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Separation of protein conformers using electrospray-high field asymmetric waveform ion mobility spectrometry-mass spectrometry

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Abstract

High-field asymmetric waveform ion mobility spectrometry (FAIMS) is a technique for continuous gas-phase ion separation at atmospheric pressure (760 Torr) and room temperature. FAIMS separates ions based on changes in mobility at high electric fields. Ion focusing in a cylindrical geometry FAIMS provides high ion transmission efficiency, thereby making FAIMS an ideal interface for transferring ions from an electrospray ionization (ESI) source to a mass spectrometer (MS). In this study, an ESI-FAIMS-MS instrument was used to study the conformers of the protein bovine ubiquitin. Multiple conformers for some charge states of bovine ubiquitin were resolved by FAIMS, including at least three for the +8 charge state. The number and abundance of the conformers of several charge states of bovine ubiquitin were dependent on solution pH and solvent composition. Mass spectra of individual conformers showed conformer-specific distributions of sodium and phosphate adduct ions. ESI-FAIMS-MS conformational data are compared with literature results that were collected using ESI drift tube mobility spectrometry/mass spectrometry. (Int J Mass Spectrom 197 (2000) 163–177) © 2000 Elsevier Science B.V.

Keywords: FAIMS; Protein conformation; Ubiquitin; ESI; Ion mobility

1. Introduction

The mechanism by which a protein spontaneously folds into a precise three-dimensional, biologically active structure has eluded researchers for many years [1]. Recently, denatured and partially folded protein conformers have received increasing attention in an attempt to gain insight into the mechanism of protein folding [2,3]. The development of electrospray ionization (ESI) [4–7] has enabled the formation of intact gas phase, pseudomolecular ions from large molecules, such as proteins. These ESI generated ions can be studied in the gas-phase free of solvent interaction, thereby providing an opportunity to look at the role of solvent in studying solution phase protein conformations [8]. The observation of the unfolding of a protein using ESI mass spectrometry (MS) was reported Smith and co-workers [9]. Since then, several groups have used ESI-MS to obtain information about protein conformations in solution [10–17].

Information on gas-phase protein conformations has been obtained using several mass spectrometry techniques. Valentine et al. divided these techniques

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into two categories [18]: chemical reactivity studies and nonreactive studies. Chemical reactivity studies, including gas-phase hydrogen–deuterium (H/D) exchange [15,19–23] and proton transfer kinetics [23–29], examine the differences of reactivity of different conformations. Nonreactive studies use collisions with inert species to derive conformational information and have been carried out in the second quadrupole of a triple quadrupole mass spectrometer [30–32] or by using a drift tube in combination with a quadrupole mass filter [33–35]. The latter arrangement has been used to study the conformations of several proteins in the gas phase [35–40]. Combinations of reactive and nonreactive studies have also been used to study conformations of equine cytochrome *c* and bovine ubiquitin [18,41].

All of the nonreactive studies described above are based on measurements of average collision cross section. Recently, a new nonreactive technique, high-field asymmetric waveform ion mobility spectrometry (FAIMS), has been described [42,43]. FAIMS separates gas-phase ions based on properties of the ion that appear to be independent of both the low-field collision cross section and the mass-to-charge ratio.

In an earlier study characterizing an ESI-FAIMS-MS instrument [44], the observed charge state distribution for equine cytochrome *c* was shown to be dependent on FAIMS variables, suggesting that FAIMS was separating ions based on their structural properties. In this article, conformers of bovine ubiquitin are investigated using ESI-FAIMS-MS. Bovine ubiquitin was selected since prior studies using ESI-MS have shown that it undergoes conformational changes with the addition of acid [15–17], changes in solvent composition [13,45], or application of heat [14]. Here, ESI-FAIMS-MS is used to examine conformational changes induced by altering the pH and solvent composition. The effect of sodium and phosphate ions on the conformation is also examined. ESI-FAIMS-MS conformational data are compared with literature results that were collected using ESI drift tube mobility spectrometry/MS. Some advantages and limitations of FAIMS for the study of conformations in the gas phase are also discussed.

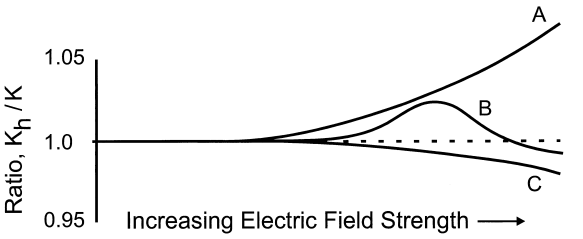

Fig. 1. Illustration of the dependence of ion mobility on electric field for three different types of ions.

2. Introduction to high-field asymmetric waveform ion mobility spectrometry

The FAIMS device separates ions at atmospheric pressure (760 Torr), and room temperature. FAIMS can be compared with conventional ion mobility spectrometry (IMS) because both techniques are based on the motion of ions induced by electric fields at atmospheric pressure. A conventional IMS [46–48] is a chamber housing a series of metal plates to which uniformly incremented dc voltages are applied (i.e. forming a drift tube). Ions are gated into the drift tube using a shutter grid assembly and are separated based on differences in their drift velocities. Conventional IMS uses low electric fields (e.g. 200 V/cm) where the ion velocity (v) is directly related to the electric field strength (E); $v = KE$, where K is the compound dependent mobility constant.

At high electric fields (e.g. 10 000 V/cm), v is no longer directly proportional to E because ion mobility is dependent on the applied electric field [47,48]. Ion mobility is therefore better represented by K_h, a nonconstant, high-field, mobility term that is given by [42,47]: $K_h(E) = K[1 + f(E)]$, where $f(E)$ describes the functional dependence of ion mobility on the electric field. This dependence of ion mobility on the applied electric field has been the basis for the development of FAIMS. Of the virtually unlimited number of possibilities for $f(E)$, Fig. 1 illustrates three trends in ion mobility, as a function of electric field, that have been observed [49]. In Fig. 1, as electric field strength increases, the mobility of a type A ion increases, a type C ion decreases, and a type B ion increases initially before decreasing. This study

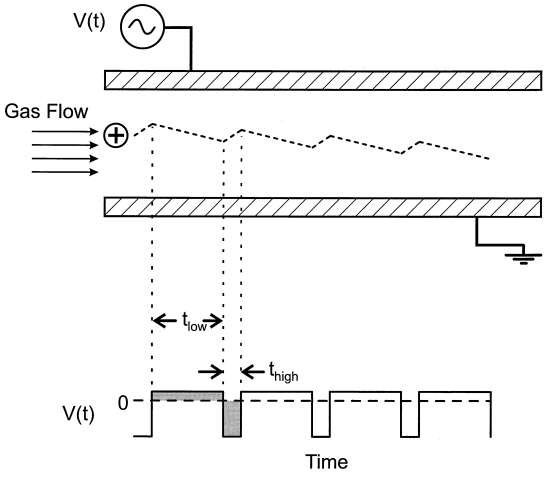

Fig. 2. Illustration of the ion motion between two parallel plates during application of an electrical potential shown as $V(t)$; the ion is transported horizontally by a gas flow (distance not to scale).

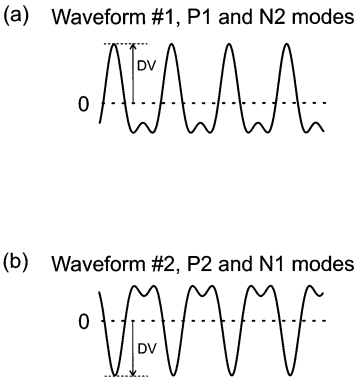

Fig. 3. Asymmetric waveforms used in FAIMS. The maximum value of the waveform is called the dispersion voltage (DV).

focuses on type C ions since all gas-phase ubiquitin ions that are discussed exhibited type C behavior.

A mathematical description of the operation of a flat plate FAIMS device is given elsewhere [42,43,50]. For illustrative purposes, consider a type C ion (Fig. 1) that is being carried by a gas stream between two parallel plates, as shown in Fig. 2. The lower plate is maintained at ground potential and the upper plate has a (simplified) asymmetric waveform, described by $V(t)$, applied to it. This asymmetric waveform consists of a high voltage (negative) component lasting a short time and a low voltage (positive) component lasting a longer time. The waveform is synthesized such that the integrated voltage–time product (thus the field–time product) applied to the upper plate during a complete cycle of this waveform is zero (i.e. the shaded areas of the waveform in Fig. 2 are equal). Since the field–time products are equal in magnitude, an ion will only experience a net displacement from its original location, relative to the plates, if the applied electric field is sufficient to cause a difference between its high-field and low-field mobilities (i.e. K_h and K). By definition, a type C ion experiences a decrease in its mobility at high electric fields (i.e. $K_h < K$). This results in a net displacement of the ion away from the upper plate as illustrated by the dashed line in Fig. 2. By applying a constant, negative dc voltage, called the "compensation voltage" (CV), to the upper plate, this net ion drift can be eliminated. If the ions derived from two compounds respond differently to the applied high electric field, the ratio of K_h to K (and thus the applied CV) is different for each compound. Under conditions where the CV is appropriate for transmission of one compound, the other will drift toward one of the plates and be lost. Thus, the FAIMS instrument is an ion filter, capable of selective transmission of only those ions with the appropriate ratio of K_h to K. To separate a mixture of ions, the CV is scanned to yield a compensation voltage spectrum (CV spectrum).

The concept described above was reported by Buryakov et al. [42] using flat plates. Later, Carnahan et al. improved the design by replacing the flat plates with concentric cylinders [51–53]. This modification has several advantages including higher sensitivity than the flat plate configuration. In an earlier report [43] we described a FAIMS interface to a mass spectrometer (FAIMS-MS) that allowed for peak identification.

Fig. 3 shows two asymmetric waveforms that are used in FAIMS. The maximum value of the waveform, called the dispersion voltage (DV), is indicated in Fig. 3. By reversing the polarity of the waveform, different types of ions will pass through the FAIMS device. This observation is a consequence of an atmospheric pressure ion focusing mechanism in the

cylindrical geometry FAIMS [43,50]. The physics behind this mechanism has been described in detail elsewhere [50]. Experimentally, these two asymmetric waveforms are considered separately, each can be used with positive (P) or negative (N) ions, for a total of four modes of operation [43]. The waveform with negative DV yields spectra of types P2 and N1, whereas the reversed polarity waveform yields P1 and N2 type spectra. In general, low mass ions (*m/z* is usually below 300) are of type A (Fig. 1) and are detected in mode 1, whereas larger ions, including the positively charged bovine ubiquitin ions studied here, are type C ions and are detected in mode 2.

Although related, FAIMS and IMS differ in several ways. (1) FAIMS is an ion filter that transmits selected ions in a continuous fashion, whereas ions are transmitted through IMS in discrete pulses. The continuous transmission of FAIMS makes it a practical interface between electrospray and quadrupole mass spectrometry (i.e. ESI-FAIMS-MS). (2) The ions in FAIMS are transported in the axial direction by a flowing stream of gas and the electric fields are applied in a direction perpendicular to the gas flow. In IMS, the ions are driven axially by an electric field. (3) When used with ESI, the FAIMS instrument operates at room temperature. Conventional IMS requires a sophisticated heated inlet system [54–56] to increase the rate of desolvation of the analyte ions produced from an electrospray source. In addition, the flight tube of a conventional IMS instrument is usually maintained at 150 °C or higher, to ensure that the ions remain fully desolvated. Desolvation in FAIMS is sufficient such that elevated temperatures in the ion source or the analyzer region are not required. (4) The cylindrical geometry FAIMS focuses the ions that it transmits [50]. This gives higher sensitivity than IMS, in which ion diffusion in the drift tube causes the ion cloud to expand, resulting in inefficient transfer of ions to the mass spectrometer.

3. Experimental

A schematic of the ESI-FAIMS-MS instrument is presented in Fig. 4. A detailed description of the hardware has been given previously [44,57]. Solutions were delivered to the electrospray needle (50 μm i.d.) by a syringe pump (Harvard Apparatus, Model 22) at a flow rate of 1 μL/min. The electrospray needle was operated at +2200 V, giving a current of about 50 nA.

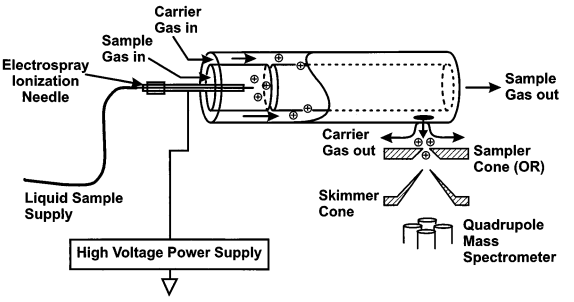

Fig. 4. Three-dimensional view of the ESI-FAIMS-MS instrument (the FAIMS and sampler cone are actually positioned at a 45 ° angle).

The FAIMS ion filter consisted of two inner cylinders that were axially aligned and positioned about 5 mm apart, and an outer cylinder that surrounded the two inner cylinders. A high frequency (210 kHz), high voltage (0–4950 $V_{p\text{-}p}$), asymmetric waveform was applied to the long inner cylinder to establish an electric field between the inner and outer tubes. All ESI-FAIMS-MS spectra were collected using P2 mode with DV = −3300 V. In addition to the high frequency asymmetric waveform, the CV was also applied to the long inner cylinder.

Nitrogen gas was passed through a charcoal/molecular sieve gas purification cylinder and introduced into the FAIMS device through the carrier in (C_{in}) port at a flow rate of 6 L/min. The gas exited through the sample out (S_{out}) port at 1 L/min and through the carrier out (C_{out}) port at 5 L/min. The sample in (S_{in}) port did not have a net gas flow. S_{in} was used to monitor the pressure within the FAIMS device. The pressure in the FAIMS was approximately 770 Torr. A fraction of C_{in} was directed radially inward through the gap between the inner cylinders and acted as a curtain gas. This portion of C_{in}, along with neutrals, exited the FAIMS device via the S_{out} port.

A custom interface was constructed for the tandem combination of a FAIMS analyzer and a PE Sciex API

300 triple quadrupole mass spectrometer. With the appropriate combination of DV and CV, ions were transferred to the vacuum chamber of the mass spectrometer through a "sampler cone" placed at the end of the FAIMS analyzer at 45° relative to the axis of the FAIMS cylinders. For clarity, Fig. 4 shows the FAIMS analyzer at 90° relative to the sampler cone. The diameter of the orifice in the sampler cone was approximately 260 μm. The sampler cone was electrically insulated and a separate voltage (OR) was applied to it. A potential was applied to the entire FAIMS unit (V_{FAIMS}) to optimize ion transmission between FAIMS and the sampler cone. V_{FAIMS}, OR, and the skimmer cone were maintained at 50, 44, and 0 V, respectively.

Ion-selective CV spectra (IS-CV spectra) were collected by scanning the compensation voltage applied to the FAIMS while monitoring a single *m/z* value. "Total ion current" CV spectra (TIC-CV spectra) show the sum of the signal for all the ions detected in a given *m/z* range as CV was scanned. A mass spectrum collected at fixed values of DV and CV revealed the identity of any ions transmitted through the FAIMS under those conditions. Unless otherwise indicated, mass spectra are sums of ten scans using a dwell time of 5 ms for every 0.5 *m/z* units.

Bovine ubiquitin was purchased from Sigma Chemical Company (St. Louis, MO) and used without further purification. Either acetic or hydrochloric acid was used to adjust sample pH. Varying volumes of HPLC grade methanol (Fisher, Nepean, ON) and distilled deionized water were used as the solvent.

4. Results and discussion

The conformers of bovine ubiquitin were investigated since prior electrospray literature has shown that multiple conformers exist for some charge states [18,22,23,28,45]. Fig. 5(a) shows a mass spectrum for a solution of 5 μM bovine ubiquitin in 50/50/0.04 water/methanol/acetic acid (v/v/v) collected using ESI-FAIMS-MS, but with the FAIMS analyzer "disabled" (i.e. DV = CV = 0 V). Fig. 5(a) resembles a

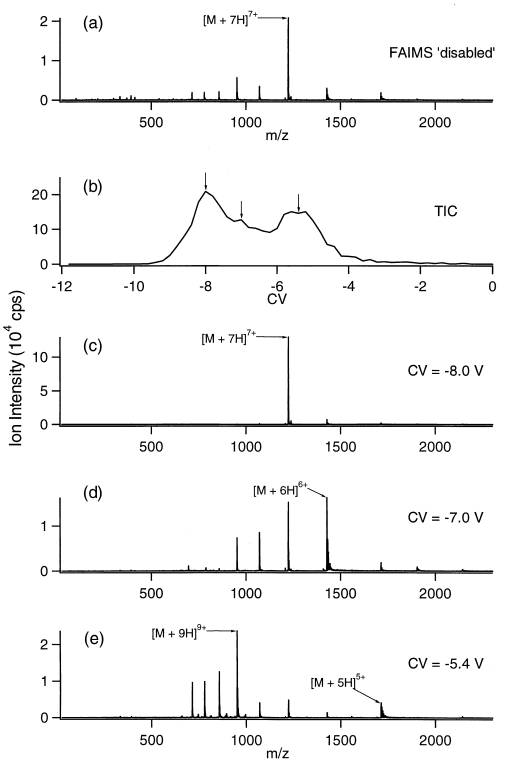

Fig. 5. ESI spectra of a solution of 5 μM bovine ubiquitin in 50/50/0.04 water/methanol/acetic acid (v/v/v). (a) Low sensitivity ESI mass spectrum obtained with the FAIMS disabled; (b) total ion current (TIC)-CV spectrum from CV = −12 to 0 V obtained over the mass range *m/z* 30–2300. Mass spectra collected at CV values of (c) −8.0 V, (d) −7.0 V, and (e) −5.4 V.

conventional ESI-mass spectrum, however the sensitivity is lower because of ion losses that occur in the FAIMS device when it is disabled. Several charge states are observed in the mass spectrum for ions electrosprayed from a solution with this solvent composition. Fig. 5(b)–(e) are ESI-FAIMS-MS spectra of the same solution with the FAIMS device in operation. Fig. 5(b) shows a TIC-CV spectrum (*m/z* 30–2300), collected by scanning the CV from −12 to 0 V. This spectrum has two distinct peaks with maxima at CV = −8.0 V and CV = −5.4 V. Fig. 5(c)–(e) are mass spectra acquired at CV values indicated by the arrows in Fig. 5(b). The mass spectrum collected at CV = −8.0 V [Fig. 5(c)] is dominated by the $[M + 7H]^{7+}$ ion of bovine ubiquitin. Unlike the conventional ESI-mass spectrum shown in Fig. 5(a), charge

states higher than +7 are virtually absent in Fig. 5(c). A mass spectrum acquired at CV = −7.0 V [Fig. 5(d)], shows a very different charge state distribution than Fig. 5(c). The $[M + 6H]^{6+}$ ion is the most abundant ion in this mass spectrum and several of the higher charge states are also observed. Finally, the mass spectrum at CV = −5.4 V [Fig. 5(e)] shows yet another charge state distribution which is quite unlike the previous two mass spectra. In this spectrum, the abundance of the $[M + 7H]^{7+}$ ion is significantly reduced compared with Fig. 5(c) and (d), and the higher charge states (along with charge state +5) have increased in intensity. The increase in sensitivity in the mass spectra collected with the FAIMS operating, Fig. 5(c)–(e), compared with the mass spectra when the FAIMS was disabled, Fig. 5(a), is a result of atmospheric pressure ion focusing [50].

The changes in the charge state distribution observed in the mass spectra collected at different CV values indicated that ion separation in FAIMS is sensitive to the structure of the protein ion. Consequently, IS-CV spectra (Fig. 6) for the individual charge states of bovine ubiquitin (+5 through +13) were collected using the same solution as Fig. 5. Some charge states (e.g. +10) show only one peak in their IS-CV spectra whereas others show multiple peaks (e.g. +8). The IS-CV spectra for the higher charge states (e.g. +10 through +13) are similar and have CV values that are consistent near CV = −5.5 V. The IS-CV spectrum for the +8 charge state includes the peaks at the most negative CV (−9 V) and the least negative CV (−5 V) observed for all the charge states.

The multiple peaks that appear in several of the IS-CV spectra in Fig. 6 are attributed to co-existing and distinct conformations of bovine ubiquitin. In the following discussion, the behavior of several of the conformers for individual charge states of bovine ubiquitin as a function of solvent composition and pH is described.

pH effects. Different concentrations of acetic acid were used with 5 μM solutions of bovine ubiquitin in 55:45 water/methanol (v/v) to investigate conformational changes as a function of pH. The composition of the solvent was selected to yield suitable mixtures

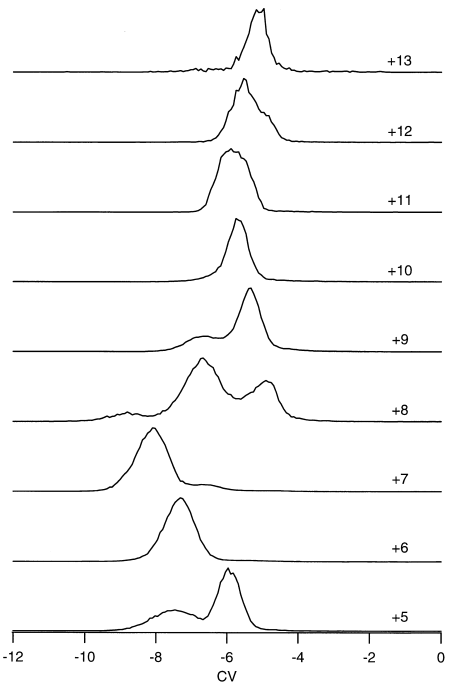

Fig. 6. Normalized ion selective (IS)-CV spectra for the charge states of bovine ubiquitin from +5 to +13 using the same solution as Fig. 5.

of conformers for illustrating the effects of pH in these experiments. Fig. 7(a) shows ESI-mass spectra, collected with the FAIMS disabled, for three acetic acid concentrations, 0.04% in trace (I), 0.4% in trace (II) and, 4% in trace (III). Fig. 7(b) shows the corresponding TIC-CV spectra (*m/z* from 30 to 2300) collected with the FAIMS in operation at the same concentration of acetic acid.

With 0.04% acetic acid, Fig. 7(a) trace (I), charge states greater than +7 are present at low abundances. This mass spectrum is similar to one reported for bovine ubiquitin in its native state [15]. The TIC-CV spectrum in Fig. 7(b) for the same solution [trace (I)], shows an intense peak at CV = −8.2 V with a small shoulder at CV ∼ −6 V. An ESI-mass spectrum collected using a solution containing 0.4% acetic acid, Fig. 7(a), trace (II), shows a second charge state distribution, centered around $[M + 12H]^{12+}$, in addition to the distribution centered at $[M + 7H]^{7+}$. This second distribution is consistent with mass spectra

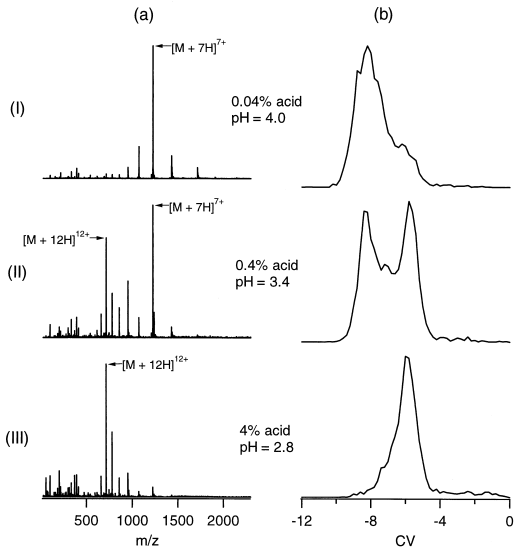

Fig. 7. Effect of the amount of acetic acid on a solution of 5 μM bovine ubiquitin in 55/45 water/methanol (v/v). (a) ESI-mass spectra collected with the FAIMS disabled, (b) TIC-CV spectra obtained over the mass range *m/z* 30–2300 with the FAIMS in operation.

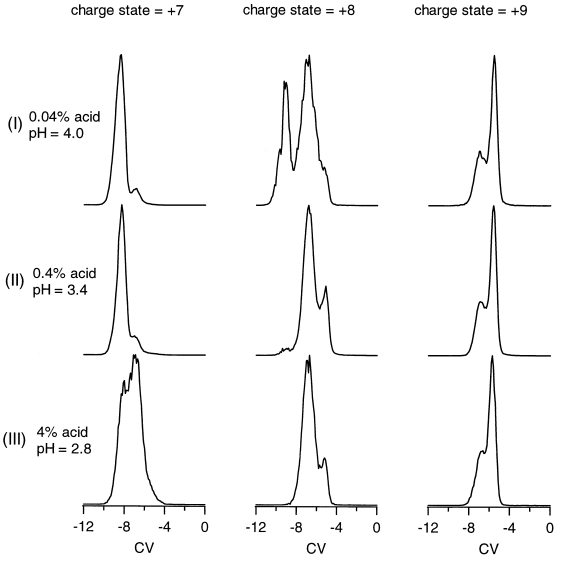

Fig. 8. Effect of the amount of acetic acid on the IS-CV spectra of charge states +7, +8, and +9 of bovine ubiquitin. The solutions in traces (I), (II), and (III) are the same as those used in traces (I), (II), and (III) in Fig. 7.

collected for bovine ubiquitin in its denatured form [15]. The TIC-CV spectrum for the 0.4% acetic acid solution [Fig. 7(b) trace (II)] contains a peak at CV $\sim$ -6.0 V, presumably due to the presence of the denatured bovine ubiquitin. Finally, at 4% acetic acid [trace (III)], the ESI-mass spectrum shows almost exclusively the higher charge states. The TIC-CV spectrum in trace (III) shows only one peak located at CV = -6.0 V. Consequently, the TIC-CV spectra collected with FAIMS reflect changes observed in the ESI-mass spectra of bovine ubiquitin.

In addition to altering the charge state distribution, changes within some charge states also occurred as a function of pH. Fig. 8 shows the IS-CV spectra for charge states +7, +8, and +9 of bovine ubiquitin as a function of pH. Traces (I), (II), and (III) were obtained using the same solutions as traces (I), (II), and (III) in Fig. 7. The IS-CV spectra for the +8 charge state, collected using 0.04% acetic acid, Fig. 8 trace (I), shows two distinct conformers at CV $\sim$ -9 V and $\sim$ -7 V. With 0.4% acetic acid, trace (II), the IS-CV spectrum for the +8 charge state changes significantly. The abundance of the conformer that was observed at CV $\sim$ -9 V is significantly reduced and a new conformer at CV $\sim$ -5 V is visible. This change suggests that as bovine ubiquitin begins to unfold, the conformation of the +8 charge state transmitted at CV $\sim$ -9 V is no longer favorable. At a concentration of 4% acetic acid, trace (III), this conformer is completely absent from the IS-CV spectrum. The +7 charge state also experienced significant changes as the concentration of acetic acid was increased. At low levels of acetic acid, trace (I), the IS-CV spectrum is dominated by a conformer transmitted at CV $\sim$ -8 V. However, when 4% acetic acid is used, trace (III), the IS-CV spectrum shows a shift that now favors the conformer that is transmitted at CV $\sim$ -7 V. The other charge states of bovine ubiquitin, such as the +9 charge state shown in Fig. 8, did not show significant changes over this pH range. Valentine et al. have also observed several distinct conformations for some charge states of bovine ubiquitin (those of intermediate size, i.e. partially folded) [18]. A detailed comparison of our results with those obtained using ESI-drift tube mobility spectrometry

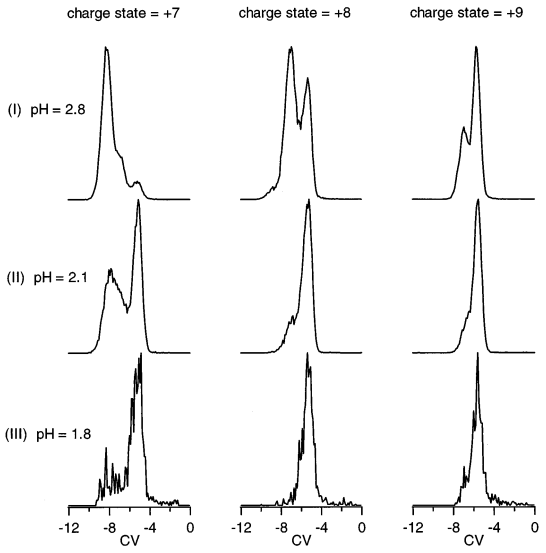

Fig. 9. Effect of adding hydrochloric acid to a solution of 5 μM bovine ubiquitin [55/45 water/methanol (v/v)] on the IS-CV spectra of the charge states +7, +8, and +9.

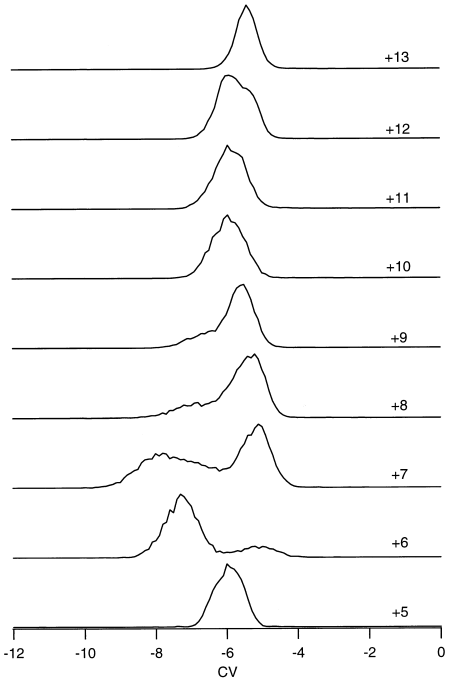

Fig. 10. Normalized IS-CV spectra for the charge states from +5 through +13 using a 5 μM solution of bovine ubiquitin [55/45 water/methanol (v/v)] acidified to pH 2.1 with hydrochloric acid.

(i.e. low pressure IMS)-mass spectrometry [18] is discussed at the end of this section.

To investigate the behavior of the conformers at lower pH values, hydrochloric acid was added to solutions of 5 μM bovine ubiquitin [55:45 water/methanol (v/v)]. IS-CV spectra collected at pH 2.8, 2.1, and 1.8 are shown in Fig. 9. Trace (I) in Fig. 9 was obtained at the same pH as the last experiment in Fig. 8 [i.e. trace (III), pH $\sim$ 2.8] to enable a comparison to be made based on changing the type of acid only. The IS-CV traces for charge states +8 and +9 at pH 2.8 in hydrochloric acid are comparable with those in Fig. 8 acquired using acetic acid at pH 2.8. The IS-CV spectra for most of the other charge states gave analogous results (not shown). The one exception was the IS-CV spectra for the +7 charge state in hydrochloric acid at pH 2.8. The IS-CV spectrum at pH 2.8, Fig. 9 trace (I), most closely resembles IS-CV spectra for acetic acid at pH 3.4 [Fig. 8 trace (II)], but with an additional conformer transmitted at CV $\sim$ −5 V that was not observed when using acetic acid.

By decreasing the pH to 2.1, trace (II), there are significant changes that are observed for all charge states shown in Fig. 9. For all charge states, a conformer transmitted at a CV between −5 and −6 V becomes favored as the conformers at more negative CV values decrease significantly in relative intensity. This trend continues as the pH is lowered even further to 1.8, trace (III). The IS-CV spectra that were obtained at pH 1.8 were noisy and much less intense, however, dominance of the conformer at the least negative CV value is still apparent.

Fig. 10 shows IS-CV spectra for the individual charge states of bovine ubiquitin (+5 through +13) for a solution acidified to pH 2.1 with hydrochloric acid [55:45 water/methanol (v/v)]. The most abundant conformers of the charge states observed using this solution, with the exception of charge state +6, are observed at CV values of $\sim$ −5 or −6 V. A comparison of the IS-CV spectra in Fig. 10 with IS-CV spectra shown in Fig. 6 [50:50:0.04 water/methanol/acetic acid (v/v/v)], show differences that reflect changes in conformation. The traces for charge states +10 through +13, in Fig. 10, are similar to those

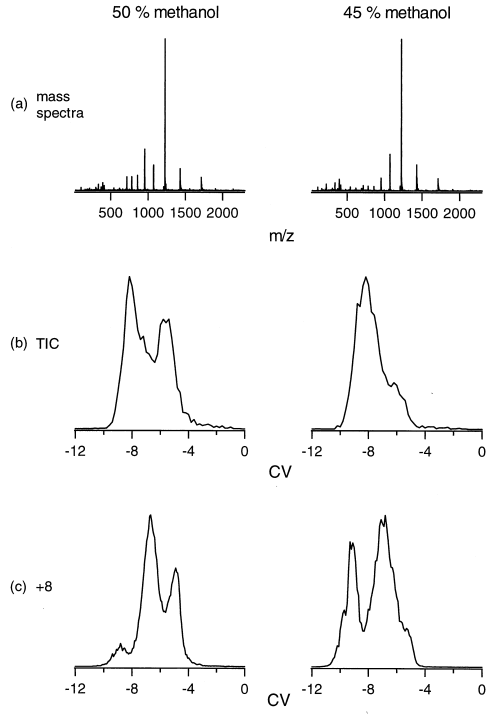

Fig. 11. Effect of solvent composition. (a) ESI-mass spectra collected with the FAIMS disabled. (b) TIC-CV spectra obtained over the mass range *m/z* 30–2300, and (c) IS-CV spectra for the +8 charge state.

observed in Fig. 6. However, the other charge states (+5 through +9) show significant differences between Figs. 6 and 10. In particular, the most abundant conformer in each of the IS-CV spectra for charge states +7 and +8 now occurs at a less negative CV value. The abundance of the conformers for the +5 and +9 charge states located at CV values of −7.5 and −7.0 V, respectively, have been drastically reduced. The abundance of the conformer transmitted through FAIMS at CV ∼ −5 V for the +6 charge state has increased.

Effect of solvent. Fig. 11 illustrates the effect of changing the solvent mixture from 50:50 water/methanol (v/v) to 55:45 water/methanol (v/v), while maintaining the same acetic acid concentration (0.04%). Fig. 11(a) shows ESI-mass spectra collected with the FAIMS disabled. The mass spectrum of the solution containing the higher percentage of organic solvent (i.e. 50% methanol) shows a slight increase in the abundance of the higher charge states. This observation is consistent with previous studies of bovine ubiquitin by Loo et al. [13] that showed that the formation of higher charge states was favored with increasing amounts of organic solvent. The TIC-CV spectra collected with the FAIMS in operation, Fig. 11(b), reflect this change in solvent composition as indicated by the increase in intensity of the peak at CV ∼ −6 V for the 50% methanol solution. The IS-CV spectrum for the +8 charge state, Fig. 11(c), also undergoes a significant change in this limited solvent range. In particular, the conformer transmitted at CV ∼ −9 V is more abundant and the conformer transmitted at CV ∼ −5 V is less abundant in the spectrum collected using the 45% methanol solution.

Effect of other species in solution. Mass spectra of the three conformers resolved by FAIMS in the IS-CV spectrum of the +8 charge state of bovine ubiquitin were collected in the presence of other salts in the sample solution. Fig. 12(a) trace (I), shows an IS-CV spectrum for the +8 charge state generated using a solution containing 5 μM bovine ubiquitin in 50:50:0.04 water/methanol/acetic acid (v/v/v). Presented in traces (II)–(IV) in Fig. 12(a) are mass spectra collected at each of the maxima in trace (I). The expanded views of the three mass spectra show that each conformer has a different affinity for sodium. Sodium ions have replaced protons, that is $[M + nH + mNa]^{(n+m)+}$, where $n + m = 8$, for each conformer of the +8 charge state of bovine ubiquitin. The conformer at CV = −4.8 V shows the replacement of up to four protons with sodium ions, the conformer at CV = −8.9 V shows virtually no proton replacement, and the conformer at CV = −6.9 V shows an intermediate level of proton replacement. These spectra suggest that the differences in the three-dimensional structures of the conformers of the +8 charge state of bovine ubiquitin have resulted in varying degrees of replacement of protons by sodium ions.

It might be argued that the location of the peaks in the CV spectrum shown in Fig. 12(a) is not a consequence of the different conformations of bovine ubiquitin, but instead that the amount of sodium dictates the location of the ion in the CV spectrum.

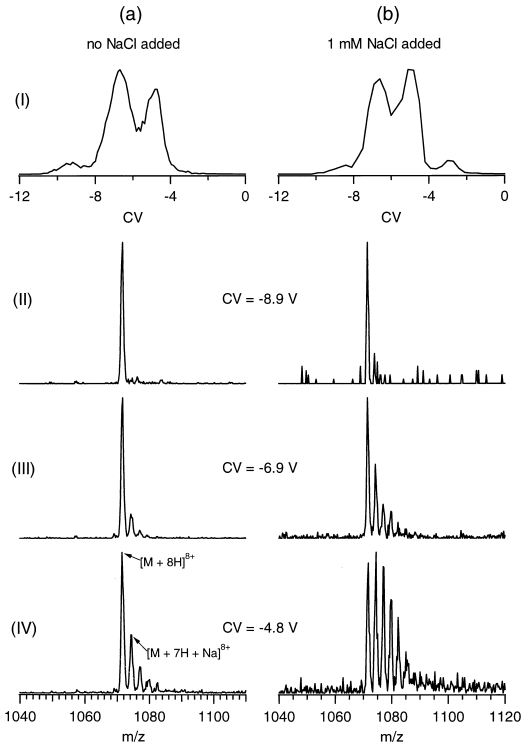

Fig. 12. Effect of adding NaCl to a solution of 5 μM bovine ubiquitin [50/50/0.04 water/methanol/acetic acid (v/v/v)] on the IS-CV spectrum for the +8 charge state. (a) No NaCl is added to the solution, (b) 1 mM NaCl is added to the solution.

Previous results with ESI-FAIMS-MS have shown that for a small peptide (e.g. leucine enkephalin, MW 555.5), adduct ions can alter the CV at which a given species is transmitted through FAIMS [44,57]. Consequently, two additional experiments are described to show that the presence of different degrees of sodium substitution is a result of the different conformations, and that in this instance, the location of the protein ion in these CV spectra is not altered by replacement of protons with sodium.

The spectra in Fig. 12(b) were obtained after 1 mM of sodium chloride was added to the solution that was used to collect the data for Fig. 12(a). Note that because the distribution of the sodium-containing ions occurs over several *m/z* values, the "IS-CV spectrum" in Fig. 12(b) was collected over a narrow *m/z* range (i.e. 1071–1080) rather than at *m/z* 1071.6 (i.e. the *m/z* of the +8 charge state). The IS-CV spectra, trace (I) in Fig. 12(a) and (b), are very similar, and the CV values of the three conformers corresponding to the +8 charge state of bovine ubiquitin have not changed significantly. The only notable change in the IS-CV spectrum shown in Fig. 12(b) is a new peak located at CV ~ -3 V. A mass spectrum taken at this CV did not reveal bovine ubiquitin, but instead a continuum of background ions. The expanded views of the mass spectra collected for the three species of the +8 charge state of bovine ubiquitin, traces (II)–(IV), have changed dramatically with the addition of sodium chloride. Furthermore, the mass spectrum for the species at CV $= -4.8$ V with no sodium added [Fig. 12(a) trace (IV)] is very similar to the mass spectra for the species at CV $= -6.9$ V with 1 mM sodium added [Fig. 12(b), trace (III)]. If the replacement of protons with sodium ions was responsible for the shift in CV, these two species should have been observed at identical locations in the CV spectra. Thus, the number of sodium replacements is concluded to be a result of the structural differences in the conformers observed for the +8 charge state of bovine ubiquitin.

The results of a second experiment that also illustrates that the number of sodium ions does not cause a shift in the CV spectrum are given in Fig. 13. Fig. 13(a) shows an IS-CV spectrum for the +6 charge state that was obtained by using a solution of 5 μM bovine ubiquitin in 50:50:0.04 water/methanol/acetic acid (v/v/v). This spectrum is dominated by a conformer transmitted at CV $= -7.5$ V, however there is also a less abundant conformer transmitted at CV $= -5.7$ V. Fig. 13(b) and (c) show mass spectra that were collected at CV $= -5.7$ V and CV $= -7.5$ V, respectively [note Fig. 13(b) represents a sum of 50 mass spectra]. For this charge state, the ion that is amenable to a higher level of sodium replacement of protons is observed at the more negative CV of the two conformers. In contrast, the +8 charge state of bovine ubiquitin (Fig. 12) indicated that the conformer with the higher number of sodium ions was observed at less negative CV values.

The effect of phosphate on the IS-CV spectra of the various charge states of bovine ubiquitin was also investigated. Fig. 14 shows ESI-FAIMS-MS data collected using a 5 μM solution of bovine ubiquitin in

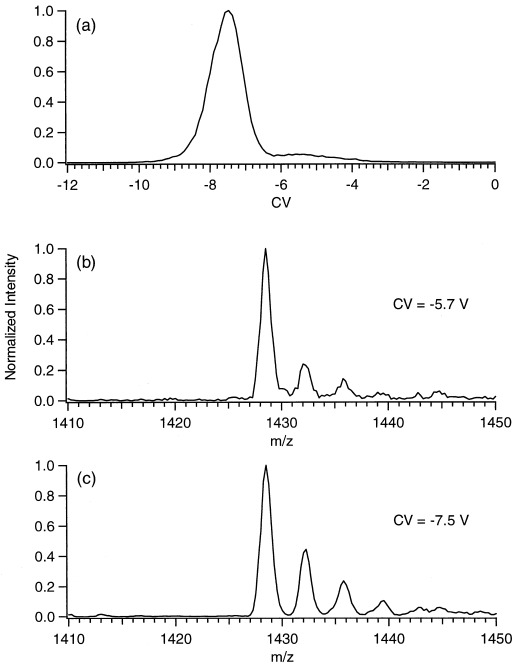

Fig. 13. Differences in the degree of proton replacement by sodium in two conformers of the +6 charge state of bovine ubiquitin. (a) IS-CV spectrum, (b) mass spectrum at CV = −5.7 V, and (c) mass spectrum at CV = −7.5 V. The solution was 50/50/0.04 water/methanol/acetic acid (v/v/v).

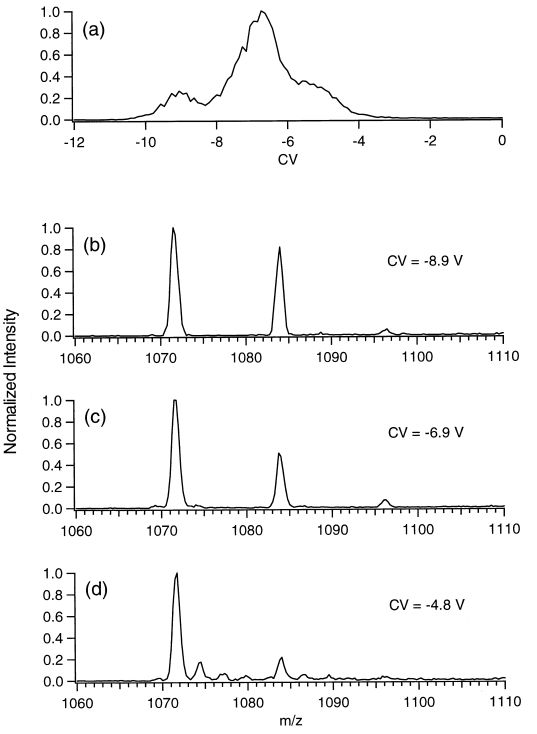

Fig. 14. Phosphate adduct ion intensity of the conformers of the +8 charge state of bovine ubiquitin. (a) IS-CV spectrum, (b) mass spectrum at CV = −4.8 V, (c) mass spectrum at CV = −6.9 V, and (d) mass spectrum at CV = −8.9 V. The solution was 50/50/0.02 water/methanol/acetic acid (v/v/v) with added KH_2PO_4.

50:50:0.02 water/methanol/acetic acid (v/v/v) and potassium di-hydrogen phosphate. Fig. 14(a) shows an IS-CV spectrum for the +8 charge state of bovine ubiquitin that is very similar to IS-CV spectra for this charge state shown in Fig. 12. The change in the relative abundances of the conformers at CV ∼ −9 V and −5 V, compared with Fig. 12(a), can be attributed to the small change in pH caused by the different acetic acid concentration and the added KH_2PO_4. Expanded views of the mass spectra, obtained at the CV values corresponding to the three maxima in Fig. 14(a), show adduct ions of the type $[M + 8H + mH_3PO_4]^{8+}$, where $m = 1$ or 2. As was observed with sodium, the degree of adduct formation is conformer dependent. However, the results in Fig. 14 show a different trend compared with the results shown in Fig. 12. That is, in Fig. 14, the conformer that appears in the CV spectrum at CV ∼ −9 V [Fig. 14(b)] shows the most intense phosphate adduct ion whereas the conformer that appears in the CV spectrum at CV ∼ −5 V [Fig. 14(d)] shows the least intense phosphate adduct ion. Note also that none of the three conformers of the +8 ion showed a tendency to replace protons with potassium ions.

These examples illustrate that by examining "spectator" ions, such as sodium and phosphate, information about the differences in the conformers may be obtained. These experiments have indicated that the replacement/addition of a spectator ion does not necessarily affect the conformation of the protein ion. However, in some cases the replacement/addition reactions may also lead to significant changes in the conformation of an ion. Such changes would be expected (in most cases) to be reflected by shifts in the IS-CV spectra. Preliminary data, in which the formation of some adduct ions results in significant changes in the IS-CV spectra [58], are being further evaluated.

Concentration effects. In a previous article, two distinct peaks were observed in IS-CV spectra that were collected while monitoring the molecular ion of leucine enkephalin using ESI-FAIMS-MS [44,57]. The isotope patterns of the mass spectra collected for these peaks identified the species as $[M + H]^+$ and $[2M + 2H]^{2+}$, both having *m/z* 556.5. To ensure that the different peaks observed in the IS-CV spectra in this study (e.g. Fig. 6) were not a consequence of the formation of multimers or other cluster ions, different concentrations of bovine ubiquitin were studied. For the concentration range from 1 to 100 μM bovine ubiquitin [in 50:50:0.04 water/methanol/acetic acid (v/v/v)], no significant changes in the shapes of the IS-CV spectra for any charge state were observed. If the multiple peaks in an IS-CV spectrum were caused by the formation of cluster ions, the relative abundances of the various peaks in the IS-CV spectra would change as a function of concentration [44,57].

FAIMS resolution. At present, FAIMS provides a low-resolution separation of the different conformations of protein ions. Variable peak widths have been observed in the IS-CV spectra presented throughout this article. This suggests that some peaks observed in the IS-CV spectra were composed of multiple unresolved coexisting conformers. Fig. 15 shows IS-CV spectra for the +12 and +13 charge states (reproduced from Fig. 9). Fig. 15(a) shows only the +12 charge state, Fig. 15(b) shows only the +13 charge state, and Fig. 15(c) shows the +13 charge state (dashed line) superimposed on the +12 charge state (solid line). Fig. 15(c) suggests that there may be at least two different conformers present within IS-CV spectrum of the +12 charge state. The presence of more than one conformer for this charge state has been reported [22,23]. The FAIMS parameters that affect resolution are currently under investigation.

Comparison with the literature. ESI drift tube mobility spectrometry/MS has been used to examine the conformations of a number of proteins. The separation of conformers in a drift tube is based on ion cross section, whereas the separation of ions in FAIMS is based on ion properties that are currently unknown. Consequently, it is expected that there will be many similarities, and also significant differences

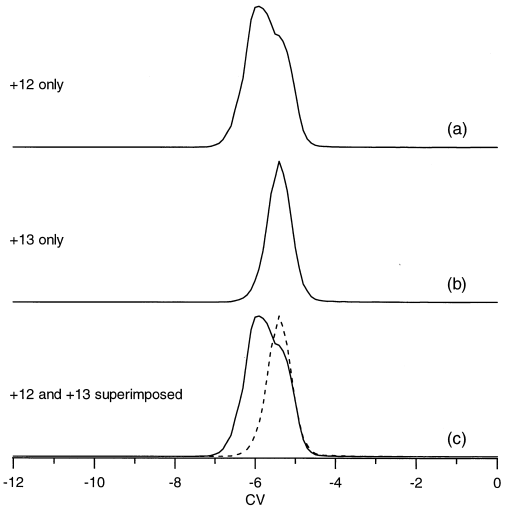

Fig. 15. Resolution limitations of FAIMS for the observation of multiple conformers in an IS-CV spectrum. (a) IS-CV spectrum for +12, (b) IS-CV spectrum for +13, (c) the two charge states are plotted together to illustrate the existence of multiple conformers.

in the array of conformations detected by these two, independent approaches.

Valentine et al. used ESI drift tube mobility spectrometry/mass spectrometry, and proton transfer reagents, to study the conformers of bovine ubiquitin [18]. In their work, the authors used their collision cross section measurements to divide the conformations of bovine ubiquitin into three types: elongated, partially folded, and compact [18]. Fig. 16 summarizes the conformational information obtained for bovine ubiquitin using ESI-FAIMS-MS by plotting the CV values for each of the conformers appearing in the CV spectrum for charge states +5 to +13. The "low acid" solution contained 55% water and 0.04% acetic acid whereas the "high acid" solution contained 55% water and the pH was adjusted to 2.1 with hydrochloric acid. The conformers that were observed in the IS-CV spectra were classified as being present in the low acid solution, the high acid solution, or both.

For charge states +10 through +13, the CV was consistently between −5 and −6 V independent of the acid concentration (pH), however the peak widths in the CV spectra varied among these charge states as noted earlier. For the lower charge states (i.e. +5

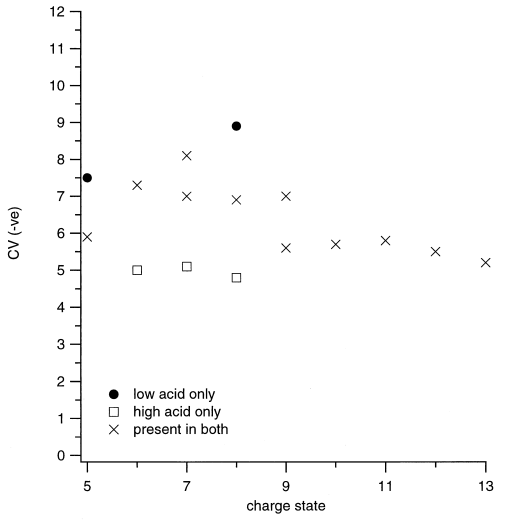

Fig. 16. Compensation voltage of the peaks of resolved conformers of bovine ubiquitin charge states from +5 to +13, obtained under different conditions of solution pH.

through +9), there were resolvable multiple conformers, and the relative abundances of these conformers were dependent on the solution conditions. The most negative CV values were observed for conformers that were present in solutions with the highest pH. For charge states +5 through +8, Valentine et al. reported the co-existence of multiple partially folded conformations [18]. For higher charge states, that is +9 through +13, spectra were apparently dominated by one conformation, although data suggested that under certain experimental conditions multiple unresolved elongated conformations may have been present [18]. Consequently, both FAIMS and drift tube mobility spectrometry identified the same charge states that have a multiplicity of conformations. The correlation between conformers detected by FAIMS and by drift tube mobility spectrometry remains to be established.

The location of an ion in a CV spectrum using FAIMS is dependent on the behavior of the mobility of an ion at high electric field. The property of the ion that controls the high-field mobility is not known, however, the results reported here indicate that the separation of protein ions in FAIMS is independent of both *m/z* and the low-field collision cross section. Thus, the FAIMS technique is truly complementary to MS and IMS techniques for studying protein conformations. Results available to date suggest that the most important ionic property that determines the location in the ESI-FAIMS-MS CV spectra of protein ions is related to the "flexibility" of the ion. An ion that appears in a CV spectrum using P2 mode (e.g. pseudomolecular ions of bovine ubiquitin) experiences a decrease in ion mobility at high electric fields compared with low fields (refer to Fig. 1, type C ions). Under the influence of a high electric field, it is possible that the protein ion will have an initial tendency to "resist" the electric field by changing its orientation relative to the direction of the field and by changing the arrangement of the internal charge distribution during ion acceleration in accordance with the field. The amount of this resistance will be a function of the ion's ability to change orientation and internal structure. Conformers that are flexible (i.e. partially folded) would be expected to permit more complex changes in structure at the onset of the electric field than the more rigid conformers, (i.e. elongated and/or compact). These structural changes in flexible conformers would result in an apparent decrease in ion mobility during the high-field portion of the asymmetric waveform. This is either a dynamic process that delays the acceleration of the ion to terminal drift velocity, or the structure of the ion is temporarily altered as a result of internal reorganization. Neither of these options is possible for ions with rigid conformations, and for example, all of the highest charge states of bovine ubiquitin shown in Fig. 16, are found at comparable CV values.

5. Conclusions

ESI-FAIMS-MS provides low-resolution separation of protein conformers at atmospheric pressure (760 Torr) and room temperature. The ionic properties that control the separation in the FAIMS device are independent of *m/z* and the low-field collision cross section of the ion thereby making FAIMS a complementary technique to MS or IMS.

Bovine ubiquitin was selected to illustrate the capabilities of the FAIMS device for obtaining con-

formational data. IS-CV spectra showed the presence of multiple conformers for several charge states of bovine ubiquitin. The number and relative abundances of these conformers were dependent on both pH and solvent composition. The addition of spectator ions, namely sodium and phosphate, provided information relating to the unique structures of the individual conformers without changing their locations in the CV spectrum.

A comparison of results using ESI-FAIMS-MS with those in the literature using ESI drift tube spectrometry/MS is given. Although the properties that control the location of an ion in a CV spectrum are currently not known, we speculate that the position of the conformer in a CV spectrum is a function of its ability to change structure in response to the applied electric field.

The existence of multiple conformers present within broadened peaks was also illustrated. As the technique matures, and as a better understanding of the ion properties that control its location in the CV spectrum is obtained, we expect that FAIMS will play an important role in studying protein conformations.

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