin modeling the basicities of the different amide sites in the pentapeptides, we calculated the proton affinities for the amide carbonyl oxygen and nitrogen atoms for the N-acetyl-amides of the four proline analogs, N-acetyl-Aze (**5**), N-acetyl-Pro (**6**), N-acetyl-Pip (**7**), and N-acetyl-NMeA (**8**) at the B3LYP/6-311++G(d,p)//B3LYP/6-31+G(d) level of theory isodesmic to N,N-dimethylacetamide (NNDMA). We also performed calculations on small model peptides N-acetyl-Pro-NHMe (**9**) and N-acetyl-Pip-NHMe (**10**) to see if there was a preference for protonation on the N-terminal or C-terminal amide oxygen atoms that would support the differences in observed fragmentation patterns for Pro- and Pip-containing peptides. Table 1 shows the total electronic energy, thermal correction, and 298 K enthalpies for the lowest energy conformers of **5**–**10**, and their protonated forms. Tabulated values of these quantities for all the low-energy conformers for **5**–**10** and **5H⁺**–**10H⁺** that we considered are given in Table S1 of Supporting Information. Low-energy structures for **5**–**10** and **5H⁺**–**10H⁺** are given in Figures S1–S6 of Supporting Information. As can be seen from the data in Table 1, the carbonyl oxygen atoms of **5**–**8** are more basic than the amide nitrogen atoms, as expected. Further, the PAs for the amide oxygen atoms in **5**–**7** are the same (916, 914, and 913 kJ/mol for **5**, **6**, and **7**, respectively) and the PA for **8** is just slightly lower at 905 kJ/mol. The uncertainties in these values are not straightforward to calculate, but based on previous studies in our laboratory with a variety of amines, alcohols, and amino acids, they are on the order of ±10–12 kJ/mol. These calculations suggest that there is no thermochemical preference for protonation of the N-terminal amide carbonyl for Pro or Aze residues compared with Pip or NMeA residues. In order to initiate amide bond cleavage, the proton must ultimately end up on the amide nitrogen. As can be seen from Table 1, the amide nitrogen atoms also have similar proton affinities with **7** and **8** having slightly larger PAs than **5** and **6**. These predictions
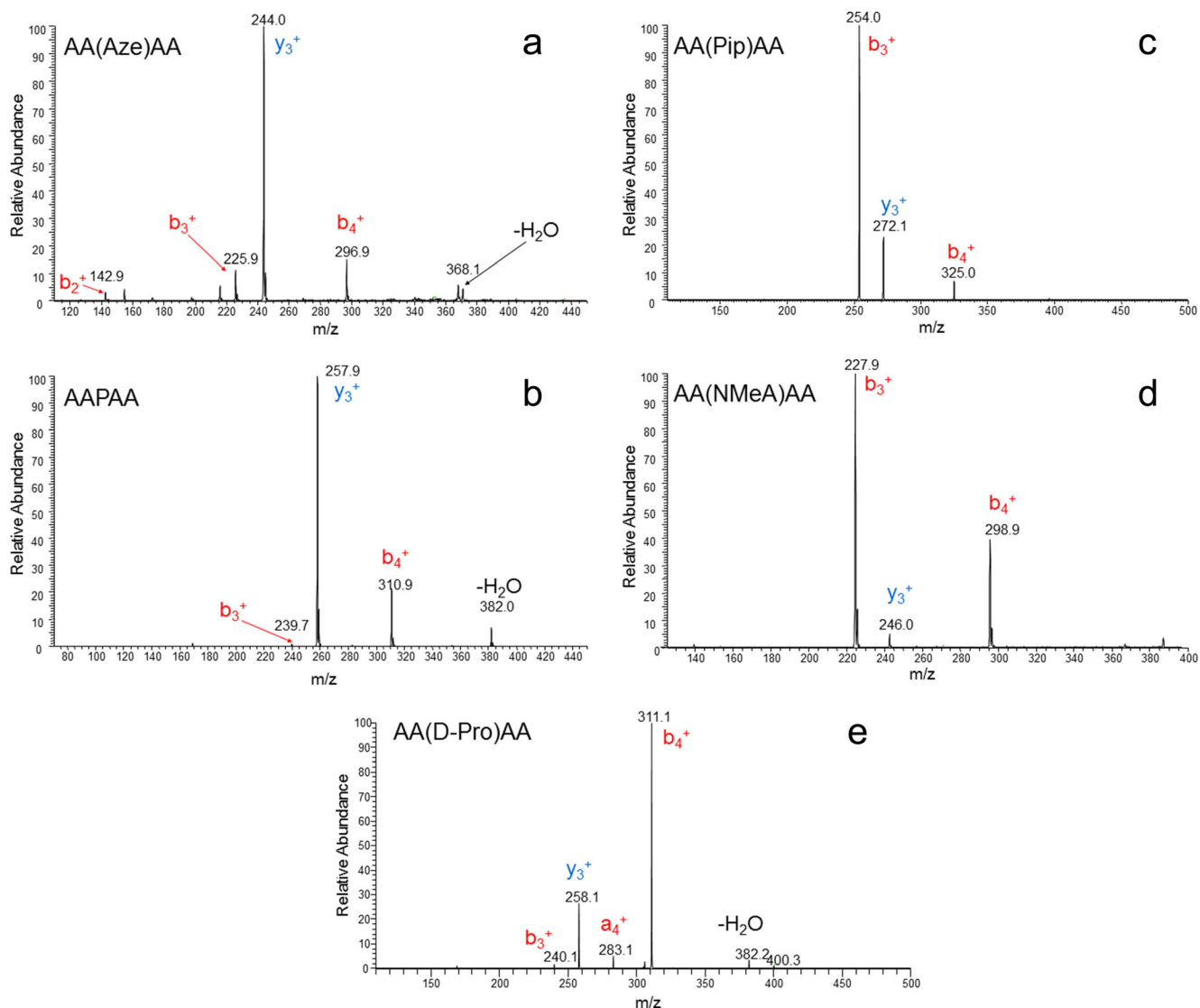
Figure 1. Product ion fragmentation spectrum for (**a**) AA(Aze)AA, (**b**) AAPAA, (**c**) AA(Pip)AA, (**d**) AA(NMeA)AA, and (**e**) AA(D-Pro)AA

suggest that if the proton affinity of the amide group N-terminal to proline is the dominating factor as suggested by Paizs and co-workers, AA(Pip)AA and AA(NMeA)AA should also fragment to give dominant y-type ions.

For model peptide **9**, *N*-acetyl-Pro-NHMe, the lowest energy structure (Figure S5 in Supporting information) for the protonated molecule involves the proton localized on the N-terminal amide oxygen with a strong hydrogen bond to the C-terminal amide oxygen atom. The orientation of the C-terminal amide is formally *cis* with respect to the N-terminal amide. Attempts to localize the proton to the C-terminal amide oxygen resulted either in proton transfer back to the N-terminal amide or in a rotation of single bonds to a *trans*-like species with hydrogen bonding between the N-terminal amide carbonyl oxygen atom and the C-terminal amide N-hydrogen atom that lies nearly 40 kJ/mol higher in energy. This indicates that the N-terminal amide is much more basic than the C-terminal amide. An isodesmic proton affinity of 948.4 kJ/mol is

predicted for the N-terminal amide for **9**, which is greatly enhanced relative to **5** because of the strong intramolecular hydrogen bond with the C-terminal amide oxygen. For model peptide **10**, *N*-acetyl-Pip_NHMe, the lowest-energy structure (Figure S6 in Supporting Information) for the protonated molecule is similar to that for **9** with a *cis*-like configuration and the hydrogen shared between the two oxygen atoms. We were able to locate an energy minimum hydrogen bonded structure with the hydrogen formally on the C-terminal amide oxygen that lies ca. 7 kJ/mol higher in energy, suggesting that the two amides have more similar proton affinities. In addition, the PA for the N-terminal amide for **10** is predicted to be 944.8 kJ/mol, which is the same as **9** within the errors of the calculations.

The computational predictions on these small model systems indicate that transfer of the mobile proton to the amide C-terminal to the pipecolic acid residue may be energetically competitive with transfer to the N-terminal amide. However, the model system suggests that both b_n^+ and y_m^+ should be

Table 1. Total Electronic Energies, Thermal Corrections and 298 K Enthalpies, and Proton Affinities for Selected Molecules

Molecule	E_{elec}[a]	ΔH_{therm}[b]	H_{298}	PA (raw)[c]	PA(iso)[c,d]
Acet-Aze	−514.641983	0.163571	−514.478412		
Acet-AzeH$^+$ (O prot)	−515.001335	0.176519	−514.824815	915.7	915.8[d]
Acet-AzeH$^+$ (N prot)	−514.978468	0.176603	−514.801865	855.4	855.5[d]
Acet-Pro	−553.995558	0.194518	−553.801040		
Acet-ProH$^+$ (O prot)	−554.354206	0.207397	−554.146809	914.0	914.1
Acet-ProH$^+$ (N prot)	−554.329344	0.207554	−554.121790	848.3	848.4
Acet-Pip	−593.318232	0.225198	−593.093035		
Acet-PipH$^+$ (O prot)	−593.676540	0.238221	−593.438186	912.7	912.8
Acet-PipH$^+$ (N prot)	−593.657977	0.238070	−593.419907	864.4	864.5
Acet-NMeA	−515.874719	0.187091	−515.687628		
Acet-NMeAH$^+$ (O prot)	−516.229756	0.199830	−516.029926	904.9	905.0
Acet-NMeAH$^+$ (N prot)	−516.215077	0.199959	−516.015118	866.0	866.1
NNDMA	−287.918386	0.139230	−287.779024		
DDNMAH$^+$ (O prot)	−288.275386	0.152921	−288.122465	907.9	−
DDNMAH$^+$ (N prot)	−288.255145	0.152898	−288.102247	854.8	−
Acet-Pro-NHMe	−573.440763	0.237223	−573.203540		
Acet-Pro-NHMeH$^+$ (O$_1$)	−573.811403	0.249010	−573.562393	948.3	948.4
Acet-Pro-NHMeH$^+$ (O$_2$)	−573.797791	0.249414	−573.547762	910.0	910.1
Acet-Pip-NHMe	−612.764159	0.267744	−612.496415		
Acet-Pip-NHMeH$^+$ (O$_1$)	−613.133298	0.279400	−612.853898	944.7	944.8
Acet-Pip-NHMeH$^+$ (O$_2$)	−613.131053	0.279739	−612.851314	938.0	938.1

[a]Values in Hartrees obtained at B3LYP/6-311++G**//B3LYP/6-31+G* level of theory
[b]Values in Hartrees obtained from unscaled harmonic frequencies obtained at B3LYP/6-31+G* level of theory
[c]Values in kJ/mol
[d]Obtained from isodesmic reaction 2 with NNDMA (PA=908 kJ/mol) as reference

formed readily for Pip-containing peptides, which is not observed in the experiments. It is likely that the model system is not capturing the complex interplay between the full structure of the pentapeptides and their dissociation behavior. Calculations on the full pentapeptide systems are beyond the scope of this paper.

Nevertheless, the experimental results point to a clear preference for transfer of the mobile proton N-terminal to the Aze and L-Pro residues and C-terminal to the Pip and NMeA residues for pentapeptides AAXAA. Figure 1e shows that substitution of D-Pro for L-Pro gives b_4^+ as the dominant peak in the fragmentation spectrum. Clearly, this difference in fragmentation behavior cannot be arising from the basicity of the residue. Rather the conformational preferences of the D-Pro- and L-Pro-containing peptides are different and are influencing the relative accessibility and energetics of the various amide sites. We postulate that the increased flexibility of the Pip and NMeA residues is allowing for peptide conformations that direct the mobile proton C-terminal to those residues and that the rigidity of Aze and Pro residues locks them into conformations such that only transfer to the N-terminal amide is favorable. For AA(D-Pro)AA, the peptide must favor a different conformation that allows selective transfer of the mobile proton to the amide residue of the fourth position of the peptide.

To test the generality of these findings and to shed further light on the systems recently investigated by Vaisar and Urban [19, 43], and by Paizs [30], we repeated the experiments with the peptides AAAXA, AVXLG, and AGXGA, X=Aze, Pro, Pip, and NMeA. Schwartz and Bursey published a fragmentation spectrum for protonated AAAPA using FAB ionization in an EbqQ instrument in their 1992 paper (Figure 2 in Reference [16]) that shows a classic "proline effect" product distribution

[16]. The y_2^+ ion is the dominant peak in this spectrum that also shows minor (less than 25% of the base peak) peaks for b_2^+, b_3^+, and y_3^+. Paizs and co-workers presented a spectrum from an ESI Q-TOF instrument that shows a similar result (Figure 1 in Reference [30]). Our ESI ion trap spectrum (not shown) is basically identical to those for earlier studies showing the expected "proline effect" peaks. Fragmentation spectra for AAA(Aze)A, AAA(Pip)A are shown in shown in Figure S7a and b of Supporting Information. These figures show that AAA(Aze)A also gives a strong y_2^+ "proline effect" peak and an equally intense y_3^+ peak. Substitution of the more flexible six-membered ring analog Pip results in a different spectrum in which fragmentation C-terminal to the secondary amide residue (b_4^+) dominates. These results indicate that the pipecolic acid residue also induces selective cleavages in peptides. This "pipecolic acid effect" seems to be at least as selective as the "proline effect."

In collaboration with the PNNL group, we previously used a data mining scheme to analyze over 28,000 peptide fragmentation spectra to try to identify structural motifs responsible for different MS/MS fragmentation intensity patterns [26, 29]. These studies found that several motifs, including CysPro, ProPro, and GlyPro, tend to resist cleavage N-terminal to Pro, whereas other residue combinations such as IlePro, LeuPro, and ValPro show enhanced cleavage N-terminal to Pro. Our findings for AAXAA and AAAXA are similar to those of Vaisar and Urban's study of the fragmentation of AVXLG peptides in which they found that for X=Pro, y_3^+ ions dominate, whereas for X=Pip and NMeA, b_3^+ ions dominate [19, 43]. We have repeated and extended these studies to include the Aze residue and also D-Pip to see if the enhanced cleavage at VX affects the selectivity differences. Figure 2 a–e show
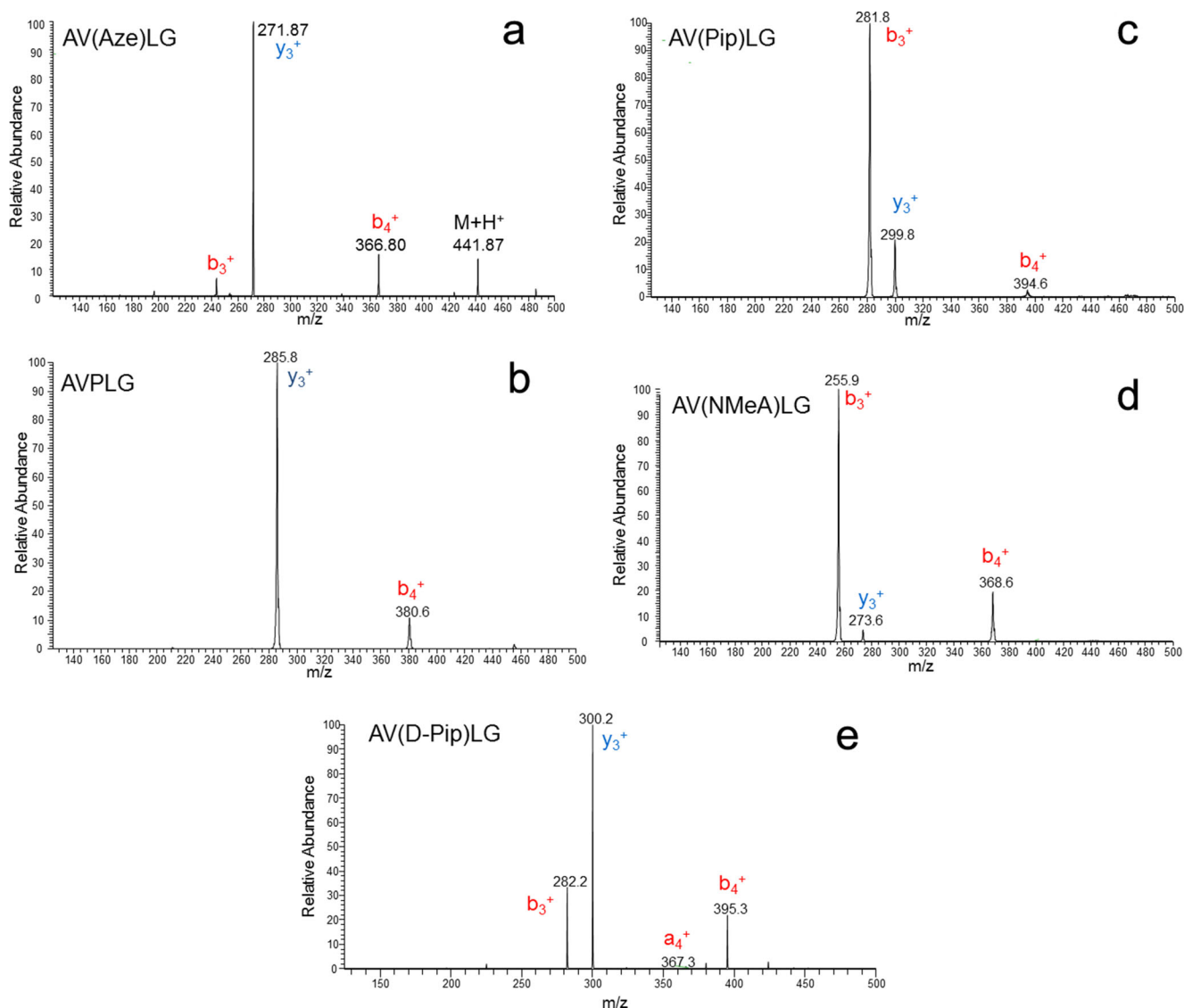
Figure 2. Product ion fragmentation spectrum for (a) AV(Aze)LG, (b) AVPLG, (c) AV(Pip)LG, (d) AV(NMeA)LG, and (e) AV(D-Pip)LG

fragmentation spectra for AVXLG peptides, with X=Aze, Pro, Pip, NMeA, and D-Pip. As with the AAXAA and AAAXA peptides, for X=Aze and Pro, the y_3^+ ion is selectively produced according to the "proline effect," whereas for X=Pip and NMeA, we observe b_3^+ as the dominant fragment ion corresponding to the "pipecolic acid effect." Interestingly, when the D-Pip residue is substituted for L-Pip, we see a change in relative abundance between b_3^+ and y_3^+ ions and an increase in b_4^+, which also cannot be arising from the basicity of the residue. Again, the conformational preferences of the different peptides are affecting the transfer of the mobile proton to different amide sites in the molecule. Interestingly, we do not see any noticeable effect of the enhanced cleavage of the VX residue; the activation amplitudes needed to reduce the M+H$^+$ parent ion to less than 5% are of the same general magnitude (25%–35% in the LCQ) as those needed to fragment the peptides with the AX residue combination. Similarly, we synthesized all four AGXGA peptides to test for effects from the

suppressing GX residue combination and saw only the minor effect that the M − H$_2$O fragments were noticeably larger in these fragmentation spectra compared with AAXAA, AAAXA, and AVXLG peptides (spectra shown in Figure S8a–d in Supporting Information). We do, however, still see the preference for b_3^+ fragment ions in Pip/NMeA peptides and for y_3^+ ions in Aze/Pro peptides, further demonstrating the "pipecolic acid effect."

Peptides with Two Pro-Analog Residues

Fragmentation of pipecolic acid-containing peptides clearly shows enhanced cleavage C-terminal to the Pip residue. In order to gauge the relative selectivity of the "proline effect" versus the "pipecolic acid effect," several pentapeptides were synthesized containing both a Pro and a Pip residue. Figure 3a–d show results from low-energy fragmentation of AProPipAA, AAProPipA, AProAPipA, and APipAProA. Fragmentation of
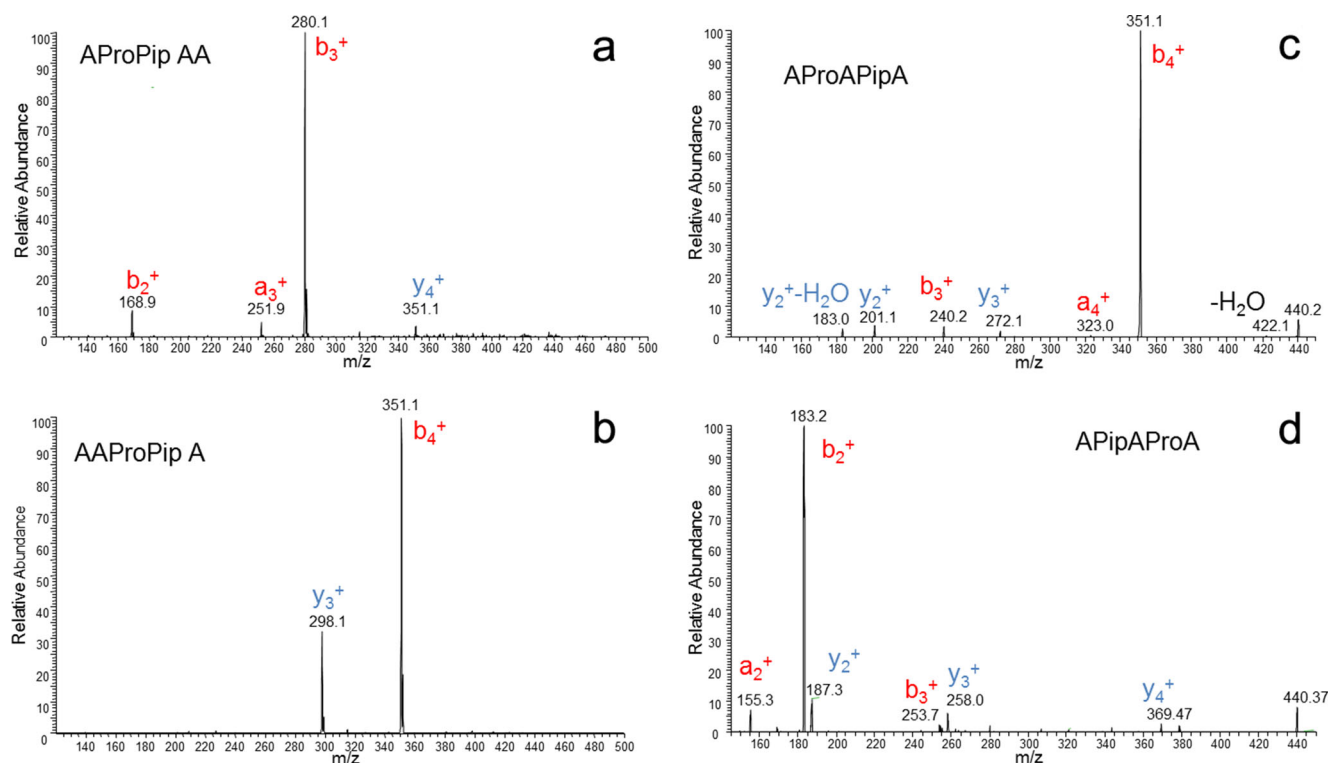
Figure 3. Product ion fragmentation spectrum for (**a**) A(Pro)(Pip)AA, (**b**) AA(Pro)(Pip)A, (**c**) A(Pro)A(Pip)A and (**d**) A(Pip)A(Pro)A

AProPipAA shows essentially one product peak corresponding to the b_3^+ ion, which is the preferred product for the "pipecolic acid effect." A small peak corresponding to y_4^+ is also seen that corresponds to cleavage N-terminal to the proline residue. Similar results are seen for AAProPipA, in which the pipecolic acid-directed cleavage product b_4^+ is the base peak and a y_3^+ peak is seen at 30% relative abundance. These data suggest that cleavage C-terminal to Pip is more selective than N-terminal cleavage to Pro. That is, the "pipecolic acid effect" is stronger than the "proline effect." Results from AProAPipA and APipAProA confirm this, as the "pipecolic acid" b_n^+-type ions are preferred over the y_n^+ type ions from the 'proline effect" for both peptides and demonstrate that the residues do not need to be adjacent in order to show the effect.

We also synthesized all four AXPAA peptides, and their fragmentation spectra are shown in Fig. 4. The ProPro motif was shown to resist cleavage in the data mining study [26, 29], and interestingly, Fig. 4a shows that APPAA does not show selective cleavage, but rather displays a strong b_n^+ series (n=2–4) as well as y_3^+ and y_4^+. Similar nonselective cleavages are found for A(Aze)PAA, as shown in Fig. 4b. In contrast, Fig. 4c and d show that A(Pip)PAA and A(NMeA)PAA exhibit selective cleavage from direction of the mobile proton to the amide group C-terminal to the Pip/NMeA residue consistent with the "pipecolic acid effect." For these peptides, the data could also be explained by initial transfer of the mobile proton to the amide group N-terminal to the proline residue consistent with the "proline effect" (step 1 of Scheme 1). Due to the quaternary nitrogen in the A(Pip)/A(NMeA) oxazolone b_2^+ ions, there is

no proton to be transferred to the Pro-containing neutral y-fragment in step 2, so b_2^+ is the dominant fragment ion in A(Pip)PAA and A(NMeA)PAA.

Fragmentation Pathways

It is clear from these results that there are two different mechanisms operating for Aze/Pro peptides and Pip/NMeA peptides. For Aze/Pro peptides, the b_2–y_3 pathway [6] is active as shown in Scheme 1. The rigidity of the Pro-reside and enhanced basicity of the amide on the N-terminal side of the proline residue directs the mobile proton to that site where it initiates cleavage [30]. Upon bond cleavage, the enhanced basicity of the prolyl amine nitrogen makes proton transfer from the nascent b_2^+ ion energetically favorable and results in y_3^+ ions. This mechanism is also active for Aze-containing peptides. Calculations indicate that the amide oxygen and nitrogen atoms in N-acetyl-Aze have similar basicity to those in N-acetyl-Pro and, therefore, the mobile proton is directed to the N-terminal side of the Aze residue giving selective bond cleavage on that side. Though the Aze amine nitrogen is slightly less basic than that of proline, it is still basic enough that proton transfer from the nascent b_2^+ ion is energetically favorable, and predominantly y_3^+ ions are observed for pentapeptides with Aze at position three. Our result from AA(D-Pro)AA shows that the most significant factor in these fragmentations is the conformational preference of the peptide itself, with different fragments dominating for D- versus L-Pro in AAXAA. AAPAA and AA(D-Pro)AA will adopt
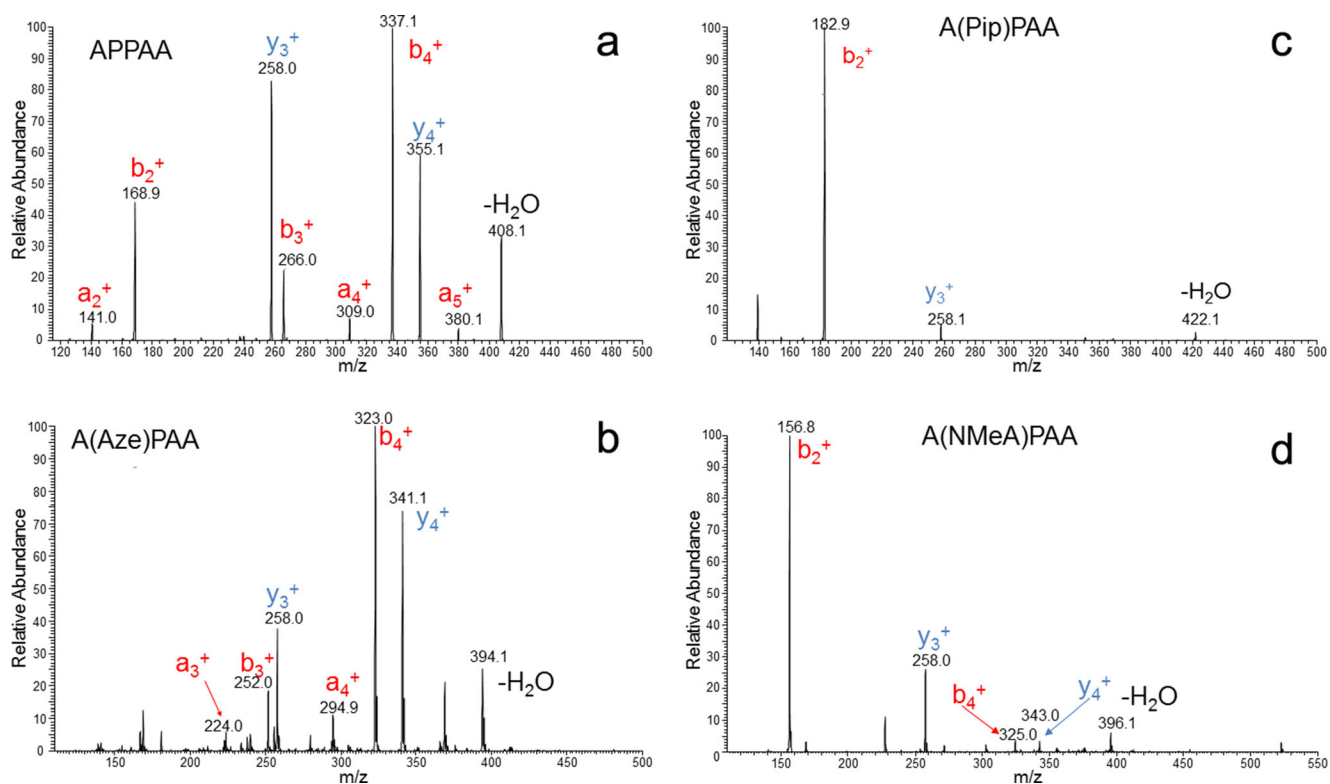
Figure 4. Product ion fragmentation spectrum for (**a**) APPAA, (**b**) A(Aze)PAA, (**c**) A(Pip)PAA, and (**d**) A(NMeA)PAA

different low-energy conformations and the relative rigidity of the Pro residue will contribute to the selectivity in the proton transfer step of the mechanism.

In contrast, in Pip-containing pentapeptides, the amide bond C-terminal to the Pip residue is preferentially cleaved. This indicates that the mobile proton is being directed C-terminal to the Pip residue and that the b_3–y_2 mechanism is active (Scheme 2). In this case, a bicyclic oxazolone species is formed with a tetra-coordinate nitrogen atom in the ring. Once the b_3/y_2 intermediate is formed through bond cleavage, there is no proton on the oxazolone to transfer to the neutral y-fragment and, therefore, the complex falls apart to give exclusively the b_3^+ fragment ion. As the basicity of the amide oxygen atoms are the same in *N*-acetyl-Pro and *N*-acetyl-Pip and the basicity of the amide nitrogen atom is actually somewhat larger in *N*-acetyl-Pip relative to *N*-acetyl-Pro, the basicity of tertiary amide of Pip cannot be controlling the site of protonation. Rather, it seems likely that the increased flexibility of Pip and NMeA residues leads to peptide conformations that promote favorable proton transfer to the amide C-terminal to the residue. The differences in the fragmentation behavior of AVPipLG and AV(D-Pip)LG can only be explained by differing conformational preferences for the pentapeptides.

Conclusions

We have analyzed the fragmentation spectrum of a set of peptides that contain proline, its four-membered ring

analog (Aze), its six-membered ring analog (Pip), or an acyclic analog (NMeA). Aze- and Pro-containing peptides fragment according to the well-known "proline effect" selectively N-terminal to the Pro/Aze residue to give y_n^+ ions. In contrast, Pip- and NMeA-containing peptides fragment selectively C-terminal to the Pip/NMeA residue to give b_n^+ ions. Analysis of the energetics of model compounds indicate that these results cannot be rationalized based only on the enhanced basicity of the secondary amine in the amino acid residue as has been previously proposed.

For Aze/Pro peptides, the rigidity of the Aze/Pro residues and the enhanced basicity of the amide moiety N-terminal to the residue causes preferential protonation at that site and subsequent cleavage. Decomposition of the b_n/y_m intermediate proceeds via proton transfer to the secondary amine of the Pro/ Aze residue to give y-type ions. The situation for Pip/NMeA peptides is more complicated. Calculations predict that the basicity of the N-terminal amide oxygen and nitrogen atoms in peptide models containing Pip and NMeA is the same or even slightly greater than those in model compounds containing Pro and Aze. Despite this, selective cleavage N-terminal to Pip/NMeA-containing peptides is not the dominant mechanism for fragmentation. Rather, these peptides undergo selective cleavage C-terminal to the Pip/NMeA residue to give b_n^+ ions. This observation is attributed to greater flexibility of these residues, which allows for peptide conformations that promote favorable transfer of the mobile proton to the amide C-terminal to the Pip/NMeA residue.

Acknowledgments

References

1. Angel, T.E., Aryal, U.K., Hengel, S.M., Baker, E.S., Kelly, R.T., Robinson, E.W., Smith, R.D.: Mass spectrometry-based proteomics: existing capabilities and future directions. Chem. Soc. Rev. **41**, 3912–3928 (2012)
2. Roepstorff, P.: Mass spectrometry based proteomics, background, status, and future needs. Protein Cell **3**, 641–647 (2012)
3. Sabido, E., Selevsek, N., Aebersold, R.: Mass spectrometry-based proteomics for systems biology. Curr. Opin. Biotechnol. **23**, 591–597 (2012)
4. Kelleher, N.L.: Status of mass spectrometry-based proteomics and metabolomics in basic and translational research. Biochemistry **52**, 3794–3796 (2013)
5. Zahang, Y., Fonslow, B.R., Shan, B., Baek, M.-C., Yates, J.R.: Protein analysis by shotgun/bottom-up proteomics. Chem. Rev. **113**, 2343–2394 (2013)
6. Paizs, B., Suhai, S.: Fragmentation pathways of protonated peptides. Mass Spectrom. Rev. **24**, 508–548 (2004)
7. Kapp, E.A., Schutz, F., Reid, G.E., Eddes, J.S., Moritz, R.L., O'Hair, R.A.J., Speed, T.P., Simpson, R.J.: Mining a tandem mass spectrometry database to determine the trends and global factors influencing peptide fragmentation. Anal. Chem. **75**, 6251–6264 (2003)
8. Cox, K.A., Gaskell, S.J., Morris, M., Whiting, A.: Role of the site of protonation in the low-energy decompositions of gas-phase peptide ions. J. Am. Soc. Mass Spectrom. **7**, 522–531 (1996)
9. Dongre, A.R., Jones, J.L., Somagyi, A., Wysocki, V.H.: Influence of peptide composition, gas-phase basicity, and chemical modification on fragmentation efficiency: evidence for the mobile proton model. J. Am. Chem. Soc. **118**, 8365–8374 (1996)
10. Wysocki, V.H., Tsaprailis, G., Smith, L.L., Breci, L.A.: Mobile and localized protons: a framework for understanding peptide dissociation. J. Mass Spectrom. **35**, 1399–1406 (2000)
11. Eng, J.K., McCarmack, A.L., Yates III, J.R.: An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. J. Am. Soc. Mass Spectrom. **5**, 976–989 (1994)
12. Perkins, D.N., Pappin, D.J.C., Creasy, D.M., Cottrell, J.S.: Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis **20**, 3551–3567 (1999)
13. Craig, R., Beavis, R.C.: TANDEM: Matching proteins with tandem mass spectra. Bioinformatics **20**, 1466–1467 (2004)
14. Elias, J.E., Gibbons, F.D., King, O.D., Roth, F.P., Gygi, S.P.: Intensity-based protein identification by machine learning from a library of tandem mass spectra. Nat. Biotechnol. **22**, 214–219 (2004)
15. Li, W., Ji, L., Goya, J., Tan, G., Wysocki, V.H.: SQID: an intensity-incorporated protein identification algorithm for tandem mass spectrometr. J. Proteome Res. **10**, 1593–1602 (2011)
16. Schwartz, B.L., Bursey, M.M.: Some proline substituent effects in the tandem mass spectrum of protonated pentaalanine. Biol. Mass Spectrom. **21**, 92–96 (1992)
17. Loo, J.A., Edmonds, C.A., Smith, R.D.: Tandem mass spectrometry of very large molecules. 2. Dissociation of multiply charged proline-containing proteins from electrospray ionization. Anal. Chem. **65**, 425–438 (1993)
18. Yu, W., Vath, J.E., Huberty, M.C., Martin, S.A.: Identification of the facile gas-phase cleavage of the Asp-Pro and Asp-Xxx peptide bonds in matrix-assisted laser desorption time of flight mass spectrometry. Anal. Chem. **65**, 3015–3023 (1993)
19. Vaisar, T., Urban, J.: Probing the proline effect in CID of protonated peptides. J. Mass Spectrom. **31**, 1185–1187 (1996)
20. Schaaff, T.G., Cargile, B.J., Stephanson Jr., J.L., McLuckey, S.A.: Ion trap activation of the $(M + 2H)^{2+}$ and $(M + 17H)^{17+}$ ions of human hemoglobin b-chain. Anal. Chem. **72**, 899–907 (2000)

21. Tsaprailis, G., Somogyi, A., Nikolaev, E.N., Wysocki, V.H.: Refining the model for selective cleavage at acid residues in arginine-containing protonated peptides. Int. J. Mass Spectrom. **195/196**, 467–479 (2000)
22. Sullivan, A.G., Brancia, F.L., Tyldesley, R., Bateman, R., Sidhu, K., Hubbard, S.J., Oliver, S.G., Gaskell, S.J.: The exploitation of selective cleavage of singly protonated peptide ions adjacent to aspartic acid residues using a quadrupole orthogonal time-of-flight mass spectrometer equipped with a matrix-assisted laser desorption/ionization source. Int. J. Mass Spectrom. **210/211**, 665–676 (2001)
23. Huang, Y., Wysocki, V.H., Tabb, D.L., Yates, J.R.I.: The influence of histidine on cleavage c-terminal to acidic residues in doubly protonated tryptic peptides. Int. J. Mass Spectrom. **291**, 233–244 (2002)
24. Breci, L.A., Tabb, D.L., Yates, J.R.I., Wysocki, V.H.: Cleavage N-terminal to proline: analisis of a database of peptide tandem mass spectra. Anal. Chem. **75**, 1963–1971 (2003)
25. Grewal, R.N., El Aribi, E.H., Harrison, A.G., Siu, K.W.M., Hopkinson, A.C.: Fragmentation of protonated tripeptides: the proline effect revisited. J. Phys. Chem. B **108**, 4899–4908 (2004)
26. Huang, Y., Triscari, J.M., Tseng, G.C., Pasa-Tolic, L., Lipton, M.S., Smith, R.D., Wysocki, V.H.: Statistical characterization of the charge state and residue dependence of low-energy CID peptide dissociation patterns. Anal. Chem. **77**, 5800–5813 (2005)
27. Harrison, A.G., Young, A.B.: Fragmentation reactions of deprotonated peptides containing proline. The proline effect. J. Mass Spectrom. **40**, 1173–1186 (2005)
28. Unnithan, A.G., Myer, M.J., Veale, C.J., Dannell, A.S.: MS/MS of protonated polyproline peptides; the influence of N-terminal protonation on dissociation. J. Am. Soc. Mass Spectrom. **18**, 2198–2205 (2007)
29. Huang, Y., Tseng, G.C., Yuan, S., Pasa-Tolic, L., Lipton, M.S., Smith, R.D., Wysocki, V.H.: A data mining scheme for identifying peptide structural motifs responsible for different MS/MS fragmentation intensity patterns. J. Proteome Res. **7**, 70–79 (2008)
30. Bleiholder, C., Suhai, S., Harrison, A.G., Paizs, B.: 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). J. Am. Soc. Mass Spectrom. **21**, 1032–1039 (2011)
31. Zhang, Q., Perkins, B., Tan, G., Wysocki, V.H.: The role of proton bridges in selective cleavage of Ser-, Thr-, Cys-, Met-, Asp-, and Asn-containing peptides. Int. J. Mass Spectrom. **300**, 108–117 (2011)
32. McGee, W.M., McLuckey, S.A.: The ornithine effect in peptide cation dissociation. J. Mass Spectrom. **48**, 856–861 (2013)
33. Roepstorff, P.: Proposal for a common nomenclature for sequence ions in mass spectra of peptides. Biomed. Mass Spectrom. **11**, 601 (1984)
34. Gucinski, A.C., Chamot-Rooke, J., Steinmetz, V., Somogyi, A., Wysocki, V.H.: Influence of N-terminal residue composition on the structure of proline-containing b_2^+ ions. J Phys. Chem. A **117**, 1291–1298 (2013)
35. Kuntz, A.F., Boynton, A.W., David, G.A., Colyer, K.E., Poutsma, J.C.: Proton affinities of proline analogs using the kinetic method with full entropy analysis. J. Am. Soc. Mass Spectrom. **13**, 72–81 (2002)
36. Tsang, Y., Wong, C.C.L., Wong, C.H.S., Cheng, J.M.K., Ma, N.L., Tsang, C.W.: Proton and potassium affinities of aliphatic and *N*-methylated aliphatic a amino acids: effect of alkyl chain length on relative stability of K$^+$-bound Zwitterionic complexes. Int. J. Mass Spectrom. **316/318**, 273–283 (2012)
37. Chan, W.C., White, P.D.: Fmoc Solid Phase Peptide Synthesis: A Practical Approach. Oxford University Press, New York (2000)
38. Millam, J.M., Daniels, A.D., Kudin, K.N., Strain, M.C., Farkas, O., Tomasi, J., Barone, V., Cossi, M., Cammi, R., Mennucci, B., Pomelli, C., Adamo, C., Clifford, S., Ochterski, J., Petersson, G.A., Ayala, P.Y., Cui, Q., Morokuma, K., Malick, D.K., Rabuck, A.D., Raghavachari, K., Foresman, J.B., Cioslowski, J., Ortiz, J.V., Baboul, A.G., Stefanov, B.B., Liu, G., Liashenko, A., Piskorz, P., Komaromi, I., Gomperts, R., Martin, R.L., Fox, D.J., Keith, T., Al-Laham, M.A., Peng, C.Y., Nanayakkara, N., Challacombe, M., Gill, P.M.W., Johnson, B., Chen, W., Wong, M.W., Andres, J.L., Gonzalez, C., Head-Gordon, M., Replogle, E.S., Pople, J.A.: *Gaussian 98* ver. A.9. Gaussian, Inc, Pittsburgh (1998)
39. *PCModel* Serena Software (2006)

40. Becke, A.D.: Density functional thermochemistry. III. The role of exact exchange. J. Chem. Phys. **98**, 5648–5652 (1993)

41. Lee, C., Yang, W., Parr, R.G.: Development of the Colle-Salvetti correlation energy formula into a functional of the electron density. Phys. Rev. B **37**, 785–789 (1988)

42. Hunter, E.P., Lias, S.G.: Evaluated gas phase basicities and proton affinities of molecules: an update. J. Phys. Chem. Ref. Data **27**, 3–656 (1998)

43. Vaisar, T., Urban, J.: Low-energy collision-induced dissociation of protonated peptides. Importance of an oxazolone formation for a peptide bond cleavage. Eur. Mass Spectrom. **4**, 359–364 (1998)