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Low energy CID and action IRMPD provide insights into a minor subpopulation of the gas-phase conformers of triply charged bradykinin

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ABSTRACT

Unique fragmentation of the IMS-resolved conformers of triply charged bradykinin is shown to occur at very low collision energies by quadrupole CID. For the major C conformation of bradykinin, the unique fragmentation appears to result from a subpopulation that has a different average CCS than the dominant C population that has been previously identified as TTC (*trans*, *trans*, *cis* at the three Pro residues). The three major gas-phase isomers exhibit minimal differences in fragmentation at collision energies greater than 12 eV, presumably because the amide bonds of the proline residues isomerize prior to dissociation at these higher energies. The differences in fragmentation observed in the low collision energy regime (<12 eV) are attributed to the conformational differences of the fragmenting subpopulations of the mobility resolved populations associated with the *cis* or *trans* isomerization state of the three proline residues. Action IRMPD of the b_2 ion supports a *cis*-Pro2 isomerization state for the subpopulation of conformation C fragmenting at low energies, assuming no isomerization was induced by the low energy CID. Substitution of N-methyl alanine, an acyclic proline mimic, independently, for each of the three prolines of bradykinin and low energy fragmentation of the minor subpopulation of conformation C allowed for tentative assignments of the isomerization state of two of the three proline resides of this subpopulation. Density functional theory calculations support the conclusion made from NMA-substitutions and together suggest that the subpopulation of conformation C is *cis* at Pro2 and *trans* at Pro7. It is not clear whether the minor subpopulation is a typical feature of triply charged bradykinin or whether unintended activation in the instrument used here produces this population. The results illustrate the utility of ion mobility coupled with low-energy CID for the determination of a minor subpopulation that was not visible by MS or IM alone.

1. Introduction

The nonapeptide bradykinin (RPPGFSPFR) is a vasodilator in the kinin class of peptides and has recently gained significant interest in the mass spectrometry community due the existence of a number of gas-phase, doubly and triply charged peptide conformers [1–3]. Through a series of ion mobility (IMS) studies in which gas-phase structure was examined following ionization from solutions containing variable amounts of organic and aqueous components, Clemmer and co-workers have established the existence of six or more gas-phase conformations of triply charged bradykinin [1–3]. Additional IMS studies using an alanine scanning approach demonstrated that the three gas-phase equilibrium conformations differ only in the *cis* or *trans* isomerization states of the three proline residues [1]. No differences in the fragmentation behavior of these conformations were reported, suggesting that activation induces isomerization prior to dissociation or that the conformers fragment identically [3]. In the present study, the *cis*/*trans* isomerization states of the proline residues in gas-phase, triply protonated bradykinin are separated by ion mobility and examined by low energy collision induced dissociation (CID). A series of Pro → N-methyl alanine (NMA) variants are additionally investigated by IM-CID in order to extract more information and the tentative assignments made from the fragmentation of the dominant drift time distribution are supported using DFT modeling.

CID fragmentation of protonated peptides most typically involves cleavage of the backbone amide bonds to generate series of "b" and "y" type ions. Action infrared multi-photon dissociation

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(IRMPD) spectroscopy, hydrogen/deuterium exchange (HDX), and computational modeling data all support the 5-membered oxazolone ring as the dominant b ion structure, although macrocyclic structures have also been shown to form [4–13]. These structures have received much interest because ring opening of the macrocycle can lead to scrambling the original peptide sequence [11,14–16]. The smallest of the b ions, the b_2 ion, has been particularly well studied because it can have either a diketopiperazine or oxazolone structure and the preferential formation of one of these structures over the other has been repeatedly shown to be related to amino acid sequence in the first three positions of the peptide [17–21]. The diketopiperazine structure is formed *via* nucleophilic attack by the N-terminus on the second carbonyl carbon and is typically more stable than the corresponding oxazolone structure. To date, systems featuring a basic residue in either of the first two positions, an amide side chain in the first position, or a proline in the second position all yield some amount of the diketopiperazine structure, a characteristic possibly related to these residues being able to play a role in proton bridging interactions that facilitate *cis/trans* isomerization [17,20,22,23].

Proline-containing peptides are well known to cause unusual gas phase fragmentation patterns [24–28]. Statistical studies in 2003 demonstrated that significantly enhanced fragmentation N-terminal to proline occurs in tryptic peptides [24,29,30]. Moreover, fragmentation C-terminal to proline residues has been shown to be significantly suppressed; the exceptions to this are specific circumstances such as b_2^+ formation when proline is the second residue or in polyproline stretches [29,30]. Because the prolyl ring restricts rotation around the N–Cα bond, the conformational flexibility of proline containing species is limited relative to non-proline containing species. In addition, the *trans–cis* isomerization barrier of the backbone amide bond of proline residues has been shown in solution-phase studies to be significantly lower than for non-proline residues and it is thought that this low barrier may contribute to the observation that diketopiperazines form in peptides containing a proline in the second position [18,23,30,31]. In addition, VUVPD of proline-containing peptides has been shown to yield unusual fragment ions depending on *cis/trans* isomerization of the proline amide bond [32].

2. Materials and methods

Bradykinin, diethyl ether, and electrospray solvents were purchased from Sigma-Aldrich (St. Louis, MO) and used without additional purification. Bradykinin variants were synthesized by solid-phase FMOC chemistry and purified by liquid/liquid extraction using diethyl ether and H_2O. Precursor peptide was dissolved in 49:49:2 H_2O:methanol:acetic acid electrospray solvent at a concentration of 10 μM and introduced into the gas phase using a nanospray ionization source composed of a platinum wire and pulled glass capillary. Ion mobility and fragmentation studies were performed on a Waters Synapt G2 ion mobility quadrupole time of flight (TOF) mass spectrometer which is composed of three traveling wave ion guides (TWIG) [33]. The first guide (trap) and third guide (transfer) are used as CID devices and the middle TWIG is used for ion mobility separations. Potential differences across all front end optics and guide voltages were kept below 2 V to minimize isomerization and fragmentation of the triply charged precursor.

Traditional ion mobility experiments separate ions of different shape and charge by exploiting their variable mobility through drift tubes to which a uniform electric field is applied. In the Waters Synapt G2 platform, however, ion mobility separation is performed using traveling waves of variable height and velocity to separate ions [34–36]. Because of this fundamental difference in separation, drift times are non-linear with respect to collisional cross section (CCS) and therefore accurate assignment of CCS necessitates external calibration methods, which have been described in detail elsewhere [37–40]. Traveling wave ion mobility experiments also utilize nitrogen as the bath gas, requiring conversion of cross-sections into the more commonly reported helium numbers. This, however, is easily done using the data tabulated by Bush et al. and the Clemmer group for a number of systems (*e.g.* polyalanine) [37,41]. Ion mobility was performed herein using nitrogen as the bath gas and typical drift cell pressures were $4–6 \times 10^{-4}$ mbar. Typical wave heights and velocities in the drift cell were 10–13 V and 250–350 m/s, respectively. In order to prevent the separated conformers from mixing in the transfer ion guide, relatively large transfer wave heights were used (7–11 V), which resulted in some ambient fragmentation of the mobility-separated conformers, even with a transfer collision voltage of 0 V.

Action IRMPD experiments were performed on a Bruker Esquire 3000^+ ion trap coupled to a free electron laser (FEL) in Orsay, France as described previously [17,22,42,43]. Ions were formed by electrospray and mass selected in the ion trap prior to irradiation with the FEL. The free electron laser was pulsed in sets of eight picosecond pulses in which typical power was 600–900 mW at 1500 cm^{-1}. This power of the beam was roughly controlled by use of 2–4 attenuators, each of which reduced the photon flux by approximately 25%. The b_2 ion from bradykinin was generated by trap CID and mass selected prior to IRMPD analysis. Spectra were generated by plotting the fragmentation efficiency of the b_2 against the irradiation frequency of the laser.

Computational modeling of triply charged bradykinin was performed using an *ab initio* approach in which starting geometries were obtained from conformational searches using the Macromodel software suite and geometry optimizations and frequency calculations were performed using the Gaussian 09 software package [44,45]. The N-terminus and two arginine side chains were considered as protonation sites and the *cis–trans* isomerization state of each proline was torsionally restricted (±100° from a 0° or 180° amide dihedral angle) during the conformational searches. The result of this was 8 unique isomers in which the *cis* or *trans* isomerization state of the three proline residues was different. An unrestricted search was also performed in order to identify the lowest energy conformers within a 10 kcal/mol window. Geometry optimization was performed at the B3LYP/3-21G and B3LYP/6-31G* levels of theory and frequency calculations were performed at the B3LYP/6-31G* level of theory. Calculated IR spectra were empirically scaled by a 0.97 scaling factor. Collisional cross sections were calculated by putting the final coordinates of each triply charged bradykinin isomer using the MOBCAL program [46,47]. Reported values from trajectory method calculations were calculated using partial charges obtained from DFT calculations.

3. Results and discussion

3.1. Ion mobility and fragmentation

Three major conformations or conformational groups of mass selected, triply charged bradykinin were separated based on collisional cross section and the drift time distribution of these conformations is shown in Fig. 1(d). The collisional cross sections of these conformers were determined to be 272, 285, and 304 $Å^2$ for conformation A, B, and C, respectively, and are in excellent agreement with major conformations A, B, and C as identified by Clemmer and co-workers [1,3]. The Clemmer group has demonstrated using variable activation energies that a roughly 70:20:10 ratio of C, B, and A, respectively, is the gas-phase equilibrium distribution of triply charged bradykinin and they proposed the structures that comprise these distributions differ only in the

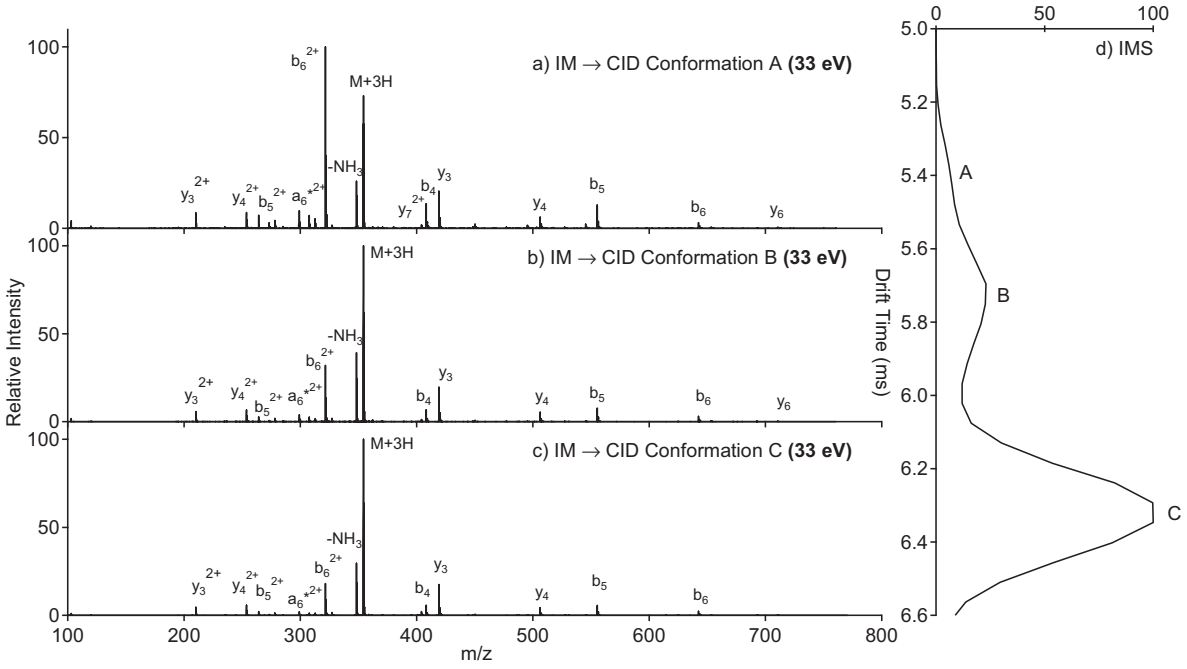


Fig. 1. IM-CID of conformations A–C of 3+ RPPGFSPFR are shown in (a)–(c) respectively using 33 eV CID in the transfer TWIG. The different conformations isomerize prior to dissociation, yielding near identical fragmentation behavior. In (d) the arrival time distribution of the ion mobility separated conformers is shown.

cis–trans isomerization states of the three proline residues. Significantly, their studies support that the three charges of triply charged bradykinin are located on the two arginine side chains and the N-terminus in all conformations. It should be noted that it is possible for multiple conformers to be present under any, or all, of the mobility-resolved peaks but higher resolution IM has not illustrated multiple conformations under the peaks. The drift time distribution shown in Fig. 1(d) is in excellent agreement with the ion mobility results shown by the Clemmer group, suggesting that the observed drift time distribution reflects the gas-phase equilibrium conformers of triply charged bradykinin [3]. In general, triply charged bradykinin exhibited a peculiar fragility in the tandem MS instrument, fragmenting at very low collision energies. To eliminate fragmentation between optical devices and transfer regions of the instrument, the instrument was tuned such that all lenses and voltage offset drops were below 2 V. Although transmission of the precursor suffered somewhat from this tuning, the base signal following this tuning was 10^4 counts/scan, which is reasonable for the experiments performed. Supporting Information Fig. S1 shows the mobilogram for triply charged bradykinin using this tuning and shows that fragmentation prior to IM was virtually nonexistent, the exception being the production of doubly charged precursor that forms *via* charge stripping of the triply charged precursor. Clemmer and co-workers have previously shown that the drift time distribution of triply charged bradykinin can be changed by varying the electrospray solution composition [2]. Attempts to repeat this experiment were unsuccessful on the Synapt G2 used in this study, which is attributed to the hotter source and optics of the Synapt, even under the gentle tuning conditions used. For this reason, the observed drift time distribution is expected to be the quasi-equilibrium distribution, but it may be possible that other conformations are present that were not measured by Clemmer and co-workers.

The MS/MS spectra in Fig. 1 show the fragmentation behavior of conformation A, B, and C using 33 eV CID to activate the drift time separated precursor in the transfer TWIG. All three spectra feature nearly identical fragments, suggesting that all three drift time distributions fragment *via* the same pathways, as reported by Clemmer and co-workers [3]. The exception to this is the b_6^{2+} fragment, which forms significantly more abundantly from conformation A than from conformation B or C. The CID spectra are largely characterized by a series of cleavages forming both singly and doubly charged ions.

Fig. 2(a)–(c) show the fragmentation behavior of conformers A, B, and C, respectively using 12 eV to activate the ions in the transfer TWIG CID region, located after the ion mobility cell. Because of the low abundance of fragments at this energy, regions in the mass spectra are zoomed in by 10- to 200-fold and are shown in the respective insets. At this relatively low collision energy, the three IM-separated distributions behave very differently by CID activation. Conformation C fragments exclusively into a b_2^+–y_7^{2+} pair. Fragmentation at this site involves cleavage C-terminal to a proline, which is typically thought of as a suppressed cleavage site [48]. Fragmentation C-terminal to proline residues in the second position of peptide, as for bradykinin, however, has been shown to be an exception to this trend [29,30]. The products generated from conformations A and B are relatively similar, with the production of the b_6^{2+} being dominant. The abundance of the b_6^{2+} relative to the precursor is dramatically higher in conformation A, however, and a spattering of fragments that presumably arise from sequential fragmentation of the b_6^{2+} are present. Interestingly, the relative abundance of the a_6^{2+} and b_5^{2+} relative to the b_6^{2+} in the MS/MS spectra from conformation A and B are different, suggesting that the a_6^{2+}, b_5^{2+}, or the b_6^{2+} ion forms *via* different pathways depending on the conformation of the precursor. Fragmentation of conformation A also yields a unique b_4 ion (unusual cleavage C-terminal to glycine), while fragmentation of conformation B yields a unique y_4^{2+} ion. There are two possibilities for the contrast of the spectra of Figs. 1 and 2. The first is that at very low collision energies, fragmentation occurs without prior isomerization, while at higher energies isomerization occurs prior to fragmentation. It is also possible that labile subpopulations are fragmented at lower energies and that fragmentation of the main population becomes more dominant at higher collision energies. It is likely that both of these explanations are contributing, and in support of the second explanation, Fig. 3 shows the extracted drift time distributions of the triply charged

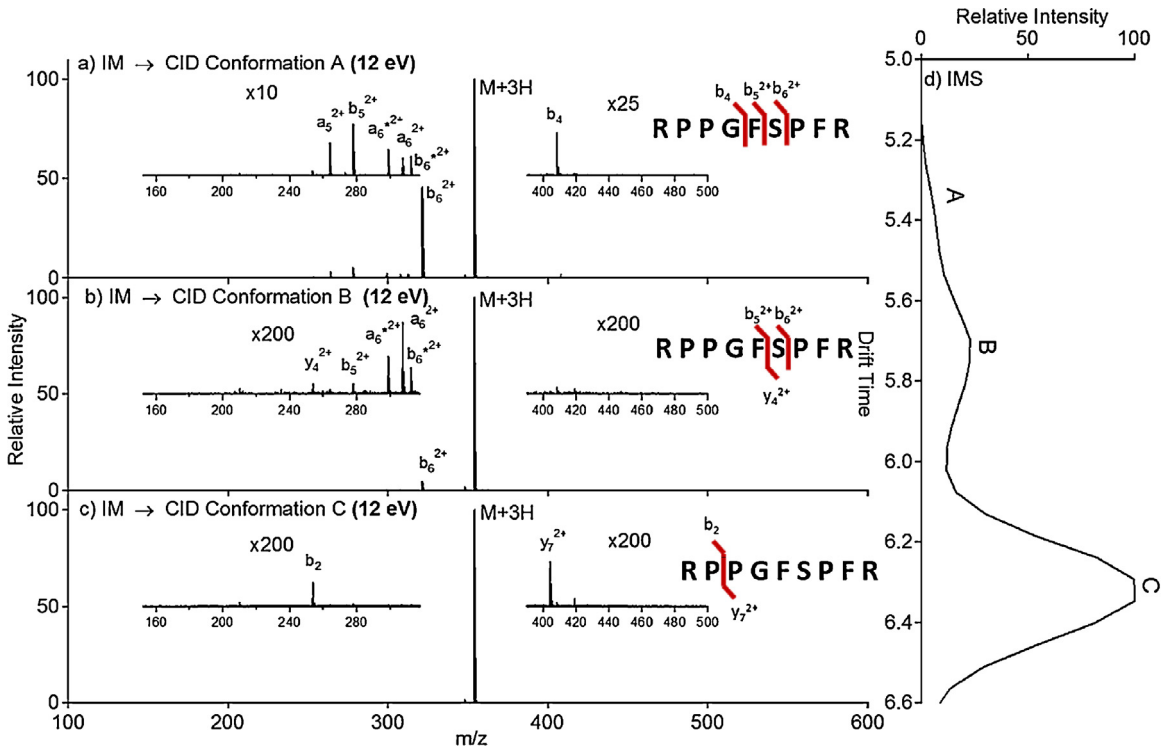


Fig. 2. CID (12 eV) of the different conformations of IM separated, triply charged bradykinin are shown in (a)–(c). Insets show the zoomed in regions of the mass spectra from which differences in fragmentation of the conformers can be observed. In (d) the drift time distribution of the three conformers is shown, with the fragmentation pattern shown in (a) corresponding to conformer A, the pattern in (b) corresponding to conformer B, and the pattern in (c) corresponding to conformer C. The * symbol denotes ammonia-loss fragments.

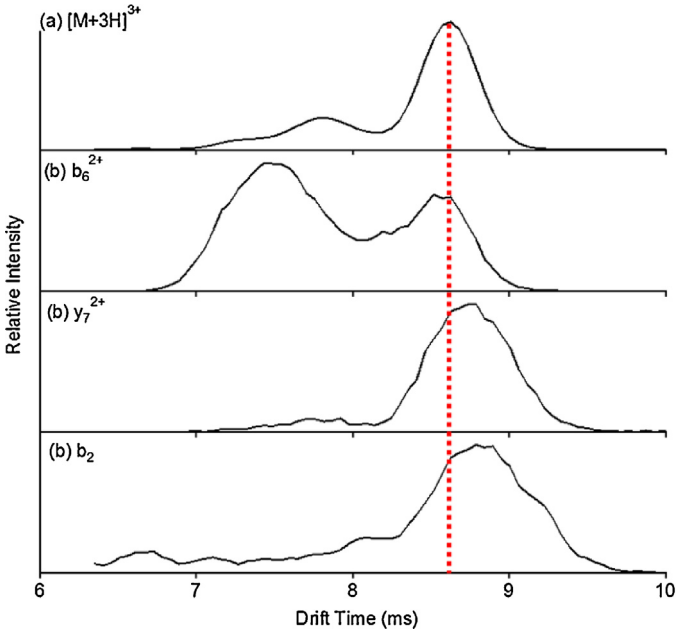
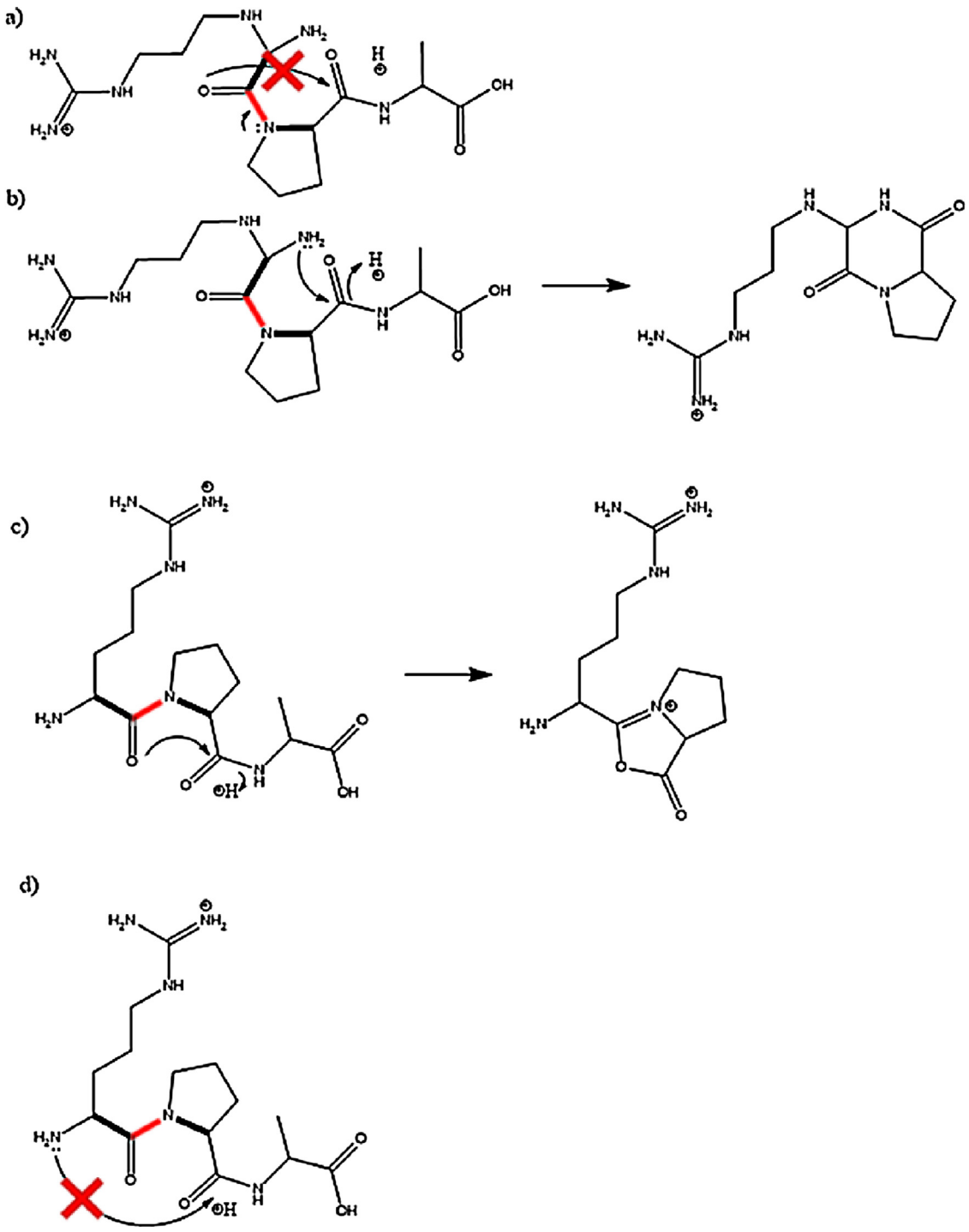


Fig. 3. Extracted drift time distributions for the triply charged bradykinin precursor, the b_6^{2+}, y_7^{2+}, and b_2 ions are shown in (a)–(d), respectively. A red line is used to highlight the shift in the center of the drift time distributions generated from the b_2 and y_7^{2+}. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

precursor, the b_6^{2+}, the y_7^{2+}, and the b_2^{2+} ions, respectively, with each trace normalized to the maximum abundance in that trace. It is clear that the same population is forming the b_2^{+}–y_7^{2+} pair and has an average drift time slightly shifted from the main C population. Although it is difficult to estimate the abundance of this population from the abundances of fragments, the low abundance of the b_2^{+} and y_7^{2+} and absence of shoulders on conformation C suggest this population is relatively small (see scaling of Fig. 2). Clemmer and co-workers have postulated that the differences in conformations A, B, and C are due to different *cis/trans* isomerization states of the three prolines [1] and we believe this also explains the structure of the minor subpopulation of C that fragments at very low energies. It is not clear whether this subpopulation is always present for triply charged bradykinin or whether the ions are heated in the instrument used here, although we have tried to minimize heating as much as possible.

Previous studies in the Wysocki group have examined fragmentation N-terminal to proline, C-terminal to proline, and proline-proline cleavages. Cleavage N-terminal to proline is typically enhanced and cleavage C-terminal is often suppressed. Cleavage between two proline residues is typically unfavorable, in part because the torsional angles of the prolyl ring restricts rotation around the N–C_α bond [26,24,29]. In the less stable *cis* state of the amide bond, formation of an oxazolone b ion is impossible. The formation of the proline oxazolone structure from *trans* amide bond isomers results in a di-ring oxazolone that has a fixed charged at the ring nitrogen. Because bradykinin has been shown to be protonated at the N-terminus and both arginines, oxazolone b ions that form from cleavage C-terminal to proline should all be doubly charged, with one charge at the newly formed oxazolone (fixed charge) and the second on the arginine side chain [1]. Note that the amino terminal mobile proton is transferred from the b fragment to the y fragment during fragmentation to satisfy valency of the departing amine group. Additional charge transfer events can occur to transfer a charge from the b ion to the y ion, particularly for structures such as the diketopiperazine, which have fewer strongly basic groups. Formation of b_2 ions in which proline is in the second position is unique, however, because the formation of a diketopiperazine structure is possible if the proline amide



Scheme 1. Pathways for oxazolone and diketopiperazine formation for an RPA peptide in which the proline amide bond is *cis* or *trans*. In (a) and (b), Pro2 is shown in the less stable *cis* state and oxazolone formation is impossible due to the orientation of the arginine carbonyl but diketopiperazine formation can readily occur, resulting in a singly charged b_2 ion after transfer of the mobile proton to the complementary y_7^{2+} fragment. In (c) and (d), Pro2 is in the more stable *trans* state and oxazolone formation can readily occur, resulting in a doubly charged b_2 with a fixed charge; in contrast, diketopiperazine formation is prohibited.

bond is *cis*. Additionally, when the first amide bond is *cis*, the oxazolone structure cannot form. The diketopiperazine structure lacks a fixed charge and is relatively non-basic (excluding the arginine side chain), and is therefore predicted to be singly charged at the Arg sidechain. Scheme 1 shows the conformational aspects of these different pathways for a peptide of sequence RPA. Thus, we make the assumption that the presence and charge state of fragments that arise from cleavage C-terminal to proline2, proline3, and proline7 can be used to probe the *cis/trans* isomerization state of the proline residues.

The b_2/y_7^{2+} pair observed to form uniquely from a subpopulation of conformation C at extremely low collision energies is the result of cleavage C-terminal to proline2. Based on the logic above, the singly charged b_2^+ is predicted to have a diketopiperazine structure. Fig. 4 shows the action IRMPD spectrum of the b_2 ion from triply charged bradykinin and a predicted IR spectrum of a diketopiperazine b_2, the agreement of which demonstrates that the b_2 is a diketopiperazine. Similar b_2 IRMPD spectra were observed for b_2 ions formed from bradykinin 1–4, 1–5, and 1–6 (not shown), suggesting that only local structure influences b_2 ion formation. It

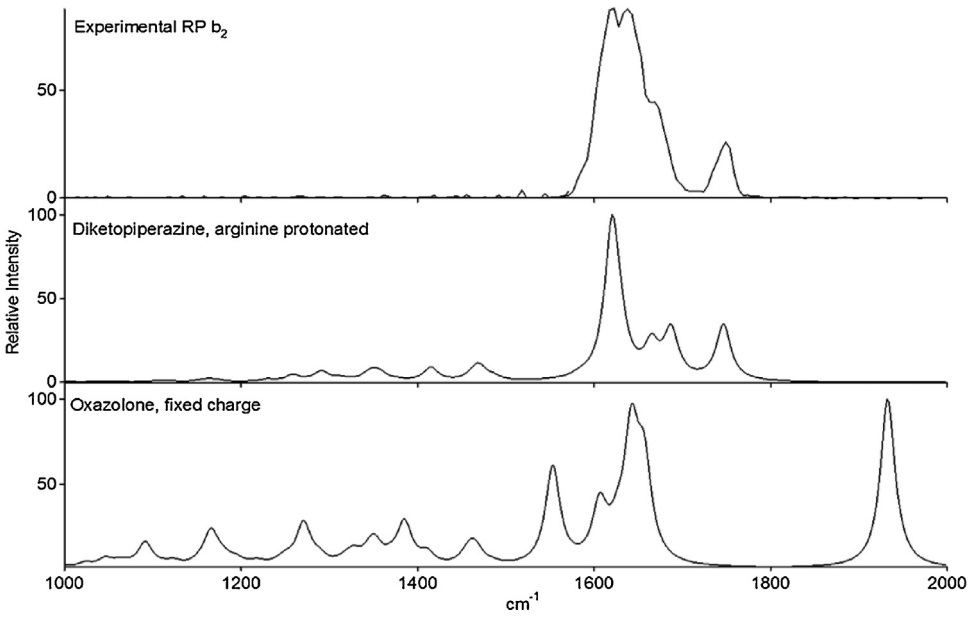


Fig. 4. Action IRMPD spectroscopy of the b_2 ion from triply charged bradykinin is shown in (a). Computed diketopiperazine and oxazolone IR spectra are shown in (b) and (c), respectively.

should be noted that although IRMPD of the b_2 ion was collected without preceding IM separation, this fragment was only observed from conformation C. The low energy nature of ion trap collisions additionally resulted in a more abundant b_2 population in the ion trap. Fragmentation therefore suggests that the subpopulation of conformation C that fragments at low collision energies (12 eV) has a *cis*-isomerized amide bond of proline2, if it is assumed that the RP bond does not isomerize during fragmentation. The absence of other fragments in the tandem mass spectrum of the subconformation of C suggests that fragmentation along the b_2–y_7^{2+} pathway is the lowest energy pathway available. Other fragmentation pathways either require more energy or require isomerization.

3.2. N-methyl alanine variants

Three peptide variants were synthesized in which Pro2, Pro3, and Pro7 were individually substituted by NMA, an acyclic proline mimic in which both the nitrogen and alpha carbon are methylated. N-methyl alanine and proline have very similar *cis/trans* isomerization barriers and have *cis* states considerably lower in energy than does the *cis* state of alanine or other non-proline residues, making NMA an excellent model amino acid for examining proline *cis/trans* behavior [49,50]. Ion mobility of these three variant peptides is shown in Fig. 5(a)–(c) with the drift time distribution of wild type BK shown in (d) for reference. The center of conformation C changes little, if at all, following substitution of NMA for any of the proline residues and suggests conformation C of the NMA variant peptides may be comprised of similar conformations. In contrast, the center of Conformation B is reduced by 0.05 ms ($\approx$5 Å^2), suggesting that compaction of the conformations occurs as a result of the NMA substitution or that different abundances or types of conformers are present. Interestingly, all NMA substitutions resulted in an increased abundance of conformation B relative to the equilibrium distribution of wild type bradykinin. The center of conformation A is too obscured by conformation B to accurately determine if any shifts occur in the drift time of the NMA variants. Substitution of Pro3 resulted in the most dramatic changes to the relative abundance of the conformations A, B, and C, as the NMA substituted peptide features a significantly more dominant conformation B than does the wild type or other variant peptides. In contrast, the Pro7 → NMA peptide features a drift time distribution

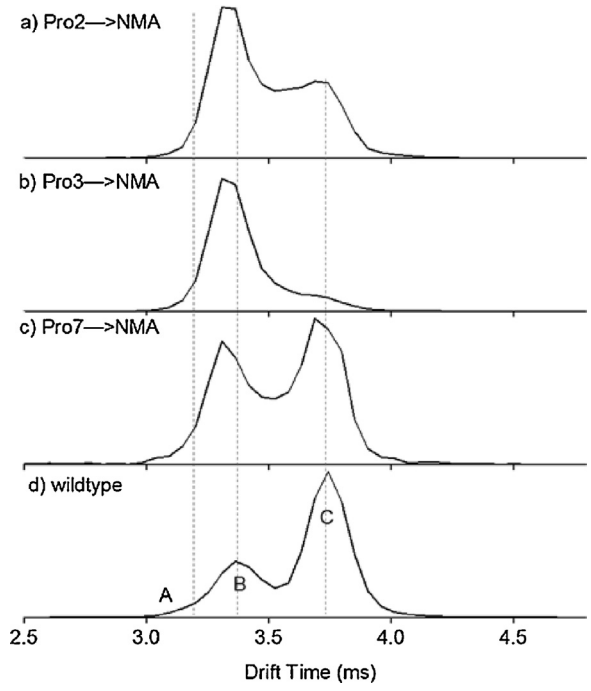


Fig. 5. Drift time distributions of triply charged Pro2 → NMA, Pro3 → NMA, and Pro7 → NMA BK are shown in (a)–(c). In (d), wild type BK is shown for reference.

in which conformation C is the most dominant distribution but has a more abundant conformation B than does wild type bradykinin. Based on the unique observation of the b_2/y_7^{2+} pair from low energy IM-CID of a minor conformation C population of wild type BK, the following analysis focuses exclusively on conformation C.

The NMA-substituted peptides were generally found to be more resistant to fragmentation at low collision energies; consequently, low-energy IMS-CID was collected using a 15 (rather than 12) eV collision energy. The resulting CID spectra for conformations C of Pro2 → NMA, Pro3 → NMA, and Pro7 → NMA are shown in Fig. 6(a)–(c), respectively. Note that the fragmentation of conformations A, B, and C of all three NMA variant peptides are shown in Supporting Information Figs. S2–S4. Consistent with

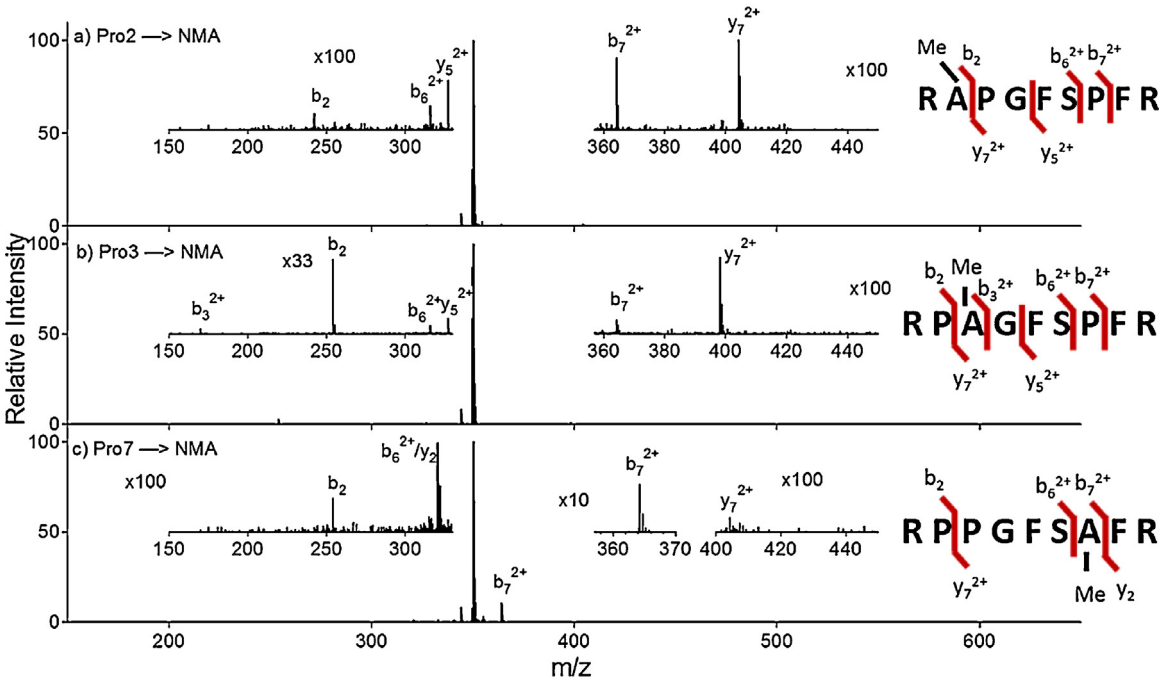


Fig. 6. IM-CID of conformation C of Pro2 → NMA, Pro3 → NMA, and Pro7 → NMA are shown in (a)–(c), respectively.

the fragmentation of the minor population of conformation C of wild type bradykinin, all three conformation C spectra feature a relatively abundant b_2/y_7^{2+} pair. These three spectra thus support that Pro2 in the subpopulation of conformation C of all triply protonated NMA-substituted variants and wild type bradykinin is *cis*. Interestingly, several additional fragment ions were present in the fragmentation spectra of conformation C of the NMA variants, including y_5^{2+} and b_7^{2+} which were observed in all three spectra. A b_3^{2+} was also observed in Fig. 6(b); however, previous studies have shown that fragmentation C-terminal to N-methyl alanine is enhanced [25]. Thus, the observation of the b_3^{2+} in Fig. 6(b) and the b_7^{2+} in Fig. 6(c) is consistent with the literature of enhanced cleavage C-terminal to NMA, although the b_3^{2+} is surprisingly low in abundance, perhaps suggesting that b_2^{+} formation kinetics are faster or b_3^{2+} formation is hindered by the cis Pro2. The abundant b_7^{2+} observed in the fragmentation of the Pro2 and Pro3 substituted peptides, however, is unexpected. The formation of oxazolone structures following fragmentation C-terminal to proline results in the formation of a fixed charge at the di-ring structure. Assuming that the two arginines and N-terminus are the protonation sites of triply charged bradykinin and that the proton at the N-terminus is the mobile proton, doubly charged b ions with an oxazolone structure will be protonated on the oxazolone ring and N-terminal arginine. In this case, all oxazolone b ions will be doubly charged. Thus, if we assume that the b_7^{2+} generated from Pro2 → NMA and Pro3 → NMA is a result of the oxazolone pathway rather than from nucleophilic attack of the N-terminus or amino acid side chain, and that no isomerization occurs prior to fragmentation, the IM-CID data suggest isomerization state of Pro7 is *trans* (see Scheme 1). Given that both arginine residues are expected to be protonated and macrocycle structures have been shown to occur only after oxazolone formation, this assumption is reasonable. IM-CID of the NMA-substituted peptides thus provides additional fragmentation that suggests that the minor population of conformation C is *cis* at Pro2, based on the singly charged b_2, and *trans* at Pro7, based on the formation of the b_7^{2+} in Pro2 → NMA and Pro3 → NMA. Verification of a b_7^{2+} oxazolone structure, however, requires additional spectroscopic study.

3.3. Computational modeling

Ab initio modeling was used to probe the gas phase conformations of triply charged bradykinin based on the different *cis/trans* states of the three proline residues. As a molecular system of 152 atoms, this presented a computational challenge. However, although *ab initio* methods are often thought to be limited to systems with tens of atoms, several characteristics of the system made it somewhat more tractable by this approach. Clemmer and co-workers have shown that acetylation of the N-terminus of bradykinin prevents the formation of the triply charged species, suggesting that the N-terminus is protonated, along with both arginine residues, in the triply charged precursor [1]. For this reason, only the N-terminus was considered as the third protonation site, which dramatically reduces the diversity of structures that need to be considered, *e.g.* backbone carbonyl protonation sites. Moreover, based on the three bradykinin protonation sites being fixed, Clemmer and co-workers postulated that the *cis/trans* isomerization states of the three proline residues are responsible for the observed conformational differences of the triply charged, gas-phase precursor. For this reason, conformational searches were performed on triply charged bradykinin using torsional restrictions (0° or 180°, ±100°) on the three proline residues. In total, 8 searches with restricted proline dihedral angles were performed to obtain collections of starting geometries. This allowed use of a 10 kcal/mol window to be used for all searches, rather than opening the energy criteria to 20–25 kcal/mol, which increased the number of discovered geometries to over 500. Restriction of rotation of the proline residues thus resulted in a significant reduction of the number of stable geometries that were discovered using a Monte Carlo approach and typically between 10 and 30 geometries were optimized by *ab initio* methods for each *cis/trans* conformer. A search in which no restrictions were placed on the proline torsional angles (energy criterion was 10 kcal/mol) was also performed to confirm the lowest energy conformations of the system.

The three unique and lowest energy geometries of each of the CCC, CCT, CTC, CTT, TTT, TTC, TCT, and TCC conformers, following optimization at the B3LYP/6-31G* are listed in Table 1 with

Table 1
Relative energies, proline dihedral angles, computed collisional cross-sections using both the trajectory method (TJM) for the top three lowest energy geometries of the *cis/trans* proline isomers of triply charged bradykinin. Different hydrogen bonding motifs are denoted a, b, and c.

Isomer	Unique hydrogen bonding motif	Relative energy (kcal/mol)	Pro^2 dihedral	Pro^3 dihedral	Pro^7 dihedral	TJM CCS (Å^2)
CCC	a	0.00	−8.65	−6.52	−18.7	284.3
	b	8.90	−0.35	−19.76	2.5	295.5
CCT	a	1.76	−9	−11.4	178.62	284.5
	b	6.38	−10.5	−6.75	171.8	262.2
	c	4.11	−8	−8.4	173.8	289.8
CTC	a	10.76	10.4	−168.8	−6.7	268.9
	b	13.08	3.5	−162.8	−5.1	269.8
CTT	a	10.22	17.4	−172.2	171.1	265.5
	b	12.17	10.6	−176.1	173.8	269.8
	c	12.69	14.9	−174.1	170.2	276.8
TCC	a	10.31	172.4	−16.3	−1.8	281.1
	b	15.12	168.7	−15.9	9.3	276.8
TCT	a	16.90	174.8	−3.9	−163.7	273.3
TTC	a	10.17	167.8	−177.7	−6.1	297.0
TTT	a	18.09	175.4	175.3	−150.9	307.1

their respective relative energies (kcal/mol) and collisional cross sections, as predicted by the trajectory method (TJM). The unique hydrogen bonding motifs are listed as a, b, or c. The proline dihedral angles are also listed. For some isomers, only 1–2 unique hydrogen bonding motifs were found in the Monte Carlo conformational search; thus, only three geometries are shown for the isomers in which three or more motifs were discovered. All geometries are shown in Supporting Information Fig. S5. All of the hydrogen bonding motifs of the CCC and CCT conformers were found to be lower in energy than all other conformations, with CCC having the lowest energy conformer. This is consistent with the results of the unrestricted search, in which only CCC and CCT conformers were found.

IM-CID of wild type and NMA substituted bradykinin suggest that Pro2 of the minor population of conformation C is *cis*, as supported by action IRMPD of the b_2 ion, and Pro7 is *trans*, based on the fragmentation of the NMA peptides. Conformation C was found to have an experimental collisional cross section of 304 Å^2. Based on predicted collisional cross sections of the computed conformers, only the CCC b, CCT c, TTC, and TTT conformations have CCSs that are within 4% of the experimental CCS of conformation C. The CCS of the TTC conformation predicted by Clemmer and co-workers agrees extremely well with the experimental CCS of the major population of conformation C, and therefore strongly supports their assignment. The minor population found by low energy IM-CID was observed to have a slightly higher collisional cross-section and was predicted by fragmentation to be CCT or CTT. Although neither of these conformations are predicted to be exceptionally close to the measured CCS of the minor conformation C population, the CCT c and CCC b conformers are within 4% of the experimental CCS, which could indicate that the minor population is comprised of one of these conformers.

4. Conclusions

We report here conformation-specific low energy CID spectra from ion mobility-separated conformers of triply charged bradykinin. At low energies, conformation A is observed to generate an unusually intense b_6^{2+} fragment, which may indicate stabilization by a hydrogen bonding interaction with Ser6. Conformation C, in contrast, generated only a small b_6^{2+} in addition to an unusual b_2/y_7^{2+} pair. Extracted drift time distributions for the b_2/y_7^{2+} pair suggest that low energy IM-CID is probing a minor subpopulation of conformation C. The charge state of the b_2 (1+), in addition to the action IRMPD spectroscopy of the RP b_2 from triply charged bradykinin suggest that this b_2 ion is a diketopiperazine and that Pro2 of this minor subpopulation of conformation C is *cis*. At higher collision energies, the fragmentation behavior of the three IMS-resolved conformations is observed to be nearly the same, suggesting that the proline amide bonds isomerize prior to dissociation at these energies and/or that the fragmentation of the major population dominates.

Individual substitution of NMA for the three proline residues of bradykinin resulted in drift time distributions that were similar in position to those in wild type bradykinin. Conformation C, in particular, showed little to no change in CCS following substitution at any one of the proline residues. The low energy fragmentation of the minor subpopulation of conformation C suggests that Pro2 is *cis* and Pro7 is possibly *trans* for both wild type and NMA-substituted bradykinin. DFT modeling of triply charged bradykinin strongly supports the TTC assignment made by the Clemmer group for the dominant conformation C population and may indicate that the minor population is CCT or CCC. Of the 8 possible *cis*/*trans* isomeric states, four had predicted collisional cross sections consistent with conformation C, including a CCT structure consistent with IM-CID. However, the predicted CCS of this structure deviated by 4% from the experimental cross section of conformation C, and may suggest that the hydrogen bonding geometry needs to be adjusted. Additional studies are needed in order to better understand the relationship between low energy fragmentation of peptides and the *cis*/*trans* isomerization state of proline.

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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.2015.09.008.

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