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DIRECT CHARACTERIZATION OF PROTEIN COMPLEXES BY ELECTROSPRAY IONIZATION MASS SPECTROMETRY AND ION MOBILITY ANALYSIS

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1.1 INTRODUCTION

Beyond its primary, secondary, and tertiary structures, the quaternary structure of a protein can be defined as its interactions and associations with other proteins, macromolecules, and ligands that conspire to define its biological function. Thus, the structural determination of protein complexes can play an important role in the fundamental understanding of biochemical pathways. Traditionally, researchers have a variety of tools at their disposal to probe and measure such interactions. These tools include ultracentrifugation, light scattering, yeast two-hybrid, surface plasmon resonance, affinity chromatography, and native gel electrophoresis, and the methods that provide an “image” of the protein complex, such as cryoelectron microscopy, nuclear magnetic resonance (NMR) spectroscopy, and X-ray crystallography. Each of these methods has its advantages and disadvantages, and each provides a defined level of information detail, from low-resolution assembly size information (e.g., dynamic light scattering) to high-resolution structure from NMR and X-ray.

Mass spectrometry (MS) is becoming a tool for probing noncovalently bound protein–ligand associations. Its popularity is increasing for several reasons, including the impressive results from a number of researchers worldwide, including Carol Robinson [1] and Albert Heck [2], who have demonstrated the capabilities of MS to measure protein complexes as large as the 2 MDa ribosome [3]. In addition, the general field of proteomics has featured prominently and has encouraged more biochemical scientists to apply mass spectrometry into their research strategies. Perhaps the greatest incentive for the increasing interest in mass spectrometry is the improvements in the technology; sensitivity, resolving power, and mass accuracy have been improving steadily, and the availability of more MS systems tailored to specific requirements (e.g., laboratory space, budget) is increased. Although most of the improvements have targeted peptide-centric analysis for protein sequencing and identification, these improved features have benefited also the analysis of intact proteins and protein complexes.

As demonstrated by the pioneering work of John Fenn, who was awarded the Nobel Prize in Chemistry in 2002 for his development of electrospray

ionization (ESI) [4], taking liquid solutions and aerosolizing them into the vapor state has unique advantages for measuring large biomolecules. Not only can the molecular weight of proteins be measured very accurately, especially with higher resolution mass spectrometers, such as the time-of-flight (TOF) analyzer, the Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer and, more recently, the OrbiTrap analyzer, but sequence information can be derived, either from the intact protein directly (e.g., top-down sequencing) or from proteolytic fragments (e.g., bottom-up sequencing) in combination with tandem mass spectrometry (MS/MS). However, the solution phase origin of ESI-MS is a unique advantage, compared to matrix-assisted laser desorption/ionization (MALDI), for the analysis of protein complexes. Most protein interactions important to biology persist in an aqueous environment under so-called “physiological” conditions. The majority of biophysical methods used to probe protein complexes *in vitro* cannot accommodate all of the biochemicals necessary to define the “physiological” state of a cell. (As an example, the next time you run across a paper describing the high-resolution X-ray crystal structure of a protein, read the conditions necessary to crystallize the protein. When was the last time one encountered polyethylene glycol in a cell?) It is assumed that the structure of many proteins in a water environment and at near neutral pH is not perturbed significantly compared to their physiological state. This allows ESI-MS to analyze directly proteins in aqueous solution at near neutral pH. In some examples, the secondary and tertiary structures can be probed by gas phase methods, such as hydrogen–deuterium exchange and ion mobility. Moreover, the protein interactions are sufficiently retained upon the transition to the gas phase that the size and binding stoichiometry can be measured. Thus, the ability of ESI to ionize macromolecules without disrupting covalent bonds and maintaining the weak noncovalent interactions is a key distinguishing feature of ESI for the study of biological complexes [5]. The molecular mass measurement provides a direct determination of the stoichiometry of the binding partners in the complex, even for multiligand heterocomplexes.

1.1.1 Historical Perspective of ESI-MS for Measuring Protein Complexes

Peptide and protein associations have been reported throughout the literature of biological mass spectrometry. From the early days of field desorption/ionization (FD and FI), fast atom bombardment (FAB or liquid secondary ionization mass spectrometry, LSIMS), particle beam and thermospray, electrohydrodynamic-based desorption/ionization (EHD), laser desorption, and californium-252 plasma desorption, curious “adducts” have been observed in the mass spectra of peptides and proteins. In many cases, adducts, or the

apparent binding of another atomic or molecular species, were associated with trace levels of alkali or alkali-earth salts, such as sodium, potassium, lithium, and calcium. The binding of ubiquitously present salts helped promote the formation of ionized peptides and proteins for their observation by mass spectrometry. Also present in the mass spectra in some cases were peaks that were assigned as a peptide “dimer,” such as $(2M + H)^+$ or $(2M + Na)^+$. Such observations were explained usually as a result of nonspecific aggregation in the gas phase. The local analyte concentrations in the desorption/ionization region of the MS source were sufficiently high to promote the formation of random associations.

Such chance associations were observed also in the early days of ESI. Myoglobin and 12 kDa cytochrome *c* were (and still are) common test proteins for ESI-MS, primarily because of their relative high purity from commercial sources and their economical prices. Myoglobin is a 153 amino acid polypeptide chain that functions as an oxygen carrier through its noncovalent association with a heme (protoporphyrin IX) molecule. Cytochrome *c* similarly binds heme, but through covalent thioester bonds. Using denaturing solution conditions to perturb noncovalent heme–protein associations, such as 50% acetonitrile or methanol and high acid concentrations (pH 3 or lower), ESI mass spectra of myoglobin show multiply charged molecules for the apoprotein, whereas cytochrome *c* retains heme binding because of its covalent association. However, sometimes a set of low abundant peaks representing the binding of heme to myoglobin were observed. In addition, peaks for protein and peptide dimers can be observed, particularly if the analyte concentrations were relatively high in solution (ca. 25 μ M or higher).

The first report of specific associations probed by ESI-MS was authored by the Cornell University groups of Ganem and Henion. The intact receptor–ligand complex between FK binding protein (FKBP) and macrolides rapamycin and FK506 [6], and the enzyme–substrate pairing between lysozyme and *N*-acetylglucosamine (NAG) and its cleavage products were reported [7]. Several reports of other biochemical noncovalently bound systems using ESI-MS detection followed shortly afterwards, including the ternary complex between the human immunodeficiency virus (HIV) protease dimer protein binding to a substrate-based inhibitor [8]. The ESI-MS of the noncovalent heme–myoglobin complex was reported first by Katta and Chait [9]; dramatic differences in the myoglobin spectra measured from aqueous solutions between pH 3.35 and pH 3.90 were observed. Myoglobin is fully denatured at pH 3.35, and the mass spectrum shows only ions for the apo or nonbinding form of the protein. The protein exists in its native configuration at pH 3.90, thus allowing the protein to fold properly, and the noncovalent binding of a heme molecule occurs. The effect of solution thermodynamics and the relative stability of the gas phase complex was studied

shortly afterwards [10, 11]. Ribonuclease S (RNase S) is composed of the hydrophobically bound 11.5 kDa S-protein complexed to 2 kDa S-peptide. In solution, S-peptide binds to S-protein with a solution binding constant, K_D , around 1–10 nM; the solution temperature dependence on the gas phase stability was predicted from thermodynamic parameters.

These early examples established not only the feasibility of the ESI-MS method, but also the design of the experiment to ensure validation of the observations. The validity of the results needs to be established in order to assess a meaningful interpretation and link to the solution phase system. These papers also helped move biology, biochemistry, and medicinal chemistry to the forefront of applications for ESI-MS technology.

As smaller protein–ligand complexes were found to be amenable to ESI-MS measurements, the ability to access larger molecular weight complexes was tested. However, as larger complexes were tested, it was found that the relative charging is low compared to the accessible mass-to-charge (m/z) range of instruments employed by most laboratories during these early days of ESI. For example, shown in Figure 1.1 is the ESI-MS of yeast alcohol dehydrogenase, a homo-tetrameric protein complex of 147 kDa; molecular ions are observed above m/z 4000.

The quadrupole mass analyzer was the system favored by John Fenn as ESI was being developed. One of the advantageous features of ESI is the multiple charging that allows most mass analyzers of limited m/z range to be used for biomolecule analysis. Most quadrupole mass analyzers are of limited

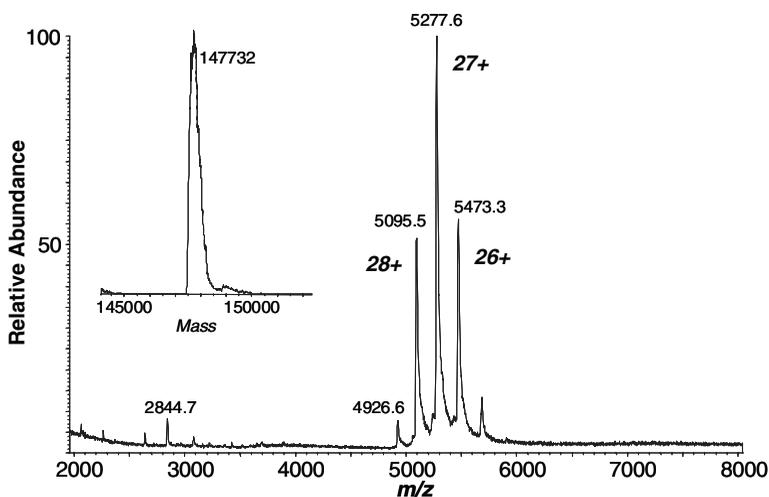


Figure 1.1 ESI QqTOF mass spectrum of yeast alcohol dehydrogenase (5 μ M, 10 ammonium acetate, pH 6.5). The inset shows the mass deconvoluted spectrum, indicating a molecular weight of 147.7 kDa for the intact tetrameric complex.

m/z range, typically less than 4000. However, multiply charged ions for complexes such as protein–protein quaternary complexes exhibit relatively low charge at high m/z . The amount of charging that a biomolecule exhibits in an ESI mass spectrum has been correlated to a global solution structure [12]. The relatively narrow charge distribution of a low-charge state (typically four to five charge states) represents retention of the higher order structure of the native protein complex, presumably because fewer charge sites are exposed and/or the Coulombic restraints restrict charging for the compact structure. A magnetic sector ESI mass spectrometer, in general, has sufficient m/z range (to m/z 10,000) to study protein complexes such as alcohol dehydrogenase and pyruvate kinase and other quaternary protein structures [13, 14]. However, the sensitivity and resolution at very high m/z of the time-of-flight (TOF) analyzer provides an ideal system for large noncovalent complexes. This was first demonstrated by Standing and co-workers with the large protein complexes from soybean agglutinin [15] and extended by a variety of other protein systems [16]. Today, the ESI-TOF and the quadrupole time-of-flight (QTOF) mass spectrometers are the systems of choice for most ESI measurements of protein noncovalent complexes [17, 18].

1.1.2 Types of Interactions that Are Probed by ESI-MS

The fundamental forces of almost all noncovalent interactions in water include hydrophobic effects, hydrogen bonds, salt bridges, van der Waals interactions, and Coulombic interactions. Yet, these types of interactions that govern noncovalent binding in solution sometimes play only a limited role in the observed MS results from the ESI-MS gas phase measurements. The transition from a high-dielectric environment (i.e., water) to a solventless vacuum environment strengthens electrostatic interactions, and thus complexes held together by electrostatic interactions are extremely stable in the gas phase. Protein–nucleic acid complexes, noncovalent complexes between a highly positively charged molecule and a negatively charged macromolecule such as human immunodeficiency virus (HIV) Tat peptide–TAR RNA complex and the NCp7 – ψ -RNA complex, are extremely stable, as the complexes are not observed to dissociate at very high collision energies [19–21]. Hydrophobic interactions in solution appear to be weakened in vacuum. For example, the relative affinities measured by ESI-MS for small molecule hydrophobic binding to acyl CoA-binding protein do not correlate with their solution affinities [22]. The differences between electrostatic and hydrophobic interactions in the gas phase are further highlighted by inhibitor binding studies to HIV-1 TAR RNA [23]. Positively charged aminoglycosides such as neomycin are known to bind to RNAs through charge–charge interactions. The neomycin–TAR RNA complex was not observed to dissociate in

the gas phase. However, inhibitors with similar solution binding affinities to TAR RNA that bind through hydrophobic-type means are extremely labile in the gas phase.

The strengthened role of charge–charge electrostatic forces in gas phase stabilities can be exploited for measuring weak, solution phase interactions. The pathological hallmark of the neurodegenerative disorder Parkinson’s disease (PD) is the presence of intracellular inclusions, called Lewy bodies, in the dopaminergic neurons of the substantia nigra. Filamentous α -synuclein (AS, M_r 14460) protein is the major component of these deposits and its aggregation is believed to play an important role in Parkinson’s disease. AS binds to natural polycations, such as spermidine and spermine. A previous NMR study suggested that spermine (M_r 202) binds to the C-terminal acidic region of AS with a solution binding affinity (K_D) of 0.6 mM [24]. ESI-MS with a QqTOF system shows the ability to measure binding affinities in the low millimolar range for the 1:1 AS–spermine complex [25]. The stability for such weakly bound ligands is enhanced in the gas phase because of charge–charge electrostatic interactions.

Likewise, protein–metal ion binding can be quite stable in the gas phase. For example, human superoxide dismutase (SOD) is a small 32 kDa homodimeric protein that binds transition metal ions, such as zinc and manganese. Each protein monomer has two metal binding sites. Figure 1.2 shows the ESI mass spectrum of human SOD in the presence of excess zinc; multiply charged SOD dimer proteins are observed to bind to four zinc metals

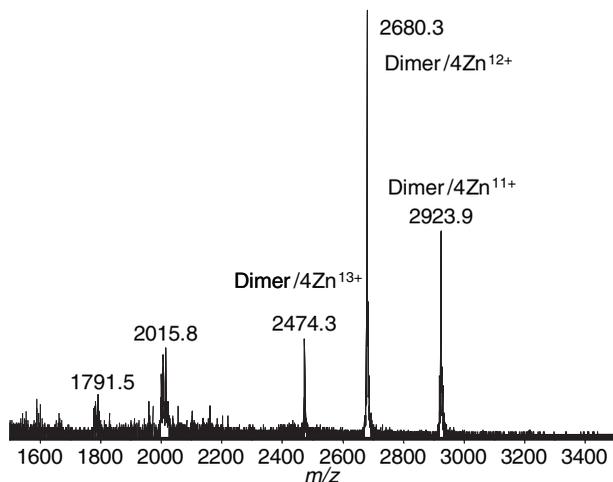


Figure 1.2 ESI QqTOF mass spectrum of human superoxide dismutase (SOD; 10 mM ammonium acetate, pH 7.5) in the presence of excess zinc chloride. Multiply charged ions for the SOD dimer bound to four zinc ions are labeled.

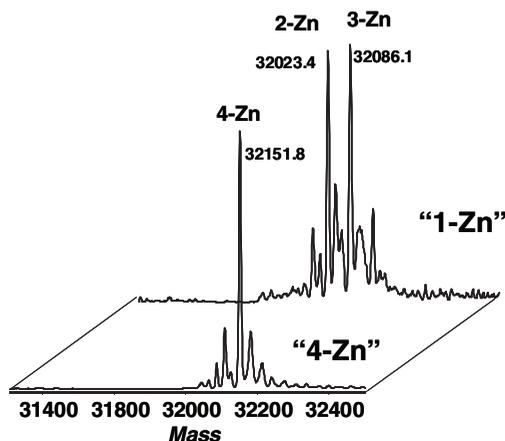


Figure 1.3 ESI QqTOF mass deconvoluted spectra of human superoxide dismutase (SOD; 10 mM ammonium acetate, pH 7.5) in the presence of excess zinc chloride (labeled as “4-Zn”) and substoichiometric zinc (labeled as “1-Zn”).

in total. One can titrate zinc metal into a solution of apo-SOD dimer to observe metal binding occupancy (Figure 1.3). Dissociating the gas phase SOD dimer–metal ion complex results in protein monomer release while retaining two zinc metal ions per monomer; that is, the protein–metal interaction is stronger than the protein–protein interaction in the gas phase.

The different relative stabilities of gas phase interactions have implications for using ESI-MS to determine solution phase absolute and relative binding affinities. For compounds that bind to a target molecule with similar type binding mechanisms, and thus may have similar gas phase stabilities, determining their relative binding affinities by ESI-MS should not be problematic. However, if hydrophobic interactions are in play, the lability of the gas phase complex may conspire to reduce the confidence of the MS data.

1.2 CRITICAL ASPECTS OF THE EXPERIMENTAL PROCEDURE

1.2.1 Instrumental Parameters

For the most part, the researchers active in the early days of ESI who explored its application for measurement of noncovalent protein complexes found the experimental parameters critical for the success of the experiments. Besides new developments in MS analyzers that offer higher sensitivity, resolution, and m/z range for larger protein complexes, the critical parameters found during these early days persist in today’s experiments.

1.2.1.1 Electro spray Ionization Source Solution flow rates of 1–10 $\mu\text{L}/\text{min}$ were common for a majority of ESI-MS applications, and this was used also for the measurement of protein–ligand complexes. However, difficulties for ESI of aqueous solutions were found until the development of nanoelectrospray. Work by Caprioli [26] and Mann [27] demonstrated the effectiveness of ESI at significantly lower flows, down to 10–200 nL/min , and this played a significant role in not only ESI-MS of biomolecules in general, but also in the study of noncovalent complexes. The advantages of nanoliter per minute analyte flow includes not only reduction of the overall consumption of precious sample without compromising signal intensity, but also the generation of smaller droplets, which results in increased signal levels.

Nanoelectrospray helps in the requirement for droplet desolvation for noncovalent complex studies. Desolvation of ESI from aqueous solutions is not as easy as found for aqueous/organic solvent mixtures because of reduced volatility. The generation of smaller diameter droplets from small-orifice ESI needles aids the desolvation process. For some examples, adding heat externally to the ESI spray region (e.g., heating of a countercurrent gas flow) may also help desolvate the aqueous droplets. However, depending on the solution and gas phase stabilities of the complex, increasing solvent/droplet temperatures may destabilize the noncovalent complex. In fact, examples have been reported that utilize solution cooling to improve the stability of the complex to be measured [28].

1.2.1.2 Atmosphere/Vacuum Interface and Pressure Nearly all types of atmospheric pressure/vacuum interfaces for ESI-MS have been used successfully for the analysis of noncovalent protein complexes. These range from nozzle–skimmer interfaces, heated metal or glass capillary inlets, to the orthogonal “Z-spray” interface used on Micromass/Waters systems. For all cases, optimal tuning of each of the various parameters associated with each interface type is critical for efficient transmission of the noncovalent protein ions. It is somewhat analogous to a restaurant waiter balancing an egg on his/her head while walking quickly between tables. The waiter could run to each table, but it does no good if the egg drops to the floor, breaking open its contents. Each lens within the interface has its optimal settings for transmitting the highest intensity ion beam, but each region between lenses can subject the fragile protein complex to collisional dissociation [13, 29]. Moreover, ion desolvation is effected in the interface region. Maximal desolvation to generate the narrowest spectral peaks is desired, while minimizing apparent dissociation of the noncovalent complex. Because of small differences in the geometry and vacuum pressures of each ESI interface, tuning conditions between instruments of the same interface may differ slightly.

Counter to traditional mass spectrometry philosophies that encourage high vacuum for establishing high performance, ESI-MS of large proteins utilizes

low vacuum in the interface region while somehow utilizing high vacuum in the measurement/detector region, which requires nine orders of magnitude in differential pumping. Perhaps it is fitting for a gas phase technique that measures analytes originating from solution, but several reports have shown that transmission of high-mass ions requires pressures in the first vacuum stages of the mass spectrometer to be increased by reducing the pumping speed or by adding a collision gas in the collision quadrupole of the QTOF. Krutchinsky et al. [30] have suggested that larger ions may acquire substantial kinetic energies (of more than 1 keV) when they are electrosprayed out of the supersonic jet. This may have a negative effect on the transfer of ions and the orthogonal extraction into the TOF region. The increased pressure in the preceding quadrupoles/hexapoles may act as a collisional dampening interface. The enhancement is most noticeable for very large assemblies observed at high m/z , as demonstrated by the reports on protein complexes in excess of 1 MDa [18, 31]. It is now widely accepted that a combination of collisional dampening, increased cooling of the ions, and more efficient desolvation is critical for the sensitive detection of large ions at high m/z [32].

1.2.1.3 Mass Spectrometry Analyzers Although the majority of current ESI-MS research projects for studying noncovalent protein complexes utilize time-of-flight (or quadrupole TOF) analyzers [33], there is no inherent operational characteristic of the analyzer that limits its use for such studies. However, the accessible m/z range (and its associated sensitivity and resolution) is the overriding factor when choosing the appropriate system. Nearly all types of mass analyzers have been used for these types of studies. These range from single and triple quadrupoles [10], forward- and reverse-geometry magnetic sector analyzers [13], quadrupole and linear ion traps [34], TOF and QTOF [35], to Fourier transform ion cyclotron resonance (FT-ICR) instruments [36]. The ion measurement timescale ranges from microseconds to milliseconds, with no apparent correlation between timescale and performance. There are distinct advantages for using analyzers with tandem mass spectrometry (MS/MS) capabilities, as dissociation of the gas phase complex can yield information on the nature of the ligand association (see later discussion).

In general, however, the m/z range and the overall sensitivity of the analyzer are the overarching factors when selecting an appropriate instrument. In general, the larger the molecular weight of the protein complex, the larger the m/z range needed to measure the full envelope of multiply charged molecules. This is especially true for native proteins and protein complexes as discussed earlier. Native protein mass spectra show typically only a few charge states and much reduced absolute charging compared to their denatured forms. Most denatured protein mass spectra show multiple charging in the 800–3000 m/z range, regardless of their size. Thus, even for a relatively small protein–ligand

complex, such as the 17.5 kDa myoglobin–heme complex, the 8+-charged molecule would be observed around m/z 2200, outside of many quadrupole and ion trap analyzers (although the 9+ and 10+ molecules would be within the available m/z 2000 range) [37]. Because of the efficient transmission of higher m/z ions, TOF and QTOF analyzers are the popular choices for measuring larger protein complexes. Standard TOF and QTOF analyzers have m/z ranges of 5000–10,000 that are well within the range necessary for many protein complexes. However, for very large protein complexes, such as larger than 0.5 MDa, m/z ranges above 10,000 may be necessary. Kaltashov has empirically found a near linear relationship between $\ln(N)$ versus $\ln(S)$, where N is average charge for a native protein and S is surface area based on available crystal structures [12]. Thus, for a 690 kDa 20S proteasome complex, composed of 14 α -subunits and 14 β -subunits, multiple charging for the fully assembled 28-mer is observed around m/z 11,000 with an average of 63+ charges [35]. Dissociation of the 28-mer generates ions for the 27- and 26-mer aggregates that span from m/z 15,000 to 35,000 (Figure 1.4).

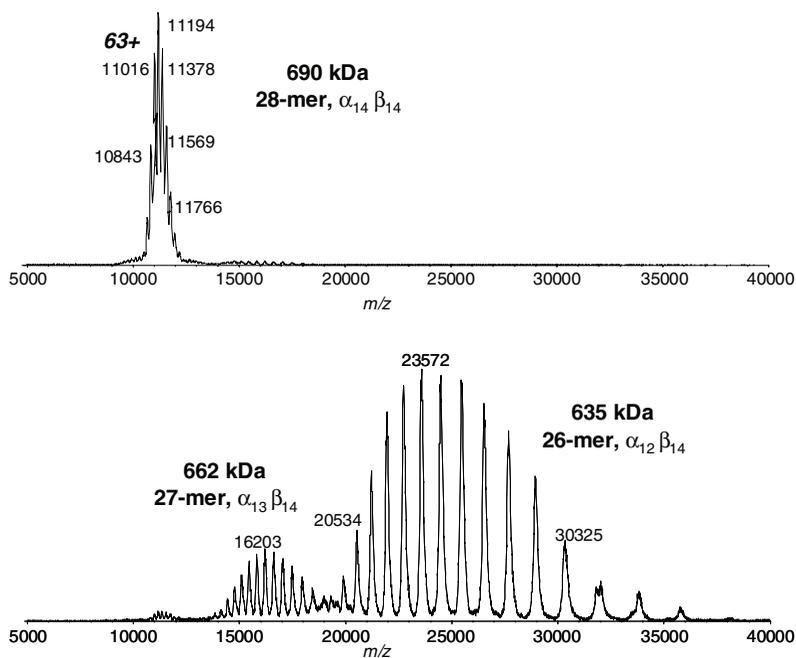


Figure 1.4 (Top) ESI-QqTOF-MS of the *M. thermophila* $\alpha_7\beta_7\beta_7\alpha_7$ 28-mer 20S proteasome with an orifice potential of +100 V. (Bottom) ESI-QqTOF-MS of the 20S proteasome with the orifice potential of +300 V. Dissociation in the atmosphere/vacuum interface of the $\alpha_7\beta_7\beta_7\alpha_7$ complex yields the liberated 27.4 kDa α -subunit (not shown) and the remaining $\alpha_7\beta_7\beta_7\alpha_6$ (or $\alpha_{13} \beta_{14}$) and $\alpha_{12} \beta_{14}$ complexes.

Although FT-ICR analyzers have demonstrated impressive resolution capabilities, well over 1 million resolving power, very few studies have demonstrated comparable high-resolution results for noncovalent protein complexes above the m/z 2000 limit. Similarly, the newer OrbiTrap analyzer is capable of resolution above 100,000 with low parts-per-million mass accuracy [38]. But to date it has not demonstrated comparable performance for measurement of ions above m/z 3000. Thus, it remains to be seen whether high-resolution FT-ICRs and OrbiTraps will be applied for studies of larger protein complexes. However, for small proteins (e.g., 20–35 kDa) binding to smaller sized ligands (e.g., products from combinatorial chemistry libraries), these analyzers may be of special utility for ligand screening [36].

1.2.1.4 Ion Mobility Analyzers Ion mobility spectrometry (IMS) is an electrophoretic technique that allows ionized analyte molecules to be separated on the basis of their mobilities in the gas phase, as opposed to separation based on their mass-to-charge ratio in conventional mass spectrometry. However, coupling IMS with MS forms a powerful combination for examining protein conformers and, potentially, protein complexes. For example, Clemmer and Cooks have combined a desorption electrospray ionization (DESI) source to an ion mobility time-of-flight mass spectrometer for the analysis of proteins [39]. Analysis of 12 kDa cytochrome *c* and 14 kDa lysozyme proteins with different DESI solvents and conditions shows similar mass spectra and charge state distributions to those formed when using electrospray to analyze these proteins in solution. The ion mobility data show evidence for compact ion structures (when the surface is exposed to a spray that favors retention of native-like structures (50:50 water:methanol)) or elongated structures (when the surface is exposed to a spray that favors “denatured” structures (49:49:2 water:methanol:acetic acid)).

Similarly, Bowers and Gray studied the protein α -synuclein, implicated in Parkinson’s disease, ESI-MS, and ion mobility [40]. It was found that both the charge-state distribution in the mass spectra and the average protein shape deduced from ion mobility data depend on the pH of the spray solution. Negative ion ESI-MS of pH 7 solutions yielded a broad charge-state distribution centered at 11⁻, and the ion mobility data is consistent with an extended protein structure. Data obtained for pH 2.5 solutions, on the other hand, showed a narrow charge-state distribution centered at 8⁻, and ion mobilities in agreement with compact α -synuclein structures. The average cross section of α -synuclein at pH 2.5 is 33% smaller than for the extended protein sprayed from pH 7 solution. Significant dimer formation was observed when sprayed from pH 7 solution but no dimers were observed from the low-pH solution.

ESI-IMS, however, has not been applied as extensively to the measurement of noncovalent complexes compared to mass spectrometric detection.

Colgrave et al. [41] reported on the noncovalent complexes formed between cyclic 12-crown-4, 15-crown-5, and 18-crown-6, and acyclic polyethers with amino acids (histidine and arginine) and peptides (MRFA, MFAR, and bradykinin) using (nano-ESI) ion mobility spectrometry. The reduced mobilities for these complexes were observed and correlate well with the mass and size of the polyether. They demonstrated the ability of IMS to distinguish between cyclic and acyclic polyethers and their complexes with biomolecules based on differences in their reduced mobilities. These differences are attributed to variations in the collision cross section arising from subtle changes in conformation in these ligand–receptor complexes.

Larger protein complexes have been analyzed by an ion mobility device, termed Gas-Phase Electrophoretic Mobility Molecular Analyzer (GEMMA) [42]. The GEMMA utilizes a differential mobility analyzer (DMA) to measure gas phase electrophoretic mobility (EM) that is proportional to the electrophoretic diameter of the particle in air. The GEMMA offers utility for the characterization of proteins, glycoproteins, protein aggregates, high-mass noncovalent protein complexes, whole viruses, and nanoparticles of biological importance [42]. For GEMMA, the biomolecules are electrosprayed followed by charge neutralization of the evaporating droplets to generate primarily neutral and singly charged molecules. Alpha-particles generated by a ^{210}Po reactor ionize gas molecules in the atmosphere, producing reactive species such as H^+ , H_3O^+ , and $(\text{H}_2\text{O})_n\text{H}_3\text{O}^+$. These primary species quickly form ionized clusters 1–2 nm in size, chiefly with water molecules in the atmosphere. The clusters diffuse to the evaporating droplets, causing their charge distribution to approach a distribution centered about zero charge. When the droplets have evaporated completely, the distribution consists almost entirely of neutral macromolecules and singly charged macroions. The singly charged protein molecules are size separated through a scanning DMA according to their EMs in air, and are detected by a condensation particle counter (CPC). Their mobilities are interpreted in terms of an “electrophoretic mobility diameter” (EMD) of the gas phase protein.

The electrophoretic mobility of a particle is governed by its size and shape, and this method has been used also to characterize proteins and noncovalently bound protein complexes, showing a correlation between the experimentally derived electrophoretic mobility diameter and its predicted molecular mass [42]. The resolving power of GEMMA is approximately 10–20 in terms of the EM diameter, but this does not preclude the utility of the GEMMA measurement for large proteins. Mass measurements are based on a simple model relating molecular weight to the diameter of a sphere and an effective density. From the GEMMA measurements by our laboratory and from those reported by Bacher et al. [42] for over 50 protein complexes ranging in size from small

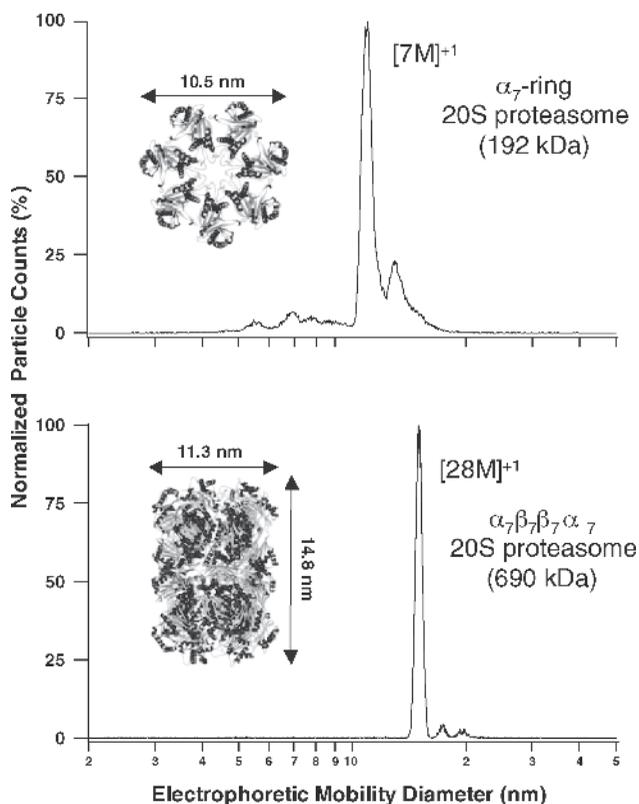


Figure 1.5 ESI-GEMMA of the α_7 and $\alpha_7\beta_7\beta_7\alpha_7$ 20S proteasome complexes from *M. thermophila*. The protein structures show the dimensions as measured by X-ray crystallography.

protein dimers to complexes as large as the 690 kDa 20S proteasome and MDa-range viral particles, an effective density of approximately 0.6 g/cm^3 can be used to estimate the molecular masses of proteins.

For example, ESI-GEMMA has been utilized to detect differences in gas phase electrophoretic mobility between the empty 20S proteasome and the 20S proteasome encapsulating protein substrates. The gas phase EMDs of the cylindrical proteasome and the disk shaped α -ring from *Methanosarcina thermophila* were consistent with crystal structure determined dimensions of the complexes from other archaea (Figure 1.5) [35]. To “trap” the substrates within the proteasome, the proteolytic activity of the complex was irreversibly inhibited prior to incubation of the complex with the protein substrates. Based on the change in GEMMA-determined molecular weight, an average of 4.5 substrate proteins were estimated to be sequestered within the complex [35]. High-resolution MS data has since shown that the 20S proteasome from *Thermoplasma acidophilum* can sequester a maximum of three or four substrate proteins of similar size [43].

1.2.2 Sample Preparation

The most critical points for the ESI-MS analysis of noncovalent protein complexes are maintaining proper solution conditions for keeping the protein complex in its folded and functional native state and effective desolvation of the ESI-generated droplets. Using the proper aqueous solvents, pH (almost exclusively near neutral physiological pH, but for some acidic proteins such as HIV protease, “native” folding and activity is maintained at acidic pH [20]), and ionic strength buffer systems are necessary to maintain complexation. Deviation from optimal solution conditions may reduce the observed relative proportion of complex formation. However, in some cases, it may be necessary to compromise solution conditions optimal for protein activity for the MS analysis because many buffers are not compatible with ESI. Volatile buffers such as ammonium acetate and ammonium bicarbonate are the most popular choices for such ESI-MS experiments because they do not often form extensive gas phase adducts with the macromolecules (as do phosphate- and sulfate-based buffers) and background ion formation is reduced without significant reduction in protein ion formation. Buffer concentrations are typically at the 5–200 mM concentration levels, but exceptions may occur, such as some protein–DNA complexes. Best sensitivity for detecting the noncovalent protein complex is obtained using nanoelectrospray sources with borosilicate or glass nanospray needles (50–200 nL/min) because of the smaller droplets formed.

Most critical to the success of the analysis is the purity and quality of the protein sample. Compared to the ESI-MS signal levels measured for pure proteins under common denaturing conditions (e.g., 50% by volume of acetonitrile or methanol with low concentrations of acetic acid or formic acid), the signals measured for proteins and their complexes can be reduced by a factor of 10 or more using “native” solution conditions, such as pH 6–8 aqueous ammonium acetate solutions. Other molecular entities that can effectively compete for the available droplet surface charges, such as peptide and protein contaminants and other small molecules, will further serve to reduce signal intensities. Extensive dialysis and the use of centrifugal membrane filtration are popular methods for salt removal and sample concentrators. All common detergents (cationic, anionic, and zwitterionic) are not tolerated well for these ESI (MS and ion mobility) experiments. Adducts formed by binding of salts (e.g., cationic sodium and potassium, and anionic phosphate and sulfate) further reduce the overall sensitivity by spreading the signal for the protein over many more channels than for the multiply protonated forms. For very large complexes in which salt adducts are not fully resolvable by mass spectrometry, adduct formation increases peak widths and can shift the peaks to higher m/z values; this reduces the ability to measure accurately

the molecular weight of the protein and protein complex. A recent strategy reported by Robinson and colleagues may help reduce some of the problems associated with adduct formation [3]. By measuring adduct formation for a variety of large protein complexes, a simulation and modeling method is developed to describe and interpret the electrospray mass spectra of large non-covalent protein complexes. Using this method, the mass accuracy for large protein complexes up to the 2 MDa ribosome is significantly improved.

1.3 SOLUTION PHASE EQUILIBRIA AND GAS PHASE DISSOCIATION

1.3.1 Measuring Solution Dissociation Constants

The correlation between the ESI-MS gas phase measurements and the solution phase characteristics has extended the application of ESI-MS to the determination of solution relative and absolute equilibrium binding constants. Competitive binding experiments, in which the total ligand concentration (single or a mixture of ligands) is greater than or equal to the protein receptor, can measure the relative binding affinities for a mixture of ligands [44]. Absolute binding constants can be derived by titration experiments and by construction of Scatchard binding plots. This was first demonstrated by Henion's group for measuring the binding constants of vancomycin antibiotics with peptide ligands [45]. The equilibrium dissociation constants of the 96 kDa dimer and 287 kDa hexameric oligomeric forms of citrate synthase binding to NADH, an allosteric inhibitor of the enzyme, was determined by Duckworth and co-workers using an ESI-TOF instrument [46]. Griffey measured the dissociation constants for oligonucleotide binding to albumin [47]. Similarly, they demonstrated the applicability of high-resolution FT-ICR mass spectrometry for measuring small molecule binding to RNA targets, and they use this strategy to screen small molecule inhibitors [48]. With ESI sources that generate stable and reproducible ion currents between multiple samples, the process of titration experiments for the purpose of measuring binding constants can be automated [49].

1.3.2 Tandem Mass Spectrometry of Protein Complexes

In general, collisionally activated dissociation (CAD) of multiply charged protein complexes held together through noncovalent bonding yields the liberated protein(s) and ligand(s). This is understandable because these are the weakest bonds found throughout the complex in solution. Also, although the results of this type of experiment may be rather "uninteresting," it can

be quite useful analytically for unknown protein–ligand systems. For example, given a solution of a single unknown protein that contains also a potential smaller molecule ligand, ESI-MS of the protein under denaturing solution conditions (e.g., 50% acetonitrile with 2% acetic acid) yields information on the molecular weight of the denatured protein. The presence of the liberated small molecule ligand may be in doubt because it is likely that many peaks appear in the m/z 100–800 range that may represent the unknown ligand. The follow-up experiment would be to acquire an ESI mass spectrum of the solution under native conditions (e.g., 10 mM ammonium acetate, pH 6.8) and measure the mass of the protein–ligand complex. This can be followed by an MS/MS experiment, in which the precursor ion for the protein–ligand complex is selectively dissociated by CAD or perhaps infrared multiphoton dissociation (IRMPD) if performed with an FT-ICR analyzer. The combination of these experiments should yield the mass of the putative binding ligand, even if multiple ligands are bound simultaneously. This is an effective experiment for mixtures of putative ligands, for example, from combinatorial libraries, as demonstrated for carbonic anhydrase and an SH2 domain protein by Smith and co-workers [50] and by Marshall and co-workers [51], respectively.

For large multiprotein complexes, MS/MS generates a liberated monomer (or a few subunits) and the remaining, much larger complex (minus the liberated molecule). For example, Figure 1.6 shows the ESI mass spectrum of the 52 kDa homo-tetrameric streptavidin complex. Although most tetrameric protein complexes are believed to be composed of a dimer of dimer proteins in solution, such as that found for streptavidin, tandem mass spectrometry of the streptavidin tetramer liberates the monomer, leaving behind a trimer

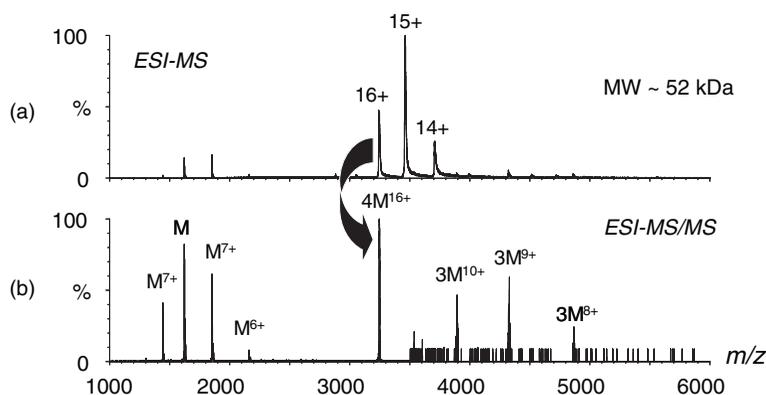


Figure 1.6 (a) ESI-QTOF-MS of streptavidin tetramer complex (10 mM ammonium acetate). (b) ESI-MS/MS of the 16+ tetramer protein, yielding product ions for the released monomer and the remaining trimer protein.

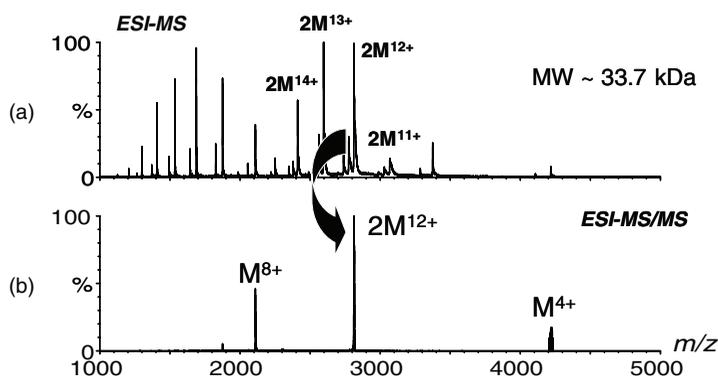


Figure 1.7 (a) ESI mass spectrum of the interferon- γ protein dimer (10mM ammonium acetate). (b) ESI-MS/MS of the 12+-charged dimer protein yields the 4+- and 8+-charged monomer products.

gas phase complex. Thus, the characteristics of the gas phase assembly may not match that found in solution. On the other hand, the dissociation mass spectrum of the 20S proteasome shown in Figure 1.4 is somewhat consistent with the general topology of the complex. Based on a $\alpha_7\beta_7\beta_7\alpha_7$ geometry, the loss of the outer α -subunits would be favored more compared to loss of the internal β -subunits because of the reduced number of potential intermolecular protein-protein contacts.

Furthermore, the distribution of charge in the products may not be evenly distributed. Jurchen and Williams [52] have reported that the asymmetric charge distribution results from unfolding of the monomer product, thus exhibiting a more flexible conformation [52]. This is demonstrated in Figure 1.7 for the MS/MS dissociation of the 34kDa interferon- γ homodimer. CAD of the 12+ precursor molecule yields 8+ and 4+-charged monomer products, rather than two 6+ products. Based on current hypotheses, the 8+ monomer is released as an unfolded, or more denatured, product. However, whether this type of experiment can yield meaningful information regarding the solution structure of the protein complex remains to be proved.

1.4 CONCLUSIONS

Mass spectrometry-based methods have the potential to provide a better understanding of the relationship between the structure of protein complexes and their biological function. Francis Collins of the National Human Genome Research Institutes states that “genes and gene products do not function independently, but participate in complex, interconnected pathways, networks

and molecular systems that, taken together, give rise to the workings of cells, tissues, organs and organisms. Defining these systems and determining their properties and interactions is crucial to understanding how biological systems function” [53]. The systematic identification and characterization of these “machines of life” will “provide the essential knowledge base and set the stage for linking proteome dynamics and architecture to cellular and organismic function” [54]. Tools based on measurement of the gas phase macromolecule will be complementary to large-scale efforts in structural biology to determine the structure of all biologically important proteins and complexes. As improvements to all aspects of the experiment, from brighter ionization sources to more sensitive and higher resolution analyzers, are made available to scientists, the ability of mass spectrometry to directly impact biomedical research will improve.

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