## RESEARCH ARTICLE

# Conformational Distribution of Bradykinin [bk+2 H]$^{2+}$ Revealed by Cold Ion Spectroscopy Coupled with FAIMS

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### Abstract

We employ cold ion spectroscopy (CIS) in conjunction with high-field asymmetric waveform ion mobility spectrometry (FAIMS) to study the peptide bradykinin in its doubly protonated charge state ([bk+2 H]$^{2+}$). Using FAIMS, we partially separate the electrosprayed [bk+2 H]$^{2+}$ ions into two conformational families and selectively introduce one of them at a time into a cold ion trap mass spectrometer, where we probe them by UV photofragment spectroscopy. Although the two conformational families have distinct electronic spectra, some cross-conformer contamination can be observed under certain conditions. We demonstrate that this contamination comes from isomerization of ions energized during and/or after their separation and not from incomplete separation of the initially electrosprayed conformations in the FAIMS stage. By varying the injection voltage of the ions into our mass spectrometer, we can intentionally induce isomerization to produce what seems to be a gas phase equilibrium distribution of conformers. This distribution is different from the one produced initially by electrospray, indicating that some of the conformers are kinetically trapped and may be related to conformers that are more favored in solution.

Key words: Cold ion spectroscopy, Tandem mass spectrometry, Photodissociation, Ion mobility, FAIMS

## Introduction

Field asymmetric waveform ion mobility spectrometry (FAIMS) is emerging as a promising analytical technique that separates gas phase ions at atmospheric pressure [1, 2]. Its separation ability is based on the difference in the mobility of an ion at high and low electric field and is independent of the $m/z$ [3], which makes it useful for the separation of analyte ions from interferences, chemical background, and isobaric species [4, 5]. FAIMS can also be used to separate gas phase conformers of proteins and peptides [6–9]. Because its separation ability does not depend on the absolute mobility but rather the change of the mobility with varying electric field [2, 3], it has a high degree of orthogonality to drift tube ion mobility spectrometry (IMS) [10]. Two-dimensional ion mobility spectra in which FAIMS has been combined with IMS have demonstrated the ability to resolve conformers that are indistinguishable when using either one technique or the other separately [11]. For example, in the case of ubiquitin, 18 and 15 gas-phase conformers were separated for all charge states using IMS and FAIMS, respectively, while by combining the two a total of 40 conformers were identified [11].

One drawback of FAIMS is that ions are subjected to high electric fields that induce collisional heating, which can cause conformational isomerization [2, 12]. IMS has been used to investigate the degree of isomerization induced during FAIMS by comparing ubiquitin ions that have passed through a FAIMS separation stage with those that have been introduced directly into an IMS drift tube [12]. It was found that for most charge states some structural transitions occur, with a net effect similar to that when heating the ions to ~75 °C prior to their introduction into the IMS stage [12].

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We have recently combined FAIMS conformer selection with cold ion spectroscopy (CIS) to investigate the nonapeptide bradykinin in its doubly protonated form $[bk+2\,H]^{2+}$ [13]. Without FAIMS separation, there is a distribution of stable conformers in the low temperature (~10 K) environment of our cold ion trap [14] and, as a result, the electronic spectrum of $[bk+2\,H]^{2+}$ is highly congested, since the spectra of different conformers partially overlap. We have used FAIMS to separate $[bk+2\,H]^{2+}$ ions into two conformational families and transmit, selectively, one family or the other into a cold trap for spectroscopic analysis [7], which allows simplification of the complex UV spectrum [13]. In the inverse sense, we have demonstrated that spectroscopy can be used to decompose an ion mobility spectrum by selectively detecting subpopulations hidden under a broad mobility peak [13].

There are two requirements for the successful use of FAIMS as a conformational filter for cold ion spectroscopy. First, the conformers or conformational families have to be clearly separated in FAIMS to allow selective introduction into the mass spectrometer and ultimately the cold ion trap. Second, the selected conformations have to maintain their structure from the time they exit the FAIMS stage until they are introduced into the ion trap and probed spectroscopically; otherwise the acquired spectrum will not be conformational family-specific. Although the ions will be heated during the separation process, if they isomerize in the FAIMS separation device, they may be filtered out, allowing only the selected conformational family to be introduced into the ion trap. However, the FAIMS-selected population may isomerize after exiting the separation stage, induced either by the energy imparted during separation or subsequently in its trajectory through our ion trap mass spectrometer, depending on the conditions used. Hence, the amount of energy imparted to the ions before they are probed spectroscopically is an important consideration in the coupling of the two techniques.

As described below, our experimental approach can provide evidence of conformational isomerization by inspecting cross-conformer contamination in the UV spectrum of a selected conformational family [13]. Here we show that the contamination is not a result of incomplete spatial separation in the FAIMS stage but rather from energizing the ions during the FAIMS separation and/or in subsequent collisions in the mass spectrometer, such that a fraction of the selected conformational population isomerizes to the other conformational family downstream from FAIMS. Moreover, our results clearly demonstrate that the distribution of conformers of $[bk+2\,H]^{2+}$ initially produced by electrospray does not represent an equilibrium distribution in the gas phase. The latter is obtained by "heating" either of the two conformational families prior to the spectroscopic investigation of the gas phase ensemble. Some of the $[bk+2\,H]^{2+}$ conformations produced by electrospray ionization appear to be kinetically trapped, a situation that may be general for large molecules, where conformational isomerization can be slow [15–18].

## Methods

We have modified our cold-ion photofragment spectrometer [19] so as to incorporate a FAIMS stage between the electrospray source and the entrance capillary of the mass spectrometer (Figure 1).

The FAIMS device that we use is of transverse cylindrical geometry ("side-to-side," Thermo Scientific) [20–22]. The process of ion separation in FAIMS has been described in detail elsewhere [2, 3, 23]. Briefly, a flow of gas carries an ensemble of ions between two electrodes across which an asymmetric voltage waveform is applied consisting of alternating components of high and low voltage of opposite polarity. Each polarity component is applied for a different amount of time so that the time-averaged voltage of the waveform is zero. The maximum amplitude of the waveform is called the dispersion voltage (DV). Under the influence of the field, the ions oscillate between the two electrodes. Because the mobility of an ion depends on the magnitude of the electric field, ions travel different distances towards the electrodes during the different segments of the waveform [2]. This results in their migration away from the gap median and a spread of ion populations perpendicular to the gas flow, eventually leading to loss on the electrodes. To compensate for the displacement of a specific ion population and to ensure its transmission through the gap, a small DC voltage called the compensation voltage (CV) can be applied to either electrode. These ion populations can be different species but can also be conformers of the same ion that have different change in ion mobility at high and low electric fields and, thus, distinct CV values at which they are transmitted. A spectrum that is indicative of the species in the gap can be recorded by monitoring the ion transmission through the electrodes as a function of the CV [24].

Because of the cylindrical geometry of the FAIMS device that we use, the electric field in the gap is inhomogeneous [20]. It has been shown that this inhomogeneity causes the ions transmitted at a specific CV value to be focused in the gap median [25, 26]. As a result, the peaks in the CV spectrum are broadened, but the transmission efficiency of a specific ion is increased [2]. However, in this particular FAIMS design, we can control the temperature of the two electrodes independently and thereby create a density gradient across the electrode gap [20], which compensates the electric field inhomogeneity. Controlling the temperature gradient thus gives us the ability to change the trade-off between transmission efficiency and resolution [2, 20]. The temperature of the inner electrode is held at 35 °C whereas that of the outer electrode is varied.

The gas that carries the ions though the FAIMS electrodes is composed of either 90 %$N_2$ and 10 % He or 100 %$N_2$ and is used at a variable flow rate. Part of the carrier gas flows out of the entrance electrode of FAIMS, counter to the electrospray droplets to ensure complete desolvation of the ions, while the rest carries the ions into the annular gap
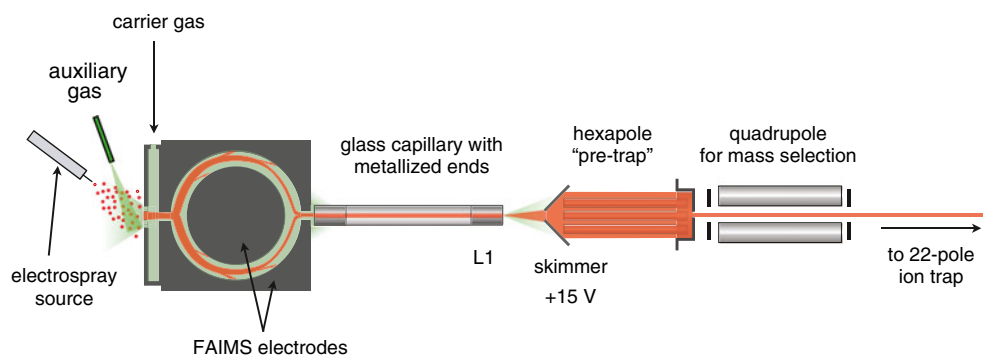
**Figure 1.** Schematic of FAIMS device coupled to the front end of our ion trap photofragment spectrometer

between the two electrodes where conformer separation and selection takes place. The dispersion voltage of the asymmetric waveform is –5000 V.

The ions transmitted through the FAIMS device are introduced into the cold ion trap tandem mass spectrometer, which is described in detail elsewhere [27–29]. They are drawn into vacuum through a glass capillary with metalized ends and then pass a skimmer before they are collected in a hexapole ion trap. We can use the voltage drop between the exit of the capillary (L1 in Figure 1) and the skimmer to vary the collision energy of the ions with the background gas upon injection [18], since the pressure in this region is 1.7 mbar. The ions are extracted periodically from the hexapole and a specific $m/z$ is selected in a first quadrupole mass filter. The parent ions are then guided into a 22-pole ion trap, where they are cooled in collisions with cold He and interrogated by UV photofragment spectroscopy. To enhance the dissociation yield after UV excitation, the ions are further irradiated with a pulse from a $CO_2$ laser [30, 31]. In the case of bradykinin, the only laser-induced dissociation channel observed is the loss of the neutral phenylalanine side chain. Parent and fragment ions are then released from the 22-pole trap, mass analyzed in a second quadrupole filter, and detected using a channeltron. The lasers fire at a frequency of 10 Hz while the trapping cycle is repeated at a frequency of 20 Hz. The laser-off parent signal is used for normalization of the laser-induced fragment signal to correct for slow fluctuations in the output of the electrospray source. Electronic spectra are recorded by measuring the number of fragment ions as a function of the UV laser frequency. CV spectra are recorded by measuring the number of trapped parent ions as a function of the compensation voltage.

## Results and Discussion

Figure 2 shows UV photofragment spectra of cold [bk+2 H]$^{2+}$ obtained without a FAIMS separation stage. The traces (e) to (a) display spectra recorded with increasing ion injection energy into the hexapole trap by increasing the voltage on the capillary exit. The voltage difference between the capillary and the skimmer is shown on the left of each trace.

The spectrum of Figure 2f was recorded under as gentle conditions in the mass spectrometer as possible that still sustains ion transmission. This means that there were no large differences in the voltages of the ion optics that are used to transfer the ions across the higher-pressure regions of the mass spectrometer. In addition, ions were not pre-trapped in the hexapole, and their translational energy entering the 22-pole was less than 1 eV. All the spectra in Figure 2 are congested, exhibiting a large number of sharp transitions. To determine which of these peaks originate from the same conformer, we have recorded conformer-specific vibrational spectra using an IR-UV double resonance spectroscopic scheme [14]. These data indicate that there are at least five different stable conformations at the temperature of our trap that all contribute to the acquired spectra. Conformational heterogeneity is thus a major source
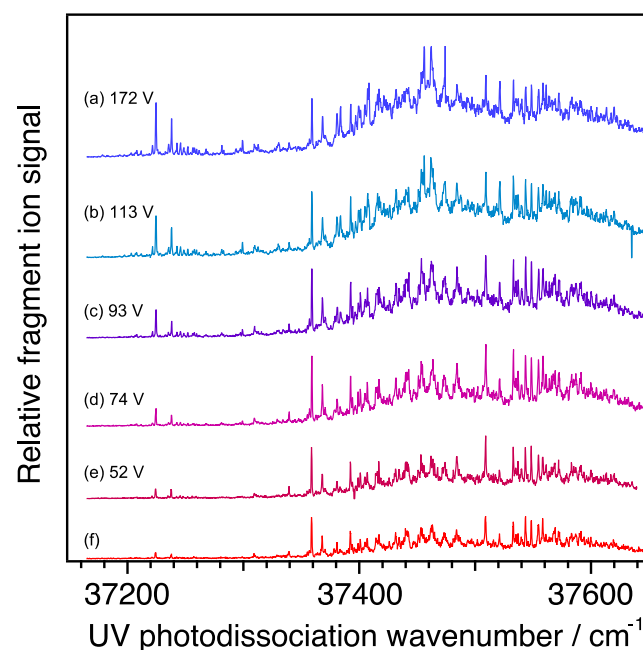


**Figure 2.** Electronic photofragment excitation spectra of [bk+2 H]$^{2+}$ obtained **(a)**–**(e)** with variable injection voltage of the ions into the hexapole ion trap, **(f)** under very gentle conditions (see text)

of congestion in the spectra of Figure 2, consistent with what one would predict on the basis of high-resolution FAIMS measurements [8].

The spectra of Figure 2 clearly show that upon increase of the ion injection energy, some peaks change in relative intensity. For example, the two transitions around 37230 cm$^{-1}$ and the transitions around 37,380 cm$^{-1}$ increase in intensity compared with the peak at ~37,360 cm$^{-1}$. Data from IR-UV double resonance spectroscopy with the UV laser set on the features at ~37,230 cm$^{-1}$ and at ~37,360 cm$^{-1}$, indicate that these transitions originate from different conformations [14]. Since the intensity of a peak depends upon the population of the initial state probed by the UV laser, the increase of the intensity of a feature reveals an increase in conformer population that gives rise to it. A change in the relative populations of the conformer distribution would necessarily involve energizing the ion population above the barrier(s) to isomerization.

The voltage drop between the capillary and the skimmer determines the injection energy of the ions and, thus, the average energy of their collisions with the background gas in this region. In our case, the increase in population of a particular conformer involves an annealing process in which the ions are first energized by collisional activation followed by cooling in collisions both with background gas in the hexapole and with cold He in the 22-pole ion trap so that the "hot" ion population relaxes into a different distribution. The increase in the intensities of the peaks around 37,230 cm$^{-1}$, 37,380 cm$^{-1}$, and 37,460 cm$^{-1}$ with increased injection energy is indicative of such a process. Over the range represented in Figure 2, the higher the collisional activation, the larger the fraction of ions that change their conformation *via* this annealing process.

In our previous study of this system, we used FAIMS to divide the stream of the electrosprayed [bk+2 H]$^{2+}$ ions into two families, A and B [13]. Interestingly, all the peaks of Figure 2 that increase in intensity originate from the conformers that constitute family B, while those whose intensity is nearly independent of the injection voltage come from family A. The fact that peaks of family B increase so significantly in intensity during the annealing process raises the question of whether the family of conformers that they represent is initially produced in the electrospray process or becomes populated only in the gas phase upon annealing. To answer this question, we recorded a UV photofragment spectrum of cold [bk+2 H]$^{2+}$ under the gentlest injection conditions possible in our mass spectrometer (Figure 2f). This spectrum shows that characteristic peaks of family B around 37,230 cm$^{-1}$ and 37,380 cm$^{-1}$ do appear but with very low intensities. This indicates that the conformers of family B are indeed present, albeit with low relative population. The conformers that give rise to the prominent peaks in Figure 2f, belonging to family A, are significantly populated without annealing and appear to be those predominantly formed in the electrospray process.

One could argue that the conditions that we employ for the transfer of ions into the 22-pole trap are not gentle enough, and that this induces their partial isomerization from the initially produced A family to B, which is a family of conformers that becomes stable in the gas phase. Another possibility may be that family A spontaneously decays to family B. However, data presented further below indicate that both of these possibilities can be excluded. We, thus, believe that the spectrum of Figure 2f reflects the distribution of populations between the two conformational families as they are produced in the electrospray process, which may represent the distribution in solution before electrospraying [10]. This would imply that in solution there are two structural families with different stabilities and, thus, different populations. When solvent is removed, the less populated family gives rise to conformational family B and the more populated to A. At the same time, the desolvation process stabilizes the structures that form family B while it leaves kinetically trapped those that form family A, since a barrier on the potential energy surface inhibits isomerization from A to B. Our observation that increasing the injection energy of the ions into our mass spectrometer induces isomerization is consistent with this interpretation. To what extent conformational families A and B produced by electrospray are related to their solution counterparts is an open question [10]; however, studies indicate that there is at least a memory of the solution conformational distribution in the nascent electrospray distribution [32]. We further investigate this scenario by first separating the conformational families using FAIMS before spectroscopically probing the final conformational distribution in our cold ion trap.

Shown in the insets of Figure 3 is a CV spectrum of [bk+ 2 H]$^{2+}$ with the temperature of inner FAIMS electrode held at 35 °C, whereas that of the outer electrode held at 95 °C. The spectrum exhibits two partially resolved peaks, one at –20.4 V and one at –16.1 V. These appear at slightly different CV than in our previous study because of small differences in the FAIMS conditions [13]. In that study, we showed that the two features in the CV spectrum give rise to two distinct electronic spectra [13]. From IR-UV double resonance experiments [14], it was shown that there are a number of conformers that contribute to each of the spectra, so we attributed the two features to two conformational families that FAIMS can separate [13]. Guevremont et al. also reached the same conclusion by combining FAIMS with H/D exchange [7]. Following the same notation as before, we refer to the peak transmitted at –20.4 V as conformational family A and the one at –16.1 V as conformational family B.

It should be noted that until recently, gas-phase conformers of [bk+2 H]$^{2+}$ were unresolved with IMS [33], while FAIMS [7] as well as kinetic studies of gas phase reactions of [bk+2 H]$^{2+}$ with hydro-iodic acid [34] indicated the existence of at least two conformations of this ion. However, with the construction of higher resolution ion mobility spectrometers it has become possible to resolve these two conformations partially in the drift time spectrum [16, 35].

Figure 3 displays UV photofragment spectra of cold [bk+2 H]$^{2+}$ in combination with FAIMS. The traces in
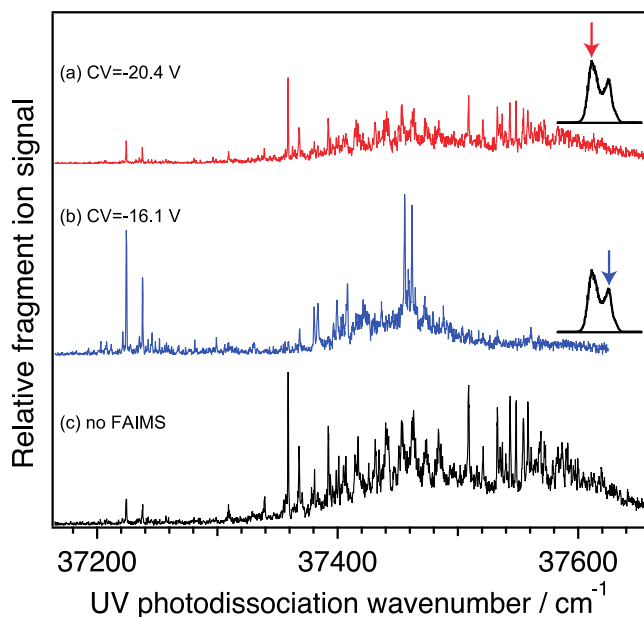
**Figure 3.** Electronic photofragment excitation spectra of $[bk+2\ H]^{2+}$ obtained **(a)** with FAIMS separation transmitting ions at CV=−20.4 V; **(b)** with FAIMS separation transmitting ions at CV=−16.1 V; **(c)** without FAIMS separation and under gentle conditions in the mass spectrometer

Figure 3a and b are recorded with the electrosprayed ions passing through the FAIMS electrodes and transmitted at CV values of –20.4 V and –16.1 V, respectively. For comparison, the spectrum recorded under gentle conditions without the use of FAIMS is included (Figure 3c). The spectra of Figure 3a and b are also recorded with low injection energy (L1:40 V) so that collisional energy imparted to the ions after exiting the separation stage is minimized. For the most part, the two spectra are distinct and represent the two conformational families.

The spectrum of conformational family A (Figure 3a) contains the peaks originating from conformers of the B family, but with much lower intensity than in the spectrum of Figure 3b [13]. However, the spectrum of Figure 3b is a conformationally clean spectrum of the B family. This cross-conformer contamination in the former case is an indication that conformational isomerization occurs between FAIMS separation and cooling in our cold ion trap [13]. From the comparison of the peak intensity at 37,224 cm⁻¹ in the spectra of Figure 3a and b, we estimate that at least at least 18 % of the molecules have undergone transition, A to B, which is a lower limit since other conformers of family B may be also formed.

During their passage through FAIMS, ions will inevitably be energized during the high voltage portion of the asymmetric waveform [12]. This warming of ions may result in their isomerization and, thus, a corresponding change in the CV value at which they are transmitted. For a large enough change in the CV value, the product will be filtered out if the isomerization happens sufficiently early in the separation process. If isomerization occurs just before the ions exit FAIMS, there might not be enough time for the new conformer(s) to be filtered out. In this case, for a specific CV value, two conformational families will be introduced into the mass spectrometer, and this will result in the cross-conformer contamination that we detect spectroscopically. In our case, we also use a temperature gradient across the FAIMS electrodes in order to increase the resolution of our device [2, 20]. While doing this allows partial separation of the ions into two conformational families, the increased temperature will raise the average energy imparted to the ions in collisions with the carrier gas. Thus, ions exiting FAIMS will be warm and, consequently, prone to isomerize downstream from the separation stage during their transfer to the 22-pole.

Heating of the ions during the FAIMS separation may not pose a problem when FAIMS is used as a stand-alone device for detecting the number of conformations of a biomolecular ion, although in cases of extreme heating, extended unfolding may lead to erroneous conclusions about the number of conformations initially produced [36]. However, when FAIMS is used in conjunction with methods in which a selected conformation is isolated for a subsequent experiment, the heating effect is undesirable and must be minimized. As discussed by Shvartsburg et al. [12], reducing the magnitude of the DV would lower the amount of field heating in FAIMS, but it would also decrease the resolution. A solution that would not affect the resolution would be to cool the FAIMS unit to compensate for the heating, which was estimated to be ~75 °C using a flat electrode FAIMS device [12]. In the case of the cylindrical geometry FAIMS device, we are obliged to maintain a temperature gradient across the two electrodes in order to achieve an acceptable resolution. This means that the amount of cooling would have to be more than 60 °C lower in order to incorporate the necessary temperature difference between the electrodes.

In order to verify the effect of the temperature gradient, we used our FAIMS device without a temperature gradient and recorded the spectrum of the ions transmitted at two different values of the CV. Figure 4 shows a CV spectrum of $[bk+2\ H]^{2+}$ with both electrodes held at 35 °C. The spectrum exhibits one peak at –14.1 V with a shoulder at –11.9 V. The smaller shoulder at –10.2 V arises from a charge reduction of $[bk+3\ H]^{3+}$ in the FAIMS device, which results in its detection as $[bk+2\ H]^{2+}$ [13, 36, 37]. As the temperature gradient is increased, the peak and the shoulder become increasingly resolved, and the CV spectrum approaches the one shown in the inset of Figure 3. We, thus, attribute the main peak in Figure 4 (–14.1 V) to conformational family A and the shoulder (–11.9 V) to conformational family B.

Although the two conformational families are not even partially resolved in this CV spectrum, operating FAIMS at reduced average temperature minimizes the thermal heating of the ions during the separation. It may be possible, by setting the CV off the main peak to more negative values of the compensating voltage (≤15 V), to introduce conforma-
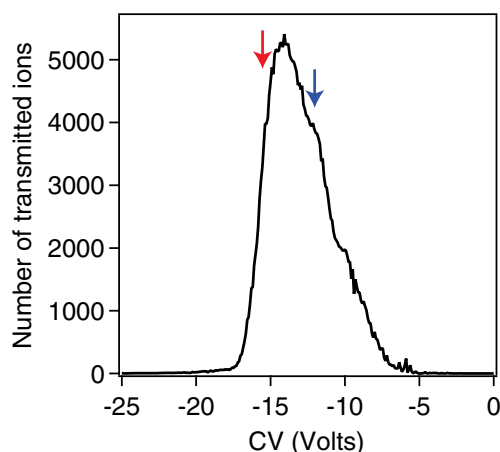
**Figure 4.** CV spectrum of $[bk+2\,H]^{2+}$ using a DV of $-5$ kV and no temperature gradient across the analytical gap. The temperature of both electrodes is 35 °C. The red and blue arrows are explained in the text. The carrier gas consists of 100 % $N_2$



**Figure 5.** Electronic photofragment excitation spectra of $[bk+2\,H]^{2+}$ obtained **(a)** with FAIMS separation transmitting ions at CV=$-15.2$ V; **(b)** with FAIMS separation transmitting ions at CV=$-11.9$ V; **(c)** without FAIMS separation and under very gentle conditions in the mass spectrometer. In spectra **(a)** and **(b)** FAIMS is used without temperature gradient

tional family A selectively into our mass spectrometer. This is because at CV $\leq 15$ V the contribution of conformational family B (shoulder, $-11.9$ V) will be reduced. Moreover, the ions of conformational family A exiting FAIMS will be heated less and thus be less likely to isomerize downstream.

Figure 5a shows the photofragment spectrum of $[bk+2\,H]^{2+}$ when the CV is set to $-15.2$ V (red arrow, Figure 4), while Figure 5b shows the spectrum when the CV is set to $-11.9$ V (blue arrow, Figure 4), both of which are recorded with no temperature gradient between the FAIMS electrodes (conditions of Figure 4). It is clear that the spectrum of conformational family A (Figure 5a) appears conformationally cleaner than that of Figure 3a, exhibiting almost no intensity for peaks belonging to family B. Thus, contrary to what might have been expected, better conformational separation in FAIMS does not necessarily produce conformationally cleaner spectra because the increased resolution is obtained by using a temperature gradient, which energizes the ions during FAIMS separation. The energized ions isomerize more rapidly in the mass spectrometer, producing the cross-conformer contamination demonstrated in the spectrum of Figure 3a.

The electronic spectrum of $[bk+2\,H]^{2+}$ exiting FAIMS at a CV value of $-11.9$ V (Figure 5b) appears to be a composite of the two conformational families. At this CV, which corresponds to the unresolved shoulder belonging to conformer family B, it is impossible to avoid the transmission of ions of the more abundant conformational family A [13].

It should be noted that spectra of Figures 3a and 5a are recorded under the same conditions in the mass spectrometer, but not in FAIMS. The carrier gas used for the separation contains 10 % He in Figure 3a, whereas in Figure 5a, pure $N_2$ is used. Use of a mixture of He/$N_2$ in FAIMS separations has been shown to increase the resolution of the separation [38] but, at the same time, heats the ions [39]. However, we estimate the amount of ion
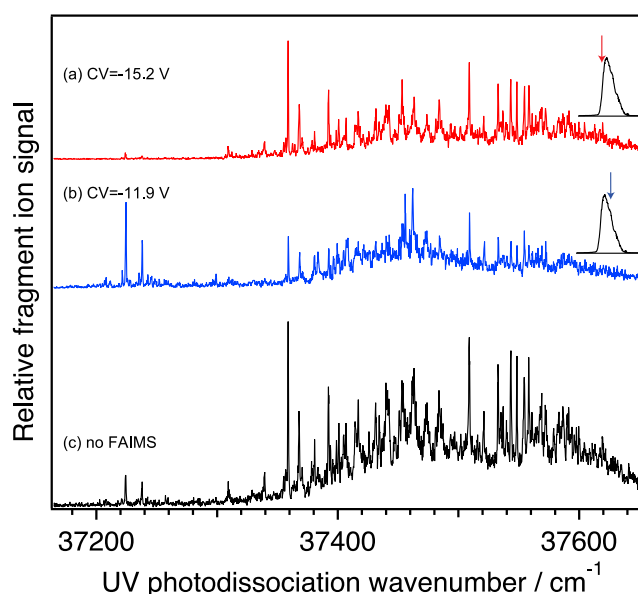
heating in a 90/10 $N_2$/He mixture to be about 4 °C greater than in a 100 % $N_2$ carrier gas and, thus, negligible compared with the increase in mean temperature when we use a temperature gradient to improve the resolution [38]. This means that the comparison between Figures 3a and 5a reveals the effect of the temperature gradient on the relative population of the conformations. The fact that we can record a conformationally clean spectrum of the A family (Figure 3a) suggests that the ions do not spontaneously isomerize to conformational family B, at least on the timescale of their transfer to the 22-pole ion trap where they are cooled. Moreover, the conditions we employ for recording the spectrum of Figure 5a are "harsher" than those of spectrum of Figure 5c (no FAIMS), and still the amount of cross-conformer contamination is negligible. This means peaks of conformational family B appear in this spectrum of Figure 5c because they are initially produced in the electrosprayed ion population and not because of isomerization from family A.

To examine conformational isomerization further, we investigated the effect of annealing the FAIMS-selected ion population by collisionally activating the ions (Figure 1) followed by cooling to 10 K in our 22-pole ion trap. The ion activation step is achieved by changing the potential difference between the capillary exit (L1) and the skimmer (see Figure 1), which changes the collisional energy of the ions as they enter the hexapole ion trap. Figure 6 shows UV spectra of the ion population after they have been cooled and trapped in our 22-pole as a function of increased injection energy into the hexapole. The left and right panels of
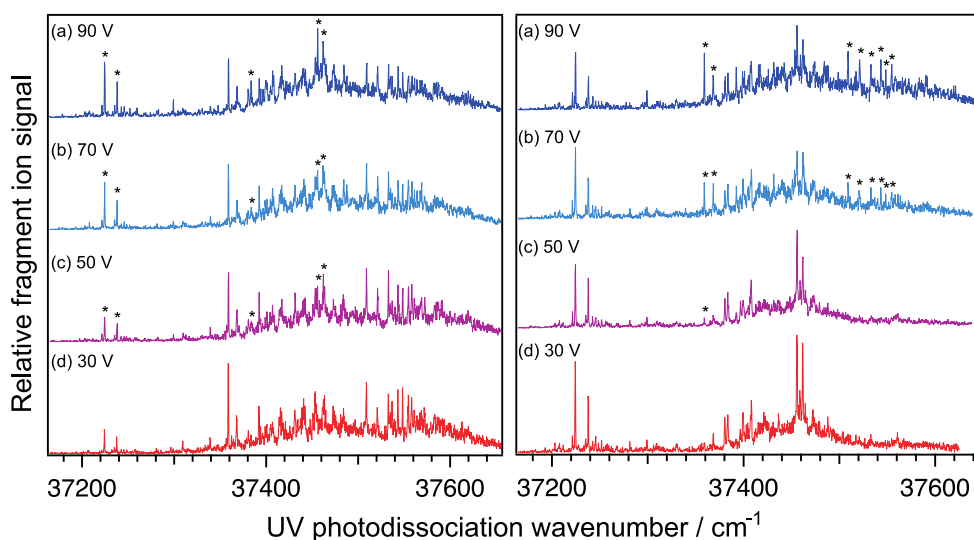
**Figure 6.** Electronic photofragment excitation spectra of $[bk+2\,H]^{2+}$ recorded as a function of the injection voltage into the hexapole ion trap. One conformational family is initially introduced into the mass spectrometer; left: conformational family A; right: conformational family B

Figure 6 show spectra when only conformational family A or B, respectively, is introduced into the mass spectrometer. The selection of conformational families is performed with a temperature gradient established between the FAIMS electrodes, which causes a small amount of contamination of conformer family B into A even at low injection voltage, as is evident from spectrum Figure 6d (left panel), where characteristic peaks of family B appear at low intensity.

As one can clearly see in Figure 6, for each conformational family, the characteristic peaks of the other family, indicated by asterisks, increase in intensity upon increase of the injection energy. This can only happen if an increased fraction of the FAIMS-selected population is excited above the barrier to isomerization from one family to the other, so that when the population is quenched in the hexapole or the cold ion trap, a new distribution is formed. At high injection energy the spectra of the relaxed population of ions in the cold trap are almost indistinguishable (Figure 6a, left and right panels). This means that the same conformational distribution of ions is formed, irrespective of which conformational family we introduce into the mass spectrometer. This is indicative of an equilibrium that is established between the two conformational families. Moreover, it is clear that this equilibrium conformational distribution is different from that observed directly from the electrospray source, when no conformational separation is used. This is evident by comparison of the spectra of Figure 6a with that of Figure 5c.

Recently, Pierson et al. investigated the dynamics of conformational isomerization of $[bk+3\,H]^{3+}$ using two dimensional ion mobility spectrometry in an IMS-IMS-MS configuration [16], with an activation region between the two mobility stages. They were able to separate six conformers of the $[bk+3\,H]^{3+}$ ion in the first stage and then selectively activate each of them and measure the subsequent distribution of conformers in the second stage as a function

of the activation voltage. They demonstrate that at high activation voltage the distribution is always the same, independent of the starting structure [16], indicating that an equilibrium is reached between the conformers at high energies. They also observe that the gas-phase equilibrium distribution of $[bk+3\,H]^{3+}$ conformers is different than the distribution initially produced by the electrospray source [16]. It thus seems that for both $[bk+2\,H]^{2+}$, as described here, as well as for $[bk+3\,H]^{3+}$, as shown in the work of Pierson et al. [16], electrospray produces a conformational distribution of ions that are kinetically trapped. Providing energy to the system induces isomerization such that the conformer populations approach gas-phase equilibrium.

The fact that the initial conformational distribution of electrosprayed ions can be different from the gas-phase equilibrium distribution is highly significant for studies of biological molecules in the gas phase. The use of vibrational spectroscopy to determine ion structure is accomplished by comparing a measured infrared spectrum with a calculated one. To determine the latter, a conformational search is typically performed using classical force fields, and then the energies of the most stable structures are refined using density functional theory. One then calculates the infrared spectra of the lowest energy structures and compares them to experiment. Since the calculations are done for isolated molecules, the structures determined by this approach should be the lowest energy gas phase structures. However, if the experiments do not produce the lowest energy gas-phase structures, this can inhibit finding a match between calculations and experiment and lead to erroneous conclusions.

From an experimental point of view, this problem can be overcome by annealing the initially formed conformer distribution as described in both our work here on $[bk+2\,H]^{2+}$, as well as that of Pierson et al. for $[bk+3\,H]^{3+}$ [16].

However, there can be important information in the conformational distribution initially produced by electrospray. Pierson et al. have shown in the case of $[bk+3 H]^{3+}$ that the nascent, gas-phase conformational distribution depends upon the solvent composition used in the electrospray process [32]. While this does not prove that the electrosprayed distribution is the same as that in solution, it clearly demonstrates that the gas-phase distribution retains a memory of the solution conditions from which it was produced.

By adding a spectroscopic dimension to ion mobility, our work should help elucidate the connection between gas-phase and solution-phase structures. The extreme sensitivity of our conformer-specific, IR-UV spectroscopic techniques puts high demands on the accuracy of calculated structures and spectra [40], and we have not yet succeeded in obtaining an acceptable match with our measured spectra of $[bk+2 H]^{2+}$ conformers [14]. Nevertheless, high-resolution spectroscopy will be key to understanding the connection between the gas-phase and solution.

## Acknowledgments

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