Protein Analyses Using FAIMS Microchips with Mass Spectrometry

Alexandre A. Shvartsburg and **Richard D. Smith**
Biological Sciences Division, Pacific Northwest National Laboratory, P.O. Box 999, Richland, WA 99352

Abstract

Differential ion mobility spectrometry (FAIMS) integrated with mass spectrometry (MS) is a powerful new tool for biological and environmental analyses. Large proteins occupy regions of FAIMS spectra distinct from peptides, lipids, or other medium-size biomolecules, likely because strong electric fields align huge dipoles common to macroions. Here we confirm this phenomenon in separations of proteins at extreme fields using FAIMS chips coupled to MS and demonstrate their use to detect even minor amounts of large proteins in complex matrices of smaller proteins and peptides.

Introduction

Gas-phase proteins were investigated[1] by ion mobility spectrometry (IMS) in conjunction with electrospray mass spectrometry (ESI/MS) since 1995. The major focus was conformational characterization by conventional IMS, where absolute mobilities K at moderate electric field (E) may be related to the ion-molecule collision cross sections (Ω) and further to possible ion geometries using *a priori* calculations.[2,3] For protein complexes or aggregates, that approach can help elucidate the global structure and subunit connectivity.[4,5] Differential or field asymmetric waveform IMS (FAIMS) separates ions by ΔK - the increment of K between two E values.[6,7] While one cannot so far correlate ΔK to ion geometries quantitatively, FAIMS shows proteins unfolding for higher charge states and upon field and/or thermal heating in consistency with conventional IMS data.[8–14] Some conformers were separated by FAIMS but not conventional IMS, illustrating substantial orthogonality of these dimensions,[12] and H/D exchange after FAIMS filtering reveals further subpopulations.[15]

In FAIMS, one measures K directly employing a periodic asymmetric field $E(t)$ in a gap between electrodes through which ions are pulled by gas flow.[6,7] For a given species, the net drift across the gap can be offset utilizing a constant "compensation field" (E_C) added to $E(t)$. Thus stabilized ions can exit the unit and be detected, hence scanning E_C produces a spectrum. The E_C values depend on the $E(t)$ amplitude ("dispersion field", E_D) and the gas temperature and composition, and ions are classified based on the $K(E)$ functional form that determines the E_C sign.[6,7] With "full-size" FAIMS devices operated at $E_D \sim 20 – 30$ kV/cm (or $E_D/N \sim 80 – 120$ Td at room temperature and pressure, where N is the gas number density), mobilities in N_2 normally increase at higher E for ions up to $\sim 200 – 400$ Da (leading to $E_C < 0$ for $E_D > 0$) and decrease for larger species including all peptides, lipids, and smaller proteins such as ubiquitin, β_2-microglobulin, cytochrome c, calmodulin, myoglobin, or trypsin (where $E_C > 0$ for $E_D > 0$).[6–17] These type A and C behaviors, respectively, reflect the contribution of the well of ion-molecule interaction potential to overall collision process.[7,18] However, proteins with masses (m) of $>\sim 30$ kDa [carbonic anhydrase, liver alcohol dehydrogenase, bovine serum albumin (BSA), or transferrin] took

up[16,17] much wider E_C segments that spanned $E_C = 0$. This has been rationalized in terms of pendularity of ion macrodipoles in FAIMS, as detailed below.

The gaps in recently introduced FAIMS microchips[19–22] have 35 μm width, or ~60 times less than 2 mm typical for full-size FAIMS used in protein analyses to date. Such narrow gaps permit E_D ~ 60 kV/cm (~250 Td at $T = 300$ K) without electrical breakdown, which enables ion filtering in ~20 – 50 μs. Here we explore these chips for intact proteins, again encountering the anomaly for larger proteins that supports the pendular hypothesis. This effect can be exploited to detect and identify even traces of large proteins in complex samples comprising smaller proteins, peptides, and other substances.

Fundamentals of dipole alignment in full-size and chip FAIMS

Virtually all macroions have permanent dipole moments (p). When the dipole energy in a field (that scales with p and E) at least compares to its rotational energy kT_R (where k is the Boltzmann constant and T_R is the rotational temperature), the dipole becomes materially aligned. The directional Ω of aspherical ions in such pendular states[23] at high E often differ greatly[16] from the orientationally averaged Ω of same ions freely rotating at low E. That disparity between Ω (and thus mobilities) in strong and weak fields would cause a giant FAIMS effect, in line with the findings for large proteins. For neutrals, T_R equals the gas temperature (T) and dipoles with any p are locked at any T by sufficient field:[16,17]

$$E > kT/(2p) \quad (1)$$

Then, with $E_D = 25$ kV/cm and $T = 300$ K, alignment requires p ~ 250 D at peak E and $p >$ ~390 D over a considerable part (20%) of the $E(t)$ cycle.[16] The p values of proteins increase with m about linearly and exceed 250 – 390 D at m ~ 8 – 20 kDa, or somewhat below ~30 kDa where the anomalous behavior is first seen.[17]

Electric fields also heat ions in gases, and ensuing rotational excitation impedes the alignment such that species with p under a critical value (p_{crit}) never align:[17]

$$p_{crit} = K(kTM/3)^{1/2} \quad (2)$$

(where M is the gas molecule mass). Species with $p = p_{crit}$ align at a single E value of[17]

$$E_{crit} = kT/p_{crit} \quad (3)$$

Alignment at other E requires larger minimum p, which we derive from eq 11 in Ref. [17] as:

$$p_{min} = [M(KE)^2 + 3kT]/(6E) \quad (4)$$

Multiply-charged ions generated by ESI for ubiquitin (Ub) and cytochrome c have $K = 0.8$ – 1.4 cm^2/(Vs) in N$_2$ at $T = 300$ K,[12,13,24] leading to p_{crit} ~ 190 – 340 D by eq 2. The p_{min} values for $E_D = 25$ kV/cm by eq 4 are naturally greater, ~290 – 360 D for alignment at the waveform peak and ~410 – 460 D over 20% of the time. The latter value is, on average, reached by proteins with m ~ 20 – 25 kDa, in good agreement with experiment.[17] *Inter alia*, this model explains why Ub ions with $p = 189$ D for the native geometry and $p < 250$ D for all simulated conformers with charge states (z) of 7, 10, and 12 behave "normally" while BSA ions with p ~ 1100 D (estimated from the native geometry of human albumin) do not.[17]

The FAIMS chips using much higher E_D let us further probe the dipole locking theory. By eq 1 where p is proportional to $1/E$, raising E_D to 60 kV/cm would align ions already for $p \sim$ 100 D at peak E and ~160 D over 20% of the cycle. In particular, a major fraction of Ub conformers would have emerged at $E_C < 0$ (in parallel to the leucine cations moving from type A at $E_D \sim$ 25 kV/cm to type C at ~60 kV/cm).[20] In contrast, eq 2 contains E only via a weak $K(E)$ dependence, and p_{crit} should change little from that for full-size FAIMS. The p_{min} by eq 4 decreases to ~190 – 340 D at peak E and ~220 – 340 D over 20% of the cycle. These values are equal or close to p_{crit} because E_{crit} of ~37 – 65 kV/cm[17] by eq 3 is almost entirely within the E_D range of present chips, which allows alignment at p_{crit}. However,[17] the p values for known Ub conformers are below ~200 D, except for rare unfolded geometries at high z for which[12] $K > 1.05$ cm^2/(Vs) and p_{crit} exceeds 250 Td. Hence Ub ions should still not align at any field. Ions of BSA, where the estimated $p \sim$ 1100 D far exceeds these p_{crit} as we mentioned, should firmly align as in full-size FAIMS. The consequent expansion of their spectra to $E_C < 0$ would then permit separation of albumin from smaller proteins such as ubiquitin and/or peptides or other biomolecules. These predictions are tested below.

Experimental methods

A FAIMS chip by Owlstone (Cambridge, UK) was mounted in front of Thermo LTQ ion trap.[20] For best separation, we employed the highest enabled $E_D \sim$ 60 kV/cm. For greatest sensitivity, the filtering time was minimized to ~20 μs by maximizing the flow through the gap: the gas (N$_2$) was pumped to the unit at 2.8 L/min and evacuated from the FAIMS/MS junction at 2.5 L/min.[20] The scan rate was 3.5 Td/min.

Samples of bovine ubiquitin and BSA were made by dissolving the purchased standards in 50:49:1 water/methanol/acetic acid to ~10 μM. To gauge the capability to select large proteins from a complex matrix, we mixed these solutions in ~100:1 Ub/BSA ratio, then added a tryptic digest of BSA (exhibiting hundreds of peptide ions even at low sensitivity)[25] and uncharacterized complex lab chemical waste. Samples were infused to an ESI emitter at 0.5 μL/min.

Results and discussion

The Ub ion envelope consists of $z = 5 – 12$ as usual (Figure 1a), all of type C (Figure 1b, c). All charge states exhibit single near-Gaussian peaks at similar $E_C/N = 2.0 \pm 0.1$ Td and widths of $0.7 \pm .01$ Td (fwhm). Besides low resolving power of these chips,[19,20] this uniformity may be attributed to near-complete protein unfolding due to extreme field heating.[12–14,25] At $E_D/N \sim$ 80 Td in full-size FAIMS, isomerization of Ub ions is controlled[13,14] by their maximum temperature T_I equal to the gas temperature plus peak magnitude of field heating, ΔT_{max}. In the two-temperature treatment:[2,7]

$$\Delta T_{max} = M(K E_D)^2/(3k) \quad (5)$$

In agreement with eq 5, the measured ΔT_{max} value at $E_D/N \sim$ 80 Td was[13,14] ~50 – 55 °C. Here the field and heating are three and nine times stronger, respectively, with estimated $\Delta T_{max} \sim$ 470 – 510 °C and peak $T_I \sim$ 490 – 540 °C. In heated drift-tube IMS[26] on the ~50 ms timescale, ubiquitin ions are folded for $z < 11$ at $T = 25$ °C, but fully unfold at ~80 – 132 °C for $z = 8 – 10$. As the Coulomb repulsion of protonated sites aides unfolding, the temperature needed increases for lower z; for the lowest reported $z = 6$, some folds survived to the highest tried $T = 132$ °C. Little is known about denaturation of protein ions on the present microsecond timescale, but perhaps at ~500 °C it could be extensive even in such short times. Then the similarity of E_C for all z in Figure 2c resembles the findings for

unfolded states of $z = 6 - 13$ in full-size FAIMS,[12] where $|E_C/N|$ was 0.18 ± 0.02 Td at the maximum $E_D/N = 90$ Td. Here, the difference of $E_C/N = 1.9$ Td for $z = 5$ from $2.0 - 2.1$ Td for $z = 6 - 13$ might indicate incomplete unfolding of $z = 5$.

The mass spectrum for BSA (Figure 1d) is close to that recorded in ESI/MS previously.[16] As for Ub, the FAIMS spectra for all observed $z = 34 - 55$ are similar and E_C slightly increases for higher z (Figure 1e, f). However, the peaks for all z are shifted to much lower E_C/N values, with the apex of total spectrum at 0.3 Td vs. 2.1 Td for Ub (Figure 1e). The BSA ions of all z include conformers with $E_C < 0$ (Figure 1f). These findings fit the above model, supporting the pendularity of larger proteins in FAIMS. The separation space for a given z is ~50% wider for BSA than for Ub (~$3.2 - 3.5$ Td vs. ~$2 - 2.4$ Td), much below the ~$5 - 10$ fold expansion in full-size FAIMS.[16,17] At least in part, this reflects the lower resolving power of present chip, where features are broad regardless of the dipole alignment.

The total FAIMS spectrum for BSA overlaps with that for ubiquitin at the margin ($E_C/N \sim 1 - 2.5$ Td), but most ions and the apexes in BSA spectra for all charge states lie outside that range (Figure 2e). Further, all peptides and other biological ions analyzed using these chips[20–22] (except smaller single amino acids such as H^+Leu)[20] come only at significantly positive E_C when $E_D/N = 250$ Td. Small ions (such as H^+Leu with $m/z = 132$) that did not yet clearly cross to type C may appear at E_C 0, but those species have $m/z < 300$ and thus are apart from BSA in the mass spectrum where the lowest detectable m/z is >1000 (Figure 2d). Therefore even a minute BSA amount should be separable from light proteins, peptides, and other small-molecule contaminants. The ESI/MS spectrum of a sample comprising Ub, protein digest, and polluted water with added BSA trace (Figure 2a) reveals the Ub charge state envelope, but BSA ions are completely obscured by omnipresent chemical noise. The FAIMS selection at $E_C = 0$ easily recovers nearly the full BSA charge state distribution by virtually eliminating all other ions except an unassigned species **x** at $m/z = 659$ that is outside the m/z range for BSA ions (Figure 2b). The non-BSA ions over the whole mass range are suppressed by ~10^4 times (~10^3 times for **x**), which is remarkable with the filtering duration of ~20 μs. As we discussed, this discrimination should broadly apply to other proteins above ~30 kDa.

In this aspect, the FAIMS chips may perform better than full-size FAIMS with superior resolving power because many medium-size ions (for example, larger amino acids) that belong to type A at moderate fields[27] convert[20] to type C and thus do not "co-elute" with large proteins. This separation capability is also unavailable from conventional IMS because larger species tend to have higher charge states and ESI-generated ions of large proteins such as BSA, small proteins such as Ub, and small and large peptides fall into the same mobility range.[5] This preliminary work has employed Generation I microchips,[19] and no detection limits have been quantified. The latest chips with similar E_D values but 100-μm wide gaps transmit nearly ~100% of heavy protein ions at their peak apexes[28] and thus should provide excellent sensitivity far exceeding that of high-resolution FAIMS devices.

Conclusions

We report the first analyses of proteins using FAIMS microchips, with small proteins exemplified by ubiquitin and large ones by BSA. For all charge states generated by ESI, ubiquitin ions behave as type C while BSA also exhibit intense type A conformers. These results match findings obtained using full-size FAIMS units and follow the theory for pendular ion states that incorporates rotational heating.[16,17] The presence of type A species for only large proteins and smallest ions with much lower m/z allows filtering of traces of large proteins (above ~30 kDa) from complex biological and environmental matrices. This capability may be useful to track protein digestion and verify its completion, and to prevent

lower-mass ions in top-down analyses of proteins and their complexes from entering an MS system (where they take up the limited charge capacity of ion traps or guides and create MS interferences).

Acknowledgments

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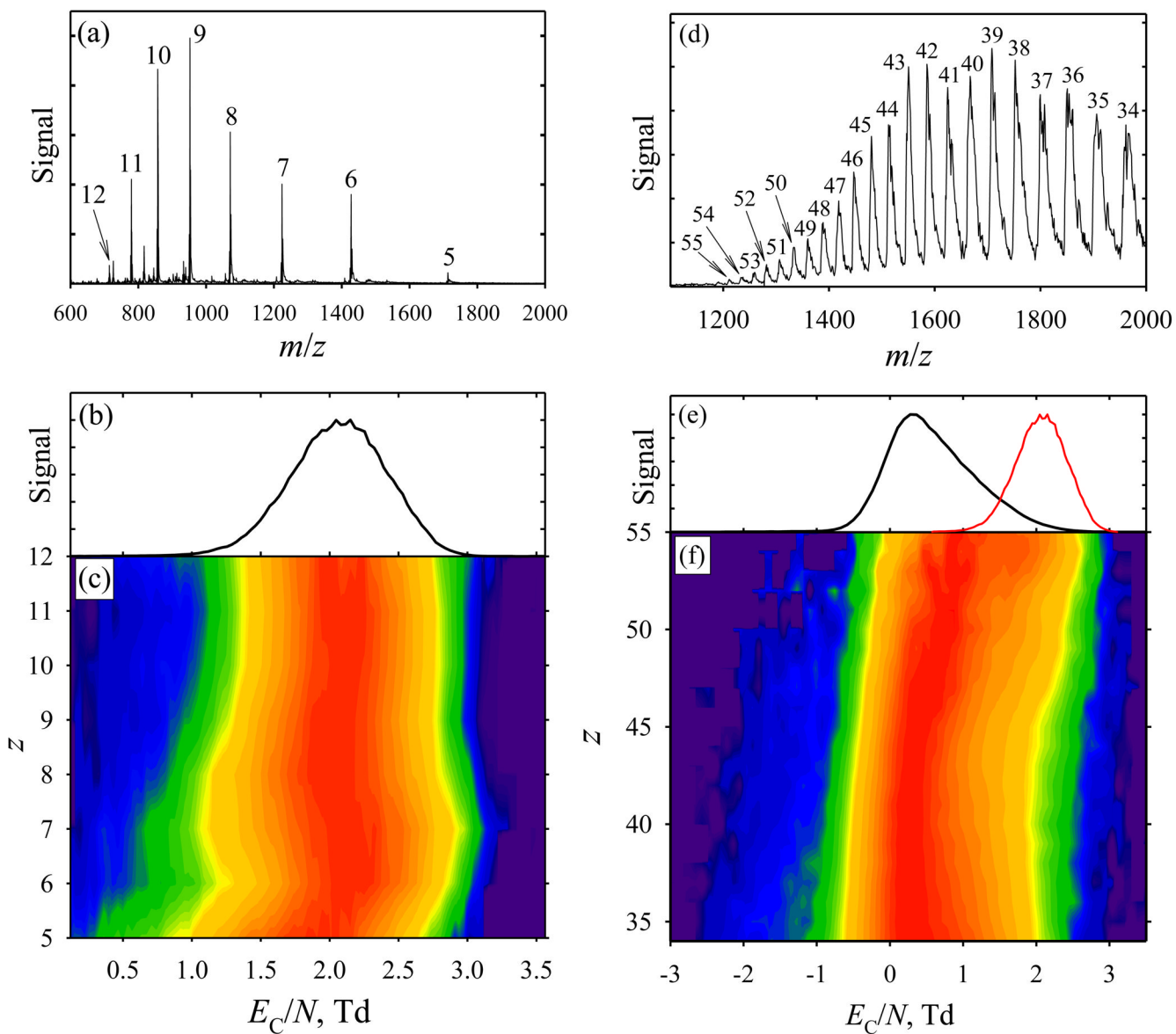
Fig. 1.
Data for bovine ubiquitin (a - c) and BSA (d - f) ions: (a, d) mass spectra with the charge states labeled; (b, e) total FAIMS spectra for all charge states (black lines); (c, f) FAIMS/MS spectra drawn as a false-color map (log scale) with interpolation between the discrete z values provided for viewing convenience. In (e), the red line copies the total spectrum for Ub from (b).
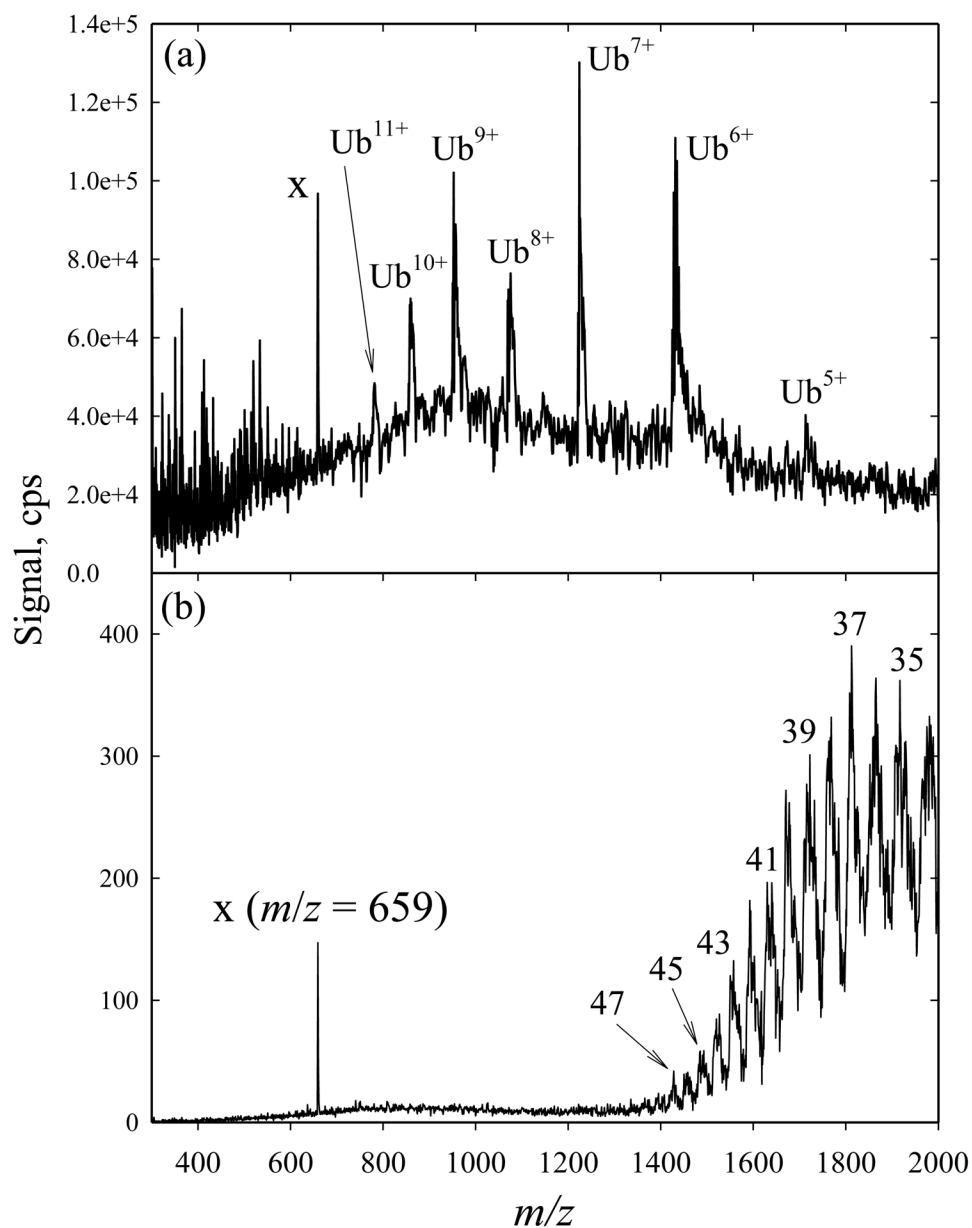
Fig. 2.
Mass spectra for the solution of ubiquitin and trace of BSA in a complex matrix measured
(a) without FAIMS (resolved Ub ions are labeled) and (b) with FAIMS at $E_C = 0$ (charge
states of BSA are labeled for odd z).