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Gas-phase structures and proton affinities of N-terminal proline containing b_2^+ ions from protonated model peptides

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ABSTRACT

In this study, we investigated the effect of the second amino acid identity of hexapeptides on gas-phase structures and the proton affinities of N-terminal proline containing b_2^+ ions produced from the fragmentation of b_6^+ ions under low-energy collision-induced dissociation (CID) tandem mass spectrometry (MS/MS). It should be noted that, among all other fragments, the b_2^+ and nominally b_4^+ (AAAA) ions ($[M+H]^+ \rightarrow b_6^+ \rightarrow b_2^+$ (PX^+) + b_4^+ ($AAAA^+$)) were mainly considered in this study. This is a unique example of consecutive cleavage of b_6^+ ions which fragments to b_2^+ and nominal b_4^+ ions. All structural and proton affinity calculations for b_2^+ ions were carried out with the B3LYP/6-31+G(d,p) level of theory. The study utilized C-terminal amidated model peptides consisting of PAAAAA-NH_2 and PXAAAA-NH_2 where X is phenylalanine (F), glutamic acid (E), tryptophan (W), and histidine (H) residue. Two main structural isomers of b_2^+ ions, namely oxazolone and diketopiperazine, have been considered for the computations. The results demonstrated that the proton affinities of oxazolone isomers of PX are greater than its diketopiperazine isomers. Higher correlation coefficient is calculated if the structure of PX is considered as oxazolone rather than diketopiperazine isomer. Additionally, a linear fit is observed between intensity ratio (PX/AAAA) and calculated proton affinities of PX ions. Additionally, MS/MS results revealed that the relative intensities of b_2^+-PA, PF, and PE- ions are lower compared to the relative intensity of AAAA fragment ion. In contrast, b_2^+-PW and PH- ions have higher relative intensities compared to the AAAA ion. This behavior is explained by the proton affinities of fragment ions computationally.

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1. Introduction

Mass spectrometry (MS) is an essential tool in protein analysis and proteomics studies [1]. Upon low-energy collision-induced dissociation (CID), protonated/multiply-protonated peptides undergo fragmentation via cleavage of amide bonds to form N-terminal *b* and *a* and/or C-terminal *y*-type sequence ions [2,3]. Understanding the gas-phase structures of these sequence ions as well as their fragmentation reactions has played a vital role for correct and reliable peptide/protein sequencing [4]. It has been previously demonstrated that b_n^+ ($n = 2–4$) ions generally have an oxazolone structure (a five-membered ring) at the C-terminal end of the peptide [5,6]. However, in some cases, it has been shown that b_2^+ ions can also adopt a six-membered ring diketopiperazine structure [7,8]. The formation of these two isomeric structures entirely depends on the amino acid composition of the peptide sequence and peptide chain length. The appearance of oxazolone isomer rather than energetically more favorable diketopiperazine can be explained by the *cis–trans* isomerization of the peptide bond [9]. The requirement of *cis–trans* isomerization makes the diketopiperazine formation pathway kinetically controlled. Most of the studies revealed that the b_2^+ ions have an oxazolone structure [10–12]. Both IRMPD (infrared-multiphoton dissociation) and quantum chemical studies by Wysocki and co-workers [10] showed that structure of b_2^+ ion of AGG adopts an oxazolone structure. Additionally, Oomens et al. [11] demonstrated that b_2^+ ion of AAA (trialanine) possesses an oxazolone structure through IRMPD spectroscopy and DFT (density functional theory) calculations. Moreover, it has been reported that b_2^+ ion from two glycine containing peptides has an oxazolone structure by comparing its IRMPD spectrum with the theoretical structure [12].

O'Hair and co-workers have showed that the b_2^+ ions of HG and GH have similar CID mass spectra compared to the cyclic-GH [13]. However, experimental and computational studies revealed that the structure of HA-b_2^+ ion was found to be a mixture of an oxazolone and a diketopiperazine [14]. Recently, Gucinski et al. [15]

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have investigated the Xxx-Pro b_2^+ ions where Xxx is Gly, Ala, Ile, Val or His by using IRMPD, hydrogen-deutrium exchange (HDX) and DFT computations. The authors emphasized that b_2^+ ion structures of GP, AP, IP and VP are dominantly an oxazolone structure whereas HP-b_2^+ ion adopts a diketopiperazine structure.

In recent years, Harrison and co-workers reported that middle-sized b_n^+ ($n \geq 4$) ions undergo macrocyclization followed by ring-opening process in low-energy CID conditions [16,17]. Numerous studies have provided a better understanding of the cyclization chemistry of *b* ions [18–24]. It is worth noting here that the presence of proline residue may affect the structure of b_2^+ ion. In line with the previous works [15,25–30], the gas-phase fragmentation reactions of peptide ions are greatly influenced by the presence and the position of proline residue. It should be noted that proline has a unique structure among the 20 amino acids, where its amine nitrogen is bonded to two carbon atoms. This specific behavior observed for proline containing b_2^+ ion can be explained by proton affinity. Proton affinity issue needs to be well known in order to better understand the gas-phase fragmentation mechanism of protonated peptides. The literature on the gas phase basicities and proton affinities of all amino acids were well reviewed by Harrison [31]. In addition, the proton affinities of the amino acids and peptides have been investigated by numerous studies in detail by several groups [32–38]. Maksic and Kovacevic [39] and Paizs et al. [40] determined the proton affinity of the twenty amino acids with computational methods and compared with experimental results.

In the current work, we have studied the structures and proton affinities of N-terminal proline containing b_2^+ ions of PX (where X is A, F, E, W, or H). To our knowledge, this study is the first report on the computational investigation of the proton affinities of oxazolone and diketopiperazine isomers of N-terminal proline containing b_2^+ ions. The structures of neutral and protonated PX were also analyzed.

2. Experimental

2.1. Mass spectrometry details

The C-terminal amidated model peptides having a sequence of PAAAAA-NH_2, PFAAAA-NH_2, PEAAAA-NH_2, PWAAAA-NH_2, and PHAAAA-NH_2 were purchased from GL Biochem Ltd. (Shanghai, China). The peptides were designed as C-terminal amide form in order to obtain b_6 ion with the highest intensity. Approximately 1 mg of each of the solid peptide samples was dissolved to a concentration of 10^{-4} M in 1:1 (v/v) mixture of HPLC-grade MeOH and deionized H_2O.

All tandem mass spectrometry experiments were conducted on a LTQ XL linear ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. A 100 pmol μL^{-1} peptide solution prepared in 50:50:1 (v/v/v) MeOH/H_2O/HCOOH was introduced via infusion at a flow rate of 5 $\mu L\,min^{-1}$ with an incorporated syringe pump. The scan range was set to *m/z* 150–800 in the positive-ion mode and at least 400 scans were averaged in a profile mode for all MS^n stages. Helium was used as the collision gas for CID and also as a damping gas. Other instrumental parameters were as follows: spray voltage: +5.0 kV; heated capillary temperature: 300 °C; N_2 sheath gas flow rate: 10 (arbitrary units); activation time: 30 ms at each CID stage; isolation width (*m/z*) for precursor ions: 1.0 and 1.8 for each CID acquisition; normalized collision energy: 20–30% for the fragmentation of precursor ion. Data acquisition was carried out with Xcalibur (ver. 2.0) software system and all data were processed using Igor Pro Software package (WaveMetrics, Lake Oswego, OR) for presentation.

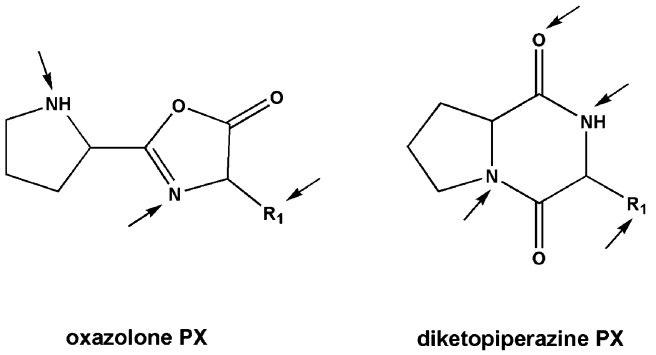

Fig. 1. The protonation sites for neutral oxazolone and diketopiperazine structures of b_2 PX ions (R_1 shows the side chain of X's amino acid).

2.2. Computational details

Two main structural isomers of b_2^+ ions, namely an oxazolone and a diketopiperazine, were considered for the computations. As Fig. 1 illustrates, the possible protonation sites are shown by arrows. All nitrogen atoms on the isomers are proposed as the protonation sites. The oxygen atoms on the diketopiperazine isomers and on the side groups, if present, were also protonated as well. Our calculations for the b_2 ions of GG and AA showed the isomers where the proton attachment site on the oxygen atoms of oxazolone ring is highly unfavored by 135.7 kJ/mol and 173.9 kJ/mol with respect to oxazolone nitrogen protonations, respectively. Previous works [12,41,42] have also not accounted for that possibility; therefore it was disregarded throughout the study.

Initial conformers of the neutral and protonated structures of b_2-PX (where X is A, F, E, W, or H) have been generated by using the molecular mechanics with Merck Molecular Force Field (MMFF) via Spartan'10 program [43]. In addition to that, different initial structures have been obtained from twisting the rotatable bonds manually. The geometry optimization was performed at the Density Functional Theory (DFT) with the Becke Three Parameter Lee, Yang, and Parr (B3LYP) [44] functional by using 6-31+G(d,p) basis set for all obtained structures. Furthermore, frequency calculations have been carried out on final structures with the same level of theory to characterize the optimum points and to obtain zero-point vibrational energies (ZPE). The Gaussian 09 program [45] was used for the all quantum chemical calculations.

Moreover, the gas-phase proton affinities of compound PX were found computationally by considering the following reaction:

$$PX_{(g)} + H_{(g)}^+ \rightarrow (PX)H_{(g)}^+$$

The following equation [39,46] was used in the proton affinity calculations where E_{elec} is electronic energy and ZPE is the zero point energy.

$$\text{Proton affinity of (PX)} = (E_{elec}(PX) + ZPE(PX)) - (E_{elec}(PXH^+) + ZPE(PXH^+))$$

3. Results and discussion

3.1. Experimental facts

The b_6^+ ions of PAAAAA-NH_2, PFAAAA-NH_2, PEAAAA-NH_2, PWAAAA-NH_2, and PHAAAA-NH_2 were selected and allowed to dissociate via low-energy CID-MS^3. Generally, each mass spectrum comprises of a series of *a* and *b* ions together with their small neutral losses (water and/or ammonia loss) (see Fig. S1, Supporting Information). In addition to these ions, the

b_6^+ ion simultaneously dissociates via cleavage of the second amide bond to form b_2^+ PX ions (where X is A, F, E, W, or H) and/or protonated AAAA fragment ion. This is a unique example of competing cleavage of b_2^+ vs. nominal b_4^+ ions during dissociations of b_6^+ ions under low CID condition. The added proton is shared by the two species in that cleavage (PX b_2^+ ion or AAAA fragment ion) and the specie having a higher proton affinity becomes more abundant in the mass spectra compared to the other.

The b_2^+ ions, namely PA (*m/z* 169), PF (*m/z* 245), PE (*m/z* 227), PW (*m/z* 284), and PH (*m/z* 235), and AAAA (*m/z* 285) fragment ions are labeled in the CID-MS^3 zoomed mass spectra, as Fig. 2 displays. The first three mass spectra exhibited that the relative intensities of b_2^+ PA, PF, and PE ions are lower than the protonated AAAA fragment. On the contrary, the relative intensities of b_2^+ PW and PH ions are higher compared to the protonated AAAA. These results clearly demonstrated that the proton affinity of AAAA fragment is greater than the proton affinities of b_2^+-PA, PF, and PE ions while b_2^+-PW and PH ions have high proton affinity than the AAAA fragment. The related proton affinity calculations and the conformational analysis for b_2^+ ions are given in detail in the subsequent sections.

3.2. Computational facts

3.2.1. Conformational analysis of the b_2^+ ions of PX

The conformational analysis of various b_2^+ PX ions has been performed in the present work. The structures and energies of all obtained conformers are given in Figs. S2–S6 and in Table S1, respectively. The represented energies are ZPE (zero point energy) corrected relative energies. The most favorable structures for both diketopiperazine and oxazolone isomers of b_2^+ ions of PX are shown in Fig. 3.

3.2.2. b_2 ions of PA

The most favorable conformer of b_2^+ PA ion was obtained as the oxygen protonated diketopiperazine structure (PA_dik_op2). In this structure, the proton is directed to the methyl group of the alanine residue (see Fig. 3). The second conformer of oxygen protonated diketopiperazine only differs for the location of hydrogen toward the proline cycle has 3.8 kcal/mol higher in energy than PA_dik_op2 (Fig. S2). Moreover, diketopiperazine isomers which have protons on the nitrogens were less feasible structures (about 17 kcal/mol); hence they were not taken into account for other b_2^+ PX ions throughout the study.

Among the oxazolone structures, the conformer where the proton was located on the proline ring (PA_oxa_pro_np) was 1.6 kcal/mol more stable than the proton on the oxazolone ring (PA_oxa_np) conformer. Furthermore, the neutral diketopiperazine structure (PA_dik) has 23.1 kcal/mol lower energy than the neutral oxazolone isomer (PA_oxa) whereas this difference became 8.7 kcal/mol for the protonated isomers. The most accessible neutral and protonated diketopiperazine and oxazolone structures of PA were almost the same (see Fig. 3). The protonation did not cause a structural change.

3.2.3. b_2 ion of PF

The oxygen protonated diketopiperazine conformer (PF_dik_op2) of PF b_2^+ ion where the proton was faced to the phenyl group was found as the most likely isomer (Fig. 3). It was 6.1 kcal/mol more stable than the second diketopiperazine conformer, in which the proton was pointed to the proline ring (PF_dik_op1) (Fig. S3). Furthermore, protonated proline conformer of oxazolone form of PF b_2^+ ion (PF_oxa_pro_np) was almost isoenergic with the oxazolone protonated isomer (PF_oxa_np). The proton always liked to be neighbors with an electron rich group, including oxygen, nitrogen or phenyl, etc.

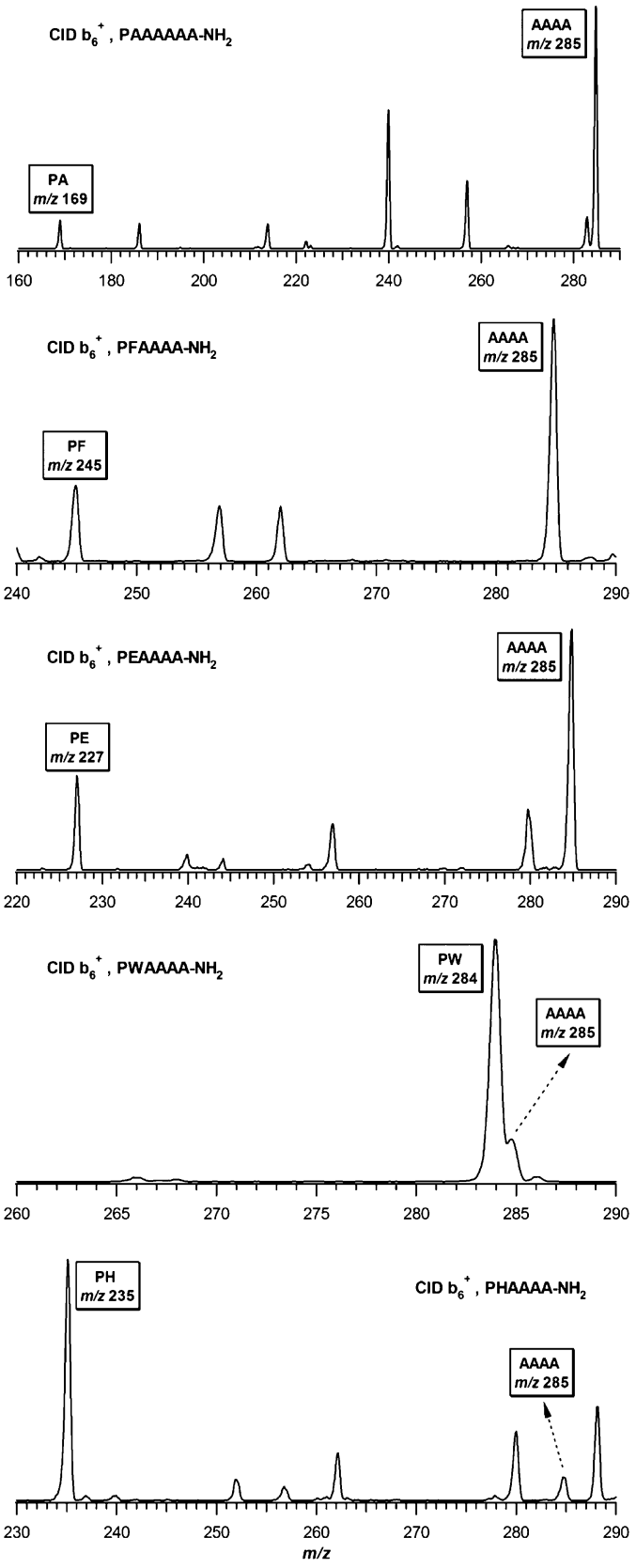

Fig. 2. Comparison of the zoomed CID mass spectra of b_6^+ ions of PAAAAA-NH_2, PFAAAA-NH_2, PEAAAA-NH_2, PWAAAA-NH_2, and PHAAAA-NH_2. The PX (X is A, F, E, W or H) stands for b_2^+ ion.

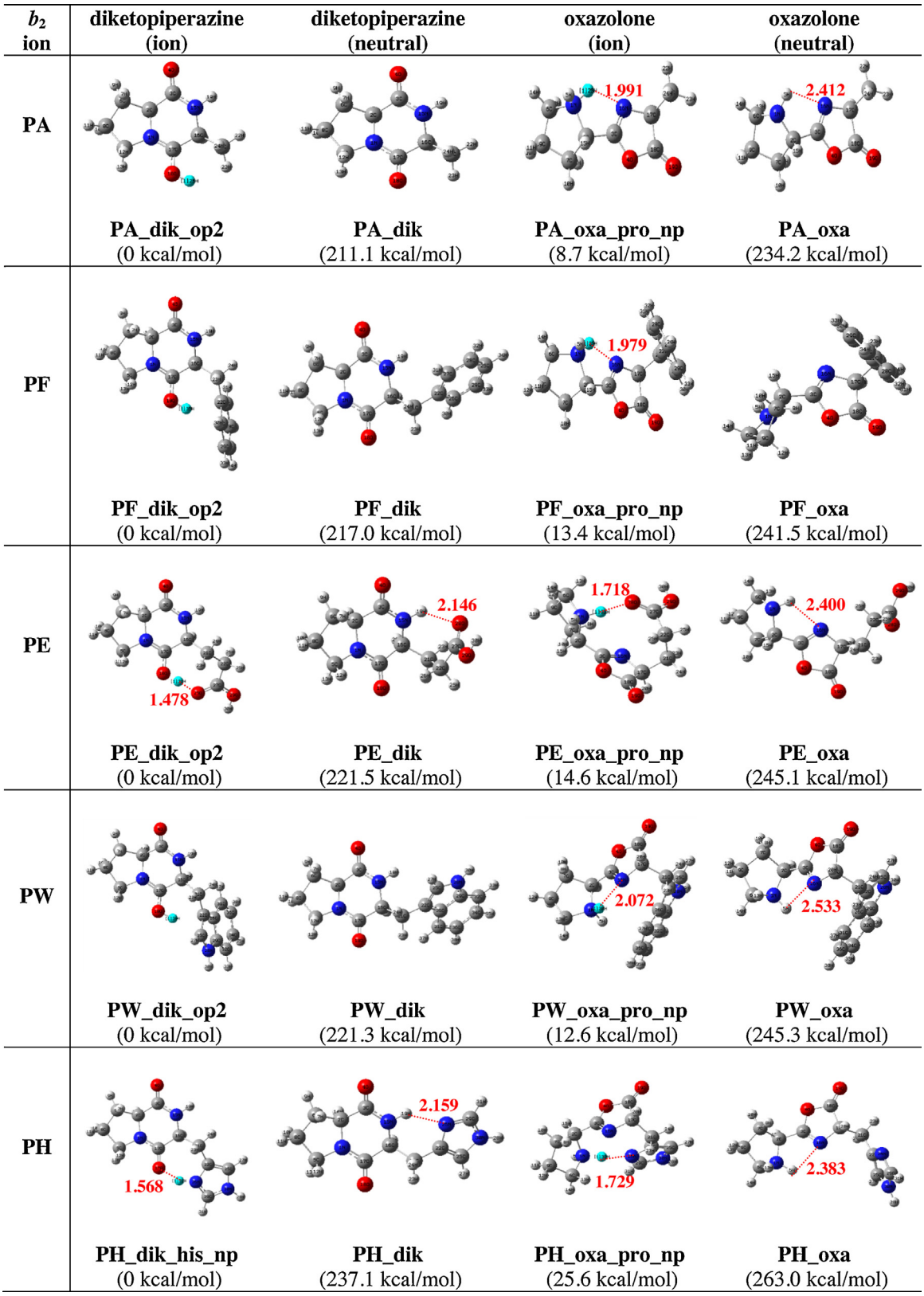

Fig. 3. The most favorable structures of diketopiperazine and oxazolone isomers for neutral and protonated b_2 PX ions (the values in parentheses are the relative zero point corrected electronic energies).

Additionally, the neutral diketopiperazine structure was 24.6 kcal/mol more stable than the neutral oxazolone isomer. However, this energy difference was lowered to 13.4 kcal/mol for protonated ions.

When the most stable neutral diketopiperazine (PF_dik) was protonated from its oxygen, the phenyl group was faced to the protonated oxygen. When neutral oxazolone (PF_oxa) was protonated from nitrogen of proline ring, it was directed to the nitrogen atom

of the oxazolone ring. Proton prefers to be close to an electron rich moiety, either phenyl group or nitrogen atom.

3.2.4. b_2 ion of PE

In this part, the structures of b_2 ion which consist of glutamic acid and proline residues were analyzed (see Fig. 3). The oxygen protonated diketopiperazine (PE_dik_op2) isomer in which the proton directed to the carboxylic acid oxygen was found as the most favorable structure. The next conformer is protonated on the oxygen of diketopiperazine faced to the proline (PE_dik_op1) which differs 5.4 kcal/mol in energy (Fig. S4). The carboxyl group protonated diketopiperazine (PE_dik_glu_op) and oxazolone (PE_oxa_glu_op) isomers were the least likely conformers. Moreover, nitrogen protonated proline isomer (PE_oxa_pro_np) has only 0.9 kcal/mol lower energy than the nitrogen protonated the oxazolone structure (PE_oxa_np).

The neutral diketopiperazine structure (PE_dik) was 23.6 kcal/mol more stable than the neutral oxazolone isomer. However, this energy difference was decreased to 14.6 kcal/mol when they are protonated. When the neutral diketopiperazine was protonated from its oxygen, the oxygen on the side chain of glutamic acid was rotated to the opposite side and faced to the protonated oxygen. On the other hand, when the neutral oxazolone (PE_oxa) was protonated from the nitrogen of proline residue, the carboxyl oxygen and proton became a neighbor.

3.2.5. b_2 ion of PW

Once again, the oxygen protonated diketopiperazine (PW_dik_op2) structure where the proton was faced to the tryptophan side chain moiety was the most stable isomer of the b_2^+ ion of PW (Fig. 3). This isomer was 7.9 kcal/mol and 19.7 kcal/mol more favorable than protonated diketopiperazine isomer (PW_dik_op1) and protonated tryptophan isomer (PW_dik_trp_np), respectively (Fig. S5). In addition, the proton usually prefers to be on either nitrogen of proline ring or nitrogen of oxazolone ring for the oxazolone conformers. However, the proton does not like to be on the tryptophan for neither oxazolone nor diketopiperazine conformers. The energy order of the protonated oxazolone structures was 12.6 kcal/mol, 14.9 kcal/mol and 43.1 kcal/mol for the protonated proline isomer (PW_oxa_pro_np), the nitrogen protonated oxazolone (PW_oxa_np) and protonated tryptophan isomer (PW_oxa_trp_np), respectively. Additionally, the neutral diketopiperazine structure was 24.0 kcal/mol more likely than the neutral oxazolone isomer. This energy difference was 12.6 kcal/mol for the most stable protonated ions. When the neutral diketopiperazine (PW_dik) was protonated from its oxygen, the side chain of tryptophan was rotated to the protonated oxygen. However, the neutral and protonated oxazolone structure of PW have similar structure.

3.2.6. b_2 ion of PH

When histidine and proline form a b_2^+ ion, the proton likes to be on the histidine unit for diketopiperazine isomer (PH_dik_his_np) rather than diketopiperazine ring unlike the other PX within this work (Fig. 3). The diketopiperazine protonated isomers (PH_dik_op1 and PH_dik_op2) were destabilized by 16–18 kcal/mol with respect to most likely conformer (Fig. S6). Despite of the previous PX b_2^+ ions, the conformer in which the proton is directed to the proline side has 2 kcal/mol less energy than the conformer where it is directed to X unit. In contrast to diketopiperazine isomers, the proton could be located in one of the nitrogen atoms of the three rings (pro, his, oxa) for the oxazolone structures. They are in competition and their energy difference is in the range of 0.8–1.3 kcal/mol.

Additionally, the neutral diketopiperazine structure (PH_dik) was 25.9 kcal/mol more stable than the neutral oxazolone isomer (PH_oxa). This energy difference was almost the same for the protonated ions. When the neutral diketopiperazine was protonated from nitrogen of the histidine, the side chain of histidine was rotated about 180° and faced to the oxygen of the diketopiperazine structure which makes a strong hydrogen bond. While the neutral oxazolone was protonated from nitrogen of proline, again the side chain of histidine was twisted to the protonation site in which the proton was surrounded by the three nitrogen atoms.

3.3. Conformational analysis of the b_2 ions of PX (X = A, F, E, H, or W)

In all b_2 ions of PX, the oxygen protonated diketopiperazine (PX_dik_op2) was the most favorable structure, where the proton was directed to the X except PH ions (Fig. 3). On the other hand, proton does not like to be on the side chain of X for neither diketopiperazine nor oxazolone conformers except PH case. In the PH cations, the histidine protonated diketopiperazine (PH_dik_his_np) structure was the most feasible isomer.

Furthermore, the proton usually prefers to be on either nitrogen of proline ring (PX_oxa_pro_np) or nitrogen of oxazolone ring (PX_oxa_np) for oxazolone isomers of PX b_2^+ ions. The former one has only 0.1–2.3 kcal/mol lower in energy; therefore they are in competition.

The energies of most favorable neutral diketopiperazine and oxazolone isomers are compared for all PX b_2^+ ions and the results showed that the diketopiperazine structures have 23–26 kcal/mol lower in energy than the oxazolone isomer. All neutral PX b_2 ions have similar and high energy difference which reflects a large contribution of diketopiperazine isomers in a sample of neutral PX b_2. On the other hand, this difference falls into a wider range of 9–26 kcal/mol when they are protonated. The largest energy difference was observed in PH b_2^+ ion while the lowest one is in the PA b_2^+ ion for the protonated isomers.

3.4. Conformational analysis of the AAAA+

3.4.1. Proton affinities of b_2 ions

The zoomed CID mass spectra of the b_6^+ ions obtained from the C-terminal amidated model peptides having a sequence of PAAAAA-NH_2, PFAAAA-NH_2, PEAAAA-NH_2, PWAAAA-NH_2, and PHAAAA-NH_2 are shown in Fig. 2. It has been observed that the fragment intensities of b_2^+-PX ions and nominal b_4^+-AAAA ion peaks in the b_6^+ion mass spectra are influenced by changing the second amino acid residue of hexapeptides. The relative intensities of b_2^+ ions with PA (*m/z* 169), PF (*m/z* 245) and PE (*m/z* 227) sequence are low compared to the protonated AAAA (*m/z* 285) fragment ion. On the contrary, the b_2^+ PW (*m/z* 284) and PH (*m/z* 235) ions have higher relative intensities compared to the AAAA fragment ion. To understand why these intensities are changing by varying X, the peak intensity ratios (I) of PX and AAAA are compared with calculated proton affinities of PX which are given in Table 1 for diketopiperazine and oxazolone structures of b_2^+ ions of PX in the ascending order. In Fig. 4, $\ln(I_{PX}/I_{AAAA})$ are plotted as a function of proton affinities of PX for both oxazolone and diketopiperazine isomers. As the proton affinity of X increases, the fragment ion intensities increase too. The correlation coefficients for a linear fit indicated that oxazolone structure has much better correlation than diketopiperazine isomer. This observation supports that most of the b_2^+ ions of PX adopt oxazolone structure in mass spectrometry experiments. For that reason, the calculated proton affinities of oxazolone structures of PX and AAAA were compared.

Proton affinity of oxazolone isomer of AAAA was calculated as 232.6 kcal/mol in this work and there is perfect agreement with the one previously reported by Paizs et al. data [48]. The DFT results demonstrated that AAAA has higher proton affinity than that of

Table 1
The DFT proton affinities of the diketopiperazine and oxazolone structures of b_2^+ ions.

b_2^+ ions	Oxazolone structure	Diketopiperazine structure	PA_{oxa}-PA_{dik}[a]
PA	225.4	211.1	14.3
PF	228.2	217.0	11.2
PE	230.5	221.5	9.0
PW	232.7	221.3	11.4
PH	237.4	237.1	0.3
AH	236.7	234.7	2.0
HA	234.8	234.7	0.1
GG	217.6	200.3	17.3
AG	219.3	202.7	16.6
AA	221.4	204.5	16.9

[a] The "PA_{oxa}-PA_{dik}" refers the DFT proton affinity difference between diketopiperazine and oxazolone structures. The proton affinity values were given in kcal/mol.

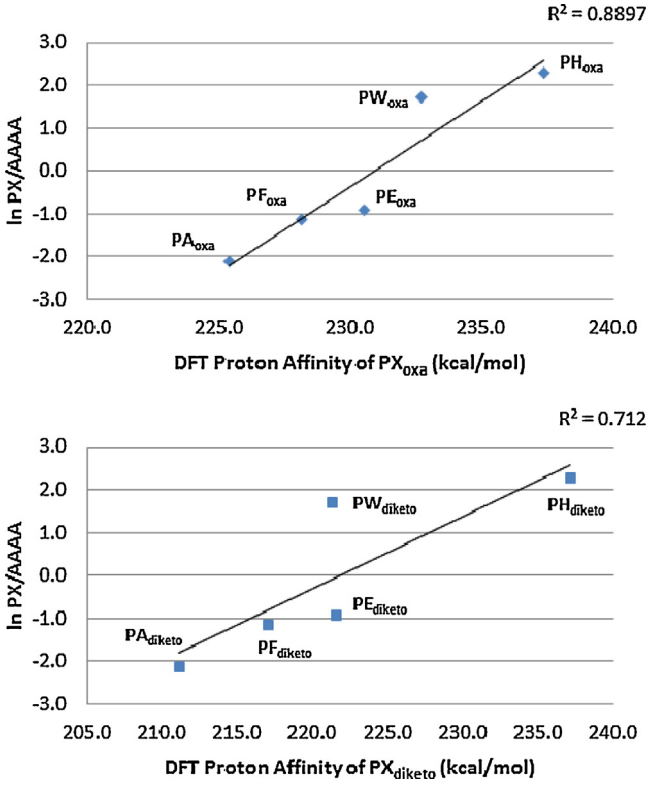

Fig. 4. The calculated peak intensity ratios (ln(PX/AAAA)) vs. calculated DFT proton affinities of the (a) PX ions (oxa) and (b) PX ions (diketo).

b_2^+ PA, PF, and PE ions. However, the proton affinity of AAAA was lower than the more basic residue containing b_2^+ PW and PH ions (see Table 1). The experimental results showed that MS/MS spectrum consists of both b_2^+-PX and AAAA fragment ions, and they are in competition with each other due to the their proton affinities. Moreover, the experimental results have been supported with DFT calculations. It has been clearly shown that proton affinities of two structures of b_2^+ PX ions have exhibited a similar trend: the proton affinities increased with increasing basicity of amino acids. Previous study by Nold et al. [47] demonstrated the experimental gas-phase proton affinities of the diketopiperazine structures of AA, LG, LA, LP and phenyl-5 oxazolone. They reported that the proton affinity of the diketopiperazine rises with increasing basicity of the amino acid. It was also noted that the diketopiperazine structures had lower proton affinity than the corresponding oxazolone isomers. Our results are in good agreement with these experimental observations. The proton affinities of the oxazolone structures were calculated approximately 11 kcal/mol higher than the

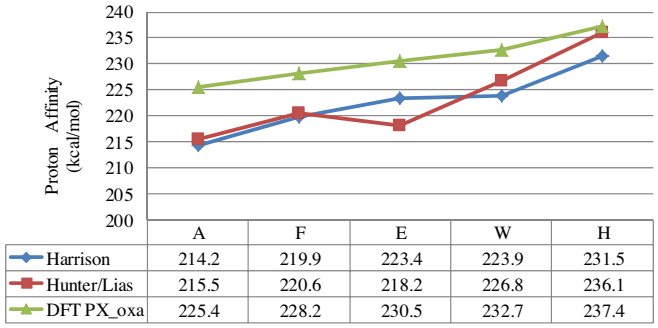

Fig. 5. The comparison of experimental proton affinities of single amino acids with calculated proton affinities of b_2^+ ions of PX. The experimental data of the proton affinity of single amino acids of A, F, E, W and H were taken from Harrison and Hunter/Lias, respectively [38].

diketopiperazine structures, except the PH b_2^+ ion where the proton affinities differ only 0.3 kcal/mol (see Table 1).

The comparison of experimental proton affinities of single amino acids with calculated proton affinities b_2^+ ions of PX was given in Fig. 5. The experimental data of the proton affinity of single amino acids of A, F, E, W and H were taken from Harrison and Hunter/Lias respectively [38]. DFT PX_oxa was referred to calculated proton affinities of oxazolone structures of b_2^+ ions of PX. Addition of proline residue from N-terminal side to the single amino acid to form the b_2^+ ions causes increase in proton affinities by 1.3–12.3 and 5.9–11.2 kcal/mol as shown by Hunter/Lias [38] and Harrison [31], respectively. Proton Affinity values of single amino acid and calculated dipeptide given by Hunter/Lias [38] were almost identical.

One of the additional results obtained in this work is the proton affinities of oxazolone isomers of b_2^+ PXs that are greater than its corresponding diketopiperazine isomers except PH. The b_2^+ PH has a unique property that has almost the same proton affinities of both isomers. Most of the experimental studies pointed out that b_2^+ ions have oxazolone structure in spite of diketopiperazine structure being energetically favored. Paizs et al. explained this behavior by performing potential energy surface calculations [9]. According to their calculation, formation of diketopiperazine structure contains *cis*-isomers along their fragmentation pathway, while *trans*-isomers are present in the oxazolone formation pathways. They also performed RRKM calculation to get the kinetic data and they found that y_1 ions formed on the diketopiperazine pathway contain a non-negligible (6–10 kcal/mol) activation energy.

To see whether there is a relation between proton affinities and the dominant structure in the mass spectra, we further calculated the proton affinities of b_2^+ ions which are exactly known to be an oxazolone or a diketopiperazine through IRMPD [10–12,14,49]. The b_2^+ ions of GG, AG, AA [10–12] have been stated as oxazolone whereas the b_2^+ ions of HA, AH, PH, PP [14,49] were a diketopiperazine or mixture of two isomers in the IRMPD. We computed the proton affinities of these b_2^+ ions for diketopiperazine and oxazolone isomers as given in Table 1. The proton affinities of HA, AH, PH for two isomers are almost same. On the other hand, the oxozolone isomers of b_2^+ ions of GG, AG, and AA have larger proton affinities than their diketopeperazine isomers. Proton affinity difference is in the range of 0.1–2.0 kcal/mol for the b_2^+ ions which have diketopiperazine or mixtures of two isomers based on IRMPD.

4. Conclusions

In this work, the effect of the second amino acid identity of hexapeptides on the gas-phase structures and proton affinities of N-terminal proline containing b_2^+ and b_4^+ (i.e. AAAA) ions produced from the fragmentation of b_6^+ ions under CID condition by using

tandem mass spectrometry, the gas-phase structures and proton affinities of N-terminal proline containing b_2 ions were investigated. It was observed that the intensity of AAAA fragment is higher than PA, PF, PE fragments; however it is lower than PW and PH fragments. In order to explain this experimental fact, proton affinities of fragment ions were computed considering both diketopiperazine and oxazolone structures of PX ions (X: A, F, E, W, H). A higher correlation coefficient was obtained when examining the PX as oxazolone compared to diketopiperazine structure, in a linear fit for the intensity ratios (ln(PX/AAAA)) of fragments ions as a function of the calculated proton affinities of the PX ions. The proton affinities of oxazolone isomers of PX were greater than diketopiperazine isomers. The proton affinity ratios of PX and AAAA are lower than 1, for PA, PF and PE and greater than 1 for PW and PH. More clearly, proton affinity of AAAA is higher than proton affinities of PA, PF and PE whereas it is lower than PW and PH fragments which supports experimental foundation.

The structures of GG, AG, and AA are predicted as oxazolone with the IRMPD experiments. However, HA, AH, PH fragments were found as diketopiperazine structure or a mixture of two isomers. Whether the structure of b_2^+ ions is oxazolone or diketo could be elucidated by comparing their proton affinities of those two isomers. The proton affinity of oxazolone isomer was found higher than diketo isomer for GG, AG, and AA. On the other hand, for HA, AH, PH fragments proton affinities of the two isomers are very close to each other. Further studies are needed to clarify.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijms.2015.09.011.

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