

High-accuracy mass measurement as a tool for studying proteins

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Electrospray ionization and matrix-assisted laser desorption/ionization, two new mass spectroscopy methods for the accurate measurement of molecular masses of individual peptide and protein molecules, are finding great utility for the solution of problems in biological research. Thus, mass spectrometry is being used for the rapid identification and detailed characterization of proteins, the determination of modifications in proteins, and the assessment of the integrity and purity of (native, recombinant, or synthetic) protein preparations. Recent data indicate that mass spectrometry can contribute significantly to the study of protein interactions and even to the investigation of aspects of protein folding and conformation.

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Introduction

The introduction of effective new methods for accurately measuring the molecular masses of individual peptide and protein molecules has vastly expanded the utility of mass spectrometry (MS) for the solution of problems in biological research [1]. Two techniques, in particular—matrix-assisted laser desorption/ionization MS [2] and electrospray ionization MS [3]—are beginning to have a significant impact on the speed and ease with which a wide variety of biological questions can be resolved. These methods enable the facile (data is obtained in minutes) and accurate (accuracies as high as 1 part in 10 000) analysis of picomole amounts of polypeptides with molecular weights up to several 100 kDa. Both techniques are still under active development and improvements are occurring at a rapid rate.

At present, the special strengths of electrospray ionization MS include the ease with which it can be coupled directly to liquid chromatography (LC), the high mass accuracy and resolving power of the technique (even for relatively large proteins), and the ability to analyze proteins directly from liquid solutions. Limitations include the deleterious effects of salts, buffers, and many impurities on the quality of the mass spectra, as well as the spectral congestion and complexity that can occur when analyzing certain mixtures. Matrix-assisted laser desorption/ionization MS, on the other hand, has contrasting strengths. These include immunity either to impurities or to additives widely used in biological research (e.g. salts, buffers, and chaotropic agents), the ease with which complex mixtures of peptides

and proteins can be directly measured, and the relatively wide applicability of the technique for the analysis of peptides and proteins. Limitations include the lower resolving power and mass determination accuracy, especially for large proteins, and the need for time-consuming calibration using internal standards. These strengths and limitations make the two techniques complementary.

This review summarizes a selection of applications of MS in protein chemistry and biology, with an emphasis on more recent developments. (Only limited reference is made to the important, but well reviewed, applications of ^{252}Cf plasma desorption MS and fast atom bombardment MS.) For reviews of previous progress in the MS field, the reader is referred to earlier publications [4-8].

Characterization of proteins

MS can be used as an alternative to SDS-PAGE for characterizing proteins through measurement of their molecular weights [1]. The mass determination accuracy (0.01-0.02%) is typically two to three orders of magnitude higher than that obtained with SDS-PAGE. This improved accuracy is of considerable value for primary characterization of unknown proteins [9], resolving ambiguities arising from anomalous migration in SDS-PAGE gels [10*], detecting mutant proteins (including silent variants) [11*,12], and elucidating post-translational modifications [1,5,8,13-18,19*,20].

Abbreviations

HPLC—high performance liquid chromatography; LC—liquid chromatography; MS—mass spectrometry; PAGE—polyacrylamide gel electrophoresis; SDS-PAGE—sodium dodecyl sulfate PAGE.

To determine modifications, the accurately determined molecular weight can be compared with the molecular weight calculated from the cDNA sequence of the corresponding gene. Any significant difference implies an error in the cDNA-deduced sequence, a post-translational modification [1,5,8,13–18,19*,20] or proteolytic processing of the protein [1,13,20,21–23]. If more detailed information concerning the nature and site of a modification or processing event is required, it is usually necessary to subject the protein to further analysis, involving enzymatic or chemically induced degradation of the protein, followed by further mass spectrometric measurement of the resulting peptide fragments. This analysis of the peptide fragments can be undertaken in several different ways, which are described below.

On-line liquid chromatography–mass spectrometry

Analysis of peptide fragments can be carried out by off-line collection of chromatographic fractions followed by individual mass spectrometric measurement of each fraction [24,25,26*]. A more facile approach involves on-line LC–MS, which at present is best carried out through an electrospray ionization interface [27,28*–30*,31,32*–34*,35]. Mass spectra are obtained continuously as the various components elute from the chromatograph, and the measured peptide masses are compared with those calculated on the basis of the cDNA sequence and the properties of the enzyme or chemicals used in the degradation. Observed differences yield information on the site and nature of modifications. Specialized strategies have been devised to facilitate rapid selective detection and analysis of glycopeptides [29*,30,31*] and phosphopeptides [32*,33*]. Improvements in the sensitivity and speed of the LC–MS analysis can be achieved through the use of narrow diameter capillary columns [34*] and appropriate packing materials, respectively [35].

Capillary electrophoresis–MS has been proposed and evaluated as an alternate approach for the ultrasensitive analysis of peptide and protein mixtures [36]. The practical applications of the technique are limited by its requirement for small injection volumes and relatively high concentrations of solute. These limitations can be ameliorated by the use of transient isotachopheresis for pre-concentrating proteins from larger injection volumes [37].

Unfractionated peptide mixture analysis by mass spectrometry

The special facility of matrix-assisted laser desorption/ionization MS for the analysis of peptide mixtures [38] allows a rapid single-step determination of the components of unfractionated protein digests [19*,38–40,41*,42,43]. The resulting spectra are readily interpreted because they are generally dominated by singly charged ion peaks. Peptide fragments having low mass (<0.5 kDa), poor solubility, or no basic amino acid

residues are often absent from the positive ion mass spectra of such mixtures, thus, leaving corresponding gaps in the peptide map ([40]; W Zhang, BT Chait, unpublished data). The high speed and sensitivity of the measurement allows easy monitoring of the time-course of the degradation reaction, providing an extra dimension of structural information as well as information on the properties of the proteolytic enzyme [43]. The high sensitivity of this approach also permits the measurement of digestion products generated from proteins separated by one- and two-dimensional gel electrophoresis ([26*,41*,44,45*]; W Zhang, R Aebersold, T Yungwirth, D Hess, BT Chait, *Proceedings of the 41st ASMS Conference on Mass Spectrometry and Allied Topics*, San Francisco, California, American Society for Mass Spectroscopy, 1993:415). The combination of two-dimensional PAGE and MS promises to be a particularly powerful means for assessing the differences between isoforms that exhibit, for example, different phosphorylation states ([41*]; W Zhang, R Aebersold, T Yungwirth, D Hess, BT Chait, *Proceedings of the 41st ASMS Conference on Mass Spectrometry and Allied Topics*, San Francisco, California, American Society for Mass Spectroscopy, 1993:415).

Sequence analysis of peptides by mass spectrometry

A number of mass spectrometric approaches have been devised for sequencing peptides that may contain modified residues. These include tandem MS [5,46], approaches that combine, wet, chemical or enzymatic degradation with MS of the resulting products [47*,48,49*], and approaches that combine classical Edman sequencing with MS [45*,50*–52*]. Tandem MS, which involves dissociation of chosen peptide ions followed by analysis of the resulting fragments, is the most mature of these MS approaches. It has the unique ability to yield sequence information on peptides blocked at their amino and carboxyl termini. The recently developed technique of protein ladder sequencing [49*] shows promise for providing rapid sequence information together with the site and nature of modifications (e.g. phosphorylation).

Identification of proteins

A pressing need exists for rapid sensitive means of identifying proteins that have been isolated, for example, on the basis of their biological activity, response to stimuli, or specific association with other biomolecules. Such identifications are now frequently made using partial protein sequence information, sometimes in combination with MS analysis [44]. The emergence of effective MS methods for determining the masses of proteins and complex mixtures of peptides [1] has led to a new strategy for protein identification that is potentially fast, sensitive, and accurate [41*,53*–55*]. This strategy involves mass determination of peptide fragments generated from the protein of interest by

an enzyme (e.g. trypsin) or chemical reagent with high specificity, followed by screening of these masses against an appropriate database of peptide fragments. The fragment database is calculated from a protein database using the known properties of the cleavage reagent. Proteins have been correctly identified using as few as 3–6 proteolytic peptides, with mass measurement accuracy between 0.1–0.01% [41*,53*–55*], and the approach has sufficient sensitivity for use with proteins separated by two-dimensional electrophoresis.

Although the molecular weight of the protein alone can be used as a means of identification, the requirements both of high accuracy and of complete information on all modifications in the mature protein often prove too stringent for practical use. On the other hand, an approximate molecular weight is likely to be a highly useful constraint in the proteolytic peptide screening strategy [53*]. In addition, the mass spectrum of the intact protein can also provide an indication of the presence of undesirable peptide and protein impurities. The effect of such impurities on the accuracy of this approach for protein identification is an important practical issue that remains to be studied. The possibility has been raised of correlating MS peptide maps of a protein both with expressed sequence cDNA tags corresponding to the protein and, ultimately, with the full-length cDNA sequence [53*].

Assessing the integrity and purity of protein preparations

It is often necessary to assess the integrity and purity of native, recombinant, or synthetic protein preparations either in a single preparation or to compare these properties in different preparations. Mass measurement provides one of the most rapid, straightforward and informative routes for such assessment, which is usually carried out by molecular weight determination followed by more detailed peptide mapping (if necessary). Examples include verification of recombinant [40,42,56] and synthetic proteins [57], assessment of the purity of proteins used to grow crystals for X-ray diffraction measurements [58], and comparison of different forms of a protein having distinct biological activities [59]. Although the mass spectra can demonstrate unequivocally the presence of impurities, the quantitative aspects of the new MS tools [1] remain to be carefully elucidated.

Probing interactions and conformations of proteins

A mounting body of data indicates that MS can contribute significantly to the study of both non-covalent and covalent protein interactions with other molecules [60*]. The MS information can be complementary to

that provided by more traditional approaches such as ultracentrifugation or gel electrophoresis. Specific non-covalent interactions (e.g. protein–protein [61*,62*], protein–peptide [63*], protein–substrate/inhibitor [61*,64], receptor–ligand [65], and protein–co-factor [66]) have been observed by direct MS determination of the mass of the intact complex. Assessment of the complex formation of metal-containing ligands [67–69] and metal ions (e.g. Zn^{2+} and Cd^{2+}) with metal-binding proteins has also proved feasible [70,71*,72]. The most suitable method for performing such measurements appears to be electrospray ionization because it is carried out on proteins extracted directly from solution and because it is a relatively gentle means of ionization [1,3]. Limitations to this approach arise both because high concentrations of many common buffers and salts quench electrospray ionization mass spectra and because the energy inserted into the complex to remove solvent molecules may be sufficient to dissociate the complex into its components. Elucidation of the energetics of gas-phase desolvation and complex dissociation is required to assess the general applicability of the method and to relate the MS observations to association stoichiometries of the interacting components in solution.

Investigations of non-covalent interactions of proteins have long been carried out by determining the solvent accessibility of amino acid residue side chains and amide hydrogens through the use of a variety of chemical and physical techniques. Side-group reactivity [73*] and the proclivity for hydrogen/deuterium exchange of particular polypeptide backbone amides [74*] can also be determined by MS. Although the quantitative aspects of these measurements remain to be explored, potential advantages of the MS approach include high sensitivity, speed, and specificity. It is more straightforward to investigate covalent interactions between proteins and other molecules because of the greater stability of covalent complexes. Thus, for example, accurate molecular weight determination has been used to probe the mechanism of inhibition of enzymes by mechanism-based inhibitors [75*,76*], to directly observe UV-crosslinked protein–nucleic acid complexes [77], to determine the multimeric states of proteins coupled through chemical crosslinks [78], and to identify crosslinked amino acids in protein pairs from complex assemblies of proteins [79].

Under appropriate conditions, MS can also be used to probe certain aspects of protein conformation. For example, changes in the conformation of proteins in solution can be detected through changes in the distribution of the charge states of protein ions observed in electrospray ionization mass spectra [80,81,82*], through observed differences in the rate or degree of hydrogen/deuterium exchange [83*–85*], or through differences in the rate of proteolysis of selected amino acid residues [86*]. Recent observations even appear to indicate that a certain degree of high-order structure is maintained into the gas phase and can be detected [87*,88*].

Prospects for the future

The new MS techniques are undergoing continued development and improvement, and are being applied to an expanding array of biological problems.

Thus, electrospray ionization MS is being used to examine ever larger [89] and more intractable [90] proteins, a trend that will likely continue. Although the robust and effective quadrupole mass analyzer endures as the mainstay of electrospray ionization MS, other types of analyzer are currently undergoing evaluation. These include the efficient, high mass-range time-of-flight analyzer [91*,92*], the high resolution double-focusing deflection analyzer [93], the ultra-high resolution Fourier transform analyzer [94*], and the versatile quadrupole ion trap analyzer.

The versatility of matrix-assisted laser desorption/ionization is being further enhanced through the introduction of improved procedures for sample preparation. These render the method ever more immune to interference by contaminants and common biological additives [95*]. The possibility of generating mass spectra directly from the blots of one and two-dimensional PAGE [96*,97*], and the use of powerful affinity-based biochemical methods, in conjunction with MS [98*], will greatly increase the power and accessibility of the technique.

The extent to which MS will impact on biological research will depend, to a large degree, both on the production of effective commercial instrumentation and on the development of methods for making MS easily accessible to biologists.

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