

Protein complexes and analysis of their assembly by mass spectrometry

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The utility of mass spectrometry for the analysis of proteins has grown enormously in the past decade. Significant advances in detection and ionization techniques are allowing questions about noncovalent assembly to be addressed by the direct observation of gas phase complexes, their assembly in real time and their disassembly by perturbation of solution or instrument conditions. These technological innovations have plainly captured the imagination of biological researchers. Recent and novel developments include the combination of mass spectrometry with isotopic labeling, affinity labeling and genomic information. Collectively, these advances are opening new doors to the isolation of complexes, the identification of their substituents and the characterization of their conformations and assembly.

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Abbreviations

ESI	electrospray ionization
FWHM	full width at half maximum
IAPP	islet amyloid polypeptide
ICAT	isotope-coded affinity tagged
ICR	ion cyclotron resonance
MALDI	matrix-assisted laser desorption ionization
MS	mass spectrometry
MT	metallothionein
m/z	mass/charge
TOF	time of flight

Introduction

A mass spectrometer can be divided in two parts. A source, which generates gas phase ions, and a detector, which measures their mass/charge (m/z) ratio. The study of protein conformation by mass spectrometry (MS) requires mild ionization conditions, which are typically accomplished by electrospray ionization (ESI) [1]. In this technique, a solution of analyte is drawn from the tip of an electrospray needle by establishing a potential difference (1–5 kV) between the capillary needle tip and a metal cone. The charged droplets that emerge evaporate down to a critical size. This is followed by desorption of the protein ions from the solution to the gas phase. Historically, this process has used high temperature and organic solvent to aid evaporation, and acid to aid charging and increase solution conductivity. All of these conditions are detrimental to the maintenance of native structure. The use of smaller orifice capillaries [2] and the substitution of volatile salts for acid allow routine ionization of macromolecules from native conditions. Of particular note are the low material

requirements of this method. Submicroliter quantities of submicromolar solutions are readily analyzed.

Two types of detector have dramatically improved in recent years. Ion cyclotron resonance (ICR) uses superconducting magnets and electric fields of sufficient magnitude that ions are captured and precess [3]. Excitation and detection of ions is performed using radio frequency techniques similar to those used in NMR spectroscopy. ICR obtains resolutions greater than 100,000 measured as mass divided by full width at half maximum (FWHM). This is sufficient to resolve isotopic peaks (resulting principally from the natural abundance ^{13}C) for proteins with masses up to 100 kDa. Time of flight (TOF) uses an electric field to propel ions down a tube, where they strike a detector. As the propelled ions have nonuniform kinetic energy, a wide distribution of flight times and, consequently, m/z ratios is measured. A reflectron at one end of the tube narrows this distribution by reflecting the ions back down the tube in a V-shaped path. The recent coupling of this technique to continuous flow ion sources such as ESI [4] allows the routine measurement of complex mixtures at resolutions of more than 5000 FWHM. The disparity in the resolution of these detectors is complemented by the disparity in their mass ranges. The practical limit of ICR detection is m/z ratios of less than 5000 Da/e, whereas TOF detects m/z ratios in the tens of thousands. In this review, I summarize a broad range of recent applications of these techniques, with particular attention given to the analysis of protein complexes and their assembly. Central to many of these analyses are exciting new approaches to making quantitative comparisons of multiple signals in a mass spectrum.

Direct measurement of protein assemblies

The ionization of proteins from native conditions results in a charge state distribution that is narrower and shifted to higher m/z ratios than proteins ionized from denaturing conditions. This effect is attributed to the increased ability of an unfolded polypeptide to stabilize charge [5]. Thus, with increasing size, the tendency toward lower charge states of folded proteins results in generally higher m/z ratios, challenging the limits of detection. Mild ionization techniques were first capitalized upon to measure deuterium incorporation at labile positions [6,7], for example, the amide NH. Provided these positions are both exposed to solvent and not participating in hydrogen bonding, they exchange with protons from water via a reaction that may be either acid or base catalyzed. Substitution of D_2O for H_2O results in an increase in mass, permitting both kinetic and thermodynamic analyses to be made [8•–11•]. Proteins may or may not unfold during or after ionization; however, preservation of label requires simply that the

timescale of ionization be fast compared with unfolding and back exchange.

The recent proliferation of noncovalent complexes observed by MS suggests that native-like conformations may persist through the ionization process. Indeed, discrete protein conformations have been measured in the gas phase of an ICR ion trap [12,13*]. The measurement of noncovalent complexes is technically challenging as a result of nonspecific adduct formation, high m/z ratios and the tendency for trapped water to contribute to and broaden signals. Nonspecific protein–protein and protein–ligand complexes readily form as artifacts of the ionization process. One or more tests are required in order to be confident that mass spectral observations reflect solution behavior. Firstly, complexes are only seen in the mass spectrum under solution conditions in which complexes are also observed by lower resolution techniques (e.g. gel filtration). Secondly, binding measured by MS is cooperative, yielding discrete states, not a binomial distribution of states. Thirdly, control ligands are used to show that nonspecific association is not inherent to the conditions of analysis.

The use of MS provides an important direct measure of metal coordination. Recent work on the prion protein (PrP) [14**] and metallothioneins [15**] provide dramatic examples. Metallothioneins (MTs) are cysteine-rich proteins whose physiological roles include cation transport and protection from toxic heavy metals. Human MT-2 ionized from native conditions at pH 6.5 in the presence of zinc yields a discrete complex incorporating seven zinc ions, with little contribution from other stoichiometries. The demonstration of cooperative binding indicates that nonspecific association of metal with the protein is minimal and that the mass spectrum is representative of the solution state. Furthermore, spectra collected as a function of zinc : MT-2 molar ratios from one to six clearly show the dominant population of a four-zinc form in advance of the formation of the seven-zinc form. The structure of MT-2 is known and possesses a four-zinc center and a three-zinc center [16]. ESI-MS was able to show that the four-zinc center is formed cooperatively and is a likely prerequisite for the cooperative formation of the three-zinc center.

Measurements of stoichiometry are obviously not limited to metal binding. For example, the stoichiometry of the 11S regulatory complex of the eukaryotic proteasome has recently been investigated by MS [17**]. The complex is formed from two homologous (~50% identity) subunits, α and β , and is particularly interesting as a homomeric complex may be formed from isolated α or β subunits. Coexpression of the two subunits in *Escherichia coli* gave rise to a complex whose mass spectrum is dominated by a heterocomplex consisting of three α and four β subunits: a total molecular weight of about 200 kDa. A heptamer is consistent with a crystal structure of the homogeneous complex formed from the α subunit alone [18]. Interestingly, the observation of subcomplexes such as

$\alpha_2\beta$, but not α , is suggestive of an assembly process involving discrete heterocomplexes. A tetramer formed nonspecifically from α and β subunits would have yielded a distribution of five components, however, only one, $\alpha_2\beta_2$ was observed. Confidence that these particular subcomponents are directly related to the assembly of the heptamer is particularly high as a result of the heterogeneous composition of the complex.

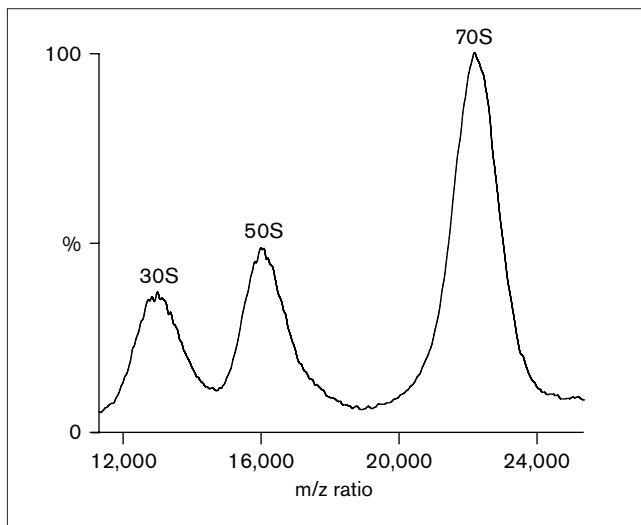
Deducing structural organization

Protein ions collide with neutral gas molecules in the source of the mass spectrometer. The energy of these collisions may be increased, for example, by raising one of the potential differences used to guide the ions through the source to the detector. These collisions can be sufficiently energetic to break covalent bonds and may be used to sequence or localize post-translational modifications [19]. They can also be used to disassemble protein complexes. For example, in the case of the 11S heptamer mentioned above, the assembly was shown to consist of a core of α/β subunits. A progressive increase in the collision energy in the source first yields an $\alpha\beta$ hexamer, suggesting relatively weak binding of the fourth β subunit.

Initial work on the *E. coli* ribosome enabled the resolution of a subset of the ribosomal components upon its disassembly in the spectrometer [20]. Not only were the masses of individual proteins observed, but a mass corresponding to a complex of five of the ribosomal subunits was also seen. These five proteins are known to be in contact in the ribosome and their observation in the mass spectrum helped to establish the utility of ESI-MS in determining protein quaternary structure. Recently, the intact 70S particle has been ionized and detected [21**]. This particle has a mass of 2.3 MDa and yielded a peak with an average m/z ratio of approximately 23,000 (Figure 1). The absence of sufficient resolution to measure individual charge states prohibited the measurement of mass directly; however, ionization of the 30S particle did provide charge state resolution, so direct measurement of mass could be made (850 kDa). The mass was consistent with the inclusion of all 21 proteins and the 16S RNA in the measured complex.

In early work, it was shown that an intact virus could be ionized [22]. Although the m/z ratio of the virus could not be detected at that time, the virus was shown to be intact by its retention of infectivity. Recently, progress has been made in measuring the supramolecular structure of a virus in an ESI-MS study of the bacteriophage MS2 [23**]. Purified monomers of the coat protein of MS2 self-assemble into a 180-unit capsid (a 2.5 MDa complex), whose mass was successfully measured to ± 25 kDa. The ability to measure the intact complex also permitted the use of gas phase collisions in the source to disassemble it. Disassembly was neither two state nor binomial in appearance, but rather revealed the cooperative release of a hexamer, as well as monomeric forms. Note that this

Figure 1



ESI-MS of the intact ribosome. At 5 mM Mg^{2+} the spectrum (not shown) is dominated by the intact 70S particle. In this spectrum, the Mg^{2+} concentration has been reduced by dilution. The complex of 57 proteins and three RNA molecules partially dissociates into components corresponding in mass to the 30S and 50S subunits identified by centrifugal sedimentation. As these complexes are large, individual charge states are difficult, but not impossible, to resolve. In this figure, each of the three peaks represents an envelope of charge states. Reproduced with permission from [21••].

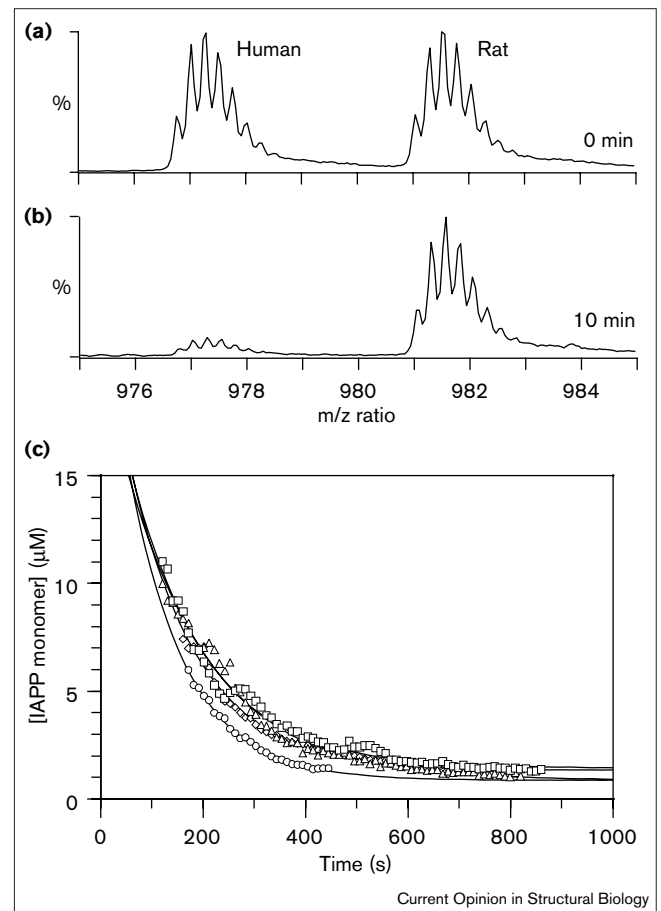
assembly information is critical to an independent determination of stoichiometry. Even though the error in the measurement (± 25 kDa) exceeds the monomer mass (13.7 kDa), it is well below the hexamer mass (82 kDa), that is, the capsid is composed of 30 hexamers.

Measuring the kinetics of assembly

Amyloid fiber formation is the process by which an ordinarily globular protein or unstructured peptide misfolds and aggregates to form unbranched assemblies rich in β sheet and heterogeneous in length [24]. These fibers are central to a number of diseases, for example, Alzheimer's and type II diabetes, and recent work has suggested that fibril formation may be a generally accessible and low-energy conformation of all proteins [25•]. Fibers are often very stable, resisting dissolution in organic solvent and chaotropic salts, thermal denaturation and proteolysis. One example is the 37-residue peptide hormone islet amyloid polypeptide (IAPP), which forms amyloid fibers in the pancreas of type II diabetics. Recently, the extreme stability of IAPP fibers has been shown to extend into the gas phase [26••]. This stability permits the real-time measurement of fiber formation kinetics by monitoring the consumption of the precursor.

IAPP from rats is nonamyloidogenic as a result of six amino acid changes, including three proline substitutions, in the putative amyloidogenic core of human IAPP. These variations also render the protein 17 Da heavier than human

Figure 2



ESI-MS of amyloidogenic (human) and nonamyloidogenic (rat) variants of IAPP. Human and rat variants ionize with identical efficiency.

(a,b) A fiber formation reaction quenched by dilution at discrete time points and mixed with a known amount of rat IAPP. The incorporation of human IAPP into fibers depletes the precursor and leads to a decrease in the human signal intensity relative to that of rat IAPP. (c) Alternatively, rat IAPP can be included as an internal standard within a fiber-forming reaction of human IAPP. Normalization of the human signal with the rat signal permits the absolute concentration of the precursor to be measured in real time during the reaction. Four independent runs are shown.

IAPP, allowing delineation in a mass spectrum (Figure 2a). The demonstration that rat and human IAPP ionize identically allows rat IAPP to be used as an internal standard to measure the concentration of human IAPP. Measurements during fiber formation can be made by removing aliquots and adding rat IAPP (Figure 2a,b). Alternatively, a reaction can be conducted in the electrospray needle, allowing the absolute concentration of nonfibrillar forms of IAPP to be measured in real time (Figure 2c). MS has enabled novel and complementary measurement of the fibrillogenesis process. Whereas other techniques for studying amyloid formation measure product formation, for example, by light scattering or the binding of histological dyes, MS can measure the consumption of precursor in real time.

These studies were facilitated by the extreme stability of IAPP fibers. It is noteworthy, therefore, that harsher ionization techniques, such as matrix-assisted laser desorption ionization (MALDI), are showing promise in the assessment of noncovalent assembly. This technique has permitted a number of recent insights into, for example, HIV-receptor interactions [27*] and epitope mapping of antibodies raised against influenza [28*].

Quantitative mass spectrometry

Central to measurements of the amyloid precursor by MS is the requirement that signal intensity be related to solution concentration. Generally, precision in biological MS is thought of in terms of the x or m/z axis. Signal intensity is highly variable from sample to sample and from scan to scan. In the case of fibrillogenesis, mentioned above, a chemically related, but nevertheless different, protein was required for direct measurement of concentration. This is advantageous as the internal standard controls variability in both instrument response and sample manipulation. Valid concerns about the relative ionization efficiency are eliminated in instances in which internal standards are chemically identical, but isotopically distinct. For example, in protein folding studies, ¹⁵N-enriched lysozymes were used to make precise comparisons of the relative folding properties of avian lysozyme variants [29] and, more recently, human amyloidogenic mutants [30].

Isotopic labeling can also be performed by growing *E. coli* in media depleted of the naturally abundant isotopes of carbon (¹³C) and nitrogen (¹⁵N) [31]. For example, a protein with 1000 carbon atoms (~22 kDa) will yield a protein with an average molecular weight that is 11 Da lighter than at natural abundance. This novel label has recently been capitalized upon to determine the relative expression of proteins in *E. coli* [32**]. Differential expression in *E. coli* was measured in response to heavy-metal (Cd²⁺) stress. Cells grown in isotopically depleted media and Cd²⁺ gave rise to proteins whose mass was dominated by a narrow, more nearly monoisotopic peak. Cells grown in normal media gave rise to proteins whose mass was sufficiently shifted that a mixture of normal and ¹³C-depleted protein could be resolved. The two cultures were therefore mixed before lysis, separation and mass analysis. As sample manipulation and ionization are, thereafter, necessarily identical, relative expression levels were obtained with a reported variability of up to 10%.

The use of differential proteome analysis by isotopic labeling and MS has also been developed and applied in yeast. Isotope-coded affinity tagged (ICAT) reagent combines a biotin for affinity tagging, a thiol reactive group for labeling reduced cysteine and a linker [33**]. The linker has eight positions that can alternatively contain aliphatic ¹H (light ICAT) or ²H (heavy ICAT). Protein is then harvested from two separate cultures, reduced and reacted with light and heavy ICAT, respectively. The proteins are then combined, digested with protease and

readily isolated using an avidin affinity column. ESI-MS of the isolated peptides yields doublets of each peptide whose areas directly reflect the relative proportion of each species. Note that there are three components of information known that aid identification: mass, protease specificity and the number of cysteine residues in the fragment. This information alone is capable of identifying a large fraction of the parent proteins without further fragmentation [34]. As a proof of principle, yeast cells were grown on either ethanol or galactose as a carbon source. Relative expression level changes of both high (100:1) and low (1.6:1) magnitude were measured with very low variability (<10%). Although many advances have been made in the measurement of mRNA expression, determination of the correlation between mRNA and protein expression levels is an active area of research [35,36]. For the direct determination of protein expression, this work marks a significant advance in the use of MS for proteome analysis.

Conclusions

The measurement of intact complexes and their disassembly in the mass spectrometer is clearly becoming a new tool for quaternary structure determination. Stoichiometry may be measured and the identification of stable subsets of protein complexes details both the structural organization and the pathways of assembly. In essence, these studies, as well as those using hydrogen-deuterium exchange labeling, are aimed at the analysis of proteins whose masses are already known. The focus, then, is on mass shifts associated with the formation of complexes or changes in signal intensity. This role for MS is often thought of as distinct from the utility of MS in identifying unknowns from protease fragments and genomic information. On an analytical level, however, several issues overlap, particularly quantitation and reproducibility. Clearly, the past year has seen a number of creative developments in instrumentation and sample manipulation, and in the scope of biological systems that have been addressed. The synergy of ideas from these varied applications of MS has greatly broadened and significantly increased the utility of MS in the postgenomic era.

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