Chapter 27. Recent Developments in the Mass Spectrometry of Peptides and Proteins.

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Introduction - The enormous current interest in both natural and synthetic peptides and proteins is placing increasing demands on the analytical methods used to characterize these materials. While many widely used techniques are very useful, they all suffer from specific drawbacks. Thus, for example, amino acid analysis cannot easily be applied to mixtures of peptides, Edman sequence analysis cannot be made on amino terminally blocked peptides and proteins, and electrophoretic molecular weight determination has limited accuracy (usually >5%). Mass spectrometry has long held the potential for overcoming many of the limitations of other methods. A number of revolutionary developments have recently been made which have converted this potential into reality, namely: (a) The discovery of several new ionization techniques for the production of intact gas phase ions from underivatized high molecular weight peptides and proteins; (b) The construction of mass analyzers capable of measuring high molecular weight compounds with good precision and sensitivity; (c) The development of tandem mass spectrometry or mass spectrometry-mass spectrometry (MS-MS), which allows the sequence of a peptide to be determined even when it is a component of a relatively complex mixture; (d) The direct coupling of liquid chromatographs (LC) to mass spectrometers.

In this review we will discuss some of these more recent developments and provide a selection of applications which illustrate the power of mass spectrometry in the analysis of peptides and proteins.

METHODS OF GAS PHASE ION PRODUCTIONS

An absolute requirement for the successful mass spectrometric analysis of any compound is the production of ions of the molecule in the gas phase. The transfer of peptides and proteins from the condensed phase into the gas phase presents special difficulties because these molecules are massive, polar and therefore highly non-volatile. Prior to the advent of the newer desorption/ionization techniques, mass spectrometric analysis of peptides was not possible without chemical derivatizations of the peptides designed to decrease their polarity and thus enhance their volatility (1). Chemical derivatizations of peptides are laborious, and require relatively large quantities of sample. In 1969 the technique of field desorption mass spectrometry (FDMS) was introduced which permitted the measurement of underivatized peptides (2). In this method, the compound of interest is ionized and desorbed from a surface by the application of a strong electric field. Although peptides of molecular weight up to ca.1500 Da have been studied by FDMS (3,4), the technique has been largely supplanted by the more recently developed ionization methods discussed below.

^{252}Cf Plasma Desorption (PD) Ionization - In this technique, peptides and proteins, in the form of a thin solid layer, are desorbed and ionized by the passage of highly energetic fission fragments through the sample (5). The fission fragments are produced from the spontaneous radioactive decay of ^{252}Cf and have energies of ca.100 MeV and masses of ca.100 Da. Samples are generally prepared for mass analysis by adsorption of monolayer amounts (10^{-11}-10^{-9} mole) of the peptide or protein onto a thin layer of nitrocellulose (6,7). The masses of the desorbed ions are determined by time-of-flight mass analysis (see later). PD mass spectrometry has been used extensively for measuring molecular weights (MW's) of peptides and proteins up to 10,000 Da and, less frequently, up to 35,000 Da (8,9). Because of the unavailability of commercial ^{252}Cf PD mass spectrometers, the use of this technique has, until recently, been limited to a relatively few laboratories. This situation has now been remedied by the introduction of a commercial instrument (10).

KeV Ion or Atom Bombardment-Induced Ionization - In this method, peptides and proteins introduced into the mass spectrometer in the condensed phase are desorbed and ionized by bombardment with ions or atoms (e.g., Cs^+, Ar, Xe) with energies in the range 5-30 keV. The sample is introduced into the mass spectrometer either as a solid (11-16) or dissolved in a low volatility liquid such as glycerol (17-19). When the sample is introduced as a solid, the technique is known as static secondary ionization mass spectrometry (SIMS), because low ion fluxes are used in order to avoid rapid sample destruction. When the sample is introduced in solution, the technique is known as liquid secondary ionization (LSI) or fast atom bombardment (FAB). The liquid sample matrix allows the use of intense bombarding ion or atom fluxes because the surface is continuously replenished with sample from the bulk of the solution. LSI is currently the most used ionization technique because of its ready compatibility with commercially available high performance magnetic deflection mass analyzers and quadrupole instruments. LSIMS has been widely used to analyze nanomole amounts of peptides and proteins up to MW 6,000 Da (20) and, less frequently, up to 24,000 Da (21). Recently, a promising method for directly coupling microbore HPLC to LSI sources has been demonstrated (18,19).

Laser Desorption and Ionization - Intense pulses of laser light can also effect the volatilization and ionization of underivatized peptides and proteins from condensed phase samples. Although the first use of laser pulses for this purpose was reported more than a decade ago (22), laser desorption has not been widely applied in this area. Three recent developments have resulted in a strong resurgence of interest in laser ionization: 1) it has been demonstrated that selected peptides desorbed by infrared laser radiation from a surface can be entrained in and cooled by a supersonic gas jet and thence ionized by multiphoton absorption of UV light from a second laser (23-25). Performing the desorption and ionization in these two separate steps provides selectivity in ionization and control over the fragmentation of the ionized molecule(s). At present the sensitivity of this method appears to be too low for many practical applications; 2) a method has been developed whereby proteins with MW's of up to 34,500 Da have been desorbed and ionized in a single step by pulsed laser irradiation (λ = 337 nm) (26). Nanomole amounts of the protein of interest are dissolved in glycerol and mixed with ultrafine metal powder. The fine powder is rapidly heated by the laser pulse causing desorption of the ionized protein. MW's were determined with a precision of ca.1%; 3) a UV laser bombardment technique has been developed in which proteins with MW's in excess of 100,000

Da can be efficiently desorbed, ionized and measured (27,28). Samples are produced by mixing picomole amounts of protein with a large molar excess of nicotinic acid in solution and then allowed to dry. Irradiation of this sample mixture with 266 nm wavelength laser pulses (a wavelength close to the absorption maximum of nicotinic acid) produces copious amounts of protein ions which are measured in a time-of-flight mass analyzer.

Thermospray Ionization - In thermospray ionization, peptides in an electrolytic solution are introduced directly into the mass spectrometer vacuum through a heated capillary tube in the form of a fine mist of charged droplets (29-31). The charge is produced by statistical fluctuations in the distribution of positive and negative ions in the droplets. Isolated peptide ions are ultimately formed in the gas phase from these charged droplets, and are subsequently mass analyzed. Special features of this mode of ionization are its ability to produce ionized peptides with no apparent fragmentation, and its natural suitability for coupling with on-line liquid chromatographic systems (30-32). Thermospray ionization has primarily been used with quadrupole mass analyzers of limited mass range (less than m/z = 2000). The availability of quadrupole mass analyzers with extended mass range (33) and the recent successful use of thermospray ionization on high performance magnetic deflection mass spectrometers (34) promise to extend the range of masses of peptides which can be analyzed with this method.

Electrospray Ionization - In electrospray ionization, peptide ions are again produced from small highly charged droplets (35-39). The droplets, in this case, are generated by applying a high electric field at the tip of a capillary through which the sample solution can flow. This procedure is carried out at atmospheric pressure and the peptide ions thus produced are sampled into the mass spectrometer through a small orifice for mass analysis. A characteristic feature of electrospray ionization is the almost exclusive formation of intact multiply protonated or natriated peptide or protein ions as shown in Fig. 1 for cytochrome c. The high average charge on the ions produced by electrospray yields mass-to-charge ratios that are sufficiently low to allow their determination by relatively modest mass range quadrupole mass analyzers. Proteins with MW's up to 40,000 have been measured with this technique (36). Sensitivities in the picomole range appear to be attainable (39) and it appears feasible to directly couple an electrospray source to a capillary zone electrophoresis separation system (37).

METHODS OF MASS ANALYSIS OF PEPTIDE IONS

Several different types of mass analyzers are used to determine the mass-to-charge ratio (m/z) of ions produced from peptides and proteins. These include the double focussing electric and magnetic deflection mass analyzer (MA), the quadrupole MA, the time-of-flight MA, and the Fourier transform ion cyclotron resonance MA. The reader is referred to standard mass spectrometry texts for details of the principles of operation of these instruments (40,41). Table 1 shows a comparison of the important properties of these various mass analyzers. The table gives the highest mass measured to date for each combination of ionization method and analyzer type. However, in most cases the quality of the mass spectral data falls off rapidly with an increase in mass. Several of the techniques given in the table are quite new and in the development stage, so that they have not been fully commercialized. Thus, for example, FAB on a Fourier transform ion cyclotron resonance mass spectro-

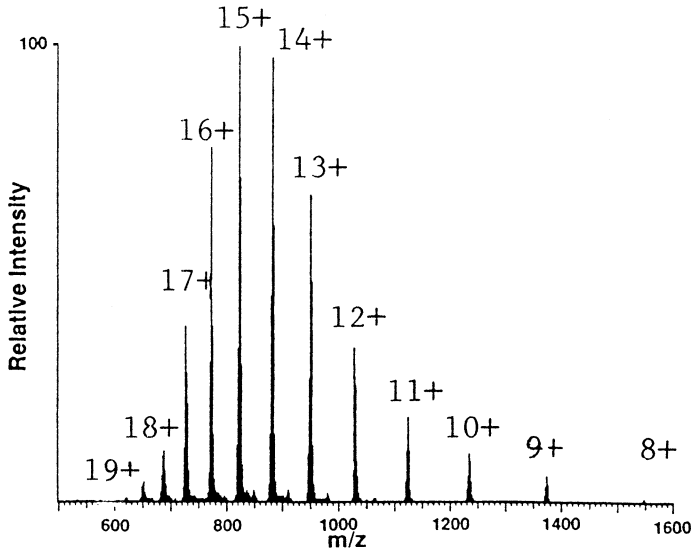

Fig. 1 Electrospray ionization positive ion mass spectrum of horse-heart cytochrome c. A gaussian distribution of multiply protonated intact cytochrome c ions is observed. The numbers above the peaks denote the number of protons attached to the molecule. Adapted from Ref. 37 with permission.

meter has only been extensively utilized for the analysis of peptides by a single research group, albeit with good success (42-44). Similarly, the use of UV-laser desorption time-of-flight mass spectrometry of large proteins has so far been limited (26-28).

TYPES OF INFORMATION AND APPLICATIONS

In this section the types of information that can be obtained from mass spectrometric analyses of peptides and proteins will be discussed, and a selection of recent biomedical applications reviewed.

Information deduced from molecular weight determinations - The most important single piece of information obtained with mass spectrometry which helps to characterize a peptide or protein is its molecular weight. The ionization techniques discussed earlier generally produce protonated positive molecule ions $(M+H)^+$ or deprotonated negative molecule ions $(M-H)^-$, where M denotes the intact molecule. In several of the techniques, multiply charged ions (e.g., $(M+nH)^{n+}$, where n is an integer) are also formed. In general, the determination of the mass-to-charge ratio of these ion species then provides the molecular weight of M. The mass spectrometric determination of peptide or protein molecular weight, alone or in conjunction with classical biochemical analytical techniques such as amino acid analysis, Edman sequencing, DNA base sequencing, etc., can provide solutions to a wide variety of biological and biochemical problems. Examples of such applications are given below:

Synthetic peptides - In recent years it has become feasible to produce routinely and rapidly by stepwise solid phase methods, synthetic peptides containing up to 50 amino acid residues (45). In response to

TABLE 1

Analyzer Type	Ionization Method	Minimum Sample Quantity Required (nmol)	Mass Range Achieved (Da)	Mass Accuracy (Da)
Deflection	FAB	0.01-5[a]	24,000	±0.3 (mass<6000)[c]
	FD	0.5-1[b]	1,500	±0.01%(6000<mass<12000)
	Thermospray	1-10	8,000	±0.3-0.7%(at mass 24,000)
Quadrupole	FAB	0.5-10	3,500	±0.3
	Thermospray	1-10	3,500	
	Electrospray	0·001-1	40,000	±1 (up to mass 17600)[d]
Time-of-flight	PD	0·002-1	35,000	±0.5 (mass<6000)
	SIMS	0·0001-.05	15,000	±2 (5000<mass<10,000)
				±0.2% (above mass 10,000)
	LD	0·0001-.01	200,000	±0.1%
Fourier Transform Ion Cyclotron Resonance	FAB	0·01-0.05	13,000[e]	±0.2 (up to mass 2000
	PD	<1	2,000	
	LD	<25	1,100	±0.2 (up to mass 1100)

[a]When an array detector is used the sample quantity required is reduced by a factor 10-100; [b]ref. 4; [c]ref. 21; [d]ref. 39; [e]ref. 44.

the strong demand from the biological community, synthetic peptides are now being produced in very large numbers. These complex biomolecules are produced by carrying out a very large number of sequential chemical operations. There are thus many opportunities for errors and modifications to occur both during and after synthesis. It is therefore imperative to have available effective means for rapidly verifying the correctness of the covalent structures of these materials. A good verification of the correctness of the structure is obtained when the measured MW agrees with the calculated MW. Conversely, any disagreement indicates the occurrence of a synthetic error or modification (46-48). Further, ion peaks in the mass spectrum in addition to those corresponding to the desired product reveal the presence of impurities which have co-purified with the product (46). The differences in MW between the desired product and undesired by-products provide important clues as to the nature and origin of the by-products.

Peptide mapping - Mass spectrometric peptide mapping has become an established and powerful structural tool for the analysis of proteins. The general strategy involves chemical or enzymatic degradation of the protein, followed by mass spectrometric MW determination of the resulting peptides (49-51). When necessary, further detailed structural information is obtained by subjecting the peptides to chemical procedures such as Edman sequencing, or to mass spectrometric sequence analysis (see later). Mass spectrometric peptide mapping has been used for the verification and correction of primary structures of proteins deduced from their DNA sequences (51-54), the structural definition of recombinant-DNA protein products (50,55-58), the identification of sites of variation in mutant and modified proteins (59-65), the determination of carbohydrate attachment sites in proteins (66-68), and the determination of peptide and protein structures (55-57, 69-76), especially in cases where the protein is modified posttranslationally.

Posttranslational modifications - One of the most important applications of mass spectrometry for protein analysis and one which has significant advantages over many classical biochemical methods is the detection and definition of posttranslational modifications. Frequently, post-translational modifications are initially detected by MW determination of chemically or enzymatically generated peptide fragments of the protein. When the MW of a given peptide fragment cannot be rationalized in terms of the sequence of the unmodified protein, the presence of such a modification is indicated. The nature and location of the modification can then be deduced, either from the mass difference between the modified and unmodified peptides, or from a further mass spectrometric structural analysis of the peptide. Examples of such mass spectrometric determinations include the detection and identification of blocked or modified amino and carboxy-termini (52,70,73-83), glycosylation sites (66-68, 84-86), phosphorylation sites (87), and others (88).

Disulfide pairing - Although only used to a limited extent, mass spectrometry appears to be of great potential utility for disulfide mapping of proteins. The mapping is generally carried out by selectively cleaving the protein under conditions where the disulfide bonds remain intact and mass spectrometrically determining the molecular weights of disulfide-containing peptide fragments. If necessary, further information concerning the identity of these peptide fragments can be obtained by amino acid analysis and Edman sequencing, and/or reducing the disulfide bond and subjecting the resultant peptides to additional mass spectrometric analysis. Mass spectrometry proved pivotal in the mapping of the seven S-S pairs in neurophysin (89-91). The task of establishing which of the 135,135 possible ways of pairing the 14 half-cystine residues in this protein was made more difficult by the necessity of using non-specific enzymes to induce release of disulfide containing peptide fragments. Mass spectrometry has also been used to locate the disulfide bonds in a series of model proteins (92), recombinant human growth hormones (93), human insulin like growth factors (94), and Paim I (95).

Miscellaneous - Mass spectrometry has also been used to provide information on the carboxytermini of proteins (78,96-98), the structure of the peptide network of pneumococcal peptidoglycans (99,100), iodination sites in cytochrome C (101) and the correct MW of proteins in cases where SDS gel electrophoresis or gel filtration gave ambiguous or incorrect results (102,103). An example of the latter application concerns the MW of the mating pheromone, Er-1, of the ciliate Euplotes raikovi (102). In urea/sodium dodecyl sulfate electrophoresis, reduced Er-1 migrated as a broad band corresponding to a MW of 29,000, a value considered to be anomalous. MW determinations of native Er-1 samples on gel filtration, which gave values of 9,000-12,000 were thought to be more reliable. The actual MW as determined by mass spectrometry was subsequently shown to be 4411.

Information deduced from mass spectrometric fragmentation - During the production of peptide ions by several of the ionization processes discussed above, large amounts of energy are deposited in the ionized molecule. A fraction of the resulting highly excited peptide ions may then undergo rapid fragmentation in the ion source of the mass spectrometer. The masses of these fragment ions can be determined and provide a partial or complete sequence of the peptide as well as information on the location and nature of any modifications present (1,72,104,105). Obtaining sequence information from these mass spectrometric fragmentation data can often be difficult because of

incomplete or weak fragmentation and interference from matrix or impurity generated ions. Tandem mass spectrometry overcomes many of these limitations and is becoming widely used for peptide structure analysis.

The technique of tandem mass spectrometry involves three sequential steps: 1) selection and separation of an ion of interest; 2) induced fragmentation of this ion; and 3) subsequent analysis of the resulting fragment ions (106). Fragmentation of the peptide ion of interest is induced by energetic collisions with neutral gas molecules or by bombardment with photons. Since a single selected ion is subjected to fragmentation, it is feasible to obtain structural information of a given peptide in a complex mixture. The total sequence can often be determined even when the amino terminus is blocked or when the peptide contains modified amino acid residues. The amount of peptide sample required for a tandem MS analysis depends on the nature of the sample as well as the instrumentation used, and varies from ca.1 nmole for two double-focussing deflection mass spectrometers operated in series (47) to ca. tens of pmoles for a tandem quadrupole Fourier transform instrument (107). It has recently been demonstrated that the sensitivity of the former instrument can be increased by a factor of 100 or more by incorporation of a so-called multichannel array detection system (108,109).

The tremendous utility of tandem mass spectrometry for providing information on the detailed structure of peptides is well documented (1,47,52,110). The technique has been especially useful for locating and defining posttranslational modifications of proteins (47,110) and for providing sequence information on proteins and peptides with blocked amino-termini, such as human lipocortin (47), type II calmodulin-dependent protein kinase (76), yeast cytoplasmic valyl-tRNA synthetase (47,53,54), cellular retinaldehyde binding protein (75), prostatic spermine binding protein (52), and prostatropin, a prostate cell growth factor (111,112). Tandem MS has also been used to determine the complete primary structures of four different thioredoxins (69,113-115), 90% of the primary structures of purple acid phosphatase and uteroferrin (116), the presence of s-farnesyl cysteine as a structural component of Saccharomyces cerevisiae mating hormone a-factor (117), the structures of cysteine-rich metal-binding polypeptides in cadmium-resistant tomato cells (118), and the structure of the polyglutamate chain attached to the folates of E. coli (119).

The tandem MS studies listed above were all carried out by inducing fragmentation of the peptide ions through collisions with neutral gas molecules. Fragmentation of a selected peptide ion can also be induced by a pulse of UV laser radiation incident on ions captured in a Fourier transform ion cyclotron resonance mass spectrometer, although such analyses have only been performed by a single research group (73,107,116,119-121). These photodissociation tandem MS analyses have yielded the complete sequence of a 10 pmole sample of a 15 residue tryptic peptide from beef spleen, purple acid phosphatase (116), have revealed that three photosystem II proteins of spinach chloroplasts contain N-acetyl-O-phosphothreonine at their amino-termini (73), have allowed the characterization of a benzyladenine binding-site peptide isolated from a wheat cytokinin-binding protein (120), and have assisted in the complete MS sequence analysis of an amino-terminally blocked, 78-residue protein (calbindin-D_{9k}) involved in calcium transport in mouse (121).

THE FUTURE

Relatively straightforward extrapolations of the present revolution in mass spectrometry lead us to predict that, in the not too distant future, the majority of peptide and protein-subunit molecular weights will be readily determinable by mass spectrometry, and that these determinations will be made rapidly (in a few minutes) on subpicomole amounts of material. Similarly, we believe that the ease and sensitivity for obtaining tandem MS structural data from peptides will also continue to increase rapidly. Thus, mass spectrometry is likely to become an increasingly important analytical tool for the biomedical scientist who works with peptides and proteins (122).

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