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Separation of Protein Conformers by Differential Ion Mobility in Hydrogen-Rich Gases

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Abstract

Proteins in solution or the gas phase tend to exhibit multiple conformational families, each comprising distinct structures. Separation methods have generally failed to resolve these, with their convolution producing wide peaks. Here we report full separation of >10 conformers for most ubiquitin charge states by the new approach of differential ion mobility spectrometry (FAIMS) employing H₂/N₂ gas mixtures with up to 85% H₂. The resolving power (up to 400) is five times the highest previously achieved (using He/N₂ buffers), greatly increasing the separation specificity. The peak widths match the narrowest obtained by FAIMS for any species under same conditions and scale with the protein charge state (z) and ion residence time (t) as $z^{-1/2}$ and $t^{-1/2}$, as prescribed for instrumental (diffusional) broadening. This suggests resolution of specific geometries rather than broader ensembles.

Introduction

Biomacromolecules have complex potential energy surfaces (PES) that feature a multitude of often abrupt local minima, many separated by large distances and high barriers (Figure S1, supporting information). Mapping those surfaces to find the principal minima and interconnecting paths is indispensable to understanding the protein folding and has become an object of enormous theoretical effort.^{1,2} The polymorphism of proteins *in vivo* and its crucial role in life processes (e.g., neurodegenerative diseases) are also increasingly appreciated in biomedicine.^{3,4} Conformations of proteins in solution have been examined by circular dichroism spectroscopy, but the information deduced is fairly generic.⁵ X-ray crystallography extracts accurate structures, but cannot handle numerous “intrinsically disordered” proteins (or their regions) that do not crystallize.^{6,7} Both methods require pure proteins in bulk quantities. The consequent low sensitivity and throughput, and inability to address conformational mixtures and structural dynamics, limit the utility of these approaches.⁸

Soon after the development of electrospray ionization (ESI) and other soft sources that generate intact gas-phase protein ions, the issue of protein structures in vacuum emerged.⁹ Fundamentally, isolated proteins with morphologies unaffected by intermolecular interactions (with solvent or nearby protein molecules) are important to test the computational models for macromolecular PES and protein folding.^{10,11} From the analytical perspective, mass spectrometry (MS) is tremendously more sensitive and faster than solution or solid-state methods, and can examine proteins present in mixtures even as minute components. An early technique was the H/D exchange, intended to identify the surface regions with accessible hydrogens as opposed to protected internal regions.^{8,11,12} However, the results are indirect and the exchange levels have poorly correlated with the shapes of gaseous proteins, e.g., folded conformers commonly exchange more hydrogens than unfolded ones with greater surface areas.^{13,14} Another approach is ion mobility spectrometry (IMS), where isomers are separated based on transport properties while driven through gas

by an electric field.^{9,13–17} Conventional IMS (based on absolute mobility K in moderate fields) and especially drift-tube (DT) IMS can aid characterizing ion geometries by matching measured mobilities to *a priori* calculations.^{9,17} With any structural probe for free proteins, a key question is the relationship to biologically relevant folds in solution. Growing research employing IMS and other tools frequently shows a memory of the global tertiary and quaternary solution structure in proteins produced by soft ESI, although the exact geometries likely differ.^{9,18,19}

An outstanding challenge to all protein separation and characterization methods has been the conformational multiplicity within each resolved feature, which broadens the peaks to widths often vastly exceeding the instrumental metrics. This effect was seen for chromatography and electrophoresis in liquids,^{20–23} and is ubiquitous in NMR.²⁴ The IMS resolving power (R), defined as peak position over full width at half maximum (w), for mass-selected small proteins in early ESI/DTIMS/MS studies was ~ 10 , close to the instrumental R (as benchmarked for multiply-charged peptides).^{25,26} Although new DTIMS technologies in the late 1990-s lifted the instrumental R to ~ 200 , the values for proteins were^{27,28} still $\sim 10 - 30$. One could readily resolve the compact, partly folded, and unfolded families, but not the precise geometries therein. This meshes with the typical protein PES depictions, where different basins are spaced apart by much higher barriers and larger distances than individual wells within each (Figure S1). Overtone mobility spectrometry, a frequency-domain mode of conventional IMS, has recently²⁹ raised R for “unfolded” protein conformers in high charge states (z) up to ~ 200 . The gain has been attributed to OMS eliminating less stable conformers that evolve during the filtering.²⁹

The mobility of any ion is a function of the field intensity (E), which underlies differential or field asymmetric waveform IMS (FAIMS)¹⁶ where a periodic asymmetric electric field filters ions by the mobility difference between high and low E . In implementation, a gas flow pulls ions through a gap between two electrodes carrying an appropriate voltage waveform of some amplitude (“dispersion voltage”, DV), which spreads ions across the gap. A superposed “compensation voltage” creates the “compensation field” (E_C) that selects ions permitted to pass the device to the next stage, here MS.¹⁶ The $K(E)$ derivative measured in FAIMS is generally much more independent of the ion mass/charge than K itself that is inversely proportional to the ion-gas molecule cross section (Ω) and thus tightly correlated to the ion size.^{16,30,31} Therefore FAIMS tends to be much more orthogonal to MS than IMS is, and usually separates more isomers at equal nominal resolving power. Early cylindrical devices distinguished up to four and five “unfolded” conformers for small proteins ubiquitin (Ub, 8.6 kDa)³² and cytochrome *c* (Cyt, 12.2 kDa)^{14,28} with high z , even at modest $R \sim 10 - 20$. These findings dovetail with H/D exchange, where Cyt ions exhibited subpopulations despite a single peak in DTIMS.³³ The FAIMS units in those experiments had a similar instrumental R , and whether the peaks were broadened by conformational multiplicity could not be ascertained.²⁸

Planar FAIMS devices with homogeneous field inherently allow higher resolution than cylindrical analogs with inhomogeneous field,³⁴ and addition of light gases in which ions are more mobile (helium^{35–37} and hydrogen³⁸) dramatically improves R . For peptide ions with $z = 3 - 7$, the highest R (at the “standard” filtering time of $t = 0.2$ s) increased from ~ 80 with N_2 gas to ~ 200 with He/ N_2 at 50% He - the maximum under the electrical breakdown threshold for DV = 5.4 kV over a 1.88-mm gap.^{35,37} Replacing He in He/ N_2 mixtures by equal H_2 molarity improves the resolution slightly, but the greater breakdown resistance of H_2 permits up to $\sim 90\%$ H_2 under identical conditions.³⁸ Use of H_2/N_2 buffers with $\sim 80 - 85\%$ H_2 has augmented the resolving power by 2 – 3 times for both multiply-charged peptides and smaller singly-charged ions.^{37–40} With the stabilized electronics providing more reproducible voltages, $R > 400$ was obtained⁴⁰ for peptides with $z = 3 - 6$.

Previously, Ub ions were analyzed⁴¹ utilizing He/N₂ mixtures with up to ~50% He. The resolving power was not improved for low z (5 – 7) but increased to ~60 – 80 for higher z (8 – 13) where the protein is predominantly or fully unfolded. However, that highest R stayed well below ~200 reached for peptides (~1.5 – 3 kDa) under same conditions as stated above, demonstrating the broadening of even the narrowest features due to conformational multiplicity.⁴¹

Here, FAIMS separations of Ub conformers using H₂/N₂ with up to 85% H₂ are explored. We achieve $R \sim 400$ and peak width unprecedented for proteins, but matching or exceeding the records for peptides in the same regime.⁴⁰ This and the functional dependences of widths on the separation time and protein charge imply that many peaks have largely instrumentally limited widths and hence represent specific protein conformers and not ensembles.

Experimental methods

The previously described platform^{35–41} consists of a planar FAIMS device³⁴ and ion trap mass spectrometer (Thermo Scientific LTQ) fitted with an electrodynamic funnel interface. The device was powered by high-definition waveform generator⁴⁰ operated at the maximum DV = 5.4 kV. The H₂ and N₂ gases were filtered, dosed by flow meters (MKS Instruments), and mixed for delivery to the FAIMS stage. The flow rate was $Q = 1 - 2$ L/min, corresponding to $t = 0.4 - 0.2$ s. Bovine ubiquitin (8,565 Da) was dissolved to ~4 μ M in 50:49:1 methanol/water/acetic acid and infused to the ESI emitter at ~0.4 μ L/min.

Results and discussion

As expected with a denaturing solvent, Ub ions with $z = 5 - 14$ were observed.^{28,42} We initially acquired the FAIMS data at $t = 0.2$ s. The results with N₂ are published^{34,41} and will not be discussed here. The spectra at 50% H₂ resemble those⁴¹ at 50% He (Figure 1), but have notably better feature resolution across the charge states. The best example is Ub¹²⁺ that comprises four major peaks (**b**, **d**, **e**, **f**). Their mean width almost halves from $w = 2.56$ V/cm at 50% He to 1.36 V/cm here. The average resolution (r) for adjacent peak pairs (**b/d**, **d/e**, and **e/f**) increases in proportion - from 1.46 to 2.64, improving the separation from about half-maximum⁴¹ to baseline (customarily set at 10% height). The gains for other z are similar, though less easily quantified. Of note in Figure 1 are complete separation of the lowest- E_C major peaks for Ub¹¹⁺ (**b** + **c**) and Ub¹⁰⁺ (**a**) from the rest with splitting for Ub¹¹⁺ (into **b** and **c**), and conspicuous splitting of the Ub⁹⁺ base peak (into **c** and **d**). Also, for $z = 8 - 11$ and 13, several discrete features are now evident (instead of continuous slopes)⁴¹ on the high- E_C sides of dominant peaks (**c**, **e**, **h**, **i** for Ub⁸⁺, **g** and **h** for Ub⁹⁺, **e** – **i** for Ub¹⁰⁺, **f** – **h** for Ub¹¹⁺, **e** for Ub¹³⁺) and their low- E_C sides (**a** for Ub⁹⁺, **a** and **b** for Ub¹³⁺). For low charge states ($z = 5 - 7$) where compact conformers prevail (at higher E_C), use of H₂ engenders new geometries at lower E_C (**a** and **b** for Ub⁵⁺, **a** for Ub⁶⁺ and Ub⁷⁺) or increases their abundance (**b** for Ub⁶⁺, **b** – **d** for Ub⁷⁺), while decreasing some at higher E_C (**c** for Ub⁶⁺).

For smaller ions, switching from 1:1 He/N₂ to 1:1 H₂/N₂ buffer has³⁸ narrowed the peaks and raised the resolving power by a marginal ~10 – 20%. This gain was rationalized as a consequence of higher ion mobility that, with fixed Ω value, is proportional to $M^{-1/2}$ (where M is the gas molecule mass).^{15,16,38} As R of planar FAIMS devices scales approximately^{43,44} as $K^{1/2}$, changing from He (4 Da) to H₂ (2 Da) would ideally raise mobilities by $2^{1/2}$ times and R by $2^{1/4}$ times or 19%. Substituting H₂ for He in gas mixtures would increase the mobility and thus R less, here by 17% and 8%, respectively (according to the Blanc's law). The real gains ought to be lower yet, because Ω for any ion is higher with diatomic H₂ than the physically smaller and less polarizable He atom.^{37,38} The last effect

would be weaker for larger ions, the Ω values for which vary with the gas properties less.¹⁶ Hence, use of H_2 should increase K and R slightly more for proteins than smaller ions. However, the resolution gain by above mechanism cannot exceed that determined for constant Ω , which is an order of magnitude below the ~80% observed for Ub^{12+} .

Alternatively, the apparent resolution can be improved by annealing of ions upon heating during FAIMS separation, which collapses the conformers into fewer energy wells. Indeed, the growth of low- E_C conformers at the expense of high- E_C ones for $z = 5 - 7$ indicates protein unfolding.^{28,45,46,47} Such isomerization can be induced by stronger collisional (field) heating in the FAIMS gap, normally caused by increasing DV ⁴⁷ or decreasing Ω values^{35,37} in agreement with the two-temperature theory. Addition of smaller gas molecules (He or H_2) to N_2 reduces the mean Ω and thus promotes unfolding of peptides and proteins,^{35,37} but replacing He by larger H_2 should have the opposite effect (and in fact has for large peptides).³⁷ Hence the unfolding of Ub ions across charge states and resolution gains when He in 1:1 He/N_2 is replaced by equal H_2 fraction remain unexplained.

The major gains for smaller species came from moving beyond 50% H_2 (see Introduction). Similarly here, increasing the H_2 content to 85% systematically narrows the peaks and shifts spectra to higher E_C , resulting in drastically higher resolving power and overall feature resolution (Figure 2). For the Ub^{12+} case, the mean width of major peaks **b**, **d1**, **e** nearly halves, dropping from $w = 1.31$ V/cm at 50% H_2 to 1.00 V/cm at 70% H_2 and 0.69 V/cm at 85% H_2 (**f** coalesces with **e** at >70% H_2 and is ignored here). The average E_C of these features meanwhile increases by 54% (from 129 V/cm to 172 V/cm and 199 V/cm, respectively), hence average R triples (from 98 to 170 and 289). The average resolution of **b/d1** and **d1/e** pairs improves in proportion, from $r = 2.77$ at 50% H_2 to 5.16 at 70% H_2 and 9.37 at 85% H_2 . These trends are replicated for other charge states in the “unfolded” range ($z = 8 - 14$). Finer separations are manifested by: for Ub^{14+} , full separation of the shoulder **b**; for Ub^{13+} , baseline resolution of **c** from **d**; for Ub^{11+} , splitting of the base peak **e** (into **e1** and **e2**), substantially better resolution of the major peaks **b** and **c**, full separation of **g**, and baseline resolution of **d** accompanied by splitting into two and then four features; for Ub^{10+} , splitting of the major peak **a** (into **a2'**, **a2''**, and minor features **a1** and **a2'''**), full separation of shoulder **d**, and baseline resolution of **e** with splitting into three features; for Ub^{9+} , baseline resolution of all significant features (**b**, **c**, **d**, **e**) with **e** splitting into **e1** and **e2**; for Ub^{8+} , full separation of shoulder **b** and splitting of **e** and **f** into three features each.

For $z = 6$ and 7 , going from 50% to 85% H_2 promotes continued unfolding, with the higher- E_C features for compact geometries (**f** for Ub^{7+} and **d** for Ub^{6+}) progressively diminishing into secondary peaks. This behavior is in line with theory, as mentioned above. However, Ub^{5+} does not visibly unfold further, probably because the Ω values with H_2 and N_2 for large macro ions are close and thus adding H_2 heats proteins only moderately. The resolution also improves for $z = 5 - 7$, especially for Ub^{7+} where the features **a**, **b**, **c**, and **f** clearly split into two - three narrow peaks. The gains are less pronounced but still obvious for $z = 5$ and 6 , where each of the four peaks (**a**, **b**, **c**, **d**) turns into two - four partly resolved features.

The resolution advantages of hydrogen-rich buffers for higher charge states are particularly striking with respect to the N_2 rather than 1:1 H_2/N_2 baseline (Figure 3). As with He/N_2 buffers,⁴¹ adding H_2 actually broadens the peaks for $z = 5$ and 6 (because early unfolding steps enrich the conformational diversity), while those for $z = 7$ roughly retain the width and those for $z > 7$ narrow as exemplified above. Combination of these trends with E_C increasing at higher H_2 concentrations has the following outcomes for the resolving power of major peak: a slow decrease for $z = 5$ and 6 (from ~20 in N_2 to ~10 at 85% H_2), slow increase for $z = 7$ (from ~10 in N_2 to ~20 at 85% H_2), and rapid increase for $z = 8 - 14$

(from ~15 – 25 in N₂ to ~200 – 300 at 85% H₂). For all $z > 7$, these values are much above those attained⁴¹ using He/N₂ mixtures (Figure 3).

The widths and resolving powers for all well-defined peaks for $z = 7 - 14$ lead to broader separation statistics across charge states (Table 1). The w values averaged for each z drop from 1.8 V/cm for Ub⁷⁺ to 1.1 V/cm for Ub⁸⁺, then gradually decrease to 0.75 V/cm for Ub¹³⁺ (a small increase for Ub¹⁴⁺ may reflect poor statistics from just two weak features). The theoretical peak width for a fixed ion geometry in planar FAIMS units is proportional to $z^{-1/2}$ (as in conventional IMS),^{43,44} and the data for $z = 8 - 14$ track that trend (Figure 4). This suggests the peak widths controlled by conformational multiplicity for $z = 7$, but mainly by instrumental parameters for $z = 8$ with mostly unfolded structures. The mean width for $z = 10 - 14$ ($w = 0.84$ V/cm) is very close to that for typical peptides with $z = 3 - 6$ under same conditions,³⁷⁻⁴⁰ for instance⁴⁰ 0.76 ± 0.05 V/cm for the 3+ ion of Syntide 2 (S2 3+).

As with other separations in media, the resolving power of planar FAIMS devices scales^{16,43} as $t^{1/2}$. The filtering time scales as the inverse gas flow rate, so reducing Q improves resolution. For example, extending t from 0.2 s at $Q = 2$ L/min to 0.5 s at $Q = 0.8$ L/min has pushed³⁶ the R values for a 3+ peptide (with 1:1 He/N₂ gas) from ~200 to >300. The price is that the signal drops and eventually vanishes as ions spread in the gap and are indiscriminately neutralized on the electrodes. The diffusion coefficient (D) for ions in gases is, like mobility, proportional¹⁶ to $M^{-1/2}$. Hence adding H₂ or He to N₂ speeds up diffusion:³⁸ by the Blanc's law, the D values for proteins in 85:15 H₂/N₂ are ~1.8 times those in 1:1 He/N₂ and ~2.8 times those in N₂. Faster diffusion accelerates ion elimination from the gap, which precluded using $t > 0.2$ s for peptides and small ions with hydrogen-rich buffers.^{38,40} However, the D values are also inversely proportional¹⁶ to Q that scales roughly as (mass)^{2/3} for similar shapes and is of course much larger for proteins than peptides and smaller species. For example, the cross sections are ~3 – 7 times greater for Ub (~1000 – 1800 Å² for $z = 5 - 11$)^{28,48} than typical 2+ and 3+ peptides such as bradykinin, angiotensin I, fibrinopeptide A, or neurotensin (~240 – 360 Å²).^{47,50} Hence proteins diffuse slower at 85% H₂ than peptides or smaller ions even in N₂, enabling extended separations with any H₂/N₂ composition.

The lowest practical Q for Ub analyses at 85% H₂ was ~1 L/min, producing $t = 0.4$ s. As usual in this regime, the sensitivity drops and many smaller features disappear, removed by “self-cleaning”⁴⁶ (upon isomerization with attendant E_C transition) or merge into the noise because of lower ion transmission (Figure 5). This applies to all features for $z = 5, 6$, and 14. However, the separation of remaining features for other z broadly improves with: (i) the major peak pairs Ub¹¹⁺ **b/c**, Ub¹¹⁺ **e1/e2**, and Ub¹⁰⁺ **a2'/a2''** baseline-resolved, (ii) the minor features Ub¹²⁺ **c1** and **c2** baseline-separated from each other and **b**, (iii) the minor feature Ub¹⁰⁺ **a1** split into **a1'** and **a1''**, and (iv) the shoulders on Ub⁹⁺ **c** and **d** baseline-separated into **c1**, **d1**, and **d3**. For the Ub¹²⁺ **b**, **d1**, and **e**, the average resolving power and resolution of two peak pairs increase by ~40 – 50%, to $R = 426$ and $r = 13.3$. This result is representative: the average R values per charge state (for $z = 8 - 13$) increase by ~30% – 70% and the mean total increases by 46%, from 212 to 309 (Table 1). These gains match the anticipated factor of $(2/1.05)^{1/2} = 1.38$, noting that R scales slightly faster than $t^{1/2}$ because of the fixed onset time for FAIMS process.³⁶ As above, the peaks tend to narrow for higher z in line with the theoretical scaling (Figure 4). Both trends further support the notion of instrumentally limited peak widths.

To verify the achieved resolution, we looped over a spectral window around the feature Ub¹²⁺ **e** (Figure S2). At 95% confidence, the peak width (for 24 replicates) is 0.500 ± 0.026 V/cm, the same as or slightly lower than $w = 0.519 \pm 0.027$ V/cm for the narrowest

heretofore reported FAIMS feature ($S2\ 3+$).⁴⁰ The resulting resolving power is 398 ± 21 , which is somewhat smaller than the highest $R = 462 \pm 29$ registered in FAIMS (also for $S2\ 3+$)⁴⁰ because of lower E_C for all Ub conformers.

Conclusions

We have applied a recently developed FAIMS approach using H_2 to ubiquitin with several known conformers in both the solution and gas phases. The resolving power (R) exceeds that achieved with He/N_2 at 50% H_2 and rapidly increases at higher fractions up to the 85% permitted by electrical breakdown, separating $\sim 10 - 15$ conformers for charge states $z = 8 - 14$ where Ub is mostly unfolded. Their mean peak width matches the narrowest for multiply-charged peptides under identical conditions⁴⁰ and scales with z properly for diffusional broadening of single peak. Slower diffusion of heavier ions allows extending the filtering beyond 0.2 s (to 0.4 s), which for smaller species is precluded at $>50\%$ H_2 by signal loss. This improves resolution across charge states by $\sim 50\%$, also as expected for diffusional broadening, with many new features revealed. The average peak widths again match the narrowest for peptides and properly scale as $z^{-1/2}$, and the statistically validated R reach ~ 400 . This is fivefold higher than R obtained using the He/N_2 compositions, a much greater gain than the ~ 2 -fold for peptides and smaller species.³⁸

The observations that peak widths for higher Ub charge states are (i) at least as narrow as those for any other ions and (ii) scale as $z^{-1/2}$ and $t^{-1/2}$ indicate that resolution is limited primarily by instrumental constraints (rather than conformational multiplicity) and convey that distinct protein conformers rather than ensembles are largely distinguished. This would be the first such separation by any method, opening the path to investigation of physical (e.g., fragmentation) and chemical (e.g., H/D exchange) properties for specific conformers.

This performance is perhaps enabled by FAIMS in hydrogen-rich buffers combining (i) a strong durable field heating that unfolds Ub for $z \geq 8$ and effectively anneals the unfolded conformers, reducing their number to under 15, and (ii) high resolving power that affords full separation of an isomeric mixture of that complexity. Also, destruction of fragile interconverting conformers by FAIMS self-cleaning⁴⁶ may leave the more stable geometries located in deeper wells. For lower charge states where compact geometries are more stable, the unfolding is massive but still incomplete even under these conditions, and the diversity of intermediates is too large for individual separation even with the present resolving power.

Can this approach work for larger proteins, which should assume a greater number of geometries within any family? The FAIMS separation space and thus instrumental peak capacity change little⁵¹ for proteins up to ~ 30 kDa and can be overwhelmed by growing multitude of conformers, restoring broad features such as seen for proteins previously. Above ~ 30 kDa, this trend would compete with a dramatic expansion of separation space (presumably due to the dipole alignment in FAIMS).⁵¹ Investigations along this direction are in progress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. FAIMS spectra for Ub ions ($z = 5 - 14$, as indicated) measured using 1:1 H_2/N_2 and $Q = 2$ L/min. The added benchmarks with 1:1 He/N_2 ([41]) are horizontally shifted for clarity. Smaller features are magnified as marked, with the original spectrum deleted. Significant peaks are labeled to relate the data across gas compositions (the labels differ from those in [41]). The signal for Ub^{14+} with He/N_2 was insufficient.

Fig. 2. Same as Figure 1, measured using H_2/N_2 with 50%, 70%, or 85% H_2 (v/v) as color-coded on top. Smaller features are magnified for 85% H_2 only.

Fig. 3. Peak widths and resolving power for the highest features in FAIMS spectra of Ub ions with $z = 5 - 10$ (left column) and $z = 11 - 14$ (right column): lines are for the peaks in Fig. 2 (as labeled), circles represent the values for $z = 5 - 13$ with 1:1 He/N₂ mixtures [41].

Fig. 4.

Mean peak widths for Ub ions: circles mark the measurements (solid for $Q = 2$ L/min, $z = 7 - 14$; blank for $Q = 1.05$ L/min, $z = 8 - 13$), dashed lines are the first-order regressions for each Q (excluding the Ub^{7+} point for 2 L/min), solid lines show the $w = z^{-1/2}$ dependence vertically scaled to match the regressed values for Ub^{8+} .

Fig. 5. Spectra for Ub ions ($z = 7 - 13$, as indicated) measured using 4:1 H_2/N_2 and $Q = 1.05 \text{ L/min}$. Smaller features are magnified as marked, with the original spectrum deleted. Selected segments are compared to those obtained with 85:15 H_2/N_2 and $Q = 2 \text{ L/min}$ (from Figure 2) in the inserts.

Table 1

Peak widths and resolving power values for significant features in the FAIMS spectra of mass-selected ubiquitin ions, measured using 85% H₂.

Charge state	Peak label	Peak width (V/cm); Resolving power	
		Standard (85% H ₂)	Extended (80% H ₂)
7			
	a1	1.14; 114	Too low
	b1	2.20; 65	Too low
	c1	1.69; 90	Too low
	c2	1.38; 114	Too low
	c3	1.66; 96	Too low
	f1	2.74; 69	Too low
	Mean	1.80; 91	n/a
8			
	a1	0.46; 338	Too low
	a2	0.81; 195	0.48; 315
	b	0.91; 175	0.66; 233
	c	0.80; 210	0.50; 326
	d/d1	1.48; 116	0.80; 209
	e1′	1.15; 152	Poorly resolved
	e2	1.50; 119	Too low
	f1′	1.20; 154	0.92; 196
	f1″	1.31; 143	Too low
	f2	0.85; 234	Too low
	g	1.28; 152	Too low
	Mean	1.07; 181	0.67; 256
9			
	a	1.02; 166	0.54; 303
	b1	Unresolved	0.88; 192
	b2	0.67; 262	0.60; 282
	c/c2	0.97; 184	0.92; 189
	c1	Unresolved	0.61; 284
	d1	Unresolved	0.60; 299
	d/d2	0.77; 237	0.59; 305
	d3	Unresolved	0.53; 343
	e1	0.86; 222	Poorly resolved
	e2	1.55; 125	0.99; 191
	g1	1.43; 142	
	Mean	1.04; 191	0.70; 265
10			

Charge state	Peak label	Peak width (V/cm); Resolving power	
		Standard (85% H ₂)	Extended (80% H ₂)
	a1'	Unresolved	0.50; 353
	a1''	Unresolved	0.55; 319
	a2'	0.78; 235	0.55; 323
	a2''	0.80; 231	0.49; 369
	b3	Unresolved	0.66; 284
	c	1.13; 171	0.61; 307
	d	1.24; 159	0.74; 257
	e1/e1''	1.04; 192	0.67; 289
	e2''	1.03; 200	0.67; 299
	g1	0.55; 389	
	h	0.81; 273	
	Mean	0.92; 231	0.60; 311
11			
	a1	0.77; 236	Too low
	a2	0.37; 506	Too low
	b	0.87; 218	0.47; 397
	c	0.83; 231	0.59; 316
	d2''	0.47; 423	0.68; 283
	e1/e1''	Poorly resolved	0.46; 435
	e2	0.98; 210	0.62; 324
	g	1.61; 132	
	Mean	0.84; 279	0.56; 351
12			
	a	1.00; 187	
	b	0.61; 313	0.41; 457
	c2	0.98; 200	Poorly resolved
	d1	0.75; 265	0.48; 400
	d2	0.85; 238	Too low
	e	0.71; 290	0.47; 420
	g	0.87; 243	
	Mean	0.82; 248	0.45; 426
13			
	a2	0.76; 252	
	b2	0.74; 262	
	c	0.74; 258	
	d	0.66; 313	0.51; 391
	e	0.84; 251	
	Mean	0.75; 267	0.51; 391

Charge state	Peak label	Peak width (V/cm); Resolving power	
		Standard (85% H ₂)	Extended (80% H ₂)
14			
	a	0.79; 250	
	b	0.87; 230	
	Mean	0.83; 240	n/a
All charge states (53/32 peaks)		1.02; 212	0.62; 309

Notes:

Data are listed for $t = 0.2$ and 0.4 s. Averages are given for each z and all $z = 7 - 14$. Empty slots stand for the peaks not reliably detected at longer t , “too low” indicates the peaks too weak for robust width determination, “unresolved” or “poorly resolved” means that the feature was not separated at half maximum.