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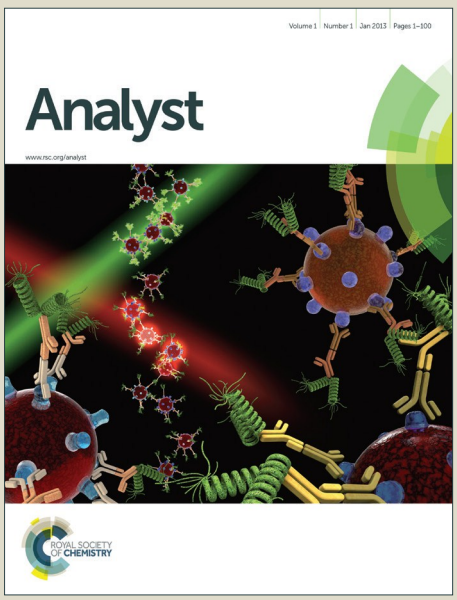

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ARTICLE

Comparing Equilibrium and Kinetic Protein Unfolding Using Time-resolved Electrospray-coupled Ion Mobility Mass Spectrometry†

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Protein unfolding intermediates are thought to play a critical role in conformational pathogenesis, acting as a 'gateway' to inactivation or pathogenic aggregation. Unfolding intermediates have long been studied either by populating partially-folded species at equilibrium using incresingly denaturing conditions, or by transiently populating 'kinetic' intermediates under fully denaturing conditions using a time-resolved approach (e.g. stopped-flow fluorescence). However, it is not clear that the folding intermediates populated under equilibrium conditions are comparable to intermediates transiently populated in kinetic experiments. In this work, we combine time-resolved electrospray (TRESI) with travelling wave Ion Mobility Spectrometry (IMS) for the first time to directly compare equilibrium and kinetic unfolding intermediates of cytochrome c. Our results show a high degree of correlation between all species populated under these substantially different regimes.

1 Introduction

The mechanisms of protein (un)folding are a long-standing challenge in structural biology.[1, 2] Intermediates that arise along the folding pathway are directly linked to the fundamental question of how proteins adopt their 'native' conformations from the initially unstructured polypeptide that is generated from the ribosome.[3, 4] Unfolding intermediates are often linked to pathogenesis,[5, 6] acting as a gateway between the functional 'native' configuration and inactive or amyloidogenic conformations. However, these protein intermediates are challenging to study as they are often transient, weakly populated at equilibrium and spectroscopically similar to the ground-state.[7, 8]

Experimental efforts to study unfolding intermediates in vitro typically involve equilibrium perturbation of protein structure over a range of denaturant concentrations.[9, 10] The advantage of populating unfolding intermediates in this way is that the sample is at equilibrium, allowing an unlimited time for analysis. Thus, it is possible to apply a wide range of analytical techniques including fluorescence,[11] SAXS[12] and notably NMR,[13] which can sometimes provide a highly detailed structural picture. However, it is not clear that species populated under a range of solvent conditions represent true unfolding intermediates, since each is a reflection of a unique conformational energy landscape.[7, 9, 14]

An alternative is to employ rapid mixing techniques to transiently populate unfolding intermediates under a single set of strongly denaturing conditions. In this case, the unfolding process is driven by a single, rapid change in the conformational landscape (e.g. due to pH jump[14] or rapid addition of denaturant[15]), and proceeds under constant conditions. This 'kinetic' approach may be more likely to generate biologically relevant intermediates, but it also presents substantial analytical challenges, since intermediates appear only for exceptionally short periods (often milliseconds or less) after mixing.[7, 8] As a result, analytical options for kinetic experiments are much more limited and the field is dominated principally by fluorescence-based approaches.[16]

Time-resolved Electrospray Ionisation Mass Spectrometry (TRESI-MS) was introduced as an alternative to optically-based stopped-flow rapid mixing experiments.[17-19] The principle advantage of TRESI is the ability to monitor virtually all reactive species simultaneously in millisecond time-resolved kinetic experiments.[18] TRESI has been used in a wide range of applications, from simple organic reactions[20] to enzyme catalysis[21] and protein folding.[22-24] In isolation, TRESI provides limited structural information about protein analytes, although a coarse-grained picture can be achieved by monitoring the electrospray charge-state distribution.[25] A more detailed picture can be achieved by combining TRESI with Hydrogen / Deuterium eXchange (HDX),[26, 27] however, such experiments are unsuitable for pH-jump unfolding measurements due to the pH dependence of HDX kinetics.

Ion Mobility Spectrometry (IMS) is a relatively new addition to the commercially-available mass spectrometry tool-kit.[28-30] The basic principle of IMS is spatio-temporal separation of gas-phase ions using low-energy collisions with a neutral gas. In most IMS techniques, retardation of ions traversing the IMS cell is directly proportional to their collision cross section, which is function of volume and shape.[28-30] The most

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straightforward implementation of IMS is the 'drift tube' in which ions traverse a high pressure cylindrical cell under the influence of a constant, relatively weak electric field. In travelling-wave ion mobility setups, ions are 'swept' through a high pressure cell by electric field 'waves' generated by successively applying a potential to a series of ring ion guides. This approach has the advantage to mobility separation can be optimized by adjusting the wave height and frequency.[31] It is also possible in most cases to calculate collision cross sections from tavelling-wave mobility data, often with use of an internal calibrant.[32]

In the present study we combine TRESI-MS with travelling wave IMS to investigate equilibrium and kinetic unfolding intermediates of cytochrome c. Cytochrome c provides an ideal model for this study because it has been extensively characterized by TRESI[22] and IMS-MS[33] independently. Our principal aim is to demonstrate the TRESI-IMS-MS approach as a powerful tool for characterizing kinetic protein (un)folding intermediates and to probe the equivalency of equilibrium vs. kinetic protein folding intermediates.

2 Experimental

2.1 Reagents and Materials

Cytochrome c (C7752), Ubiquitin (U6253), and Myoglobin (M1882) were all purchased from Sigma Aldrich (St. Louis, MO). Protein solutions were made to 10μM concentrations from 300μM protein stock solutions which were stored at 4°C. Solutions were made in HPLC water (Fischer Scientific) and the pH was adjusted with LCMS-grade ammonium hydroxide (Sigma-44273) and acetic acid 99.7% (Sigma Aldrich,St. Louis, MO).

2.2 Ion Mobility Measurements

Mass spectra and traveling–wave mobility spectra were acquired via electrospray ionization on a Waters Synapt G1 (Manchester, UK) equipped with an 8k quadrupole. Nitrogen gas was used for both the source and IMS region, and argon gas was used in the trap region of the mass spectrometer. The traveling-wave mobility separation was calibrated using 10μM ubiquitin and 10μM myoglobin in a 49:49:2 water, methanol, and acetic acid solution.[29] Settings such as capillary voltage, sample cone, extraction cone, trap/transfer collision energy, and trap DC bias voltages were optimized to allow for optimal ion transmission with minimal activation of the protein. It is important to note that a certain degree of ion activation is necessary for transmission, but too much activation can lead to fragmentation and erroneous mobility measurements. Discussion of this topic is beyond the scope of this paper, we direct the reader to the following excellent reviews.[29, 34] The resulting source conditions used for all measurements are as follows: Capillary 2.25 kV, sample cone 30 V, extraction cone 1.0 V, source temperature 80°C, desolvation temperature 150°C, desolvation gas flow 200 L/h, cone gas flow 25 L/h. The pressure in the source region was left unchanged at 200 Pa. The trap and transfer collision energies were set to 10 V each, with a trap pressure of 3.99 Pa.

Protein solutions were infused at a rate of 10 μL per minute using Harvard 11 Elite syringe pumps (Holliston, Massachusetts). Time-of-flight mass spectra were acquired for 3 minutes with a 2 second scan duration with mass-to-charge (m/z) range of 500-4000, and showed no fragmentation with excellent transmission of both ubiquitin and myoglobin. Optimal traveling-wave mobility separation was achieved by using a 300 m/s IMS wave velocity and 7.5 V IMS wave height, with an IMS pressure of 48.6 Pa. The pusher frequency of the TOF was set to 90μs, which coincided with a Transfer T-wave velocity of 200 m/s to ensure any “ripples” or “beats” in the mobility spectrum are eliminated.[29] Transfer wave height was held at 8 V. Spectra were acquired for 5 minutes with a 5 second scan duration. Mass and mobility data were processed using MassLynx 4.1 (Waters Ltd. Manchester, UK).

2.2 Equilibrium Unfolding

Equilibrium unfolding studies were carried out by running a series of pH adjusted cytochrome c solutions and recording their mass spectrum and mobility separation. Solutions of 10μM cytochrome c in water were pH adjusted to pH 2.4, 2.5, 3, 4, 5, 6, 7, 8, 9, 10 and infused into the ESI source of the Synapt at a rate of 10 μL per minute. Identical instrument settings were used for these experiments, ensuring no instrument-derived systematic error in measured drift times. Spectra were acquired in triplicate for 5 minutes with a 5 second scan duration.

2.3 Kinetic Unfolding Using TRESI

Kinetic unfolding of cytochrome c was done using a custom-built time-resolved ESI (TRESI) source that interfaces directly to the commercial heated electrospray source of the Synapt (Figure 1). The TRESI interface is acts as an adjustable millisecond timescale reactor, facilitating the mixing of two solutions which are then electrosprayed into the mass spectrometer.[35] Analyte solutions are infused through two separate concentric capillary lines which mix in a small volume region that can be adjusted by pulling back the inner most capillary.

The time-resolved interface was constructed as per Liuni *et al*.[36] with some minor changes. A 33 gauge stainless steel metal capillary (I.D.=132.6 μm, O.D.=203.2 μm, length=18 cm, McMaster-Carr, Aurora, OH) served as both the outer capillary and electrospray capillary. Cytochrome c at 10μM was infused into the inner glass capillary (I.D.= 40μm, O.D.=109.2 μm, length=40 cm, Polymicro Technologies, Phoenix, AZ) at a rate of 5μL per minute and a 10% acetic acid solution was infused into the outer metal capillary at a rate of 5μL per minute. Reaction profiles for the acid-induced unfolding of cytochrome c were acquired by steadily pulling the inner capillary back from end of the outer capillary. For instance, a 1 mm distance between the ends of the inner and outer capillaries corresponds to a reaction time of 136 ms (this reaction time is calculated taking into account laminar flow, see ref.[19]). To

acquire a time-course, the inner capillary is withdrawn in steps of 1mm, with 5 minute acquisitions at each step.

3 Results and discussion

3.1 IMS-MS of equilibrium unfolded cytochrome c.

Cytochrome c is among the most-well studied proteins in the context of (un)folding.[37] This is owed principally to the fact that cytochrome c is an exceptionally good analyte, with distinctive chromophoric properties arising from its covalently-bound heme group and a tendency to ionize easily in electrospray mass spectrometry. There is consequently a wealth of literature on cytochrome c unfolding, including equilibrium and kinetic studies using optical detection[38] and even TRESI-MS.[39] Cytochrome c was also among the first protein IMS analytes and is now an exceedingly well characterized in terms of IMS collision cross section and other parameters, including equilibrium unfolding.[33]

A summary of our equilibrium unfolding data are shown in Fig. 2. One interesting feature of these data compared to previous measurements is the persistence of m/z peaks corresponding to a partially-folded species at low pH. Even at pH 2.4 (the lowest value attainable with acetic acid), cytochrome c exhibits an equilibrium between the fully unfolded state and a distribution of peaks centred on 8+, which is generally thought to correspond to a molten-globule intermediate. While this molten-globule is often observed as a low-populated species in pH 2.4 cytochrome c mass spectra, its apparent persistence in this case may be attributable to the enhanced sensitivity of z-spray type ion sources for globular species relative to extended (unfolded) species in electrospray.

In general, the observed equilibrium unfolding is in-line with what has been reported previously, consisting of three principle components: A 'folded' species (corresponding to charge states 5^+ - 7^+, centred on 7^+) , an intermediate 'molten-globule' species (charge states 8^+ - 11^+, centred on 9^+) and an 'unfolded' species (charge states 12^+ - 19^+, centred on 16^+). With the inclusion of IMS, it is possible to observe the extent to which these charge state distributions overlap in terms of the species from which they arise (Fig. 3, top row). The 8^+ peak, for instance, includes a significant contribution from the 'folded' species in addition the 'intermediate' species to which it is assigned based on the charge-state distribution alone. Similarly, the 10^+ peak arises from both 'intermediate' and 'unfolded' species.

3.2 Unfolding kinetics of cytochrome c using TRESI-IMS-MS

TRESI-MS has been used to monitor folding and unfolding kinetics in a number of proteins, including myoglobin,[23] haemoglobin,[40] ubiquitin[41] inducible nitric oxide synthase.[42] The latter study in particular offers an excellent example of the mechanistic detail that can be derived from TRESI measurements alone, provided that there are changes in mass due to ligand dissociation or complexation. Kinetic cytochrome c unfolding has been monitored by TRESI-MS in the introduction of a TRESI-based microfluidic device. In this study, a kinetic intermediate, identified as a transiently populated species during time-dependent unfolding, was observed in the 9^+ and 10^+ charge states. TRESI-MS data from the current study are summarized in Fig. 4. The results are broadly in agreement with those of Rob *et al.*,[43] except that kinetic intermediate behaviour is also observed in the 8^+ charge state. Here again, the data are indicative of a three-component system, with a 'folded' species centred on 7^+, an 'intermediate' centred on 9^+ and an 'unfolded' species centred on the 16^+ charge state. The transition from the 'folded' to 'intermediate' charge-state distribution occurs within the dead-time of the TRESI apparatus, so that the observed kinetics are associated with the transition from the 'intermediate' to 'unfolded' state.

Without IMS, the observed kinetic profile of each *m/z* peak is an average of the individual profiles of all contributing species. In the current study, IMS resolution allows each contributing species to be monitored independently, as shown in Fig. 3 (bottom row). What is striking about the temporal profiles of the kinetic mobility data (Fig 3., bottom row, IMS peak intensity *vs*. reaction-time profiles and heatmaps) is that some IMS-distinguishable species track with the dominant peak in the profile, while others do not. For instance, the 10^+ profile consists of three IMS-distinguishable species (drift times 5.94, 7.38 and 8.46 ms) the former two of which show substantially different kinetic behaviour compared to the dominant IMS peak at 8.46 ms drift time. The many IMS peaks associated with the 8^+ charge state, in contrast, exhibit virtually identical time-dependent behaviour (after adjusting for ion intensity). While both 8^+ and 10^+ species are clearly kinetic intermediates, it may be that the kinetically-distinct species in the 10^+ profile are reflective of true solution-phase structures, while the observed 8^+ species are a consequence of gas-phase unfolding during transit through the IMS cell.[44, 45]

3.3 Comparison of equilibrium and kinetic unfolding intermediates

One of the principal objectives of this work was to determine if the intermediates populated during equilibrium and kinetic unfolding of cytochrome c are structurally distinguishable. In both the equilibrium and kinetic unfolding experiments described above, the unfolding process involved 'folded', 'unfolded' and an 'intermediate' species that is presumed to correspond to a well-established molten globule. When the IMS profiles of equilibrium and kinetic unfolding are compared, taking into account the fact that the kinetic data proceed from the 'intermediate' (which is maximally populated at pH 3 under equilibrium conditions), the similarity for all charge-states is striking (Fig. 5).

The most straightforward profiles correspond to the unfolded species (*e.g.*, 14^+), which exhibits a unimodal IMS peak and a monophasic exponential increase over the pH range 2.75 - 2.4 in the equilibrium case, or 210 ms to 1.71 s in kinetic unfolding. Intermediate charge states show a bimodal IMS profile with a faster exponential decay (either as a function of pH or time) for the smaller cross-section species. In the case of intermediate charge-states with a significant 'folded' component (*e.g.*, 8^+), the IMS profile is complex,

consisting of five species (Fig. 3). However, in contrast to the 10^+ charge-state, all of these species exhibit identical time- or pH-dependent behaviour (the implications of this are described above). Finally, charge-states corresponding to the 'folded' species exhibit a unimodal IMS profile with rapid monophasic exponential decay as a function of pH or time.

Critically, even charge-states with the most complex IMS profiles exhibit precisely the same set of collision cross sections in equilibrium and kinetic unfolding experiments. This in itself provides substantial support for the notion that equilibrium and kinetic unfolding intermediates of cytochrome c are structurally identical. Furthermore, the equivalence of the apparent thermodynamic and kinetic stabilities of species populated during cytochrome c unfolding, leading to qualitatively similar pH-dependent and time-dependent profiles for cross-section-matched species, also points to the equivalence of kinetic and equilibrium unfolding intermediates. Taken together, these data represent substantive evidence that cytochrome c unfolds through a similar mechanism whether in response to progressively increasing denaturant concentrations or time exposed to fully denaturing conditions.

4 Conclusions

In this work, we have introduced a new hyphenated mass spectrometry technique, TRESI-IMS-MS, which is suitable for a broad range of applications. Specifically, TRESI-IMS-MS can provide cross-section based structural analysis of species that are transiently populated during (bio)chemical reactions. The selected application in this work was a comparison of equilibrium and kinetic unfolding intermediates of cytochrome c, with the aim of determining if the species populated under equilibrium conditions were identical to those transiently populated during time-dependent unfolding. Our data strongly suggest that these species are indeed equivalent, at least in the sense that they generate identical IMS profiles at each charge state. A more thorough examination of this issue could include hydrogen / deuterium exchange implemented either in solution through the TRESI source or in the gas phase during IMS separation. Ultimately, though, our current data indicate that even when protein folding landscapes are substantially different (due to different solvent conditions), they share common local minima.

Conflict of Interest

The authors have declared no conflict of interest

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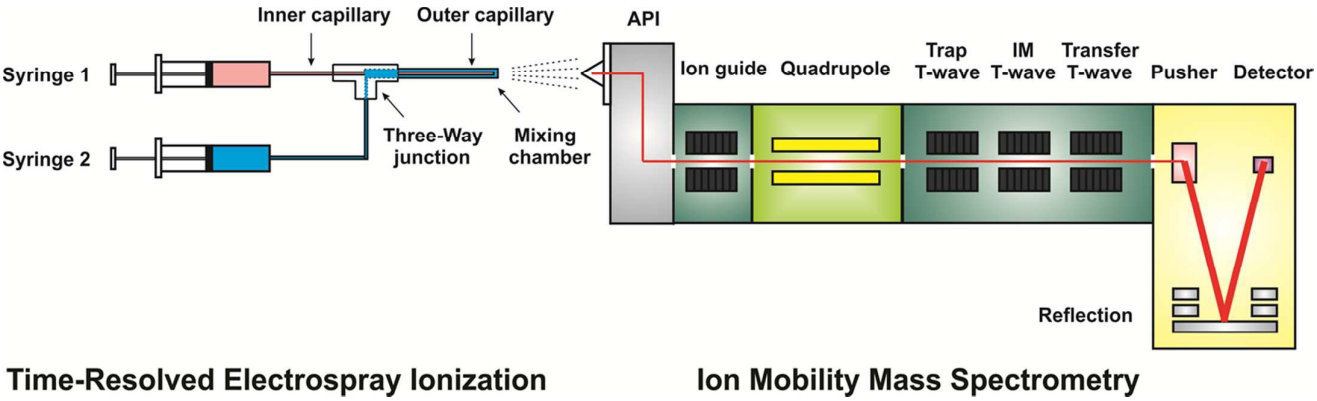

Fig. 1 A schematic depiction of the TRESI-IMS-MS apparatus. Rapid mixing of 'folded' cytochrome c (Syringe 1) and denaturing solution (10% acetic acid in H_2O, Syringe 2) occurs near the end of the inner capillary. Unfolding reaction time is adjusted by withdrawing the inner capillary from the end of the outer capillary, which serves as the electrospray source.

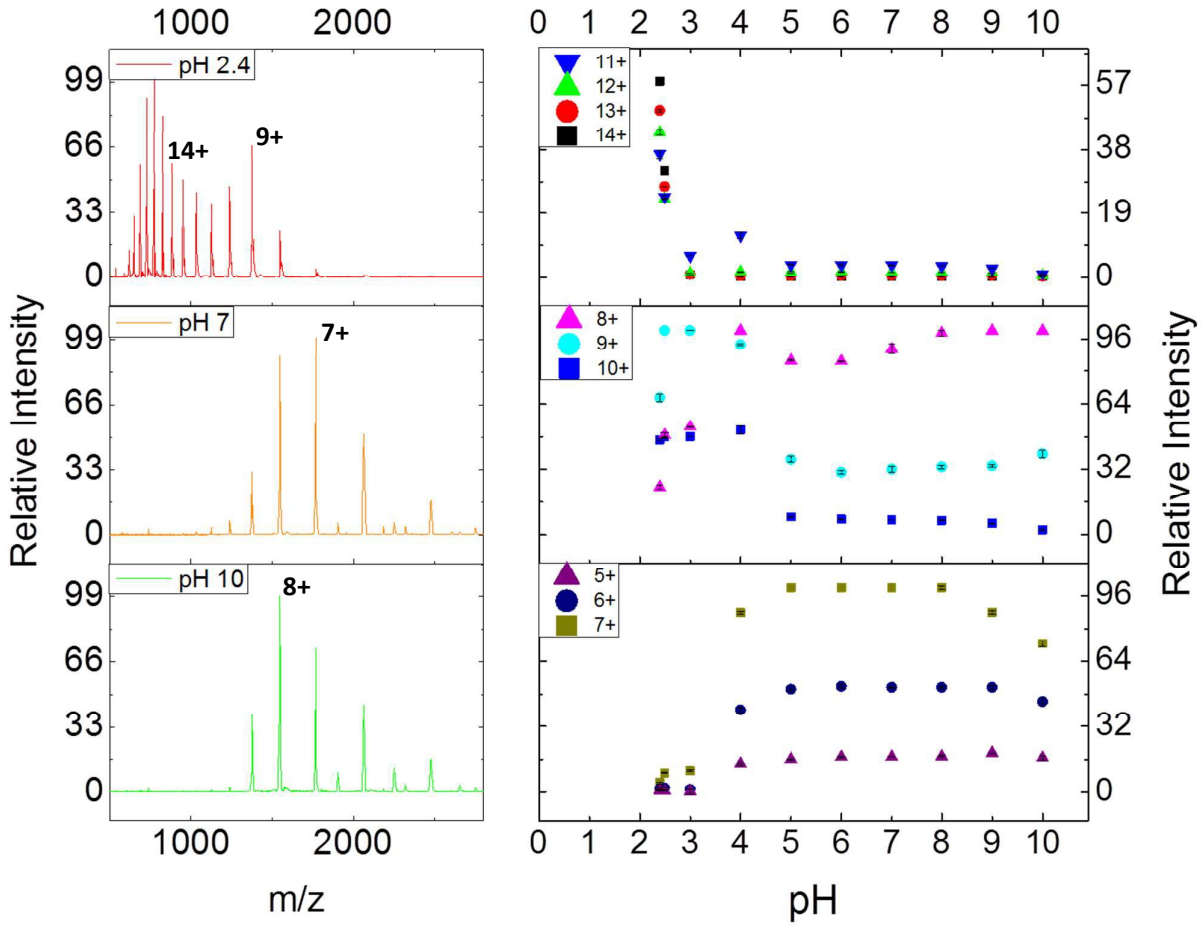

Fig. 2 An overview of cytochrome c equilibrium unfolding data. Raw mass spectra show shifts in the charge state distribution associated with unfolding (left). Charge-state specific pH profiles (right) are grouped by dominant contributing species

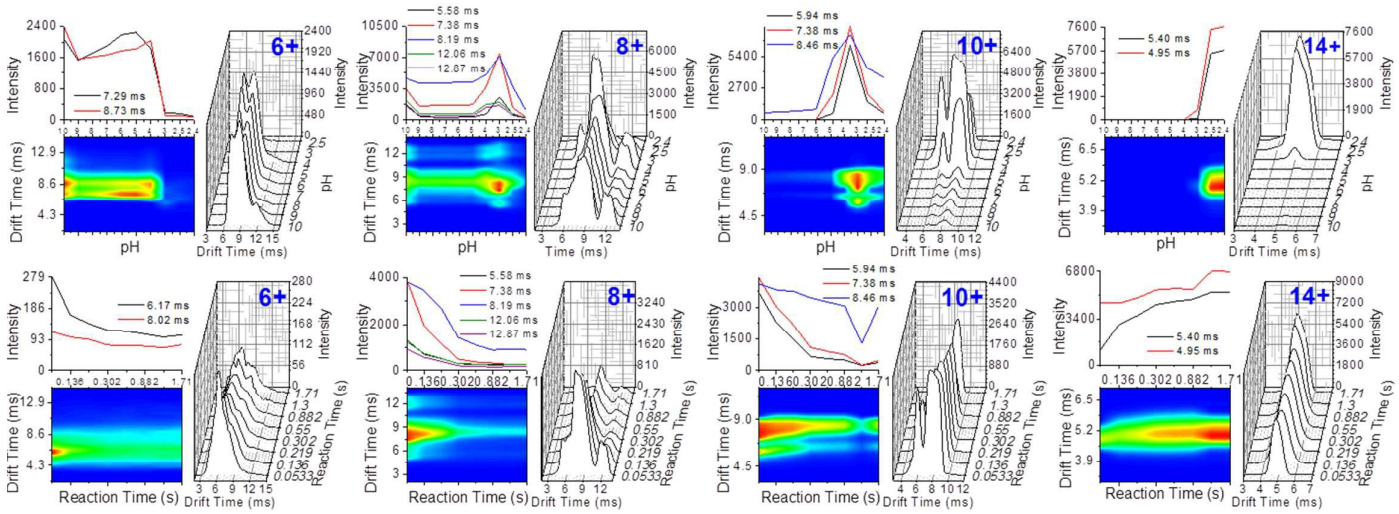

Fig. 3 Equilibrium (top row) and kinetic (bottom row) IMS profiles for selected charge states. Each panel includes a spline trace illustrating the pH or time-dependent profiles for specific drift times (top left), a heat map providing a 'top-down' view of the pH or kinetic IMS profile and a 3D view of the pH or time-dependent IMS profile for each charge state.

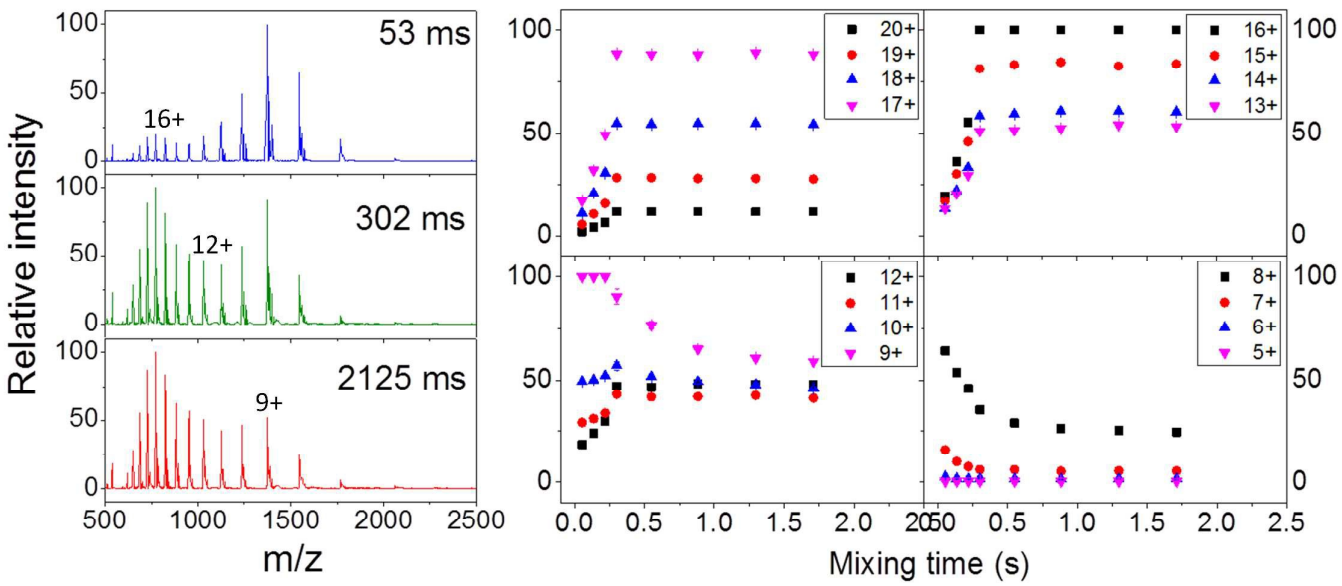

Fig. 4 An overview of cytochrome c kinetic unfolding data. Raw mass spectra show shifts in the charge state distribution associated with unfolding (left). Charge-state specific intensity-time profiles (right) are grouped by dominant contributing species

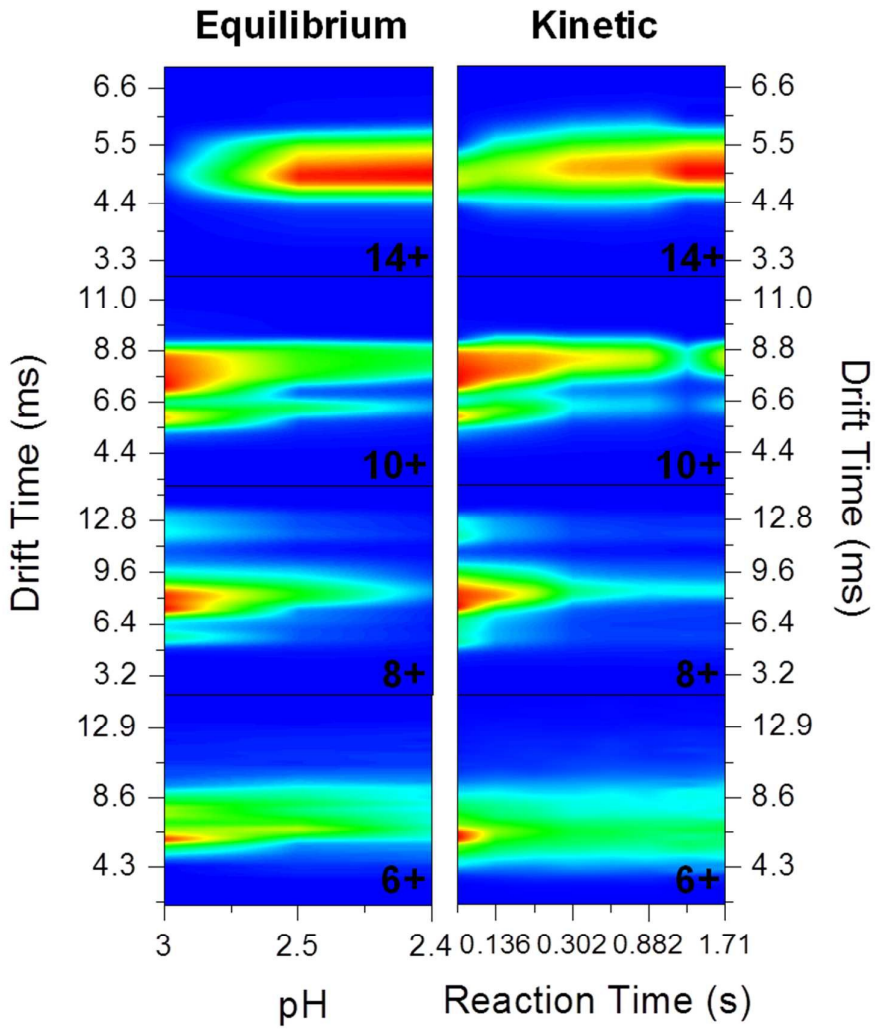

Fig. 5 A direct comparison of the pH or time-dependent IMS profiles of selected cytochrome c charge states in the course of unfolding. The pH scale was adjusted to focus on the 'intermediate-to-unfolded' transition that can be directly monitored in the kinetic experiments. All equilibrium species (left column) line up with cross-section corresponding kinetic species (right column) suggesting that the conformations populated during equilibrium and kinetic unfolding of cytochrome c are identical. Moreover, cross-section matched species exhibit similar thermodynamic and kinetic stability profiles.