Evidence for Many Resolvable Structures within Conformation Types of Electrosprayed Ubiquitin Ions

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A new two-dimensional ion mobility spectrometry approach combined with mass spectrometry has been used to examine ubiquitin ions in the gas phase. In this approach ions are separated in an initial drift tube into conformation types (defined by their collision cross sections) and then a gate is used to introduce a narrow distribution of mobility-separated ions into a second drift tube for subsequent separation. The results show that upon selection a narrow peak shape is retained through the second drift tube. This requires that at 300 K the selected distribution does not interconvert substantially within the broader range of structures associated with the conformation type within the ∼10−20 ms time scale of these experiments. For the $[M + 7H]^{7+}$ ion, it appears that many (∼5−10) narrow selections can be made across each of the compact, partially-folded, and elongated conformer types, defined previously (*Int. J. Mass Spectrom.* **1999**, *187*, 37−47).

Introduction

Understanding the structures of proteins in the absence of solvent is important for a number of reasons. Fundamentally, the relationship of amino acid sequence to structure and function is only partially understood; although statistical algorithms for predicting structure have emerged, calculations of structure for such large systems are still challenging—in part because of issues relating to sampling such a large system as well as force field limitations. Studies of conformations for isolated proteins in the gas phase can help to delineate contributions to structure that are intrinsic to the polypeptide chain.[1−13] Comparison of gas-phase results with structural information from solution provides insight about how solvent−molecule (and solvent−solvent) interactions influence structure. Practically, ion structure in the gas phase has become an important factor in the development of new analytical techniques for studying mixtures of proteins.[14−18]

During the last 10 years, evidence from several types of measurements (ion mobility separations,[6−8,11,19−26] isotopic exchange measurements,[1,5,9,14,27−32] thermodynamic studies,[4,10−12,33,34] and electron capture dissociation studies[15,35−37]) suggests that within an individual charge state there are typically a few dominant conformation types that can be resolved. For example, electrospray ionization (ESI)[38] of ubiquitin[2,20−22,29,35,39−42] (a small protein comprised of 76 amino acids, studied extensively in the gas phase) appears to exist in the gas phase as three dominant structural types:[22,29,31,35] a compact form (identified in mobility studies as those ions with cross sections less than 1120 Å^2, observed for the +4 to +7 charge states); elongated states (having cross sections larger than 1500 Å^2, observed for the +6 to +13 states); and, partially-folded structures (having cross sections between these values, observed for the +7 to +10 charge states).[20,22,29]

In addition to different physical dimensions that allow them to be separated, some conformation types appear to convert into others. For example, when stored in a Paul trap (or upon high-energy injection into a drift tube) compact conformations of the $[M + 7H]^{7+}$ ion will convert into partially-folded and elongated states.[22,29] Remarkably, at 300 K, partially-folded and elongated structures of the $[M + 8H]^{8+}$ ions do not interconvert,[22,31] even when stored for 30 s.[22] Other studies of ions stored in an ion cyclotron resonance cell suggest that conformation types may be stable over much longer times (as much as an hour).[31−33,35] Overall, at 300 K, these general structural types do not appear to reach equilibrium; rather, structures evolve to favored gas-phase conformations from the states that existed earlier in solution.[23]

Although general conformation types have been defined, little is known about the nature of states within a conformer type. Recent studies of ubiquitin using a hybrid high-field asymmetric waveform ion mobility spectrometry (FAIMS)/isotopic exchange approach[32] and a FAIMS/ion mobility spectrometry (IMS) approach[43] provide evidence for resolvable structures within a conformer family (as defined from the original IMS studies of this system). The shapes of peaks from ion mobility measurements are too broad to correspond to a single structure. This introduces two possible explanations:[6,8,44] (1) multiple stable conformations with similar cross sections are present but not resolved (resulting in a broad peak corresponding to the distribution of structures), or (2) structures within a conformation type interconvert on time scales that are similar to the time required to make measurements (usually a few milliseconds).[6,22,45]

In the present paper we examine the conformations of ubiquitin ions using a new two-dimensional ion mobility spectrometry method (IMS−IMS coupled with time-of-flight (TOF) mass spectrometry).[46] In this approach it is possible to separate ions within a charge state into the structural types (characterized previously)[20,22,35] and then select a narrow range of ions (within the broader peak) for further separation in a second drift tube. The results show that the narrow range of structures that is selected is retained through the second separation, requiring that these ions do not interconvert significantly within the broader range of conformations associated with the structural type.

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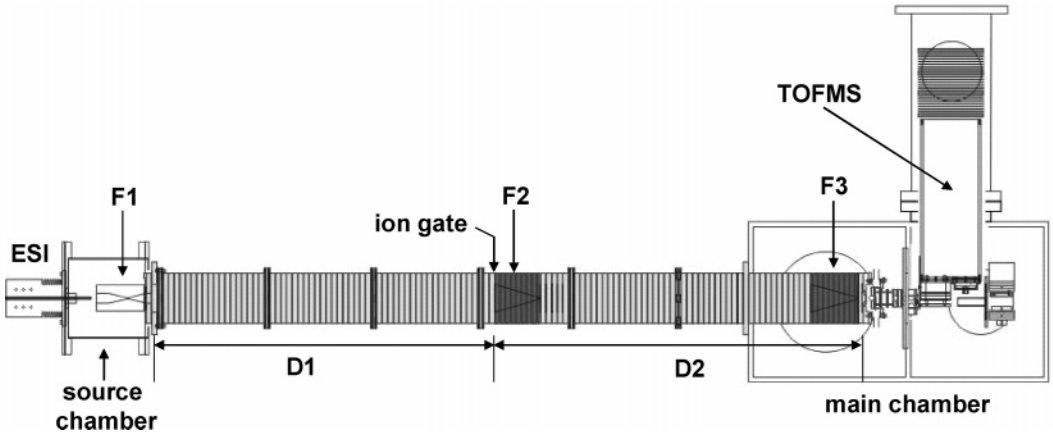

Figure 1. Schematic diagram of the IMS−IMS-TOF instrument used to measure high-resolution ($R = 80-120$) ion mobility distributions. The drift tube assembly consists of three ion funnels (F1−F3) and two drift regions (D1 and D2). Two-dimensional ion mobility experiments are performed by mobility selecting (at the ion gate) a narrow pulse of ions from the D1 separation into the second drift tube (D2). See text for details.

Experimental Section

Two-Dimensional Ion Mobility Measurements. Ion mobility spectrometry techniques and methods for obtaining structural information from cross section measurements are reviewed elsewhere.[7,11−13,17,47] Here we focus the discussion on the two-dimensional IMS measurement. A schematic of the instrument used to make measurements is shown in Figure 1. Briefly, positively charged ubiquitin ions were produced by electrospraying a 3×10^{-5} M protein solution and 49/49/2 (% vol) water/acetonitrile/acetic acid, into the source region of an ion mobility/mass spectrometer. The continuous beam of ions is guided under the influence of a weak field into an initial ion funnel (designated as F1 and operated at 17 V cm^{-1}, 60 V_{p-p}, 475 kHz) where it is accumulated into a concentrated packet (for ∼35 ms). The packet of ions is then released as a 100 μs pulse into the first drift region (∼87-cm-long), and different charge states and structures separate under the influence of a uniform electric field (12 V cm^{-1}) according to differences in mobilities. As mobility-dispersed ions exit the first drift region (D1), they pass through an ion gate region that can be operated in a mode in which all ions are transmitted into a second region or in a mode where only a narrow distribution of ions having a specified mobility are transmitted. Selection of ions having a specified mobility is described in more detail below. Those ions that are transmitted enter a second ion funnel (F2, operated at 14 V cm^{-1}, 100 V_{p-p}, and 480 kHz) after the ion gate where they are focused through the ∼0.5 cm ion funnel exit aperture and into the second drift region. The selected ions are separated again under the influence of a uniform electric field (12 V cm^{-1}) and focused through a third ion funnel (F3) operated at 18 V cm^{-1}, 90 V_{p-p}, and 480 kHz. The entire drift tube assembly is 181-cm-long and was operated using 3.1 Torr of helium buffer gas at 300 K.

Ions exit the drift tube and are focused into a reflectron time-of-flight mass spectrometer where flight times, which can be used to derive mass-to-charge (m/z) values, are measured. As described previously,[48] flight times are recorded in a nested fashion such that mobility distributions for all ions are recorded in a single experimental sequence.

Isolation of Narrow Distributions within Protein Ion Structural Types. As mentioned above, we and others have previously recorded IMS data for individual charge states of ubiquitin. The instrument in Figure 1 is designed such that there is a good separation of conformation types near the midpoint of the drift tube. At this point, a narrow region within the IMS distribution can be isolated. To isolate a select group of ions, a repulsive field of 20 V (biased with respect to the drift tube voltage at this point) is applied to a gating electrode, preventing ions from entering the second drift region. This repulsive field is dropped to the drift voltage for 50 μs to introduce a short pulse of ions. The selected ion pulse is delayed with respect to the initial introduction pulse such that an ion having any mobility can be selected and transmitted into the second drift tube. The ion gate timing is controlled by a pulse delay generator (model DG535, Stanford Research Systems, Inc.) triggered by the initial pulse in the first ion funnel.

Ion drift times in the first drift tube range from ∼6 to 15 ms for ion distributions presented here. A systematic study of voltages used to trap and focus ions (as well as for gating of ions) suggests that structures are not influenced significantly under the conditions used in these experiments. By varying the fields it is possible to change structures such that noticeable differences in mobilities are observed; in the extreme case, the voltages can be increased to the point where substantial dissociation is observed. In the present studies, low voltage conditions, in which there is no evidence for structural changes, are employed.

Determination of Collision Cross Sections. For comparison with previous results, it is useful to plot data on a cross section scale. For an individual charge state, drift time distributions can be converted to cross section scale using the relation[49]

$$\Omega = \frac{(18\pi)^{1/2}}{16}\frac{ze}{(k_B T)^{1/2}}\left[\frac{1}{m_I}+\frac{1}{m_B}\right]^{1/2}\frac{t_D E}{L}\frac{760}{P}\frac{T}{273.2}\frac{1}{N} \quad (1)$$

where z, e, k_B, m_I, and m_B correspond to the charge state, electron charge, Boltzmann's constant, and masses of the ion and buffer gas, respectively, and N is the neutral number density. The parameters E, L, P, and t_D can be measured precisely such that collision cross sections from any two measurements will typically agree to within ±1% (relative uncertainty). The total drift time of an ion is the sum of the time the ion spends in each region of the instrument. The accuracy of the cross section scale is influenced by how well each parameter is known experimentally. In this case, the scale is accurate to within ±2% (compared with measurements in other instruments).[24,46,50]

Results and Discussion

Figure 2 shows two nested drift(flight) time data sets for ubiquitin ions. The first of these data sets is obtained when all

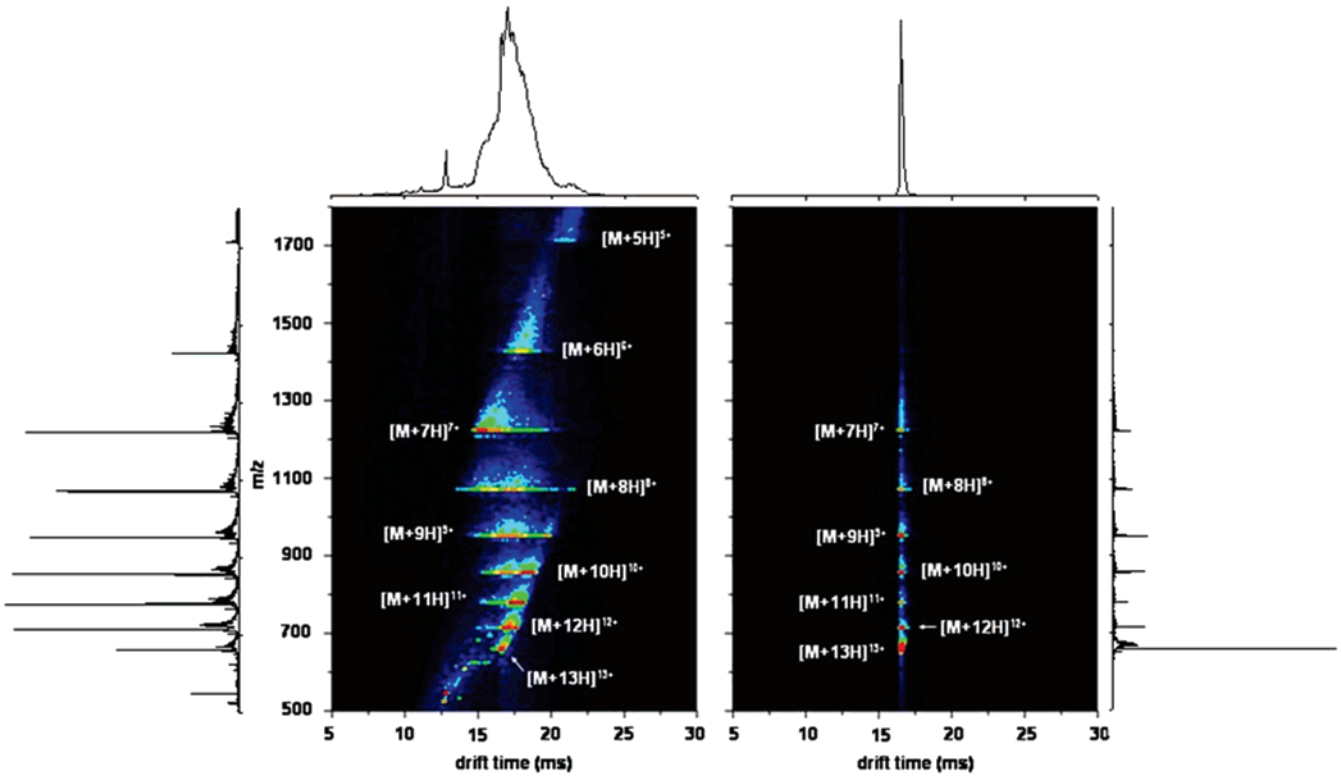

Figure 2. Nested drift(flight) time distributions showing the total distribution of electrosprayed ubiquitin ions (left) and a narrow distribution of mobility-selected ions that were gated into D2 at 7.8 ms (right). Also shown are the summed mass spectra (sides) and summed drift time distributions (top) obtained by integrating the two-dimensional data across all drift times and all *m*/*z* values, respectively. The drift time represents the total time required for the ions to travel through D1 to the TOF source.

ions are transmitted through both drift regions and is essentially indistinguishable from data measured previously for this system.[20,22] We observe evidence for the +5 to +13 charge states of ubiquitin, and the range of compact (+5 to +7), partially-folded (+7 to +10), and elongated (+9 to +13) conformations is consistent with previous measurements.[20,22,29] It is worthwhile to note that in this instrument the original ion packet is gated rather than injected into the drift region. From a series of studies in which the gating conditions are varied, it appears that the conditions that are employed here are relatively gentle. Therefore the present distributions are most similar in appearance to those data that we have recorded at our lowest injection energies (in previous studies)[22] as well as data obtained from our high-pressure drift tube (where no injection is employed).[20]

The second data set shows the two-dimensional nested drift(flight) time distribution that is obtained when a narrow range of ions at a specified mobility is transmitted into the second drift region. These ions are selected in the gate region 7.8 ms after release of the initial ion packet. Within this selection, the +7 to +13 charge states are observed, and each of these charge states has a total drift time of 16.57 ms and is observed as a single sharp feature (along the drift time dimension) in the distribution. To have the same mobilities, these different charge states must have different conformations. The +5 and +6 charge states are not observed in the selected ion data set because none of the conformations of these charge states has mobilities within the range of the selection.

The most striking feature of the selected-mobility distribution recorded in Figure 2 is that each of the charge states (and conformations) displays a single very sharp peak in the drift time distribution. As shown in more detail below, this peak shape appears to be typical for ubiquitin ion charge states (as well as charge states for two other systems that we have examined so far, cytochrome c and bradykinin). That is, at most selection times, most ions appear as a single sharp peak. In a few cases, some ions appear as broader peaks, even when selected using a narrow pulse. This type of behavior is still under investigation and will be presented elsewhere.

Figure 3 illustrates the shape of the IMS and IMS−IMS peaks on a cross section scale for the +7 charge state. The single dimension IMS measurement shows evidence for the three conformer types that we have discussed previously: a compact state, a partially-folded state, and elongated state. A selection of a narrow region of mobilities in the compact region leads to a single sharp peak that is much narrower than the range of compact structures associated with the peak in the single dimension experiment. When all ions are transmitted through both drift tubes (a single dimension of IMS separation), the +7 charge state maximum is observed at ∼1060 Å^2 (corresponding to compact ions) and a broad feature that peaks at ∼1250 Å^2 and extends to ∼1500 Å^2 (corresponding to partially-folded ions). The cross section distribution for this charge state is essentially indistinguishable from data published previously from a high-pressure, high-resolution drift tube measurement.[20] We estimate the full width at half-maximum (fwhm) for the peak associated with the compact conformer type in Figure 3 to be ∼100 Å^2. When a narrow range of structures, having cross sections that are near the maximum of the distribution for this charge state, is selected, the peak is much narrower, having a fwhm of ∼9 Å^2, near the 8 Å^2 distribution that is calculated from the transport equation for transmission of a single structure.[49] This result requires that these ions exist as a much narrower range of cross sections than what is observed within the range of the compact conformer type. Additionally, these

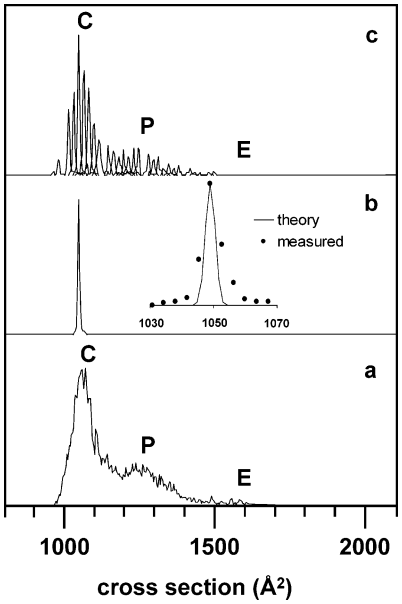

Figure 3. Drift time distributions of the $[M + 7H]^{7+}$ of ubiquitin. Mobility distributions were obtained by integrating drift time intensities over a narrow range of *m*/*z* values corresponding to the $[M + 7H]^{7+}$. (a) The total mobility distribution shows that the +7 charge state exists mostly as compact structures (C), some partially-folded structures (P), and minimal elongated structures (E). (b) A single peak is observed when a narrow distribution (50 μs) of the compact structure is isolated with mobility selection at 7.8 ms. The inset compares the theoretical to the experimentally measured peak shape (see text). (c) The total mobility distribution for the +7 is reconstructed with 28 mobility-selected distributions acquired every ~0.125 ms.

structures do not appear to change structure significantly during the time scale of the measurement.

By variation of the selection time associated with the ion gate, it is possible to select regions of each conformer type. Figure 3 illustrates this by showing a distribution of mobility-selected data sets corresponding to transmission of different structures within the compact and partially-folded conformer types of the total ion distribution (elongated structures have been examined in other charge states). When superimposed, the narrow peaks that are observed take on the appearance of the broader distributions that are observed from the total ion distribution. This distribution shows that it is possible to isolate narrow ranges of cross sections (and presumably conformations) within the broader conformer types.

Within each of the broader conformation types associated with the +7 charge state we have been able to easily select ~5−10 narrow regions that do not appear to interconvert substantially. Clearly, many conformers may still exist, even within the narrower range of selected ions.

Conclusion

We have shown that the broad peaks that are typically observed in ion mobility separations can be comprised of many isolable structures that are not fully resolved rather than a system of structures that interconvert during separation. For the $[M + 7H]^{7+}$ at the resolution of the present system, it is possible to isolate from 5 to 10 narrower distributions within the broader conformation types.

An important issue involves the origin of the many closely related structures within a conformer type. Because conformations do not interconvert significantly within the range of conformer types at 300 K, one might imagine that it is possible that the structures may reflect those present in solution prior to and during electrospray ionization. In this case, such structures could be stabilized if they are effectively "frozen out" during the evaporative cooling process associated with ion formation from the charged electrospray droplets.[51−53] It is intriguing to consider that, to the extent that this is true, the number of isolable gas-phase structures may reflect the nature of the local equilibrium of states that exists in solution prior to formation of the isolated ion.[51−53]

The present results may also cause us to reexamine our previous definitions of conformation types. Had it been the case that a narrow selection led to a broad peak that reflected the larger distribution of a previously defined conformer type, then we would have concluded that peaks within a conformer family sampled many related conformations across the family. The narrow peaks observed in the present studies appear to be stable on the time frame of these experiments. It is possible that although the cross sections are similar (to the extent that conformations cannot be resolved without selection) the structures associated with these narrow distributions correspond to very different types of folded structures. Further experiments are underway to test these ideas.

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