Ion Mobility Spectrometry of Macromolecules with Dipole Alignment Switchable by Varying the Gas Pressure

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Supporting Information

ABSTRACT: Since inception in the 1980s, differential or field asymmetric waveform ion mobility spectrometry (FAIMS) was implemented at or near the ambient gas pressure (AP). Recently, we developed FAIMS at 15–30 Torr within a mass spectrometer and demonstrated it for small and medium sized ions, including peptides. The overall separation properties mirrored those at AP, reflecting the shared underlying physics. Here we extend these analyses to macromolecules, namely, multiply charged proteins generated by electrospray ionization. The spectra for smaller proteins (ubiquitin, cytochrome c, myoglobin) again resemble those at AP, producing features for one or a few adjacent well-defined conformers with type C behavior. Large proteins (single aldolase domain and albumin) now follow, with no broad bands for type A or B species that dominated at 1 atm. Those unique behaviors were ascribed to pendular ions with electric dipoles reversibly locked by the strong field in FAIMS. Disappearance of those bands shows loss of alignment predicted by first-principles theory, further supporting dipole locking at AP. The capability to modulate dipole alignment by varying gas pressure at constant normalized field provides the basis for determining the ion dipole moment and direction within the molecular frame from the pressure of onset and characteristics of spectral drift. This new approach to alter FAIMS separations of proteins could make a powerful tool for structural biology and be useful for proteomics and imaging.

Ion mobility spectrometry (IMS) can separate and characterize charged species based on the properties of their transport through gases driven by an electric field of some intensity (E). In the classic drift-tube (DT) IMS implementation, a moderate fixed E is established over a known distance inside a gas-filled enclosure. Ions travel through it at constant velocity v that is proportional to E over the gas number density (N):

\[ v = KE = \frac{K_0N_0E}{N} \]  

(1)

Here, \( N_0 \) is N under standard temperature and pressure (2.687 \( \times 10^{25} \) m\(^{-3} \)), \( K_0 \) is mobility, and \( K_0 \) is K at STP, the descriptor of an ion/gas molecule pair set by their identities and gas temperature (T).

As ions are normally free to rotate, \( K_0 \) is determined by the ion–molecule collision cross section averaged over all partner orientations (\( \Omega_{avg} \)). Other pertinent properties, such as the magnitude of ion heating by above-thermal collisions and threshold for electrical breakdown in gas, are also governed by \( E/N \) expressed in Townsend (1 Td = \( 1 \times 10^{−17} \) V \( \times \) m\(^2 \)). Subsequent IMS configurations utilized a dynamic field in stationary gas (traveling-wave and transverse-modulation IMS) or static field in moving gas (aspiration or trapped IMS). These linear IMS methods separate ions by \( K_0 \) at moderate \( E/N \), where \( K_0 \) does not materially depend on \( E/N \) and \( v \) is proportional to \( E/N \) by eq 1.

Coupling to mass spectrometry (MS) in the 1960s opened the door to practical IMS applications such as isomeric analyses of aromatics. Emergence of soft ionization sources in the 1990s revolutionized IMS/MS, extending it to proteomic, metabolomic, petroleomic, forensic, and environmental analyses. The ability to extract ion geometries from IMS data by matching measured \( \Omega_{avg} \) with molecular dynamics calculations brought IMS to nanoscience and structural biology. Recent commercialization of diverse IMS/MS platforms expanded the technology to the broad user community. Huge gains of resolving power in designs employing a dynamic field and/or gas flow permitted separating structures with a <1% mobility difference. Nonetheless, the (linear IMS)/MS combination has two intrinsic limitations. First, \( \Omega_{avg} \) is tightly correlated to ion mass (m); large ions tend to be heavy. This results in poor utilization of IMS/MS separation space: the contribution of IMS to 2-D peak capacity (pc) is much below its own pc. Second, orientational averaging smears out the geometry details, reducing the utility of IMS for structural mass spectrometry.

As was learned early on, the mobilities of all species in any gas depart from \( K_0 \) above certain \( E/N \). For polyatomic ions of...
analytical interest, usual deviations within several percent do not influence separations. Therefore, high-field mobility remained an academic curiosity until the invention of differential or field asymmetric waveform IMS (FAIMS) based on the difference between mobilities in high and low field segments \((\Delta K)\). In FAIMS, a periodic waveform of some amplitude (dispersion voltage, DV) spreads ions by \(\Delta K\) perpendicularly to the gap between two electrodes. The species with sought \(\Delta K\) are equilibrated by added compensation voltage (CV) and pass to a detector, while others are deflected to electrodes. Scanning CV reveals the spectrum of species entering the gap. To compare the data for different gap widths \((g)\), the DV and CV are often expressed as dispersion field \(E_D = (DV)/g\) and compensation field \(E_C = (CV)/g\). To further adjust for the gas pressure, these are normalized to \(E_D/N\) and \(E_C/N\).

The FAIMS/MS approach is gaining currency in bio and environmental analyses, especially for targeted applications such as isomer separations. A key advantage is substantial orthogonality between the dimensions, commonly exceeding 4,22 that in linear IMS/MS by \(\sim 3-4\times\). That lets FAIMS to distinguish isomers better than linear IMS of equal resolving power. Examples are peptides with inverted sequences 23 or variant localization of post-translational modifications 26 and lipids with permuted acyl chains or different double bond positions or symmetries. 24,25 The \(K(E/N)\) for all ions vary with the gas nature 21 and temperature 29 in a complex poorly predictable manner. The dependence on the gas pressure was not really probed, as FAIMS was essentially an ambient-pressure (AP) technique for three decades since its advent 8 in 1982.

The first work beyond AP 30 covered the nearby range \((P = 0.4-1.5\text{ atm})\) in a setup derived from AP systems. Recently we demonstrated FAIMS at 15-30 Torr within the MS instrument. 31 The separations for all species tried 30,31 (amino acids, other small ions, and peptides up to 6 kDa) copied those at 1 atm. This is because nearly all phenomena aggregated in the FAIMS effect depend on \(E\) and \(N\) as \(E/N\) ratio. Those include: (i) dependence of scattering on the distribution of ion–molecule velocities \((v_{\text{rel}})\), (ii) collisional alignment of nonspherical ions, also controlled by \(v_{\text{rel}}\) distribution, 32 (iii) periodic changes (“breathing”) of ion geometry as a function of internal temperature \((T_{\text{ion}})\) oscillating in the waveform cycle, and (iv) reversible adsorption of gas molecules or vapor dopants on ions, 33 also controlled by \(T_{\text{ion}}\). The weights of each depend on the ion and gas properties, for example, (iii) is important for flexible biomolecules 36 but not rigid ions such as fullerenes 37 and (iv) is critical for organic vapors 34,35 but not \(N_2\) (at ambient temperature). That matters not, as \(T_{\text{ion}}\) is fixed by the \(v_{\text{rel}}\) distribution determined by \(E/N\). The simplest two-temperature (2-T) model, 2 generally valid in FAIMS, says

\[
T_{\text{ion}} = T + \Delta T = T + M(N_0K_0(E/N))^2(E/N)^2/(3k)
\]

where \(M\) is the molecule mass and \(k\) is the Boltzmann constant.

Equation 2 breaks down at extreme \(E/N\), where the \(v_{\text{rel}}\) distribution shifts away from Maxwellian and the ion temperature is ill-defined, 2 but the ion heating is still controlled by that distribution and thus \(E/N\).

The sole FAIMS effect not governed by \(E/N\) is the alignment of electric dipoles that forces ions into pendular states with mobility governed by directional cross sections \((\Omega_{\text{dir}})\). 39-41 The dipole is a superposition of permanent and polarized components, but the latter is negligible. 39 Unlike with fixed dipoles, the torque seeking to lock orientation competes with field heating flowing into rotations. This results in the minimum dipole moment \((p)\) lockable 40 at any \(E:\)

\[
\frac{p_{\text{crit}}}{K} = K(\frac{KTM}{3})^{1/2}
\]

Electrospray ionization (ESI) of smaller proteins produces multiply charged ions with typical \(K = 0.8-1.3\text{ cm}^2/(Vs)\) in \(N_2\) at ambient conditions. 38 Then eq 1 yields 40 \(p_{\text{crit}} \sim 200-300\) Debye (D). As significant impact requires alignment over a reasonable waveform segment rather than peak field, the practical minimum \(p\) with a sinusoidal waveform is greater \((\sim 250-350\text{ D})\). 40 Larger proteins statistically have higher moments (because of greater number of partial charges and possible distances between them), with \(>400\text{ D}\) prevailing above \(\sim 30\text{ kDa}\). In fact, the carbonic anhydrase II \((29\text{ kDa})\), alcohol dehydrogenase \((44\text{ kDa})\), bovine serum albumin (Alb,

Figure 1. Total FAIMS spectra (top) and \(E_c/charge\) state diagrams for myoglobin (a) and albumin (b). Reproduced from ref 39. Copyright 2006 American Chemical Society. The x axis units should be V/mm (an error in ref 39).
66.7 kDa), and transferrin (Tr, 78 kDa) exhibited (Figure 1) major “type A” peaks at $E_C < 0$, meaning a steep rise of $K(E)$. This otherwise occurs only for species under ~300 Da, where this behavior ensues from the attenuating role of attractive ion–molecule interactions for more energetic collisions.\textsuperscript{23,39,40}

With macro ions, this must follow from alignment of dipoles (likely pointed along the principal ion axis).\textsuperscript{39,40}

The dipole moments also depend on the conformation, probabilistically growing for elongated geometries with more distant charge sites.\textsuperscript{40} For instance, tiny features at $E_C < 0$ exist for bovine heart cytochrome c (Cyt, 12.23 kDa) and equine skeletal myoglobin (Myo, 16.95 kDa) in higher charge states ($z$),\textsuperscript{31} but not ubiquitin from bovine erythrocytes (Ub, 8.57 kDa) in any $z$.\textsuperscript{42–44} Indeed, all Ub conformers in any $z$ have\textsuperscript{44} computed $p < 250$ D, while some unfolded Cyt and Myo conformers in high $z$ have projected\textsuperscript{39} $p = 300–400$ D versus 220–280 D for native conformations ($p_{\text{nat}}$).

The picture with FAIMS microchips\textsuperscript{39} employing extreme $E_D \sim 60$ kV/cm ($E/N \sim 250$ Td) tracks that with full-size devices using $E_D \sim 25$ kV/cm ($E/N \sim 100$ Td): only large proteins display\textsuperscript{39} intense signal at $E_C < 0$. This supports the existence of $p_{\text{nat}}$ regardless of the field strength.\textsuperscript{40} The above observations dovetailing with theory amount to strong yet lower in the LP case. The actual mobilities in eq 3 scale in proportion, causing slight overestimation of $p_{\text{crit}}$ above. However, this error is minor and perhaps compares to the inaccuracy of 2-T model at the extreme $E/N$ in LP-FAIMS.

In summary, most multidomain proteins and complexes over ~1 MDa and some unfolded conformers over ~400 kDa should lock dipoles at $P = 15$ Torr. However, no protected ions under ~100 kDa (including all found locked at AP)\textsuperscript{39–41,46} can align under any assumptions. That prediction is verified here.

## EXPERIMENTAL METHODS

As in the baseline ESI/LP-FAIMS/quadrupole MS platform, a planar-gap FAIMS device was inserted in the first vacuum region prior to the Q-array. A conical element attached to the inlet capillary terminus directs the expanding flow of gas (here $N_2$) from inlet to the middle of FAIMS gap.\textsuperscript{31,41} This flow carries ions into and through the gap. The near-perfectly rectangular waveform had a frequency ($\nu$) of 200 kHz and an aspect ratio ($j$) of 4. The pressure was regulated by leaking $N_2$ through a valve and registered by membrane manometer, independent of the gas identity (Omega). The quadrupole $m/z$ limit was 2000.

The optimum $g$ is commensurate with the terminal beam width for desired ions.\textsuperscript{4} That width, mainly determined by diffusion (including its anisotropic component), approximately scales\textsuperscript{4,31} as $p^{-1/2}$. Hence, operation at the bottom of the previous 15–30 Torr pressure range is favored by widening the gap. Here, we augmented $g$ from 5 mm in the baseline platform\textsuperscript{31} to 7.5 mm, the largest ever in FAIMS. The electrodes were maintained at 40 °C (measured by a thermocouple).

By Paschen law, the breakdown threshold $E_{B/N}$ in any gas is controlled by $P_g$ and always elevates at lower $P_g$. Either $P$ or $g$ in FAIMS can be reduced. The $P_g$ values at AP were ~75–190 Torr × cm for “full-size” devices with $g = 1.0–2.5$ mm (e.g., by Ionalytics, Thermo, Sciex, and Heartland MS),\textsuperscript{22,24,35,39,48} 38 Torr × cm for MEMS devices with $g = 0.5$ mm (by Sionex),\textsuperscript{30,33} and 2.7–7.6 Torr × cm for microchips with $g = 0.035–0.10$ mm (by Owlstone).\textsuperscript{45,49–51} Accordingly, the threshold (in $N_2$) increased from ~140 Torr in full-size devices to ~700 Torr in the narrowest-gap microchips (limited to $E_{B/N} = 270$ Torr by the power supply constraints).\textsuperscript{45} At low pressure, we have $P_g = 7.5–15$ Torr × cm for the baseline system\textsuperscript{31} at $P = 15–30$ Torr and $P_g = 11$ Torr × cm presently at $P = 15$ Torr. The bottoms of these ranges match that for microchips with $g = 0.10$ mm (ultra-FAIMS),\textsuperscript{31} leading to $E_{B/N} \sim 350$ Torr. Unlike with chips, these metrics are easily attained at low pressure: the engineering constraints are lifted by moderate voltages even for extreme $E/N$, modest frequencies (200 kHz versus 28 MHz in chips)\textsuperscript{45} allowed by wide gaps, and low capacitance of those gaps. For instance, the DV to create maximum presently used $E_{B/N} = 284$ Torr is ~960 V. To compare, the benchmark system required 1160 V at $P = 30$ Torr, despite a lower $E_{B/N} = 250$ Torr and narrower gap.\textsuperscript{41}

The FAIMS resolving power ($R$) scales as $P^{1/2}$; as faster diffusion at lower pressure broadens peaks.$^3$ The resolution loss inherent in LP-FAIMS is offset\textsuperscript{31} by raising $E/N$ with $R$ crudely proportional to ($E/N$)$^{0.6}$ and upgrading the waveform from bisinusoidal to rectangular to double the key ($F_1$) factor.\textsuperscript{41}

The $R$ metric also scales as the square root of ion residence time ($t$).\textsuperscript{31,52} Hence, one can improve resolution by extending $t$ using longer gap and/or slower gas flow.$^3$ Here we elongated the cell from 100 mm in the baseline platform\textsuperscript{31} to
126 mm (the largest in FAIMS) and reduced the gas speed from 13 m/s (at 25 Torr)\textsuperscript{31} to 9.8 m/s. The consequent increase of \( \tau \) from 8 to 13 ms incremented \( R \) by \( \sim 25\% \).

The load capacitance decreased as the greater gap width has more than offset the larger area. With lower voltage and capacitance, the near-rectangular waveform was readily implemented by digital switching.\textsuperscript{31}

The papaverine standard and proteins were purchased from Sigma (Dorset, U.K.). The number of observed protein charge states was maximized by promoting protonation in a highly acidic ESI solvent, 50:49:1 water/methanol/formic acid (pH = 2). The samples diluted to 1 \( \mu \)M were infused at 9.5 \( \mu \)L/min.

We scanned \( E_C/N \) range in increments of 0.042 Td with 10 ms dwell time. This scan speed (4.2 Td/s) is \( \sim 5 \times \) faster than in the benchmark system.\textsuperscript{31} Thus, the scan over full range (20 Td) involved 480 steps, taking 4.8 s. Complete \( E_C/N \) (\( E_D/N \)) palettes with 50 \( E_D/N \) steps of 5.8 Td were acquired in 4 min. The \( E_C/N \) spectra were smoothed using the moving average method.

\section*{RESULTS AND DISCUSSION}

\textbf{Performance Validation.} We benchmarked the modified system versus original\textsuperscript{31} at two pressures in the middle of 15–30 Torr range using the \( H^+ \) papaverine ion (\( m/z = 340 \)). The \( E_C(E_D) \) curves are close (Figure 2a). The slight systematic shift is likely due to the temperature difference between the present cell (40 °C) and the previous cell (uncontrolled, but Ohmically heated by surrounding circuitry to an extent depending on DV). The device heating is a greater concern in LP- than AP-FAIMS because of slower heat dissipation at low pressure. Present data at known fixed temperature must be more accurate.

Present peaks are narrower at either pressure (Figure 2b), by \( \sim 20\% \) on average. (The previously seen slight peak broadening at maximum \( E_D/N \) was likely an artifact of unaccounted heating mentioned above.) This confirms the scaling of \( R \) with \( t^{1/2} \), established at AP\textsuperscript{32–34} and lights the path to yet higher resolution in LP-FAIMS using larger cells.

Figure 2. Separation parameter (a), fwhm peak width (b), and resolving power (c) obtained for papaverine with present and benchmark\textsuperscript{31} systems, depending on the dispersion field (vertical axis).

Broadening the gap decreases \( E_{BR}/N \) per the Paschen law, to \( \sim 300 \) Td here from \( \sim 350 \) Td at \textit{g} = 5 mm.\textsuperscript{31} However, the resulting greater ion transmission provides sufficient signal to somewhat higher \( E_D/N \) (>250 Td versus previous 200–250 Td).\textsuperscript{31} Hence, the data cover a wider \( E_D/N \) range, and the maximum resolving power significantly improves (Figure 2c).

Overall, these comparisons validate proper performance of present LP-FAIMS system.

\textbf{Protein Mass Spectra.} We have explored four proteins representing diverse FAIMS behaviors reviewed above: Ub with no type A ions, Cyt and Myo with type A conformer traces, and Alb with dominant type A species. The rabbit muscle aldolase not investigated by FAIMS previously was also studied.

The mass spectra (Figure 3) correspond to the charge state envelopes in severely denaturing ESI regime:\textsuperscript{39,55} \( z = 6–13 \) (Ub), 7–19 (Cyt), 9–27 (Myo), and 34–60 (Alb). The maximum \( z = 27 \) for Myo exceeds previous \( z = 23 \), perhaps because of lower solvent pH (2.0 vs 2.5–3 previously). Under these conditions, noncovalent protein complexes routinely

Figure 3. Measured protein mass spectra. Charge states are labeled, and those selected for FAIMS analyses are in red.
dissociate into constituent domains. The aldolase comprises four identical 39.26 kDa domains (Ald), seen in the data with $z = 26 - 60$. Except for Ub, the distributions are bimodal with a lesser hump at lower $z$ due to incompletely denatured (unfolded) conformers. The similar average and maximum $z$ for Ald and substantially heavier Alb are somewhat unusual.

The minor tails of envelopes for Myo and Alb likely extending to $m/z > 2000$ were not measurable. As the same applied at AP, the results directly compare.

**Heating and Survival of Intact Proteins at Extreme Field.** We acquired the FAIMS spectra for lowest, intermediate, and (except for Cyt) highest sufficiently intense charge states (Figure 3) with quadrupole in the selected ion monitoring (SIM) mode (Figures 4, 5, and S1–S3).

In all cases, the signal with $s/n > 10$ persisted to at least $E_D/N = 250$ Td, about the maximum in prior LP-FAIMS or microchips. The lower charge states of all proteins exhibited enough signal up to the breakdown threshold.

Assuming $K(0) = 1.0$ cm$^2$/Vs measured for unfolded Ub conformers ($z = 10 - 13$) after AP-FAIMS stage, the ion heating and temperature at maximum $E_D/N = 284$ Td by eq 2 are $\Delta T = 542 \degree C$ and $T_{ion} = 582 \degree C$. However, the $\Delta T$ in the

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**Figure 4.** $E_C/E_D$ palettes for ubiquitin with $z = 6, 10, 13$. The vertically scaled FAIMS spectra at highest $E_D$ with good signal (marked by arrows in palettes) are on top, with peak widths and resolving power values listed. The scaling factors convey peak heights relative to the charge state with greatest signal (here 10+). The $E_C(E_D)$ curve bend areas are in white rectangles.

**Figure 5.** Same as Figure 4 for albumin with $z = 36, 47, 55$. 
low-field segment (nominally taking 80% of the waveform period) is 1/16 of the above, that is, just 34 °C. Then the average ΔT over cycle is 135 °C for \(T_{\text{ion}} \) = 175 °C. The real (maximum and average) heating would be weaker because of (i) lower \(K(0)\) values due to greater unfolding\(^{38,57}\), upon stronger heating in present fields compared to \(\sim 100\) Td in full-size AP-FAIMS systems and (ii) decreasing high-field mobilities, as discussed above.

The isomerization of Ub ions in AP-FAIMS is mainly controlled by maximum heating.\(^{38,57}\) Presumably, the unfolding proceeds by hopping between adjacent basins on the potential energy surface during brief high-field segments (lasting 1 µs here). The associated ion temperature here is well above 130 °C that causes extensive unfolding of Ub in even the lowest \(z = 6\) in a heated capillary with transit time under the present total spent by ions at high field (2.5 ms). The average \(T_{\text{ion}}\) also exceeds 130 °C. Hence, at least the smaller proteins here must be substantially unfolded.

With any protein, the signal decay at greater \(E_D\) was faster for higher charge states. Except with the largest protein Alb (Figure 5), the data for highest \(z\) end short of the breakdown field (Figures 4 and S1–S3). For example, the maximum useful \(E_D/N\) decreases from 284 Td for Ub 6+, Myo 9+, or Ald 36+ to \(\sim 260\) Td for Ub 13+, Myo 26+, or Ald 56+ (Figures 4, S2, S3).

The practical limitation of \(E_D/N\) range by ion loss seen here may be caused by (i) dissociation promoted by stronger field heating, (ii) “self-cleaning” of isomerized heated ions upon \(E_D\) move beyond the peak width, wherein the altered species are removed by FAIMS action,\(^{38,57}\) (iii) faster diffusion of hotter ions, especially its anisotropic component along the field (i.e., across the gap),\(^{52}\) and (iv) effective gap narrowing with increasing oscillation amplitude in the cycle (about proportional to \(E_D/N\)).\(^{52,53}\) The interplay of these factors in LP-FAIMS of proteins will be discussed in a future publication.

In summary, macroions can broadly endure FAIMS process at fields up to \(\sim 300\) Td for \(>10\) ms. This new regime ought to allow many novel analyses. Here we focus on the crucial dipole alignment aspect.

**FAIMS Separations Showing No Alignment.** The palettes for all proteins (at any \(z\)) are now analogous: the \(E_C(E_D)\) functions smoothly increase up to the maximum \(E_D\) with positive second derivative (Figures 4, 5, and S1–S3). This type C behavior, where \(K\) continually decreases at higher \(E_D/N\), is classic for peptides and nonaligned small proteins at AP,\(^{26,39,44,46,55}\) and peptides at low pressure.\(^{37}\) No signal was found at \(E_C < 0\) or (at relevant \(E_D\)) \(E_C \sim 0\). These findings copy those at AP for small proteins (e.g., Figure 1a), but totally differ from those for Alb (Figure 1b).

Further, the curves lie close across proteins. At the maximum \(E_D/N\), all come at \(E_C/N = 4.5–6\) and 5–8 Td for the lowest and highest \(z\), respectively. The difference between the curves for Alb and mean trend for small proteins (not exhibiting alignment at AP) is less than the scatter around trend for those proteins. For example, the \(E_C/N\) for Ub (5.5 Td), Cyt (5.4 Td, mean of two conformers), and Myo (4.6 Td) differ by 0.9 Td, but average to same 5.2 Td as \(E_C/N\) for Alb at lowest \(z\).

The peak widths for Alb are likewise similar to those for small proteins. The \(w\) for highest \(z\) is 0.4 Td, same as the mean for Ub (0.4 Td), Myo (0.3 Td), and Ald (0.6 Td). The mean \(w\) over all charge states increases from 0.4 Td for Ub to 0.6 Td for Myo, but not further to Ald and Alb. In contrast, at AP,\(^{38,40}\) the band abruptly expands by >10-fold around 30 kDa. The instrumental peak widths in planar-gap FAIMS scale\(^{2,53}\) approximately as \(K^{-1/2}\). As larger protein ions are somewhat less mobile despite higher \(z\), a modest gradual peak broadening is expected and does not suggest alignment or structural heterogeneity.\(^{41}\)

Like for small proteins at AP, the peak widths narrow at higher \(z\) on average, from 0.7 Td for the lowest \(z\) to 0.5 Td for highest \(z\). This presumably happens as complete unfolding reduces polymorphism.\(^{38,59,44}\) On the contrary, the bands for aligned proteins did not narrow at higher \(z\), but often got wider with firmer alignment.\(^{39,40}\)

All these findings indicate no alignment in LP-FAIMS and, conversely, confirm that of large proteins in AP-FAIMS.\(^{39–41,46}\)

At AP, the \(E_C\) values maximized in lower \(z\) for small proteins\(^{38,55,57}\) (e.g., 7+ or 8+ for Ub and 9+ for Myo, Figure 1a) and remained near-constant for large proteins (e.g., Alb, Figure 1b). That was due to shift \(E_C\) drop upon unfolding at intermediate \(z\), only partly offset by \(E_C\) increase at higher \(z\) for fixed geometries.\(^{38,57,55}\) Here, a substantial unfolding already at lowest \(z\) (induced by more severe field heating) apparently leaves less room for unfolding over the probed \(z\) range.

We still see structural transitions and coexisting distinct conformers. For Ub and Myo with lowest \(z\), the \(E_C(E_D)\) curves kink toward lower \(E_C\) as \(E_D/N \sim 200\) Td (Figures 4 and S2). For Ub with highest \(z\) and Myo (and prominently Cyt with lowest \(z\) (Figures S1 and S2), two conformers appear in same \(E_D/N\) range and last to the breakdown (Figure 6). The lack of multiple peaks or curve inflections for Ald and Alb is common for large proteins, where many similar conformers allow less discrete transitions through pathways with more intermediates.\(^{39,40}\)

The curve bending to lower \(E_C\) may suggest unfolding.\(^{38,57}\) However, the coexistence of conformers at maximum \(E_D/N\), modest \(E_C\) difference between them, and their occurrence for highest \(z\) (fully unfolded even for much colder ions in AP-FAIMS or linear IMS with no field heating)\(^{38,55}\) all point to the multiple unfolded conformers discovered by AP-FAIMS.\(^{34,55}\) Their origin is uncertain, but feasibly manifests protomers (peptides with alternative protonated residues) that would interconvert less readily than conformers within a protonation scheme.\(^{35}\) Anyhow, not merely proteins but specific conformers broadly survive to at least 284 Td despite peak heating to \(\sim 600\) °C.

![Figure 6. Selected FAIMS spectra showing conformer resolution (parameters labeled as in Figures 4 and 5).](image-url)
At $R > 100$ in AP-FAIMS, smaller proteins (Ub, Cyt, Myo) with high $z$ featured $\sim 10$ distinct peaks with widths close to those for peptides and thus corresponding to essentially single conformers. The widths for Ub$^{13+}$ and Myo$^{26+}$ here (0.3–0.4 Td, Figures 4 and S2) only slightly exceed $w = 0.2–0.3$ Td for peptides at same 15 Torr.31 This reveals no hidden structural broadening for larger proteins), 41 but does not prove its heterogeneity (considering the above-mentioned trivial peak fusion instead of conformational multiplicity).38,63

**CONCLUSIONS AND FUTURE PROJECTIONS**

We report first analyses of macromolecules employing the recently engineered low-pressure FAIMS (integrated with mass spectrometry).31 Separations of small proteins, peptides, amino acids, and other small ions track the AP benchmarks. The behavior of large proteins drastically differs: there is no signal at $CV < 0$ for species with mobility rising in stronger fields. This dichotomy reflects the locking of electric macrodipoles of large proteins at ambient but not low pressure, while smaller species with weaker dipoles are free rotors in either case. These findings accord with a priori theory for dipole alignment of ions in gases, requiring (at any field) a minimum dipole moment proportional to the inverse gas pressure.39–41,46 Then alignment in low-pressure FAIMS would occur for $p$ over $\sim 10^4$ D projected for MDA-size biomolecules.

Present results further prove the dipole locking in high-field IMS and enable characterizing the dipoles for isolated macromolecules by tracking the pressure-dependent evolution of nonlinear ion mobility spectra. The point for onset and characteristics of spectral drift would reveal the dipole moment and orientation in the ion, respectively. Given the exceptional structural specificity of dipole magnitude and direction within the molecular frame, a path to deduce them opened by present work is anticipated to benefit integrative structural biology.

The option to modify FAIMS spectra by tuning the dipole alignment may also facilitate (i) advancing proteoform separations33,26 to larger proteins and top-down workflows and (ii) use of FAIMS in tissue imaging, where pulling the pendular large proteins apart from small proteins and other species alleviates spectral congestion and increases the number of identified proteins.60–62

The linear IMS spectra for macromolecules are now routinely annotated in terms of structures.17,63 This is achieved by relating the measured mobilities to those computed for high-field IMS and enable characterizing the dipoles for isolated macromolecules by tracking the pressure-dependent evolution of nonlinear ion mobility spectra. The point for onset and characteristics of spectral drift would reveal the dipole moment and orientation in the ion, respectively. Given the exceptional structural specificity of dipole magnitude and direction within the molecular frame, a path to deduce them opened by present work is anticipated to benefit integrative structural biology.

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**ASSOCIATED CONTENT**

5 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.9b00525.

The $E_C/E_p$ palettes for cytochrome c, myoglobin, and aldolase (PDF)

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The authors declare the following competing financial interest(s): The corresponding author is a consultant to Shimadzu Research Laboratory.

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