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Mass spectrometry of peptides and proteins

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Abstract

This tutorial article introduces mass spectrometry (MS) for peptide fragmentation and protein identification. The current approaches being used for protein identification include top-down and bottom-up sequencing. Top-down sequencing, a relatively new approach that involves fragmenting intact proteins directly, is briefly introduced. Bottom-up sequencing, a traditional approach that fragments peptides in the gas phase after protein digestion, is discussed in more detail. The most widely used ion activation and dissociation process, gas-phase collision-activated dissociation (CAD), is discussed from a practical point of view. Infrared multiphoton dissociation (IRMPD) and electron capture dissociation (ECD) are introduced as two alternative dissociation methods. For spectral interpretation, the common fragment ion types in peptide fragmentation and their structures are introduced; the influence of instrumental methods on the fragmentation pathways and final spectra are discussed. A discussion is also provided on the complications in sample preparation for MS analysis. The final section of this article provides a brief review of recent research efforts on different algorithmic approaches being developed to improve protein identification searches.

Keywords: Mass spectrometry; Proteomics; MS/MS; Peptide sequencing; Protein identification; Gas-phase chemistry; Fragmentation

1. Introduction

Mass spectrometry and tandem mass spectrometry (MS/MS) experiments are major tools used in protein identification. Mass spectrometers measure the mass/charge ratio of analytes; for protein studies, this can include intact proteins and protein complexes [1], fragment ions produced by gas-phase activation of protein ions (top-down sequencing) [2–6], peptides produced by enzymatic or chemical digestion of proteins (mass mapping) [7,8], and fragment ions produced by gas-phase activation of mass-selected peptide ions (bottom-up sequencing) [9]. The application of mass spectrometry and MS/MS to proteomics takes advantage of the vast and growing array of genome and protein data stored in databases. The information produced by the mass spectrometer, lists of peak intensities and mass-to-charge (m/z) values, can be manipulated and compared with lists generated from "theoretical" digestion of a protein or "theoretical" fragmentation of a peptide. Applications to analyze ever smaller quantities of sample are driving the development of more sensitive mass spectrometers, as well as low flow, high resolution separation technologies, to provide structural information on individual components in complex mixtures of thousands of proteins derived from biological samples. Protein identification by mass spectrometry requires an interplay between mass spectrometry instrumentation (how molecules are ionized, activated, and detected) and gas-phase peptide chemistry (which bonds are broken, at what rate, and how cleavage depends on factors such as peptide/protein charge state, size, composition, and sequence). This brief tutorial article provides an overview of peptide and protein fragmentation in mass spectrometers.

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2. Instrumentation

A rich variety of different MS/MS instrument configurations (with different capabilities in terms of speed, ionization method, resolution, sensitivity, and mass/charge range) have been developed both in research laboratories and in the marketplace for application to proteomics. (For a tutorial on mass spectrometry instrumentation, refer to http://staging.mc.vanderbilt.edu/msrc/tutorials/ms/ms.htm.) This large number of instrument types has developed because no one instrument type has all of the features desired for an ideal proteomics experiment. High performance instruments are often large and expensive; small, inexpensive instruments may compromise on resolution and mass accuracy but are robust workhorse instruments that allow analysis of large numbers of samples in a small amount of time.

2.1. Ionization

A major advance that enabled examination of protein structure by MS and MS/MS was the introduction of soft ionization techniques to 'volatilize' biomolecules, in particular electrospray ionization (ESI) and matrix assisted laser desorption/ionization (MALDI). In ESI, charged droplets are produced by passing a solubilized sample through a high voltage needle at atmospheric pressure. Desolvation occurs prior to entrance into the high vacuum of the mass spectrometer [10–12]. This approach is often coupled with a chromatographic system, typically reverse phase chromatography or capillary electrophoresis, allowing analysis of very complex samples. ESI typically induces a range of charge states; because most mass spectrometers actually detect mass/charge or m/z (actually $(M+z)/z$, where M is the uncharged mass of analyte), the resulting spectra may have many ions for each analysis. A recent variant, called electrosonic spray ionization (ESSI), produces ions with one or only a few charge states [13]. In MALDI, samples are cocrystallized onto a sample plate with a small organic matrix compound that usually has a conjugated aromatic ring structure, and thus can absorb at the wavelength of the laser [14,15]. Although multiply charged ions can be produced, more typically, only singly charged ions are observed in MALDI. Charging can be induced by addition or loss of protons (at acidic or basic pH values for the delivering solvent, respectively) to form the MH^+, MH_2^{2+}, MH^-, $MNaH^{2+}$, etc., by loss or gain of electrons (uncommon in ESI or MALDI), or by adduction of small ions, such as Na^+, acetate, ammonium, etc.

It is important to remember the limitations of these methods when interpreting results of analysis of a sample. For example, after digesting a protein with trypsin, the analysis of the digest by MALDI will produce only a limited subset of the expected peptide ions. Peptides must be able to cocrystallize efficiently with the matrix, for instance, with α-cyano-4-hydroxycinnamic acid (CHCA) matrix, the majority of the ions observed are 800–1800 Da, while with sinapinic acid (SA) matrix, larger ions give better signals. Although a tryptic digest typically produces more peptides with lysine at their C-termini, arginine-containing peptides are more intense than lysine-containing peptides in MS spectra. We typically observe peptides covering only 30–45% of the full protein sequence in tryptic digests of proteins [16].

In ESI, higher voltages favor lower charged forms, and if a peptide is large, the lower charged forms may not be within the mass range of the mass analyzer (or may exceed the mass limit). However, lower voltage is better for smaller analytes. Instrument and ionization parameters are a compromise, when looking at complex samples such as peptide digests that have widely varying chemical properties. Another issue in ESI is competition between analytes for charge as they are extruded from the spray droplets. If a protein digest is analyzed by infusion, with no separation of the peptides, only a small number of the most easily ionized peptides are observed. To detect more ions, peptides are separated by a chromatographic method directly coupled to the MS. Thus, only a few peptides elute at the same time, and nearly complete coverage of a protein can be achieved, although the chromatography has its limitations. For instance, the most commonly used method is reverse phase chromatography, where peptides bind to beads packed into a column and binding is via hydrophobic interactions with alkyl-terminating chains covalently bound to the beads. When carbon loading is high, smaller or more hydrophilic peptides are recovered in high yield, but the larger or more hydrophobic peptides are poorly recovered; when carbon loading is lower, the larger or more hydrophobic peptides give higher yield, but the smaller, hydrophilic peptides do not bind. To get around these issues, the easiest approach is to produce different types of digests, to achieve complete coverage of a protein. Although it is not yet widely used in practical proteomics studies, ion mobility, in which ions of different cross sections, charge states, and m/z are separated by collisions with a bath gas in a uniform electric field, is being explored as an additional separation that may improve the numbers of peptides that can be successfully identified from a digest of a protein mixture [17].

2.2. Mass analysis

ESI and MALDI interfaces are combined in various ways with different mass analyzers. Four types of mass analyzers are commonly used with biomolecules (1) **Quadrupole mass analyzers** resolve m/z by applying radio frequency (RF) and DC voltages, allowing only a narrow mass/charge range to reach the detector

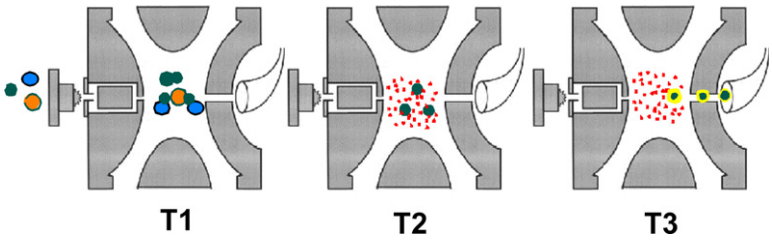


Fig. 1. Illustration of MS/MS in a quadrupole ion trap. Figure provided by Linda Breci, University of Arizona.

[18,19]. Quadrupoles are usually limited in mass range and have low resolution, particularly when used for ESI, where vacuum quality is compromised. Commercially available instruments usually have mass/charge limits ranging from 0 to 4000 *m/z* and at best are normally set to resolve the various ^{13}C isotope peaks for a singly charged ion (which differ by one Da), although the resolution may be intentionally degraded to improve sensitivity. In ESI, multiple charging enables quadrupole mass measurement of molecules >100,000 Da, if the molecule can be charged sufficiently. (2) **Time-of-flight** (TOF) analyzers accelerate the ions by using a short voltage gradient and measure the time ions take to traverse a field free flight tube; the flight time is proportional to the square root of the *m/z* [20]. Ion manipulations are used to increase resolution (delayed extraction of ions from the source, two stage sources with complex voltage gradients, and reflectron technology) and a commercial TOF instrument can typically achieve resolution of 10,000 or greater (separate *m/z* 1000.0 from *m/z* 1000.1) [21]. (3) **Quadrupole ion traps** focus ions into a small volume with an oscillating electric field; ions are resonantly activated and ejected by electronic manipulation of this field [22]. Inexpensive quadrupole ion traps, relying primarily on RF fields, have proven very useful in analysis of high complexity samples because they can rapidly shift between scanning for masses of the analytes (MS scan) and generating fragmentation spectra (MS/MS scan) of the ions detected in the MS scan. Ion traps are very sensitive, because they can concentrate ions in the trapping field for varying lengths of time. Linear ion traps are a recent improvement in quadrupole ion traps, with higher scan ranges, larger electronic trap fields, and higher resolution [23,24]. (4) **Fourier transform ion cyclotron resonance** (FTICR) MS use high magnetic fields to trap the ions and cyclotron resonance to detect and excite the ions, with resolution >1,000,000 (separate *m/z* 1000.000 from *m/z* 1000.001) [25,26].

An important aspect of mass spectrometry is the development of tandem (MS/MS) or MS^n instruments that can select specific ions, induce their fragmentation, and measure the *m/z* of the fragment ions. Fragmentation occurs at the most chemically labile bonds, and by adding more energy, energy dependence of fragmentation can be explored. Initially MS/MS was performed by combining mass analyzers in a tandem configuration; for instance, in a triple quadrupole MS instrument, the ion to be sequenced is selected in the first quadrupole mass analyzer and activated by collision with argon in a second quadrupole, then the fragments are analyzed in the third quadrupole mass analyzer. The fragmentation spectrum is usually referred to as an MS/MS or MS^2 spectrum, because of the tandem mass analyzers in the instrument. In ion traps, there is only one mass analyzer operated in different electronic modes to first trap the ions in the sample, then electronically destabilize all ions, except the ion to be sequenced (T1 in Fig. 1). This ion is then activated electronically to produce fragments (T2), and the trap is then shifted to analysis mode to 'read out' the fragment ions by sequentially ejecting them from the trap for detection (T3). Although only one mass analyzer is used, the resulting spectra are usually still referred to as MS/MS spectra. In an alternative scan mode (MS^3), the ion trap has the additional functionality of destabilizing all fragment ions except one, and then producing a subfragmentation spectrum. This approach can be extended to multiple isolation and fragmentation stages (MS^n).

3. Top-down sequencing: protein fragmentation in the gas phase

An approach that involves direct protein sequencing in the gas phase is referred to as top-down sequencing and has been demonstrated [2] and developed [3–5] over the past dxsecade. This approach is an alternative to the commonly used bottom-up sequencing (see next section). In the top-down approach, the protein sample is not subjected to enzymatic digestion, but instead transferred into the gas phase intact. Subsequent measurement of the protein molecular weight and fragmentation of the intact protein using various techniques, combined with database searching, lead to identification of the source protein. The main advantages of this approach include the potential for 100% sequence coverage of the protein and improved detection of post-translational modifications [27,28]. With the application of ECD and IRMPD in an FTICR mass spectrometer,

the ''top-down'' approach is gaining momentum as an alternative to the ''bottom-up'' approach but is not yet well established. This approach suffers from limited availability of large, expensive FTICR instruments that utilize high field magnets. The application of this approach is thus limited to a small set of academic groups. Recently, an approach based on ion–ion chemistry in quadrupole ion traps has been demonstrated with promising results [29]. Upon further development, this approach could be an alternative to the existing methods using FTICR, and lower the barriers to ''top-down'' sequencing even further.

4. Bottom-up sequencing: peptide fragmentation in the gas phase

The more popular approach to protein identification relies on peptide sequencing and is referred to as bottom-up sequencing. This approach requires accurate sequence analysis of the MS/MS spectra of the proteolytic fragments so that protein identification can be made and typically relies on algorithms for amino acid sequence assignments.

4.1. Ion activation and fragmentation kinetics

Scheme 1 lists parameters that contribute to the overall appearance of MS/MS spectra. It is commonly accepted that activation and subsequent unimolecular dissociation of the activated ion are distinct events that occur upon and following collisional activation of a projectile ion in MS/MS, i.e., an ion typically collides with a gaseous target, energy is redistributed in the ion, and fragmentation occurs. This has important implications for instrument design and/or appearance of spectra, because instruments designed for analysis of large molecules with a large number of vibrational degrees of freedom require larger observation ''time windows'' than those designed for fragmentation of smaller molecules. Pathways available for fragmentation vary with the molecular structure and the energy distribution deposited depends on the activation method employed.

Activation + unimolecular dissociation:
Internal energy distribution

Molecule:
Different reaction pathways
Energy dependent rate constants

Instrument Configuration:
Time window for observation of reaction
Slow heating vs. energy sudden

Scheme 1. Parameters contributing to MSMS spectra.

When calculations are performed using RRKM unimolecular dissociation theory and the best known values for activation energies and densities of state, the predicted reaction rates plateau at values too low to explain the rich fragmentation patterns that are detected for instruments that have only a microsecond observation time window (e.g., sectors, TOF–TOF, and triple quadrupoles) and yet the fragmentation does exist and is detected routinely for peptides [30–32]. One complication of these calculations is that researchers generally perform calculations from one protonated form of the molecule and no intramolecular transfer of the proton(s) is modeled. In reality, protonated peptides in the gas-phase have a high degree of intramolecular solvation and, especially after activation, consist of dynamic populations that may include different ways of distributing protons in the same molecule (different protonation motifs) and that may allow for intramolecular proton transfers that initiate dissociation.

Several MS/MS activation methods that are alternatives to gas-phase collision-activated dissociation are under investigation in research laboratories and include infrared multiphoton dissociation (IRMPD) [33,34], blackbody infrared dissociation (BIRD) [35,36], surface-induced dissociation (SID), photodissociation [37], and electron capture dissociation (ECD) [38–42]. There are several motivations for the examination of alternative activation methods including (1) the prominence of tandem mass spectrometry as a structural analysis tool, (2) the need to understand the analytical capabilities of activation techniques that are complementary to gas-phase collisions, (3) the need to better understand how and why unimolecular dissociation patterns differ when different excitation methods (and different instrument configurations) are applied to a given type of molecule, and (4) the ability of mass spectrometry to determine energetics and mechanisms of unimolecular dissociation [43]. Although most commercial instruments utilize collisions with a gaseous target, IRMPD is gaining in popularity in both quadrupole ion traps and FTICR [44]. Marshall and co-workers [45] have shown that IRMPD can be a valuable tool to study unimolecular dissociation of gas-phase molecules, owing to its clearly defined energy deposition. Application of IRMPD has also been demonstrated in structural elucidation of oligosaccharides [46,47]. The recent development of electron capture dissociation for fragmentation of ions now allows facile collection of MS/MS data in FTICR MS, greatly increasing their analytical usefulness [48]. An advantage of ECD is that peptide bonds can be broken in the presence of modifications such as glycosylation; for example, when ECD is combined with IRMPD, it is possible to obtain carbohydrate structure from the IRMPD experiment and peptide sequence from the ECD experiment [48,49]. A related promising development in quadrupole ion traps is electron transfer dissociation (ETD). ETD

makes use of ion–ion chemistry to produce spectra containing **c** and **z** ions (described in the next section) [39] which are analogues to those observed in ECD. In their work, Hunt and co-workers [29] demonstrated for the first time the use of singly charged anthracene as the electron carrier to react with multiply protonated peptides containing phosphorylation. The resulting spectra show extensive fragments along the peptide backbone, whereas spectra obtained from the same peptides using CAD are dominated by fragments corresponding to the loss of phosphoric acid. More importantly, the experiment is conducted in a modified quadrupole linear ion trap instrument, making ETD a potentially less expensive alternative to ECD.

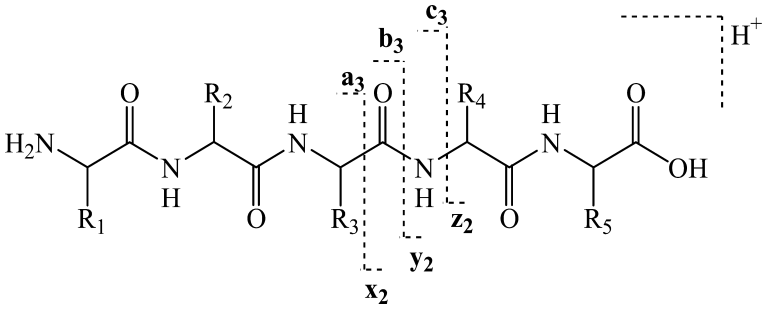
4.2. Fragment ion types

Peptide sequence identification by mass spectrometry involves fragmentation of a peptide to produce smaller *m*/*z* fragments; ideally, measured *m*/*z* values of these pieces can be assembled to produce the original sequence. Cleavage is commonly accepted to occur predominantly through charge-directed pathways, i.e., cleavage is initiated by a charge that is transferred to the vicinity of the cleavage site. The mobile proton model [50] is a general description of the need to transfer a proton or protons intramolecularly to cleavage sites throughout the peptide. These transfers are facilitated by the proton affinity of heteroatoms (e.g., backbone carbonyl oxygen). This interaction of the charge and the heteroatoms is more important in the gas phase because the solvent molecules that typically stabilize the molecules in the solution phase are absent in the gas phase so internal groups in the molecule serve the role of "solvent." For peptides in which all protons can be bound to basic residues, in particular Arg, cleavage often occurs selectively at the C-terminus of Asp (Asp–Xxx cleaves) or Glu (sometimes referred to as a charge remote mechanism because added proton is not directly required [50], although the proton is derived from the acidic side chain). If the number of protons exceeds the number of Arg residues, cleavage occurs at other sites with especially strong cleavage at the N-terminal side of Pro (Xxx–Pro cleavage).

A nomenclature [51–53] exists that is used to describe the fragment ion types that are produced by cleavage of different bonds along the peptide backbone and/or side chain (Scheme 2). Typical ion structures will be illustrated below, although actual structures of a particular fragment ion are often only inferred from model studies.

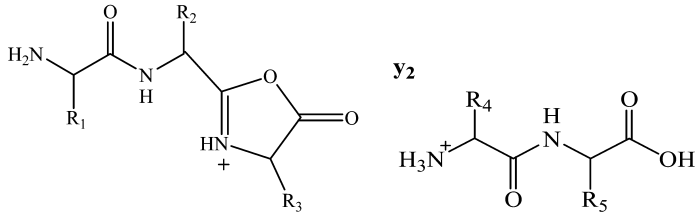
Cleavage of the backbone typically occurs at the peptide amide bond to produce **b** ions, if the amino terminal fragment retains the charge, or **y** ions, if the carboxy-terminal fragment retains the charge (Scheme 3).

In the case of multiply charged ions, a charge separation can occur to produce complementary ion pairs (e.g., a doubly charged ion can fragment to produce a $\mathbf{b}_n/\mathbf{y}_m$ ion pair where $n + m =$ total residues in peptide). Both partners of the complementary pair are not always detected in equal abundance, because they are not equally stable against further fragmentation or because instrument discrimination may enhance or diminish one partner of the pair. Although **b** and **y** ions are considered to be the most useful sequence ion types, because they correspond to cleavage of the amide bond, other ion types are observed and used in spectral interpretation



Scheme 2. Nomenclature of common ion types.

b3: oxazalone



Scheme 3. Representative structures of **b** ions and **y** ions.

or database searches. These include **a** ions (Scheme 4), which correspond formally to loss of CO from a **b** ion; a *m/z* difference of 28 between two peaks suggests an **a–b** ion pair and is useful in identifying the ion series to which the peaks belong.

The **y** series is sometimes accompanied by peaks formally corresponding to loss of NH_3 from the **y** ions, allowing designation of the higher *m/z* ion of each delta 17 pair as belonging to the **y** ion series. Ions that correspond to immonium ions (Scheme 5), or fragments of immonium ions, of individual amino acid residues in a peptide are often detected, even for residues from the internal portion of the sequence [54]. The characteristic *m/z* values of these ions are often used as sequence qualifiers that allow one to exclude sequences from a candidate sequence list if those ions are not present (see Table 1 *m/z* values of common immonium ions).

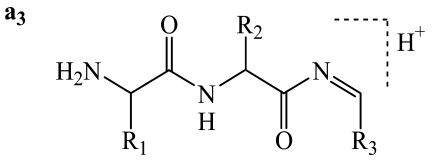
Ions that correspond to cleavage of a side-chain bond in addition to backbone cleavage(s) are referred to as side-chain cleavage ions. These are designated as **d**, **v**, and **w** ions, and allow distinction between isomeric or isobaric ions (e.g., Ile from Leu) (Scheme 6).

Internal ions are produced by cleavage of bonds to both the N- and C-terminal sides of the resulting fragment ion (Scheme 7).

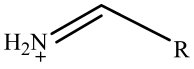
Whereas traditional activation methods give cleavages at peptide bonds and produce **b/y** ion pairs, ECD produces **c/z·** ion pairs with extensive cleavage along the peptide backbone for multiply-charged precursor ions. Homolytic cleavage at the N-C_α bond produces **c** ions when charges are present in the amino-terminal fragment, and **z·** ions when charges are present at the carboxyl-terminal fragment. The **z·** ions produced in ECD are unique and different from the generic z ion structure shown in Scheme 8 in that they are odd electron radical cations. No charge is retained directly at the cleavage site in the ECD **z·** ions; charges in **z·** ions come from additional charges originally present in the fragment 39. Ions of type **x** have recently been reported for photodissociation experiments [55] (Scheme 8).

The types of ions detected in an MS/MS or MS^n experiment vary with the peptide, the activation step, the instrument's observation time frame, and/or instrument discrimination factors. See Table 2 for ion types used in the database searching program, MASCOT [56], based on fragmentation spectra acquired from different instruments. Quadrupole ion traps, for example, discriminate against product ions that are less than 30% of the *m/z* of the precursor ion. This means that immonium ions and low *m/z* sequence ions that could be used in sequence determination are generally not present in ion trap spectra.

How the energy is deposited into an ion also alters the appearance of the fragmentation spectra of peptides. Activation in ion traps, for example, is considered to be a slow-heating method; energy is added in multiple low-energy increments until enough energy is deposited to induce fragmentation. When this is coupled to the long millisecond–second time frame of the trap MS/MS experiment, it becomes clear that ion traps often favor low-energy fragmentation pathways (e.g., internal ions are strong in trap MS/MS spectra). A method that deposits energy in 1–2 large steps, an "energy-sudden" deposition such as that in the high energy (keV) CAD of the MALDI TOF–TOF, may induce higher energy pathways such as side-chain cleavage. See Fig. 2 for an illustration of how the two types of activation can influence fragmentation. This does not mean, however, that ion traps and TOF–TOF instruments produce completely different fragmentation patterns. Fragmentation chemistry is dependent on molecular structure and some overarching fragmentation characteristics are detected regardless of instrument type and activation method: Arg ending singly charged peptides are more difficult to fragment than Lys ending peptides. Enhanced cleav-



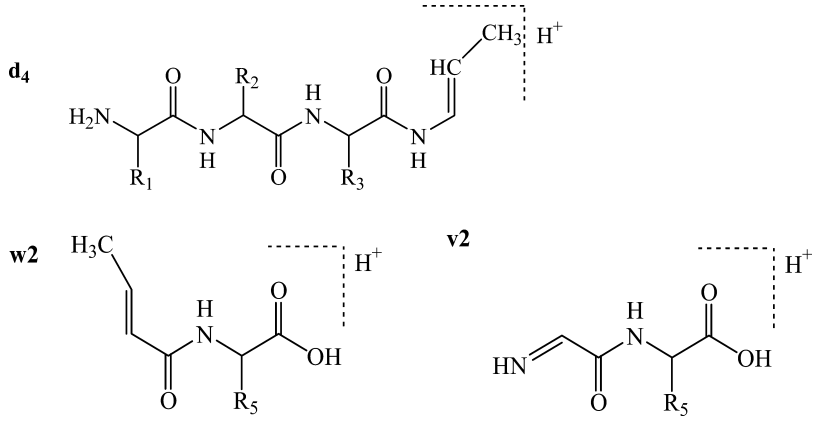
Scheme 4. Structure of **a** ions.



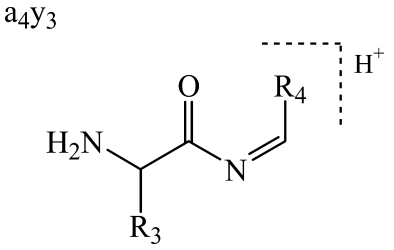
Scheme 5. Structure of immonium ions.

Table 1
m/z values of common immonium ions

Immonium ion (*m/z*)	Amino acid residue	Major (M) or minor (m) peak
60.04	S	M
70.07	R or P	M
72.08	V	M
73.00	R	m
74.06	T	M
84.08	K or Q	M
86.1	I or L	M
87.09	N or R	M
88.04	D	M
100.09	R	m
101.11	K or Q	M
102.06	E	M
104.05	M	M
110.07	H	M
112.09	R	M
120.08	F	M
126.06	P	M
129.1	K or Q	m
136.08	Y	M
138.07	H	m
159.09	W	M

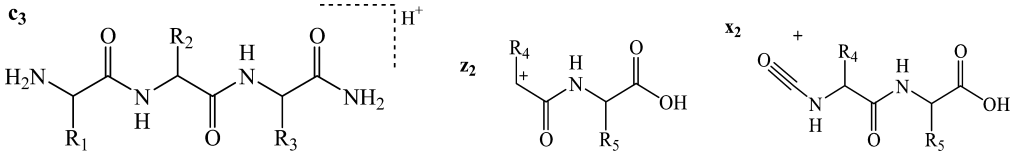


Scheme 6. Structure of fragments involving side-chain cleavage (**d**, **v**, and **w** ions), with Val as residue 4.



Scheme 7. Structure of internal fragments of a pentapeptide.

age at Asp can be detected in a variety of instrument types as long as there are no "mobile" protons that can induce cleavage at the backbone amide bonds. Enhanced cleavage at Pro often occurs in a variety of instrument types, if mobile protons are present [57]. When there are no Pro in the middle of the sequence (Pro only near N- or C-terminus) and mobile protons are present, stronger cleavages may be detected C-terminal to branched aliphatic residues (Ile–Xxx, Leu–Xxx,



Scheme 8. Structures of **c**, **z**, and **x** ions.

Table 2
Default ion types for corresponding instrument configurations and restrictions placed on the ion types for Mascot searches [56]

	Default	ESI Q-TOF	MALDI TOF–PSD	ESI Trap	ESI QQQ	ESI FTICR	MALDI TOF–TOF	FTMS ECD	MALDI Q-TOF
1^+ fragments	X	X	X	X	X	X	X	X	X
2^+ fragment if precursor 2^+ or higher	X	X		X	X	X		X	X
2^+ fragment if precursor 3^+ or higher									
Immonium ions			X				X		X
a series ions	X		X				X		
a–NH^3 if fragment includes RKNQ	X		X				X		
a–H^2O if fragment includes STED			X				X		
b series ions	X	X	X	X	X	X	X		X
b–NH_3 if fragment includes RNKQ	X	X	X	X	X	X	X		X
b–H_2O if fragment includes STED		X	X	X	X	X	X		X
c series ions									
x series ions									
y series ions	X	X	X	X	X	X	X	X	X
y–NH_3 if fragment includes RKNQ	X	X		X	X	X	X		X
y–H_2O if fragment includes STED		X		X	X	X	X		X
z series ions									
z + H series ions								X	
Internal $\mathbf{y}_b < 700$ Da							X		X
Internal $\mathbf{y}_a < 700$ Da							X		X

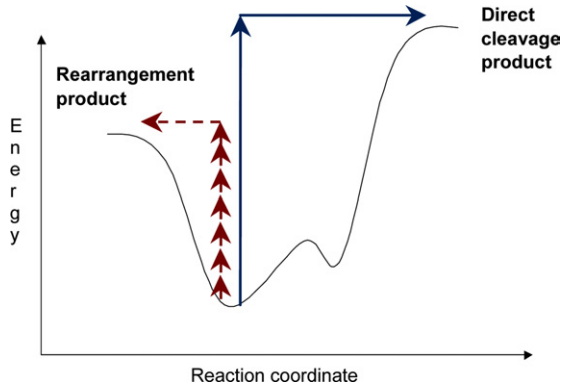


Fig. 2. Activation by slow-heating (e.g., quadrupole ion trap) vs. a single large step, energy sudden activation (e.g., keV CID) may highlight different fragmentation pathways.

and some Val–Xxx) [58]. Although Gly is often used in model peptides examined experimentally and computationally, this is risky if one is attempting to determine general fragmentation behavior because Gly has been noted to have decreased cleavage at its C-terminus and enhanced cleavage at its N-terminus compared to many other general amino acid residues [57,59].

4.3. Ion structures

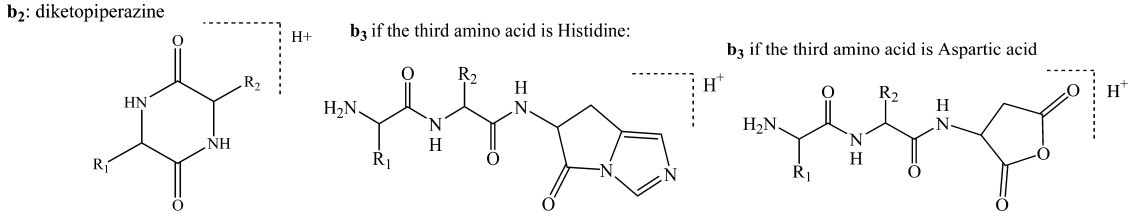
The actual structures of the common fragment ion types have been established for a few model systems only. Often, structures presented in the literature and illustrated above have been proposed based on knowledge of the *m*/*z* of the fragment plus chemical intuition, without detailed structural studies. Although it is common to think of a particular ion type as having a common structure, it has been shown that the structure can depend on the particular amino acid residues at the cleavage site. Ions of type **b** are commonly thought to be protonated oxazolones (Scheme 3) formed when the carbonyl O of the R_{n-1} residue attacks the carbonyl C of the R_n residue [60]. In the special case of $\mathbf{b}_2$ ions, where the amino terminal N can serve as a nucleophile, the **b** ion may be a protonated diketopiperazine [61] (Scheme 9), although this may depend on the chemical identity of the first three amino acid residues in the peptide. When the side chain contains a nucleophile, side-chain attack on the backbone carbonyl may also be involved (e.g., see structures involving His or Asp) (Scheme 9) [50,62]. It is accepted that ions of type **y** have the structure of truncated peptides.

Because product ions may be produced by more than one pathway, it is risky to assume that any one ion type has a single structure. This is readily seen for **b** ions, but holds true for other ion types. An **a** ion, for example, may be produced by loss of CO from a **b** ion or, under high energy conditions, may be produced through a radical process involving an **a** + **1** ion. That **a** + **1** ion might also serve as a precursor to a **d** ion [52]. A formal ammonia loss peak, e.g., a **b**–$\mathbf{NH_3}$ might be produced by ammonia loss from a **b** ion or an [$\mathbf{MH^+}$–$\mathbf{NH_3}$] might be formed first and fragment to a **b**–$\mathbf{NH_3}$, as has been shown by double resonance and MS^3 experiments. In spite of the complication that many structures may exist for a given ion type, it is still helpful in practical sequencing to assign a generic structure to a given ion type. In other words, while knowing the atom content of a residue is necessary to properly assign peaks labels in the tandem mass spectra, and knowledge of 3D structure of fragments may help improve our ability to predict spectra in the future, it is not necessary to know the actual 3D structure of the fragment to assign the peak "label."

Although quantum chemical calculations have greatly aided our understanding of gas-phase fragment ion structures, unfortunately even modern computational capabilities can only model relatively small and simple systems well. This is an active area of research and it is expected to continue to contribute strongly to our knowledge of peptide fragment ion structures and fragmentation pathways [63–66].

4.4. Interpreting MS/MS spectra

Knowledge of peptide chemistry is helpful for interpreting MS/MS spectra. Methods of digesting proteins produce cleavages at specific sites; for instance, trypsin cleaves specifically and nearly quantitatively at the C-terminus of lysine or arginine, but rarely between Lys-Pro and Arg-Pro. Because the amino terminal residue of each peptide (except for the C terminal peptide) is basic, tryptic peptides are usually doubly or triply charged in ESI. This produces multiply charged fragment ions, complicating interpretation. For example, in Fig. 3,



Scheme 9. Structures of different **b** ions.

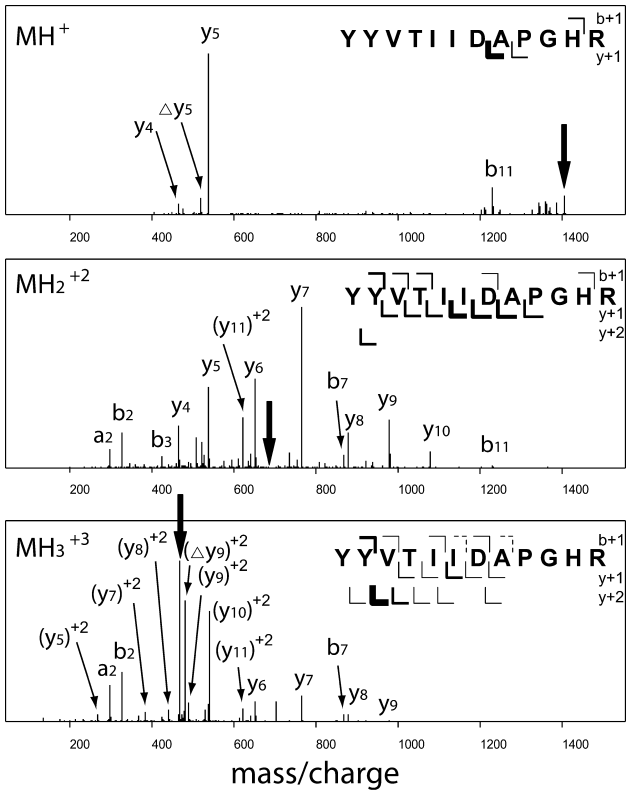


Fig. 3. Peptide MS/MS spectra obtained on a quadrupole ion trap. The precursor ions selected for fragmentation (indicated by bold arrow) have 1, 2, or 3 charges (top to bottom). Major fragment ions are labeled, as described in the text, numbered from N-terminus for **b** and **a** ions and from C-terminus for **y** ions. Dehydrated fragment ions are indicated by Δ. Doubly charged fragment ions are labeled with a superscript +2, singly charged fragment ions are not specifically labeled with their charge. In the upper right-hand corner of each panel, the peptide sequence is shown, with the ions that are detected indicated by ⌝ for **b** ions and ⌞ for **y** ions. Ion intensity is indicated by the thickness of the line, and the singly and doubly charged **y** fragment ions are indicated in separate rows.

the 3D ion trap MS/MS spectra for singly, doubly, and triply charged states of a single peptide sequence are shown.

The sequence of the peptide, YYVTIIDAPGHR, has two basic residues and the amino-terminus, consistent with observing up to three charges. The gas-phase chemistry of the peptide changes in interesting ways as the charge state changes which influences the appearance of the spectra. There are few fragment ions in the singly charged precursor (top panel). This is because this peptide contains an arginine, which sequesters the proton due to its very high gas-phase basicity. Thus, there are almost no cleavages initiated by "mobile protons" where a proton is transferred to the cleavage site by internal motions in the peptide. The observed fragment ions are diagrammed on the sequence in the upper right-hand corner of the panel. The singly charged precursor ion yields one major **y** type fragment ion from cleavage between D and A, and to a much lesser extent between I and D, consistent with an alternative mechanism called "charge remote fragmentation," where the proton is derived from the side chain of the aspartic acid (glutamic acid acts similarly). Cleavage at Asp–Xxx is sometimes referred to as "charge remote" cleavage to indicate that the cleavage occurs when the added proton is not mobile. Although it has been shown that it can occur in the absence of an added proton, it is clear that an acidic H (which some might consider as a charge) is involved, and it is possible that the charge from elsewhere in the molecule (e.g., protonated Arg) could assist the cleavage. Note the complexity of neutral losses observed from the precursor, which includes various losses from arginine, including complete loss of arginine, represented by the $\mathbf{b}_{11}$ ion.

In contrast, the doubly charged precursor ion (middle panel) produces a relatively rich fragmentation pattern. A series of **y** ions is observed from $\mathbf{y}_4$ through $\mathbf{y}_{10}$; this rich fragmentation pattern is typical of doubly charged ions, even with an arginine C-terminus, because one proton is free to initiate the cleavages. In addition, a few, weaker **b** ions are observed, including $\mathbf{b}_2$, $\mathbf{b}_3$, $\mathbf{b}_4$, and $\mathbf{b}_8$—this pattern of strong **y** ions and weak **b** ions is typical in MS/MS of doubly charged tryptic peptides. Water or ammonia loss can also be observed, usually as minor peaks, but this case shows a large amount of the dehydrated $\mathbf{b}_4$, because of the presence of threonine (T) at the C-terminus of that **b** ion (these are the pair of ions at 509 and 527 Da that are not labeled in the figure). This spectrum also shows a large $\mathbf{b}_2$ ion (normally $\mathbf{b}_2$ ions are not observed in a 3D ion trap, but the large $\mathbf{b}_2$ size allows observation in this case), consistent with the ease of formation of the oxazolonium or diketopiperazine ion at the N-terminus. A fairly substantial $\mathbf{a}_2$ ion is also observed (a **b** ion without the C-terminal CO). A few doubly charged ions are present, but they are weak (except for the doubly charged $\mathbf{y}_{11}$). The most common distribution of charge in this case is one on the arginine and one on the **b** ion. The smaller **b** ions are generally lost from the trap, while the larger **b** ions are often unstable, and produce additional cleavages giving small products that are lost from the trap.

The bottom panel shows the fragmentation observed when this peptide is triply charged. In this case, the most likely distribution of charge is two protons on the arginine and histidine, and this is reflected in the fact that for nearly every $\mathbf{y}^+$ ion observed, the corresponding doubly charged **y** ion is also observed. The $\mathbf{b}_2$ ion is increased relative to the other ions, and the $\mathbf{a}_2$ ion is also higher in intensity. Overall, the intensity of the doubly charged **y** ions is significantly higher than observed for the same ions in the doubly charged precursor. There are a few high mass triply charged ions; although they are relatively weak in this spectrum, in some spectra, they can be relatively intense. Consequently, in some

cases, there may be ambiguity regarding fragment ion assignments, particularly when there are several possible charge forms for each fragment ion and the resolution of the instrument used in the experiment is not high enough to determine the charge state.

4.5. Modified peptides

Mass spectrometry is ideally suited for mapping post-translational and other modifications of analytes, because most modifications produce a diagnostic mass difference when present vs. absent. Thus, it is also possible to detect unusual or unexpected modifications. A list of known modifications is available at the website developed by Ken Mitchelhill (http://www.abrf.org/index.cfm/dm.home). In complex digests, identifying modifications often proves difficult, particularly if the stoichiometry is low. Gas-phase chemical reactions can be exploited to identify the modified peptides. An understanding of the chemistry of the modification is essential in development of analytical protocols. For example, two ESI/MS scanning methods exist for selectively detecting phosphopeptides. One utilizes the propensity for neutral loss of phosphoric acid after ion activation, scanning for the appearance of a fragment ion with mass 98 Da less than the singly charged precursor (for instance, if precursor is doubly charged, then scan for [precursor − 49] Da). An alternative method uses high orifice voltage conditions to generate fragment ions, PO_3^{3-} (79 Da) and PO_2^{2-} (63 Da), which are detected by scanning in the negative ion mode. New hybrid instruments such as the ABI QTrap, which have a quadrupole mass analyzer coupled to an ion trap with a collision cell in between, are ideally suited for this approach.

Care must be exercised that peptides have not undergone artifactual modification during sample preparation. Commonly observed mass changes are +16 or +32, reflecting methionine oxidation to sulfoxides or sulfones, respectively, and can be observed at Trp, Cys, and His as well. Labile peptides can undergo dehydration (−18), loss of ammonia (−17 Da), or deamidation of Gln and Asn, respectively, to Glu and Asp (+1 Da) during preparation, in the acidic MALDI matrices, or during ESI. Free Cys can react with alkylating agents, including acrylamide (+71 Da), oxidized acrylamide (+86 Da), or sulfhydryl reductants from preparation of the sample (e.g., +76 Da for 2-mercaptoethanol). Alkylation with iodoacetamide at lower pH values (e.g., pH 6.8) may produce a −48 Da artifact, due to loss of CH_3SH from Met [67].

5. Fragmentation mechanisms and algorithm development

In the early 1990s, computer search algorithms for identifying proteins from peptide mass spectral data became available allowing for the high-throughput identification of unknown proteins [68–72]. Pioneering studies in this area utilized in-gel digestion protocols and began to establish databases of proteins expressed in human myocardial cells, melanoma cells, and yeast [73,74]. The term "proteomics," used to describe the sum of proteins expressed in a given cell type, was coined by Williams, Humphery-Smith, and co-workers [75,76], and ensuing publications have popularized this exciting new field. More recently, several groups have begun analyzing more complex samples, including tryptic digests of whole cell lysates (shotgun proteomics), utilizing multidimensional chromatography to partially separate the peptides. This approach has been successful in characterizing the protein composition of organisms such as *Saccharomyces cerevisiae*, where 25% of ORFs contained in the genome were observed [77].

To identify peptides, database searching programs compare each MS/MS spectrum against theoretical spectra of candidate peptide sequences represented in a protein database, and a score is assigned to rank the most likely peptide assignments [78]. However, current scoring methods are poor at distinguishing correct from incorrect sequence assignments, leading to high false positive and false negative rates [79]. For example, two commonly used search programs (Sequest and Mascot) validated less than half of potentially identifiable MS/MS spectra from shotgun analyses of the human erythroleukemia K562 cell line [80]. In large part, this is because of inadequacy in the theoretical spectra. Algorithms use mainly *m*/*z* information (e.g., all the **y** ions are given equal relative abundance in the theoretical spectra) and not intensity information. Several groups are attempting to determine whether the inclusion of expected abundance information might improve the success rate of sequencing algorithms. These studies may involve adaptations of established algorithms to include new fragmentation models or rules or may involve using an uninterpreted dataset as a training set to calculate the relative likelihood of a spectrum given a candidate sequence [57,81,82]. The inclusion of abundance information in established algorithms requires knowledge of expected fragment ion abundance information, requiring that studies on fragmentation patterns and algorithm development be coupled.

Several research groups are investigating peptide fragmentation pathways and the corresponding expected relative abundances for cleavage. Traditionally, these studies have been performed for a set of model peptide structures. The "mobile proton model," which was described briefly above and which has been defined and expanded on in several publications [83–86], grew out of the work of many investigators [52,87,88] and was developed as a qualitative description of peptide fragmentation. Various investigators are now expanding from this model to explain fragmentation more quanti-

tatively; this work includes the "Pathways in Competition" model of Paizs [66] and the kinetic model of Zhang [82]. An alternative approach to examining fragmentation of sets of model peptides is to examine large bodies of spectra that have been collected; these collections provide an excellent experience base for understanding gas-phase unimolecular dissociation chemistry of peptides. Several research groups are now statistically analyzing fragmentation information in spectra for which sequence has been assigned with high confidence [57,58,89–91] and evaluating whether the inclusion of the expected abundance information might improve the success rate of sequencing algorithms. One must be cautious, however, and understand that if the set of spectra used for statistical analysis was assigned a sequence by a current algorithm, then a biased set of data is being used. Those spectra that were not assigned a sequence are not represented in the set. It is also not yet clear how much difference there is in gas-phase chemistry between different instruments or with different instrument settings. Careful comparisons of information between different datasets will be an important informatics feature of these studies.

6. Concluding remarks

In the last 15 years, mass spectrometry applications have revolutionized analysis of proteins, moving from simple studies of purified proteins, blocked N-termini, modified peptides, and analysis of peptide synthesis reactions, to the current dizzying array of new methods and instruments, as well as inspiration for the new field of systems biology. Proteomics is now a multibillion-dollar enterprise. In the same time, we have shifted from an era where our understanding of protein and peptide gas-phase chemistry was built up slowly, to an era where large datasets can now be mined for rules that rapidly increase our understanding of the fundamental chemical processes. This should greatly enhance our ability to identify components of complex samples and yield significant advances in medicine and biology. The great reliance of the field on mass spectrometry for protein characterization has spurred many advances in mass spectrometry instrumentation, separations technology, and software, and data management capabilities. Yet, this amazing growth has only whetted our appetites, and we can look forward to even more powerful instrumentation and algorithms used in creative ways to yield a rich information avalanche in the future.

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