Gas-Phase Separations of Protease Digests

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A mixture of peptides from a complete tryptic digest of ubiquitin has been analyzed by ion mobility/time-of-flight mass spectrometry techniques. All components of the mixture were electrosprayed and ions were separated in the gas phase based on differences in their mobilities through helium before being dispersed into a time-of-flight mass spectrometer for mass-to-charge analysis. The data show that ions separate into families primarily according to differences in their charge states and, to a lesser extent, differences in conformation. This approach reduces spectral congestion typically associated with electrosprayed mixtures and provides charge assignments for mass-to-charge ratio data. Gas-phase separations of ions appear to provide a new physical basis for characterizing components of biological mixtures. (J Am Soc Mass Spectrom 1998, 9, 1213–1216) © 1998 American Society for Mass Spectrometry

An ion mobility/mass spectrometry technique [1] has been used to separate and identify peptides from a tryptic digest of ubiquitin. The mixture was electrosprayed into the gas phase and ions were separated by differences in their mobilities through He prior to mass-to-charge ratio analysis. The results show that ions separate into families primarily because of differences in charge states. Variations associated with different gas-phase conformations are observed, but are less pronounced. This approach reduces spectral congestion, and peptide masses can be determined directly from mass-to-charge ratio data because peaks belong to charge-state families. Gas-phase ion separations appear to be a new physical approach for characterizing complex mixtures.

Identification of peptides from protease digests by liquid chromatography/mass spectrometry methods [2] and matrix-assisted laser desorption ionization approaches [3] provides information about binding, tertiary structure, mutations and modifications, and sequences [4]. Generally, direct electrospray of mixtures leads to congested mass spectra: complicated by charge state distributions for individual components. Data can be simplified by using algorithms that distinguish charge state distributions [5] or by the use of ion/ion reactions for charge reduction [6]. Here, we present the first study showing that ion mobility separation can be used to delineate components of complex mixtures. The approach is rapid and provides independent information about the ion charge state (important for determining masses from mass-to-charge ratio values) and conformation (that can allow peptides with identical amino acid compositions, but different sequences, to be distinguished [7]). Ion mobility methods have been used to detect small analytes [1d, 8], characterize particle sizes [9], determine cluster geometries [10], and measure cross sections for selected biomolecular ions [1f, 11].

Experiments were performed using an injected-ion mobility/time-of-flight instrument that has been described previously [12]. Peptide digests were prepared by addition of 150 μL of 0.2 mg/mL trypsin (Sigma, St. Louis, MO, sequencing grade) solution in 0.2 M ammonium bicarbonate (EM Science) to 0.5 mL of a 20 mg/mL solution of ubiquitin (Sigma, bovine, 90%). After incubation for 20 h at 37°C the trypsin was filtered from the digest using a microconcentrator (microcon 10, Amicon) and the peptides that remained were lyophilized [13]. Protonated peptide molecules were formed by electrospraying solutions containing 0.08 mg/mL of the lyophilized peptide mixture in 49:49:2 (% volume) water:acetonitrile:acetic acid. Pulses (100 μs wide) of ions were injected at 70 V into a drift tube containing ~2.0 torr of He buffer gas. At 70 V, most noncovalent aggregates dissociate to form monomer ions and typically less than a few percent of monomer ions fragment [14]. Ions drift across the instrument under the influence of a weak electric field (11.13 V cm^{-1}) and are separated based on differences in their mobilities. High-charge states experience a larger electrostatic force and have higher mobilities than lower charge states [15]. Compact conformers (of a given charge) have higher mobilities than elongated ones. Ions exit the drift tube into a source region of a time-of-flight mass spectrometer. High-voltage pulses synchronous with initial injection pulses are used to initiate time-of-flight mea-

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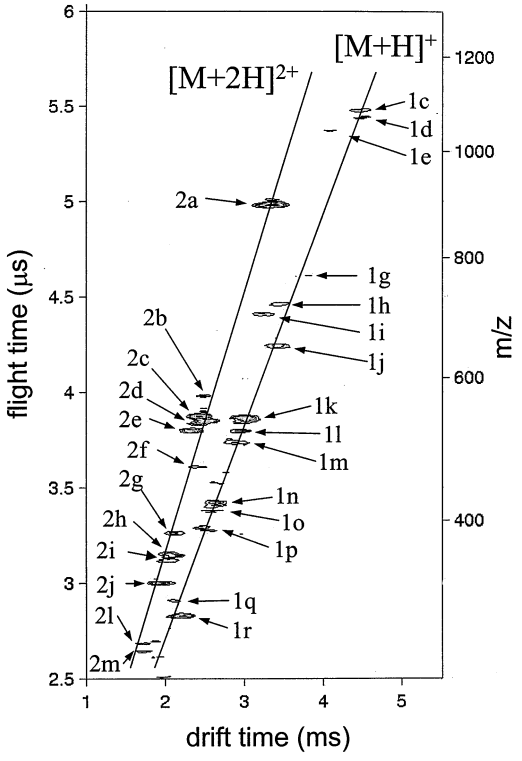

Figure 1. Contour plot of drift times and flight times for direct electrospray of the peptide mixture obtained from a tryptic digest of ubiquitin. The resolving power along the flight time axis is typically 300 ($m/\Delta m$ of a peak for a singly charged ion where Δm is determined at half maximum). Ion mobility separation was carried out using a buffer gas pressure of 2.00 torr and the drift field was 11.13 V cm^{-1}. The flight time axis has been scaled by using a three point calibration to a bradykinin spectrum that was recorded immediately before this data set. The mass accuracy can be assessed by comparison of experimental and expected values in Table 1. The contours are shown on an eight point scale; this effectively removes all drift time and flight time coordinates where fewer than eight ions were collected.

surements in the mass spectrometer. Flight times in the evacuated flight tube are much shorter than drift times, allowing hundreds of mass spectra to be recorded with respect to each injection of ions. This makes it possible to measure mass-to-charge ratios for all components of a mixture of mobility-separated ions simultaneously.

Figure 1 shows a two-dimensional plot of drift and flight times for ions formed from the digest. Peaks fall into two families; similarities within each family can be deduced by assigning several peaks. Flight times of peaks 1e (5.371 μs, m/z = 1041.4), 1d (5.440 μs, m/z = 1068.3), and 1c (5.478 μs, m/z = 1083.3), in the lower-mobility family are assigned to several singly protonated peptides that are expected from the digest: [EGIPPDQQR+H]$^+$ (mol. wt. = 1039.5); [ESTLHLVLR+H]$^+$ (mol. wt. = 1067.5); and [TLSDYNIQK+H]$^+$ (mol. wt. = 1081.6), respectively. Peaks 2a (4.980 μs, m/z = 895.3) and 2c (3.873 μs, m/z = 540.5) in the higher-mobility family are associated with formation of doubly charged [TITLEVEPSDTIENVK+2H]$^{2+}$ (mol. wt. = 1787.9) and [TLSDYNIQK+2H]$^{2+}$ (mol. wt. = 1081.6), respectively. To assign the remaining peaks, mass-to-charge ratios determined from flight times were converted to masses by assuming that all ions within each family correspond to [M+H]$^+$ or [M+2H]$^{2+}$ ions. These masses are compared with molecular weights that have been calculated for peptides expected from the digest in Table 1. Nine peaks in the lower-mobility [M+H]$^+$ family can be assigned to singly protonated peptides expected from the digest. The only expected ion that was not observed in this family is [TITLEVEPSDTIENVK+H]$^+$ (m/z = 1788.9). Peaks in the higher-mobility [M+2H]$^{2+}$ family also correspond to expected peptides. Most of the anticipated peptides were observed in this family (Table 1). The only exceptions are [LR+2H]$^{2+}$, [GG+H]$^+$, and [AK+H]$^+$ that are expected but not observed because their flight times are below a delay associated with detector activation following the high-voltage pulse. Masses determined from peaks in both families are in good agreement, providing a valuable cross measurement.

Advantages of reduced spectral congestion can be seen for the [TLTGK+H]$^+$ (3.796 μs, m/z = 520.2) and [EGIPPDQQR+2H]$^{2+}$ (3.798 μs, m/z = 520.7) ions (peaks 1l and 2e, respectively) shown in Figure 1. Identifying these ions by mass spectrometry alone is challenging because of overlapping isotopic distributions. Mobility-based separation allows these ions to be easily resolved and assigned.

Several peaks are not readily assigned to tryptic fragments. The mobility-based charge determination is extremely useful in identifying these ions. Peak 1i (4.411 μs, m/z = 702.4) and 2i (3.120 μs, m/z = 351.4) in the [M+H]$^+$ and [M+2H]$^{2+}$ families, respectively, give an average mass of 701.1, consistent with AKIQDK (mol. wt. = 702.4). This peptide is formed in a region where two lysines are separated by a single amino acid, a situation that can lead to incomplete digestion. The [M+H]$^+$ ion, 1k (3.865 μs, m/z = 539.3), has no corresponding [M+2H]$^{2+}$ ion. This peak could be because of MQIF (mol. wt. = 537.7) formed from mild chymotryptic activity (i.e., cleavage on the carboxyl side of phenylalanine). The [MQIF+H]$^+$ ion may be prevalent here because of its accessibility as the first four residues in the ubiquitin sequence; the presence of only a single protonation site (the N-terminal amino group) explains the absence of a [M+2H]$^{2+}$ peak. Finally, a low intensity series of [M+H]$^+$ peaks (<3% of the total signal), 1n to 1r (Figure 1 and Table 1) is observed. These can be explained by collision-induced dissociation of other peptides; but, unique assignments cannot be made and we cannot rule out sample impurities.

The results presented above show that charge state primarily determines the mobility family for each peptide ion. Peptides do not appear to fall into families because of differences in conformation, although some deviations from a smooth increase in drift time with mass-to-charge ratio are observed. Observation of only [M+H]$^+$ and [M+2H]$^{2+}$ families from electrospray of this mixture can be understood by considering that

Table 1. Flight times, masses, and assignments of ubiquitin tryptic digest peptides

$[M+H]^+$ family		$[M+2H]^{2+}$ family					
Peak label[a]	t_f (mass)[b]	Peak label[a]	t_f (mass)[b]	Neutral mass [M][c]	Assignment[d]	Calculated molecular weight[e]	Fragment position[f]
1r	2.828 (288.7)			287.7	LR	287.4	73–74
1q	2.909 (305.5)			304.5			
1p	3.287 (390.0)			389.0			
1o	3.376 (411.4)			410.4			
1n	3.421 (422.5)			421.5			
1m	3.736 (503.9)	**2m**	2.643 (504.4)	502.7	IQDK	502.6	30–33
1l	3.796 (520.2)	**2l**	2.684 (520.2)	518.7	TLTGK	518.6	7–11
1k	3.865 (539.3)			538.3	MQIF[g]	537.7	1–4
1j	4.244 (650.2)	**2j**	3.00 (649.8)	648.5	LIFAGK	647.8	43–48
1i	4.411 (702.4)	**2i**	3.12 (702.8)	701.1	AKIQDK[g]	701.9	28–33
1h	4.461 (718.4)	**2h**	3.146 (714.6)	715.0	QLEDGR	716.6	49–54
1g	4.612 (767.9)	**2g**	3.260 (767.4)	766.2	MQIFVK	765.0	1–6
		2f	3.608 (939.8)	937.8			
1e	5.371 (1041.4)	**2e**	3.798 (1041.4)	1039.9	EGIPPDQQR	1039.1	34–42
1d	5.440 (1068.3)	**2d**	3.849 (1069.6)	1067.5	ESTLHLVLR	1067.3	64–72
1c	5.478 (1083.3)	**2c**	3.873 (1083.0)	1081.7	TLSDYNIQK	1081.2	55–63
		2b	3.981 (1144.2)	1142.2			
		2a	4.980 (1790.6)	1788.6	TITLEVEPSDTIENVK	1788.0	12–27

[a]Peaks assigned in Figure 1.
[b]Measured flight times (t_f). Masses for $[M+H]^+$ and $[M+2H]^{2+}$ given in parentheses. Estimated uncertainties are ±0.5 au.
[c]Mass of the neutral peptide. In cases where both $[M+H]^+$ and $[M+2H]^{2+}$ were observed, an average mass value is reported.
[d]Unless otherwise noted, assignments are for sequences that are expected for complete tryptic digest of ubiquitin. The GG and AK peptides were not observed in these studies. See [16].
[e]Molecular weights are the isotopic average for each peptide.
[f]Position of the peptide fragment in the ubiquitin sequence.
[g]See text for discussion.

cleavage at all basic residues results in peptides that have two protonatable sites: the basic residue and the N-terminal amino group. The $[M+H]^+$ family is resolved from the $[M+2H]^{2+}$ family because of differences in cross section-to-charge ratios. The 22.26 eV/cm force experienced by +2 ions is twice the 11.13 eV/cm force experienced by the +1 ions. However, cross sections for $[M+2H]^{2+}$ ions are always less than a factor of 2 larger than cross sections for analogous $[M+H]^+$ ions. The 100% increase in force between +1 and +2 ions compared with a smaller corresponding change in cross section for +1 and +2 charge states of each peptide leads to separation into charge state families. This will not be the case for all charge states and systems. If conformations expand with increasing charge to reduce coulomb repulsion (as observed for highly charged proteins) [11c, 16] then mobilities for high charge states can fall below those for lower charge states. The present approach is suited for analysis of low charge states where there are large relative changes in drift force between charge states.

Some examples of the influence of conformation upon mobility (within a charge state family) are observed. The $[AKIQDK+H]^+$ ion (peak 1i, $m/z = 702$) has a shorter drift time than $[LIFAGK+H]^+$ (peak 1j, $m/z = 650$). Cross sections [1f] for these ions are 181 and 187 Å^2, respectively; the lower mass-to-charge ratio ion has a more open conformation. Recent improvements in the resolution of ion mobility instruments have made it possible to resolve species that differ only slightly in mobility [11b, 17] (<1% in some cases). Peptides with identical residue compositions but different sequences have been separated [7]. Concurrent efforts to calculate accurate biomolecular ion conformations and include realistic ion-buffer gas potentials [18], scattering interactions [19], and dynamics of the biomolecule [11a] in mobility calculations, suggest that even very subtle differences in mobility (such as those arising from different conformations associated with sequence inversions) will be assignable. Gas-phase separation of ions into conformation types and charge families will provide an important new analytical tool for characterizing mixtures.

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