STUDYING NONCOVALENT PROTEIN COMPLEXES BY ELECTROSPRAY IONIZATION MASS SPECTROMETRY

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Electrospray ionization mass spectrometry has been used to study protein interactions driven by noncovalent forces. The gentleness of the electrospray ionization process allows intact protein complexes to be directly detected by mass spectrometry. Evidence from the growing body of literature suggests that the ESI-MS observations for these weakly bound systems reflect, to some extent, the nature of the interaction found in the condensed phase. Stoichiometry of the complex can be easily obtained from the resulting mass spectrum because the molecular weight of the complex is directly measured. For the study of protein interactions, ESI-MS is complementary to other biophysical methods, such as NMR and analytical ultracentrifugation. However, mass spectrometry offers advantages in speed and sensitivity. The experimental variables that play a role in the outcome of ESI-MS studies of noncovalently bound complexes are reviewed. Several applications of ESI-MS are discussed, including protein interactions with metal ions and nucleic acids and subunit protein structures (quaternary structure). © 1997 John Wiley & Sons, Inc., Mass Spectrom Rev 16(1), 1–23, 1997

I. INTRODUCTION

Proteins are friendly in nature. They like to form partners with other molecular species. In some cases, they act as baggage carriers, providing transport to important destinations. In other cases, proteins require a partner to fulfill their crucial task in life. Generally, proteins as a class are nondiscriminating, interacting with a variety of species, such as other proteins, peptides, oligonucleotides, metal ions, and other small molecules. Several protein systems are functionally active only when one or several copies of itself are in the vicinity (the quaternary structure of a protein). However, as with the macroscopic world, each individual protein species may have preferences for a particular partner.

These are only a few of the characteristics that make proteins interesting for study by biochemists and molecular biologists. A recent analytical tool employed by protein chemists is mass spectrometry (MS). More specifically, electrospray ionization (ESI) (Fenn et al., 1989) has been a workhorse for the mass spectrometric analysis of peptides and proteins and has been used to study noncovalently bound protein complexes (Loo, 1995a; Smith & Light–Wahl, 1993). ESI is a gentle ionization method, yielding no molecular fragmentation (unless induced in the atmosphere/vacuum interface) and allowing intact weakly bound complexes to be detected. From the beginning of

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the development of ESI, it was evident that these weakly bound complexes could be detected. In some of the early examples reported, adducts onto protein ions attributed to salt and solvent species were observed (Edmonds et al., 1989; Whitehouse et al., 1985). Aggregates of the protein analytes were observed, especially at higher protein concentrations (Smith et al., 1992). Even complexes between protoporphyrin IX (heme) and heme-containing polypeptides such as myoglobin are evident in some of the earlier ESI-MS spectra (Covey et al., 1988; Edmonds et al., 1989). Not until articles by Katta and Chait on the globin–heme interaction of myoglobin (Katta & Chait, 1991) and the receptor–ligand complex reported by Ganem, Li, and Henion (Ganem et al., 1991a) was it suggested that *specific* noncovalent interactions could be detected by ESI-MS. Since these initial reports, several other types of protein–ligand binding have been studied, including antibody-antigen (Chernushevich et al., 1995), protein-cofactor (Drummond et al., 1993), enzyme–substrate pairings (Ganem et al., 1991b), and protein–DNA complexes (Cheng et al., 1996d; Greig et al., 1995). Intermolecular noncovalent interactions are responsible for the aggregation of folded polypeptide chains into multimers that determines a protein system's quaternary structure. Protein subunit complexes have been the study of several ESI-MS studies (Eckart & Spiess, 1995; Fitzgerald et al., 1996; Light–Wahl et al., 1994; Light–Wahl et al., 1993b; Loo, 1995b; Loo et al., 1993b; Schwartz et al., 1995a; Schwartz et al., 1995b; Schwartz et al., 1994; Smith et al., 1996b; Tang et al., 1994).

Evidence from the growing body of literature suggests that the ESI-MS observations for these weakly bound systems reflect, to some extent, the nature of the interaction found in the condensed phase. However, control experiments are necessary to rule out ubiquitous nonspecific interactions (i.e., nonspecific aggregation) and caution needs to be exercised when interpreting data from such ESI-MS experiments (Smith & Light–Wahl, 1993). For example, for a variety of ligands of different solution binding strengths available for testing, the protein–ligand interaction found in the ESI-MS experiment should reflect the expected solution phase measurements. This situation was found for the ribonuclease S-protein/S-peptide system, where the protein–peptide noncovalent complex with an S-peptide analog showed a weaker attraction as expected from solution-phase binding experiments (Ogorzalek Loo et al., 1993).

The purpose of this report is to review the literature to date on the direct observation of noncovalent protein complexes by ESI-MS. Several other reviews of the subject have appeared in the literature in recent years and interested readers are directed to those reports as well (Loo, 1995a; Przybylski, 1995; Przybylski & Glocker, 1996; Smith & Zhang, 1994; Smith et al., 1996a; Smith & Light–Wahl, 1993). Other types of biochemical, nonprotein noncovalent complexes, such as inclusion complexation (Camilleri et al., 1994; Camilleri et al., 1993; Penn et al., 1997; Selva et al., 1993) and oligonucleotide duplex assemblies (Bayer et al., 1994; Cheng et al., 1996a; Doktycz et al., 1994; Gale et al., 1994; Gale & Smith, 1995; Ganem et al., 1993; Light–Wahl et al., 1993a) have been observed by ESI-MS. Practitioners of ESI have varied views of the application of noncovalent complexes. There are three camps of opinion: believers, nonbelievers, and undecided. In my opinion, the believers hold their view because they had an early success in the laboratory and found ESI-MS to be useful for studying noncovalent complexes. On the other hand, nonbelievers had an early failure in the laboratory and, therefore, projected that the technique cannot possibly be used for this application; i.e., ''It doesn't work for my project, so how can it be useful as a general technique?'' The shrewd undecided majority have not actually attempted any (or many) experiments of this type and are waiting until more examples are reported to convince them to dive in. I am a *cautious* believer. Therefore, many examples will be cited to support the use of ESI-MS for studying noncovalent protein complexes. However, as I and others have found in the laboratory, the use of ESI for this endeavor is not without complications. These ''features'' will also be brought up throughout the discussion.

II. WHY USE ESI-MS TO STUDY NONCOVALENT COMPLEXES?

There are several established instrumental methods that have been applied for the study of macromolecular interactions, such as spectroscopic approaches (e.g., circular dichroism, light scattering, and fluorescence), differential scanning calorimetry, and isothermal titration calorimetry (Hensley, 1996). Each method has its strengths and weaknesses. A comparison of MS to other biophysical techniques for characterizing protein interactions is depicted in Fig. 1. Modern analytical ultracentrifugation is a powerful tool for determining molecular mass, shape, and equilibrium constants (Hensley, 1996; Schuster & Toedt, 1996). The development of analytical ultracentrifugation was awarded the Nobel Prize in 1926 to Svedberg. Ultracentrifugation was used in 1926 to correctly determine the assembly of hemoglobin as a 66.8 kDa tetramer from 16.7 kDa monomers (although it was not known that hemoglobin is composed of 2 α-subunits and 2 β-subunits, with each polypeptide binding one heme molecule; it was thought that all proteins had masses that were some multiple of a basic unit of weight of approximately 17,000) (Svedberg & Fahraeus, 1926). The mass accuracy of the method does not compare well with modern MS. However,

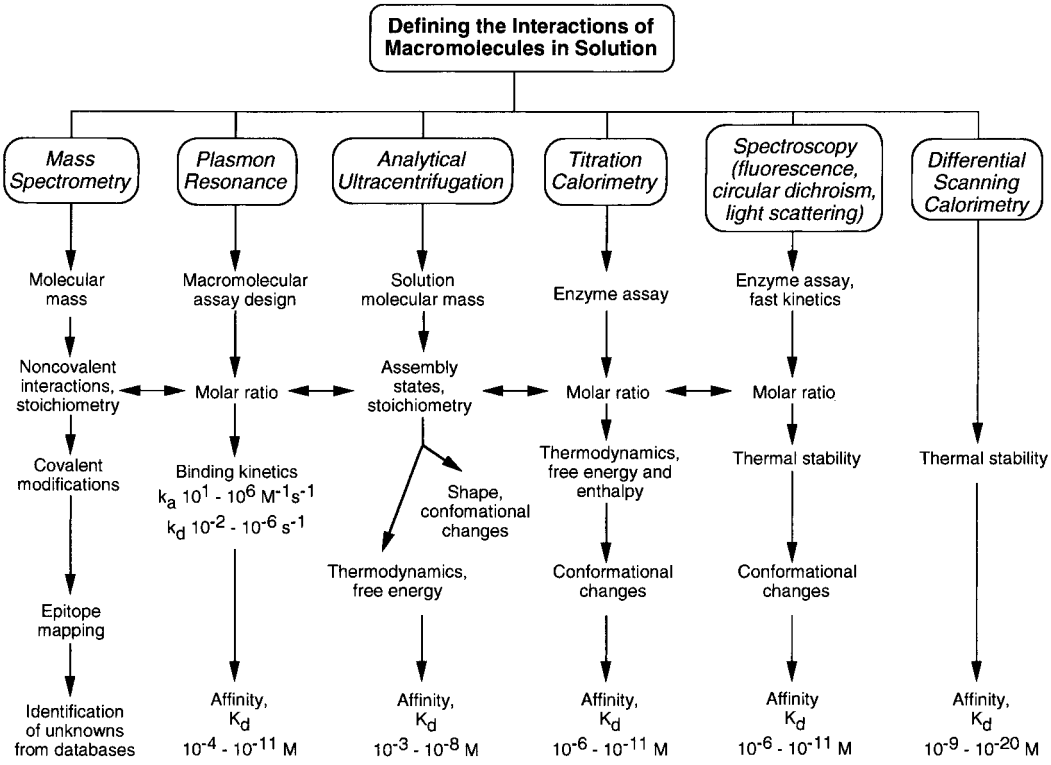

FIGURE 1. Biophysical methods for characterizing protein binding and assembly events in solution. Reprinted from (Hensley, 1996) with permission from Current Biology Ltd. and Dr. Preston Hensley. Copyright 1996 by Current Biology Ltd.

ultracentrifugation does provide important stoichiometry information for complexes.

A method used to measure rate constants (i.e., association rate constants, k_a, and dissociation constants, k_d) is surface plasmon resonance (SPR) (Szabo et al., 1995). One of the binding partners is immobilized on a surface. As the second binding partner in solution flows to the immobilized ligand, the refractive index change on the surface is measured. Thus, the binding interaction is monitored as the event occurs. Drawbacks to SPR include a lower molecular weight limitation of around 180–200 Da, and immobilization may modify the protein; that modification can lead to inactivation.

Chromatographic- and electrophoretic-based assays, such as size exclusion chromatography and gel electrophoresis, have traditionally been used to determine the stoichiometry of protein complexes; e.g., quaternary structure. However, the elution or mobility times are sensitive to the shape and physical properties of the protein. Thus, the measured molecular weight values can greatly depend on the choice of calibrating proteins. For the chaperone protein, SecB, molecular weights ranging from 64–115 kDa have been measured by size exclusion chromatography and gel electrophoresis; the ESI-MS mass spectra of SecB indicate a molecular weight of 68.6 kDa, which is consistent for a tetrameric protein composed of 17.2 kDa monomers (Smith et al., 1996b).

Smith and Zhang have compared the merits and disadvantages of mass spectrometry with respect to other biophysical methods, such as NMR, X-ray crystallography, and other spectroscopic techniques (Smith & Zhang, 1994). Data from spectroscopic methods such as UV, IR, and fluorescence indicate changes in the three-dimensional structure. NMR and X-ray crystallography yield unparalleled high resolution structures of biomolecules and complexes from the solution phase and solid phase, respectively. In general, the structures from NMR and X-ray crystallography agree, although there have been exceptions noted in the literature (Svergun et al., 1997). Both of these techniques require relatively large quantities of material (multimilligram scale) and are slow. These materials may be precious; i.e., difficult to obtain or produce. Often, the protein may precipitate at the high concentrations necessary for NMR. NMR is limited to approximately 40 kDa, although larger magnetic fields allows access to larger molecular weight complexes. X-ray crystallography can provide high-resolution protein structures. However, not all proteins are easy to crystallize. ESI-MS does not provide direct structural data in the same sense as does NMR and X-ray crystallography. However, MS

makes up the difference by providing important stoichiometry information, including systems in which there may be heterogeneity in the stoichiometry of the complex, from much less material in a much shorter time frame.

Mass spectrometrists tend to extend an MS-based methodology to solve virtually every scientific problem, even though methods based on other advanced technologies may have certain important advantages. However, much can be learned from this exercise. It is an important task for all analytical chemists to improve ''their'' technique. In the case of using ESI-MS to study noncovalent complexes, several important features of MS can be exploited over the other more traditional methods. McLafferty has often referred to the ''S'' advantages of mass spectrometry for solving problems: specificity, sensitivity, and speed (McLafferty, 1981). Each one of these virtues can apply to the application of noncovalent complexes.

Specificity is a critical advantage of ESI-MS. Protein complexes come together by specific interactions, based on key structural and/or energetic features, and nonspecific interactions (e.g., aggregation). Smith and coworkers used this feature to study the binding of zinc-loaded carbonic anhydrase and a 289-component library of benzenesulfonamide-based inhibitors by detecting the noncovalently bound complexes (Cheng et al., 1995a). The mass and relative abundance of each released inhibitor by a subsequent tandem MS (MS/MS) experiment provided information regarding the identity and relative binding constant. Small structural changes in a ligand can dramatically affect solution binding. The binding of the $pp60^{v\text{-}Src}$ SH2 (Src homology 2) domain protein is specific towards phosphopeptides and is variable in its amino acid sequence immediately C-terminal to the phosphotyrosine residue. An ESI-MS study showed the expected greater affinity of phosphopeptides to Src SH2 relative to an unphosphorylated peptide of the same sequence (Loo et al., 1997).

Speed and *sensitivity* are the most obvious advantages of a MS-based method to study noncovalently bound complexes. Results from Smith's laboratory using Fourier transform mass spectrometry (FTMS) (Schwartz et al., 1995a) and Standing's reports with time-of-flight (TOF) analyzers (Fitzgerald et al., 1996) show low picomoler-to-femtomoler sensitivity for such experiments. The recent advantages of low-flow ESI sources (commonly referred to as ''nanoelectrospray,'' nano-ESI (Wilm & Mann, 1996)) offer the potential for impressive sensitivity enhancements.

A discussion of ''S'' advantages should include the term ''*stoichiometry*.'' The number of ligands that form a unique and biologically relevant complex is an important issue in many systems. The stoichiometry of a complex can be easily obtained from the resulting mass spectrum, because the molecular weight of the complex is directly measured. Whether a protein's quaternary structure involves formation of a monomer, dimer, trimer, tetramer, and so on can be determined by ESI-MS. Multimeric proteins present an opportunity for ESI-MS to study very large molecular weight complexes. Many enzyme systems are composed of identical and nonidentical subunits that associate together to form the fully active species. Studying the nature of the interactions that maintain the quaternary structure of enzymes is essential for the understanding of cellular functions at the molecular level. MS experiments with protein oligomers can yield information on the stoichiometry and molecular nature of subunit interactions. The accuracy of the mass measurement is more than adequate to differentiate complexes composed of, for example, a dimer rather than a trimer.

ESI-MS results on tetrameric protein complexes such as avidin (64 kDa) (Light–Wahl et al., 1994; Schwartz et al., 1994) and concanavalin A (102 kDa) (Light–Wahl et al., 1994; Light–Wahl et al., 1993b; Loo et al., 1993b) have been previously discussed (see Table 1). In these experiments, only tetramer associations were observed. The absence of trimer and pentamer species suggest that the ESI-MS data reflect the specific solution-phase interactions that are known to occur. Furthermore, succinylated concanavalin A is known to form dimers as the highest aggregate order; the ESI mass spectra show the dimer as the largest mass species (Light–Wahl et al., 1994; Loo et al., 1993b).

III. CAN A GAS-PHASE MEASUREMENT REVEAL SOLUTION-PHASE BINDING CHARACTERISTICS?

Some aspects of the gas-phase studies need to be consistent with the solution-phase world in order for the ESI-MS experiments to have some utility for biochemists. As discussed above, complex stoichiometry is easily provided by MS. In most published reports, the observed stoichiometry is consistent with the expected result. Sometimes, more ligand is observed to bind to the protein receptor, perhaps due to nonspecific gas-phase aggregation. The prevalence of nonspecific aggregation can be decreased by reducing the solution concentration of the analytes (Smith & Light–Wahl, 1993; Smith et al., 1992). However, a survey of the published literature does not always reflect the true situation in this field of research. Negative results, experiments that ''failed'' to observe a noncovalent complex that was either ''expected'' or unknown, are rarely reported (Huang & Wang, 1996; Knight et al., 1993). Nonetheless, the numerous ''successful'' reports suggest that ESI-MS has utility for studying noncovalent complexes.

An indication that solution-phase binding events are being monitored is the ESI-MS reports on the determina-

tion of the relative and absolute strength of these solution-phase interactions. The binding of various peptide inhibitors to Src SH2 domain protein (12.9 kDa) was examined by Loo (Loo et al., 1997). From mixtures of peptide inhibitors, where the total peptide concentration is much greater than the protein concentration (competitive binding conditions), the relative abundances of the Src SH2 protein–phosphopeptide complexes observed in the ESI mass spectrum were consistent with their measured solution-phase binding constants. Henion's group (Lim et al., 1995) demonstrated that data from ESI-MS experiments can be used to construct conventional Scatchard plots for measuring the binding constants of vancomycin antibiotics with tripeptide ligands. Their gas-phase measurements were in reasonable agreement with previously reported solution-phase values. Likewise, the association of albumin protein with oligonucleotides was measured to have dissociation constants in the micromolar range by Scatchard analysis of ESI-MS data, and independently verified by capillary electrophoresis experiments (Greig et al., 1995).

The nature of the noncovalent interaction may have an effect on the ''success'' of the mass spectrometry experiment. There are at least four types of noncovalent forces involved in protein folding and interactions: ionic interactions, hydrogen bonds, the hydrophobic effect, and van der Waals forces. Going from solution to a solventless gas phase environment has many ramifications. Electrostatic interactions may be strengthened in vacuum. Feng studied the binding of highly basic spermine to an acidic spermine-binding peptide (SBP, 1607 Da) containing 4 glutamic acid residues (Feng, 1995). Despite a weak binding constant (10^4 M^{-1}) in solution, the spermine–SBP complex could be readily detected by ESI-MS. Moreover, the complex is unusually stable in the gas phase, as demonstrated by collisionally activated dissociation (CAD) studies. As the collision energy was increased, covalent bond dissociation occurred before dissociation of the noncovalent complex was observed. The avidity of the gas-phase complex may or may not be similar to that found in solution. For the spermine–peptide complex, a solvent-free environment may enhance Coulombic stabilization of opposite charges.

However, most gas-phase complexes are relatively fragile. ESI interface (atmosphere/vacuum) conditions typically need to be as gentle as possible to maintain the intact complex. Enough energy needs to be expended to reduce solvation of the complex for sensitive detection. Figure 2 depicts the process of a solution-phase noncovalent complex as it emerges into the gas phase. In some cases, depending on the interface, instrument, and the complex, there is a very fine line between sufficient desolvation of the gas-phase complex and dissociation of the complex. However, there are examples at both extremes of the scale. Protein complexes with oligonucleotides usually involve extensive electrostatic forces. Studies of protein–DNA complexes (Cheng et al., 1996c; Cheng et al., 1996d) and protein–RNA complexes (Sannes et al., 1996) are examples where dissociation of the gas-phase complex is very difficult; i.e., the multiply charged ions for the complex are stable at high interface energies. At the other extreme, Robinson's work with acyl coenzyme A-binding protein and acyl CoA analogs illustrates an example where the ESI-MS data are not perfectly faithful to the solution-phase characteristics (Robinson et al., 1996). Acyl CoA ligands with different solution dissociation constants to the protein were not differentiated by the ESI-MS experiment. A combination of hydrophobic, electrostatic, and nonpolar stacking interactions maintain protein–ligand binding in this example. Changes in the length of the hydrocarbon acyl chain, although greatly affecting solution binding, did not appear to affect the stability of the gas-phase complex. Similarly, the gas-phase stabilities of noncovalent complexes between bovine carbonic anhydrase and para-substituted benzenesulfonamide inhibitors were found to have no direct correlation with the hydrophobicity of the inhibitors (Wu et al., 1997). The off-rates for the complex in solution correlated with hydrophobicity for this system. As noted by Robinson (Robinson et al., 1996), ''Where hydrophobic interactions have been shown to play a major role in the solution state of other systems, for example, the leucine zipper peptides and the receptor–ligand interactions, the fraction of complexed species in the gas phase is low (typically ~10–20%) since such interactions are in large part attributed to the role of solvent.'' Caution is needed when interpreting results from ESI-MS experiments. On the other hand, perhaps ESI-MS can be used to assess the type of bonding interaction that keeps complexes together.

What do the results of these studies suggest about the structure of the *gas-phase* complex? Multiply charged ions for complexes such as protein–protein quaternary complexes exhibit relatively low charge state at high m/z. Standing and coworkers have determined an empirical relation for the most abundant charge state (Z) for over 30 noncovalent complexes studied in their laboratory: $Z \sim (m)^K$ (where m is mass and $K = 0.55$) (Chernushevich et al., 1996a). Furthermore, the distribution of charge states is typically rather low; e.g., 3–5 charge states. For example, Fig. 3 shows the ESI mass spectrum of tetrameric streptavidin (13.3 kDa monomer, 53.1 kDa tetramer) from a pH 8.6 solution (Schwartz et al., 1995a). Only three charge states, 13+–15+ at m/z greater than 3500, are observed. For several tetrameric proteins, Light–Wahl et al. noted that the average charge per subunit has the order: monomer > dimer > tetramer (Light–Wahl et al., 1994). The amount of charging that a biomolecule exhibits in an ESI mass spectrum has been correlated to a global solution structure. It has been noted that disulfide-containing pro-

TABLE 1. Noncovalent protein complexes observed by electrospray ionization mass spectrometry.

Complex	Ligand/interaction and comments
Polypeptide–metal ions	
Adenylate kinase [Briand et al., 1997]	Zn
Amino acids [Canty et al., 1994]	alkali metals, Hg
Amyloid precursor protein fragment [Multhaup et al., 1996]	Cu
Angiotensin peptides [Hu & Loo, 1995b; Loo et al., 1994b; Sullards & Adams, 1995]	Zn
Brain protein (bovine) [Fundel et al., 1996]	Zn; 31 kDa protein monomer
Calbindin D_{28K} [Veenstra et al., 1997]	Ca, Tb; EF-hand protein
Calmodulin [Hu & Loo, 1995a; Hu et al., 1994; Lafitte et al., 1995a; Lafitte et al., 1995b; Nemirovskiy et al., 1996]	Ca, Mg, Tb; EF-hand protein
Carbonic anhydrase [Cheng et al., 1995a; Gao et al., 1996; Wu et al., 1997; Zaia et al., 1996]	Zn
Cys-containing 27-residue peptide [D'Agostino et al., 1996]	Hg
Cytochrome c oxidase subunit [Kelly et al., 1993]	Cu
Desulforedoxin [Czaja et al., 1995]	Zn, Fe
Estrogen receptor DNA-binding domain [Allen & Hutchens, 1992; Hutchens & Allen, 1992; Hutchens et al., 1992a]	Cu, Zn; zinc finger protein
Ferredoxin [Armengaud et al., 1995; Breton et al., 1995; Jaquinod et al., 1993a; Petillot et al., 1995a; Petillot et al., 1995b]	Fe
Glucocorticoid receptor DNA binding domain [Witkowska et al., 1995]	Zn, Cd
Glutathione [Canty et al., 1994; D'Agostino et al., 1996]	Hg
Hemerythrin [Lei et al., 1996]	Fe; protein octamer
Histidine-rich glycoprotein [Hutchens et al., 1992b]	Cu
Integrin α_{IIb} subunit peptides [Haas & Plow, 1996]	Tb; α_{IIb} peptide-β_3 peptide heterodimer binds Tb
Integrin β_3 subunit peptides [D'Souza et al., 1994; Haas & Plow, 1996]	Tb
Lysozyme [Moreau et al., 1995]	Cu, Zn
Matrilysin [Feng et al., 1995]	Zn, Ca; matrix metalloproteinase; drug inhibitor binds to metalloprotein
Metallothionein [Beattie et al., 1996; Pleasance et al., 1990; Yu et al., 1993; Zaia et al., 1996]	transition metals
Myoglobin [D'Agostino et al., 1995]	Hg, Ag, Zn, Cd, Pb, La, Tb; adducts for ion charge state determination
Nucleocapsid protein (NCp7) [Fabris et al., 1996; Fenselau et al., 1994; Hathout et al., 1996a; Hathout et al., 1996b; Loo et al., 1996; Surovoy et al., 1992; Surovoy et al., 1993]	Zn; zinc finger protein
Parvalbumin [Hu et al., 1995; Hu & Loo, 1995a; Hu et al., 1994]	Ca; EF-hand protein
PECAM-1 peptides (platelet endothelial cell adhesion molecule-1) [Jackson et al., 1997]	Tb
($Tyr^{5,12}$, Lys^{7})-Polyphemusin II [Tamamura et al., 1996]	Zn; anti-HIV peptide T22
Prenisin [Surovoy et al., 1993]	Zn
Pseudoazurin [Aplin & Robinson, 1996]	Cu
Rubredoxin [Jaquinod et al., 1993a; Kazanis et al., 1995; Petillot et al., 1993]	Fe, Zn, Ga
Rubrerythrin [Lei et al., 1996]	Fe; protein tetramer
SPARC peptide [Lane et al., 1994]	Cu; extracellular matrix-binding protein
Stromelysin catalytic domain [Bauer et al., 1995; Hu et al., 1994]	Zn, Ca; matrix metalloproteinase
Ubiquitin [Jiao et al., 1995; Senko et al., 1993]	Cu, Hg, Ag, Zn, Cd, Pb, La, Tb; adducts for ion charge state determination
Protein–small molecule	
Acyl coenzyme A binding protein [Robinson et al., 1996]	Acyl CoA derivatives; ESI-MS results did not correlate with acyl chainlength
Albumin [Bakhtiar & Stearns, 1995]	FK506/FK520
β-Amyloid (1–40) [Camilleri et al., 1994]	β-cyclodextrin
Antithrombin III [Tuong et al., 1994]	heparin fragment; glycoprotein-pentasaccharide complex

TABLE 1. *Continued.*

Avidin/streptavidin [Chernushevich et al., 1996a; Eckart & Spiess, 1995; Light–Wahl et al., 1994; Schwartz et al., 1995b; Schwartz et al., 1994]	protein tetramer binds to biotin
Bradykinin [Penn et al., 1997]	permethylated β-cyclodextrin
Carbonic anhydrase [Cheng et al., 1995a; Gao et al., 1996; Wu et al., 1997]	inhibitors derived from combinatorial chemistry bind to Zn-loaded protein
Catalytic antibody (single chain) [Siuzdak et al., 1994]	hapten
Cytochrome b_5 [Hunter et al., 1997]	heme
Elastase [Aplin et al., 1994]	peptidic substrates/products
FKBP [Baczynskyj et al., 1994; Ganem & Henion, 1993; Ganem et al., 1991a; Henion et al., 1993; Hsieh et al., 1995; Li et al., 1994]	FK506, rapamycin; receptor-ligand
Hemoglobin [Aplin & Robinson, 1996; Henion et al., 1993; Konishi & Feng, 1994; Li et al., 1993a; Loo et al., 1993b; Schnier et al., 1996]	heme
HIV-1 protease [Baca & Kent, 1992]	protein dimer binds to inhibitor
Lysozyme [Ganem & Henion, 1993; Ganem et al., 1991b; Henion et al., 1993; Lumb et al., 1992]	*N*-acetylglucose hexasaccharide; enzyme-substrate/ enzyme product
Methionine synthase [Drummond et al., 1993]	cobalamine
Myoglobin [Chernushevich et al., 1996b; Ganem & Henion, 1993; Hunter et al., 1997; Jaquinod et al., 1993a; Katta & Chait, 1991; Konishi & Feng, 1994; Li et al., 1993a; Loo et al., 1993a; Loo et al., 1993b; Loo et al., 1993c; McLuckey & Ramsey, 1994; Przybylski & Glocker, 1996; Schnier et al., 1996]	heme
Peptide–antibiotics [Hamdan et al., 1995; Lim et al., 1995]	vancomycin, ristocetin; measured K_D by ESI-MS (Lim et al., 1995)
Spermine–binding peptide [Feng, 1995]	spermine
Protein/peptide–peptide	
Albumin [Baczynskyj et al., 1994]	growth hormone releasing factor
Antisense peptide [Loo et al., 1994a]	specific heterodimerization
Calmodulin [Nemirovskiy et al., 1996]	melittin binds to calcium-bound protein
$F(ab')_2$ (from IgG antibody) [Chernushevich et al., 1995]	immunocojugate with carboxypeptidase G2 (183 kDa complex)
Fyn SH2 domain [Chung et al., 1996]	phosphopeptide inhibitors
Glutathione-*S*-transferase [Przybylski & Glocker, 1996]	glutathione (noncovalent and covalent complexes)
Integrin α_{IIb} subunit peptides [Haas & Plow, 1996]	heterodimer with β_3 subunit peptide
Integrin β_3 subunit (residues 118–131) [D'Souza et al., 1994]	heterodimer with Arg-Gly-Asp-containing peptides
Leucine zipper peptide [Li et al., 1993b; Przybylski & Glocker, 1996; Wendt et al., 1995; Witte et al., 1996]	dimer and trimer formation
Margatoxin [Bakhtiar & Bednarek, 1996]	peptides from potassium channel
Ribonuclease *S*-protein [Goodlett et al., 1994; Ogorzalek Loo et al., 1993]	*S*-peptide
SP-C lung surfactant protein [Przybylski et al., 1994]	dimerization
Src SH2 domain [Anderegg & Wagner, 1995; Bruce et al., 1996; Loo, 1995a; Loo et al., 1997; Loo et al., 1995]	phosphopeptide inhibitors
$TAF_{II}42(11\text{-}95)/TAF_{II}62(1\text{-}82)$ [Xie et al., 1996]	heterodimer; TATA box-binding protein-associated factors
Trypsin [Kraunsoe et al., 1996; Mar et al., 1996]	protein inhibitors
Various peptides [Busman et al., 1994; Smith et al., 1992]	multimer formation
Protein subunit complexes	
Alcohol dehydrogenase [Loo, 1995b; Potier et al., 1997; Van Dorsselaer et al., 1996]	dimer (horse, 80 kDa), tetramer (yeast, 148 kDa)
Avidin [Light–Wahl et al., 1994; Schwartz et al., 1994]	tetramer (64 kDa)
Catalase HP II [Chernushevich et al., 1995; Chernushevich et al., 1996b]	tetramer (339 kDa)
Citrate Synthase [Krutchinsky et al., 1996]	dimer (95.8 kDa), hexamer (287 kDa)
Concanavalin A [Light–Wahl et al., 1994; Light–Wahl et al., 1993b; Loo et al., 1993b; Wang et al., 1996]	tetramer (102 kDa)
Estrogen receptor (ligand-binding domain) [Witkowska et al., 1996]	dimer (57 kDa)

TABLE 1. *Continued.*

Gene V protein [Cheng et al., 1995b; Cheng et al., 1996b; Cheng et al., 1996d]	dimer (19.5 kDa)
gp45 [Ganem et al., 1994]	dimer (49.7 kDa)
Hemerythrin [Lei et al., 1996]	octamer (110 kDa)
Hemoglobin [Aplin & Robinson, 1996; Light–Wahl et al., 1994; Loo et al., 1993b; Wang et al., 1996]	$\alpha\beta$ dimer/$\alpha_2\beta_2$ tetramer (64.5 kDa); each chain binds; one heme molecule
Hexakinase [Eckart et al., 1996]	dimer (108 kDa)
HIV-1 protease [Baca & Kent, 1992]	dimer (21.5 kDa)
Lectin, catfish roe [Murayama et al., 1996]	trimer (95 kDa)
4-Oxalocrotonate tautomerase [Fitzgerald et al., 1995; Fitzgerald et al., 1996]	hexamer (40.9 kDa)
Pyruvate kinase [Loo, 1995b]	tetramer (261 kDa)
Rubrerythrin [Lei et al., 1996]	tetramer (87 kDa)
SecB [Smith et al., 1996b]	tetramer (68 kDa); chaperone protein
Soybean agglutinin [Chernushevich et al., 1995; Tang et al., 1994; Wang et al., 1996]	tetramer (116 kDa)
Streptavidin [Chernushevich et al., 1996a; Chilkoti et al., 1995; Eckart & Spiess, 1995; Schwartz et al., 1995a; Schwartz et al., 1995b; Schwartz et al., 1994]	tetramer (52 kDa)
Transthyretin [Green & Oliver, 1995]	tetramer (55 kDa)
Protein–Nucleic Acid Complexes	
Adenylate kinase [Briand et al., 1997; Loo et al., 1993c]	AMP, Ap_5A
Albumin [Greig et al., 1995]	DNA; measured K_D by ESI-MS
Aldose reductase [Jaquinod et al., 1993b; Potier et al., 1997; Van Dorsselaer et al., 1996]	NADPH
Citrate synthase [Krutchinsky et al., 1996]	NADH; protein dimer and hexamer
DNA-binding domain of transcription factor PU.1 [Cheng et al., 1996c]	double-stranded DNA
Elongation factor-Tu (EF-Tu) [Przybylski et al., 1995]	GDP
Gene V protein [Cheng et al., 1995b; Cheng et al., 1996b; Cheng et al., 1996d]	DNA; protein dimer
Peptide nucleic acid (bis) [Griffith et al., 1995]	DNA; PNA-DNA duplex
Ras protein [Ganguly et al., 1993; Ganguly et al., 1992; Taveras et al., 1997]	GDP
regA protein (bacteriophage T4) [Harms et al., 1996]	RNA
Ribonuclease A [Camilleri & Haskins, 1993; Haskins et al., 1994]	CMP
Tat protein [Sannes et al., 1996]	TAR RNA (31-mer)

teins show less average charging than their disulfide-reduced counterparts, presumably because either fewer charge sites are exposed, or the Coulombic restraints restrict charging for a more compact structure (Katta & Chait, 1993; Loo et al., 1990a; Loo et al., 1990b). The same rationale may apply to subunit protein complexes. The narrow charge distribution of a low charge state represents retention of the higher order structure of the native protein complex.

It has yet to be conclusively demonstrated that a gas-phase dissociation energy (or thermodynamics) can be used to predict or even to reflect the solution-phase binding strength. ESI-MS studies on protein subunit complexes show gas-phase complexes that are not stable species in solution. For example, the dissociation of tetrameric concanavalin A (Light–Wahl et al., 1994; Light–Wahl et al., 1993b), avidin (Light–Wahl et al., 1994; Schwartz et al., 1994), streptavidin (Eckart & Spiess, 1995; Schwartz et al., 1995a; Schwartz et al., 1995b; Schwartz et al., 1994), hemoglobin (Light–Wahl et al., 1994), and chaperone protein SecB (Smith et al., 1996b) yielded ions for the non-physiological trimer species. Likewise, CAD of hexameric 4-oxalocrotonate tautomerase (4OT) produces ions that are consistent for the pentameric and tetrameric ion states; the crystallographic data show 4OT to be composed of a trimer of dimers (Fitzgerald et al., 1996). It has been speculated that this phenomenon occurs by ''a Coulombically driven process in which a monomer species becomes 'unraveled' and ejected from the aggregate with a disproportionately large share of the charge (Light–Wahl et al., 1994).'' Thus, tandem MS does not appear to be particularly promising for learning about the solution-phase assembly of complexes. Research by Henion's laboratory showed no trend (i.e., no direct correlation) between the collision en-

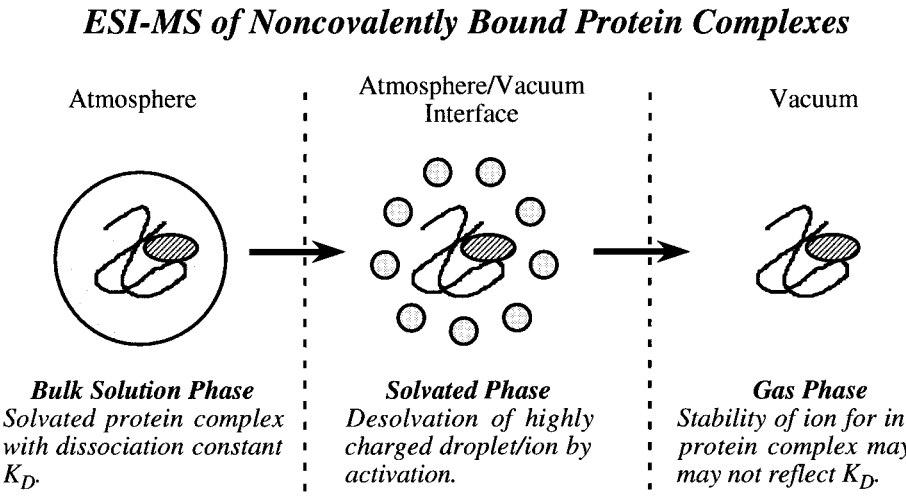

FIGURE 2. The process of solvent evaporation as the noncovalently bound complex is transferred from solution to the gas phase. Gentle desolvation conditions are generally required to maintain the intact gas-phase complex. For several protein systems, the ESI-MS data may be consistent with the solution-phase binding constants. Although some features of the solution structure may be preserved by the gas-phase ions, the stability of the gas-phase complex ion may not be reflected by the solution-phase binding constant.

ergy threshold (in a triple quadrupole system) for complex dissociation and solution-phase dissociation energies between the FKBP receptor protein and a mixture of rapamycin analogs with a ninefold difference in solution dissociation constants (Li et al., 1994).

However, recent binding studies have suggested that the stabilities of the gas-phase and solution-phase complexes are similar for selected systems. The relative gas-phase stability of heme binding to variant forms of apomyoglobin and apocytochrome b_5 was compared to solution kinetic data (Hunter et al., 1997). For a series of proteins in which the number of hydrogen bonds between the heme prosthetic group and the polypeptide is systematically reduced (from 3–0), the energy that gave 50% heme dissociation for the holoprotein correlated with the solution-phase activation energy. FTMS with blackbody infrared radiative dissociation data show the greater stability of the myoglobin–heme gas-phase complex compared to the α-hemoglobin–heme system; that stability is consistent with the trend found in solution (Schnier et al., 1996). An investigation of the enzyme trypsin and complexation with a variety of protein inhibitors, such as bovine pancreatic trypsin inhibitor (BPTI), soybean trypsin inhibitor, and other inhibitor analogs revealed ESI-MS data that suggested that the CAD data reflected the order of the strength of the known binding affinity in solution (Kraunsoe et al., 1996). Although these studies on the use of ESI-MS and CAD to probe the relative stability of noncovalent complexes are encouraging, it is not clear whether similar studies on other biochemical systems will yield similar results. The general applicability of the method has yet to be determined.

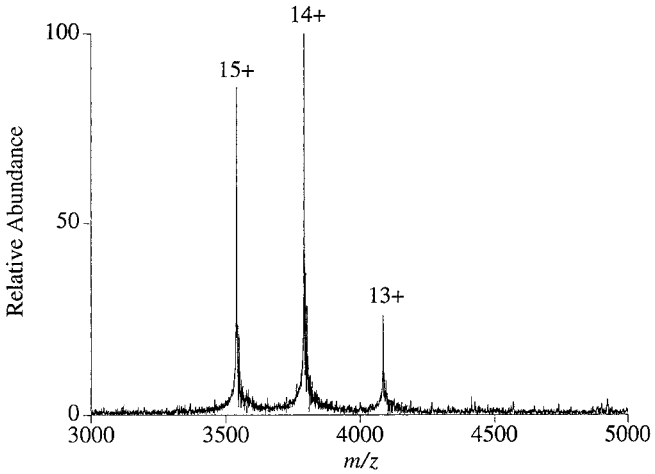

FIGURE 3. ESI-FTMS mass spectrum of streptavidin tetramer in pH 8.6 aqueous solution (10 mM ammonium acetate). Reprinted from (Schwartz et al., 1995a) with permission from Elsevier Science, Inc. Copyright 1995 by the American Society for Mass Spectrometry.

IV. EXPERIMENTAL

For most cases, the solution conditions that are needed to maintain an intact complex are not optimal conditions for ''normal'' ESI operation. For maximum sensitivity, a solution pH of 2–4 for positive ionization and pH 8–10 for negative ion ESI are typical for polypeptide analysis. Moreover, the addition of an organic modifier, such as

methanol or acetonitrile, also enhances sensitivity and ion signal stability. However, these conditions are not generally tolerated when trying to maintain a noncovalent complex. Many protein complexes are denatured in solution at a pH value outside of the pH 6–8 range, and the addition of a high concentration of an organic co-solvent can likewise disrupt the stability of the complex. This test can act as a simple control experiment for an ESI-MS study.

Although quadrupole ESI mass analyzers were the first systems used to detect noncovalent complexes, essentially all of the major types of mass spectrometers, such as magnetic sector instruments, ion traps, and FTMS systems, and TOF instruments have been successfully used. Analyzers with high mass-to-charge range (greater than *m/z* 4000) have advantages for studying the full range of biochemical complexes because of the tendency of many noncovalent complexes to exhibit relative low charge states.

Every type of atmospheric pressure ionization (API) interface has been utilized to observe such complexes (differentially pumped nozzle-skimmer interface, heated glass or metal capillary inlet with or without a countercurrent bath gas). Careful control of the various instrumental settings associated with each interface must be maintained. Variables that affect droplet/ion desolvation and ion activation can affect the success of the experiment. Solvent molecules need to be stripped from the ion prior to detection. Desolvation is accomplished through a variety of methods, including the use of a counterflow of nitrogen gas, heat (either a warm countercurrent gas, a heated capillary inlet, or a warm interface chamber), and CAD downstream of the initial interface. However, some gas-phase complexes are extremely fragile and the amount of activation (voltage difference between the capillary-skimmer or nozzle-skimmer lens elements) must be reduced to maintain the intact complex. The gas-phase complex between acyl CoA binding protein and acyl CoA derivatives was found to be sensitive to the ESI source temperature; increasing the temperature from 20–80°C reduced the proportion of complexed species to zero (Robinson et al., 1996). Best results were obtained by cooling the ESI nebulizer gas and the analyte solution (Robinson et al., 1996).

The use of low-flow ESI sources may prove to have advantages for electrospraying aqueous solutions and for studying complexes; however, there have been few reports on this subject. Standing's results with nanoelectrospray and the 4OT hexameric enzyme complex suggest that the nano-ESI-MS combination may be more gentle than higher flowrate sources (Fitzgerald et al., 1996). However, more extensive experiments for a variety of systems must be performed to draw any conclusions.

V. SELECTED EXAMPLES

There are several types of noncovalent interactions involving polypeptides that have been reported in the MS literature in recent years (Table 1). For the sake of organization, these examples have been grouped into the following categories: protein–peptide, polypeptide–metal ion, protein–small molecule, protein–protein (quaternary structure), and protein–nucleic acid complexes. A few selected examples that illustrate the types of problems that can be solved will be discussed.

A. Protein–Peptide Interactions

An example of a protein–peptide interaction studied by ESI-MS is the ribonuclease S (RNase S) system (Goodlett et al., 1994; Ogorzalek Loo et al., 1993). Ribonuclease S cleaves phosphodiester bonds within ribonucleic acids. RNase S is a modified form of RNase A in which limited proteolysis by subtilisin yields two polypeptides: S-peptide (residues 1–20) and S-protein (residues 21–124). The fully active form of RNase S is composed of the noncovalent association of S-peptide and S-protein with a dissociation constant (K_D) of approximately 10^{-10} M (pH 7, 0°C). RNase S represents a particularly challenging complex for ESI-MS study because of its thermal instability. Smith's group investigated a number of experimental variables affecting the solution-phase and gas-phase stability of the RNase S complex (Goodlett et al., 1994; Ogorzalek Loo et al., 1993). Different ESI interfaces were utilized, including a conventional differentially pumped nozzle-skimmer interface and a heated metal capillary inlet for ESI-MS, and capillary electrophoresis-ESI-MS. The temperature of the interface and countercurrent gas and the nozzle-skimmer potential greatly affected the relative abundance of the RNase S complex ions (Goodlett et al., 1994). The ''softest'' conditions were necessary to maintain stability of the complex (i.e., low nozzle-skimmer bias, low temperature gas and/or capillary). However, some activation is necessary to desolvate the ions prior to MS detection. This situation creates a ''balancing act'' that the experimentalist needs to maintain; activation is required to observe desolvated ions, but too much energy may dissociate the gas-phase complex. Even the temperature of the RNase S solution affected the results; incubation of RNase S at 60°C prior to ESI-MS resulted in very low abundance RNase S complex ions, indicating thermal denaturation of the protein complex in the liquid phase.

The noncovalent binding of various peptide ligands to $pp60^{src}$ (Src) SH2 domain protein has been used as a model system for development of ESI-MS as a tool to study noncovalently bound complexes (Loo et al., 1997). SH2 motifs in proteins are critical in the signal transduction pathways of the tyrosine kinase growth-factor receptors, and they recognize phosphotyrosine-containing proteins and peptides. ESI-MS with a magnetic sector instrument was used to detect the protein–peptide complex with low-picomole sensitivity. The relative abundances of the

multiply charged ions for the complex that formed between Src SH2 protein and several nonphosphorylated and phosphorylated peptides were compared and were found to correlate well with the measured binding constants that derived from solution-based methods, indicating the applicability of the method to assess the affinity of such interactions. Modified phosphopeptides, which included sequence stereoisomers that resulted in a wide range of binding affinities, were examined by ESI-MS. From competitive binding experiments, relative affinities for even D/L-isomeric peptides could be determined. Solution-phase equilibrium constants were determined by measuring the amount of bound and unbound species as a function of concentration for construction of a Scatchard graph.

ESI-MS of a solution containing Src SH2 with a mixture of phosphopeptides showed the expected protein–phosphopeptide complex as the dominant species in the mass spectrum. Figure 4 shows the results from the ESI-MS analysis of an equimolar mixture of six synthetic peptides with Src SH2 protein. With the total protein concentration nearly equal to the total peptide concentration, the relative abundance of multiply charged ions for four protein–peptide combinations are very similar (Fig. 4a). Two out of the six peptides have very low affinities to Src SH2 protein and were not observed to form a complex with Src SH2 protein. By increasing the total peptide concentration to establish a more competitive solution-phase binding condition, ESI-MS showed the expected noncovalent complex as the dominant species in the mass spectrum (Fig. 4b). For mixtures of much more complexity, either an ESI-mass spectrometer with higher resolution (see, for example, the binding of carbonic anhydrase to a mixture of 289 small molecule inhibitors by ESI-FTMS (Cheng et al., 1995a)), or simplification of the mixture by a pre-separation step are necessary for ligand identification by this method.

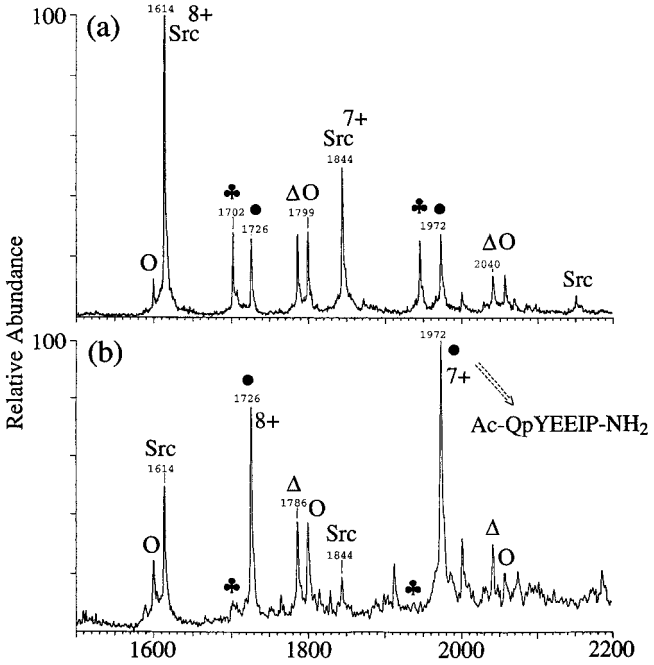

FIGURE 4. ESI-MS binding experiment between a 6-component peptide mixture and Src SH2 protein in 10 mM ammonium acetate, pH 6.9. The concentration of Src SH2 was maintained at 10 μM and the concentration of each peptide in the mixture was (a) 2 μM (noncompetitive binding) and (b) 15 μM (competitive binding). The following symbols represent peaks for the Src SH2 protein complex with IC_{50} values (concentration required to inhibit specific binding by 50%) measured from solution: Ac-Gln-pTyr-Glu-Glu-Ile-Pro-NH_2 (●, 0.7 μM), Ac-pTyr-Glu-*D*-Tyr-Ile-NH_2 (♣, $>$ 100 μM), Phe-Asp-Asp-Pro-Ser-pTyr-Val-Asn-Val-Gln-Asn (Δ, 7.8 μM), and Phe-Leu-Pro-Val-Pro-Glu-pTyr-Ile-Asn-Gln-Ser-Val (○, 41.8 μM). Two additional peptides (Ac-Gln-Tyr-Glu-Glu-Ile-Pro-NH_2, $IC_{50} > 100$ μM; O-phospho-Tyr, $IC_{50} > 1000$ μM) did not form a detectable complex with Src SH2 protein. Reprinted from (Loo et al., 1997) with permission from Elsevier Science, Inc. Copyright 1997 by the American Society for Mass Spectrometry.

B. Polypeptide–Metal Ion Complexes

Metal ions are essential to the catalytic function and structural stability of many metalloenzymes. Techniques used to study the interaction between metal ions and proteins include absorption spectroscopy, circular dichroism, electron paramagnetic resonance spectroscopy, and nuclear magnetic resonance spectroscopy. The potential of ESI-MS to determine peptide–metal ion stoichiometry is promising, as illustrated by the many examples listed in Table 1. Hu et al. (Hu et al., 1995; Hu & Loo, 1995a; Hu et al., 1994) and others (Lafitte et al., 1995a; Lafitte et al., 1995b; Veenstra et al., 1997) described the application of ESI-MS to determine the calcium-binding stoichiometry of Ca^{2+}-binding proteins that contain a common helix-loop-helix structural motif (also called an EF hand). Bovine calmodulin, rabbit parvalbumin, and bovine α-lactalbumin were found to bind specifically to four, two, and one Ca^{2+} ions, respectively, in agreement with previously reported results obtained by other physical methods (Hu & Loo, 1995a; Hu et al., 1994). For calmodulin (CaM), the relative population changes of the coexisting species carrying various number of Ca^{2+} ions was monitored during a Ca^{2+} titration study (Hu & Loo, 1995a). Upon increasing calcium concentration, the abundance of $CaM \cdot Ca_4$ increased and $CaM \cdot Ca_0$ decreased steadily, whereas the abundances of $CaM \cdot Ca_2$ and $CaM \cdot Ca_3$ remained low. Calbindin D_{28K} contains six putative EF-hand domains; results from spectroscopic methods suggested that the protein binds anywhere between 3–6 mol of calcium for each mol of protein. A recent ESI-MS study conclusively showed that the protein binds 4 mol of calcium per mol of protein; i.e., two of the EF-hands do not participate in binding calcium (Veenstra et al., 1997).

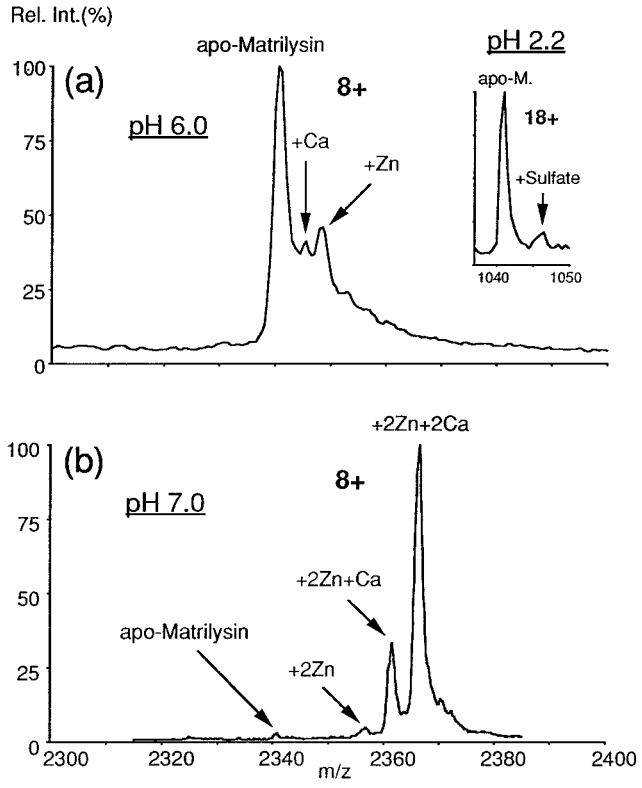

FIGURE 5. ESI quadrupole partial mass spectra of the matrix metalloproteinase, matrilysin (18.7 kDa), from various pH solutions. Binding stoichiometry for zinc and calcium are indicated. Reprinted from (Feng et al., 1995) with permission from Elsevier Science, Inc. Copyright 1995 by the American Society for Mass Spectrometry.

Matrix metalloproteinase enzymes are believed to be involved in connective tissue degradation and have been implicated in diseases such as arthritis and cancer. This enzyme family requires zinc and calcium for activity. The zinc- and calcium-binding stoichiometry for stromelysin, a member of this family, was measured by ESI-MS by Loo and coworkers (Hu et al., 1994). Another matrix metalloproteinase, matrilysin, was found to simultaneously bind to two Zn^{2+} and two Ca^{2+} ions at physiological pH (Fig. 5); only ions for the apo-enzyme were observed at a pH below 4.5 (Feng et al., 1995). Moreover, noncovalent binding of several inhibitors to the 2 Zn/2 Ca form of matrilysin was observed in the ESI-MS experiments.

Zinc finger proteins contain Cys and His ligands that are believed to coordinate zinc and to participate in protein–nucleic acid interactions (Klug & Schwabe, 1995). Many transcription factors include zinc finger structures that appear to be well-suited to DNA recognition. A few zinc finger proteins have been studied using ESI-MS, as shown in Table 1. Nucleocapsid protein NCp7 contains two zinc fingers that are involved in the encapsulation of genomic RNA during HIV (human immunodeficiency virus) viral assembly. Surovoy et al. (Surovoy et al., 1992; Surovoy et al., 1993) and others (Fenselau et al., 1994; Loo et al., 1996) have demonstrated that ESI-MS can determine the zinc stoichiometry for NCp7. The ejection of zinc from NCp7 caused by the covalent binding of various inhibitors to the protein can be monitored by MS (Hathout et al., 1996a; Hathout et al., 1996b; Loo et al., 1996). Witkowska et al. (Witkowska et al., 1995) have studied the zinc- and cadmium-binding characteristics of the DNA-binding domain of the glucocorticoid receptor, which contains two zinc fingers, each with four cysteine residues coordinated to a zinc atom. Accurate mass measurements and chemical modification experiments have determined that two thiol groups are deprotonated upon zinc complexation by CysCysHisCys-type zinc finger structures (i.e., two protons are lost for each zinc ion complexed) (Fabris et al., 1996).

C. Subunit Protein Structures

ESI-MS has shown tremendous utility for determining the assembly states of the quaternary structure of proteins. Protein complexes can be homocomplexes or heterocomplexes. The mass measurement afforded by mass spectrometry can differentiate between these complexes. Of all the protein structures in the Brookhaven Protein Databank, 33% exist as some form of multimeric state (Jones & Thornton, 1996). Out of these multimeric proteins, 80% are composed of either dimers or tetramers. Determining the number of subunits in the quaternary ensemble is a task that is well-suited to MS.

Loo (Loo, 1995b) reported results for alcohol dehydrogenase (ADH), a zinc metalloenzyme responsible for the interconversion of acetaldehyde and ethanol. Yeast and mammalian ADHs are distinctly homologous, yet only 25% of all residues are conserved. Equine liver ADH is dimeric, yet yeast alcohol dehydrogenase exists as the tetrameric complex for the active species. ESI mass spectra of ADH in acidic pH solution showed only ions for the monomer form. Acidic pH is known to cause dissociation for a number of dehydrogenases. The ESI mass spectrum of horse ADH in water (pH $\sim$ 6) showed multiply charged molecules for the monomer and dimer species of relatively low charge state. The relative abundance of the two forms was dependent on a number of experimental factors, including the amount of energy imparted to the ions in the ESI interface. At higher energies, CAD of the complex resulted in the dissociation of the complex to the monomeric species. The mass spectrum of bakers yeast ADH showed only ions for the monomer and a tetramer species. No yeast ADH dimer molecules were evident; that result is consistent with the specific solution-phase characteristics of the protein; i.e., a monomer-tetramer equilibrium.

Standing and coworkers, using ESI with a TOF mass

analyzer, studied the oligomeric structure of the enzyme 4OT (Fitzgerald et al., 1996). The high mass-to-charge range of the TOF system is important for such studies because of the relatively low charging observed for noncovalent protein–protein complexes. Quadrupole analyzers modified for high *m*/*z* transmission (Light–Wahl et al., 1994; Light–Wahl et al., 1993b), FTMS (Schwartz et al., 1995a), and magnetic sector instruments (Eckart & Spiess, 1995; Loo, 1995b; Loo et al., 1993b) have been used to study high *m*/*z* protein complexes. By gel permeation chromatography and ultracentrifugation, 4OT was estimated to be a pentamer. However, the ESI-TOF-MS studies demonstrated that 4OT (62-residue monomer) exists as a hexamer in solution; that result is consistent with recent X-ray crystallography experiments. Moreover, analogs of 4OT were used to demonstrate the importance of specific residues for maintaining the hexameric state. The analysis of (Met(O)^{45})4OT and (des-Pro^{1})4OT showed predominantly monomeric ions (Fig. 6); those data are consistent with structural studies by circular dichroism spectroscopy.

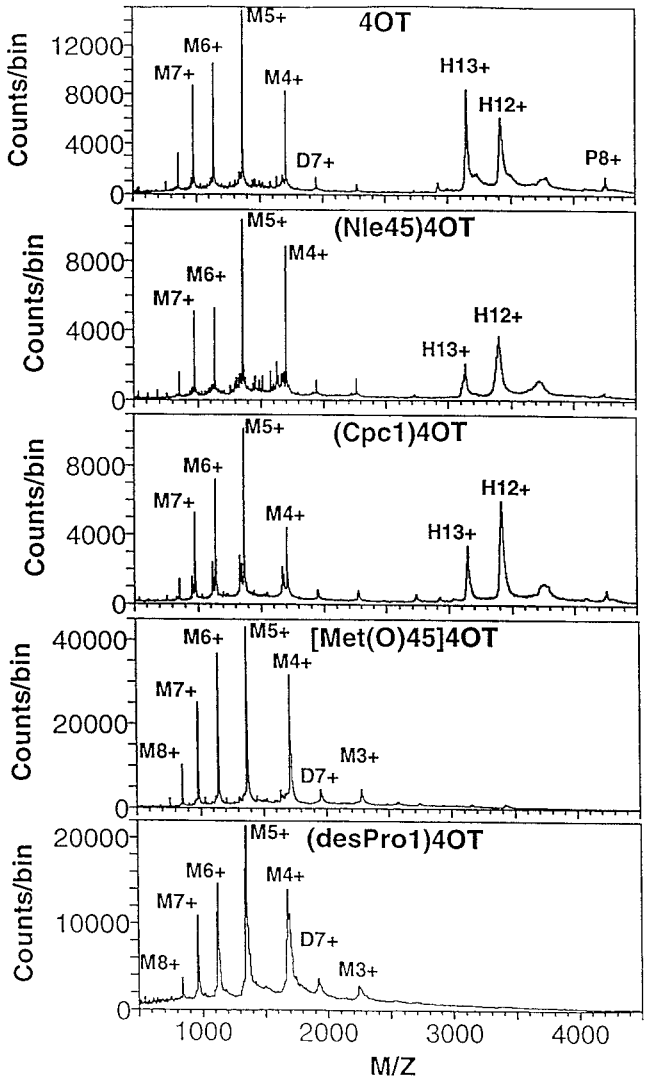

FIGURE 6. Electrospray ionization mass spectra (time-of-flight analyzer) of 4-oxalocrotonate tautomerase (4OT, 6.8 kDa monomer) and 4OT analogs in 5 mM ammonium bicarbonate buffer (pH 7.5). Hexameric protein ions were detected for 4OT and analogs, except for the des-Pro^{1} and Met(O)^{45} analogs. Reprinted with permission from (Fitzgerald et al., 1996). Copyright 1996 by the National Academy of Sciences, U.S.A.

D. Protein–Nucleic Acid Complexes

The expression of the genetic information found in nucleic acids is dependent upon the specificity of their interaction with proteins. Proteins serve as the regulators of the genetic information provided by nucleic acids. Thus, the development of techniques to study and understand the molecular details and function of protein–DNA/RNA interactions has broad interest and utility. The observation of intact noncovalent protein–DNA complexes by ESI-MS has been recently reported. Positive ion and negative ion ESI mass spectrometry have both been used to observe the association of a polyanionic oligonucleotide and a polycationic protein. The binding of albumin and oligonucleotides and the measurement of dissociation constants by Griffey and coworkers (Greig et al., 1995) was discussed above. Gene V (from bacteriophage f1) is a single-stranded DNA-binding protein (9.7 kDa) and exists as a homodimer under physiological conditions; the protein dimer binds to DNA with a 1:1 stoichiometry for approximately every eight DNA bases. Addition of a solution of $d(pT)_{13}$ yields the formation of the gas-phase protein dimer-DNA complex; the observation of the protein dimer–DNA complex is consistent with the solution-phase results obtained from NMR and gel-shift assays (Cheng et al., 1996d). MS experiments with a $d(pT)_{18}$ sample produces the gas-phase complex composed of a pair of protein dimers bound to one DNA molecule. Again, the observed gas-phase stoichiometry is consistent with the known solution binding stoichiometry. Figure 7 shows the binding of a 16-mer oligonucleotide to the gene V dimer. Competitive binding studies between $d(pT)_{13}$ and $d(pA)_{14}$ were consistent with the known greater affinity of poly(dT). Smith and coworkers have also investigated the binding of transcription factor PU.1 to double-stranded DNA (Cheng et al., 1996c).

ESI-MS has proved to be useful for studying protein-ribonucleic acid recognition, which is important in the replication cycle of HIV-1 and in the effect of targeted drug inhibitors on these complexes (Sannes et al., 1996). Tat protein from HIV is a viral trans-activator that is essential for viral replication. Tat is required to increase the rate of transcription from the HIV long terminal repeat (LTR), and its action is dependent on the region near the start of transcription in the viral LTR called the trans-

activation responsive (TAR) element. ESI-MS was used to study the complex formation between Tat protein (9.8 kDa) and TAR RNA (31-mer, 9.2 kDa) (Sannes et al., 1996). Tat protein contains an arginine-rich region that is essential for RNA binding. A 40-residue peptide containing this basic region has very similar binding characteristics to TAR RNA compared to the protein. Figure 8 shows the positive ion ESI mass spectrum of Tat peptide binding to TAR as the 1:1 stoichiometry complex. TAR RNA contains a three-nucleotide pyrimidine bulge that is essential for Tat binding and activity. Under competitive binding conditions, ESI-MS spectra show that Tat peptide affinity for TAR RNA is greatly reduced for the bulge-less 28-mer RNA; those data are consistent with solution-phase measurements. The Tat protein-TAR RNA complex highlights the importance of RNA structure in protein recognition of RNA. Neomycin, an aminoglycoside antibiotic, is known to bind specifically to RNA molecules. ESI-MS data obtained from pH 7 aqueous solutions in positive- and negative-ion modes show that several neomycin molecules bind noncovalently to the Tat peptide–TAR RNA complex prior to dissociation of the complex. Interaction between the antibiotic and TAR RNA was also observed in the ESI-MS data (Sannes et al., 1996).

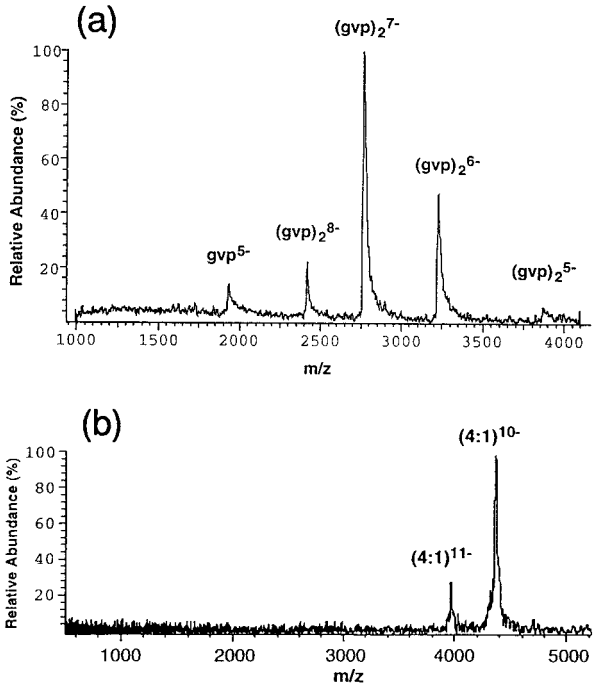

FIGURE 7. Negative ion electrospray ionization mass spectra of (a) gene V protein (9.7 kDa monomer), showing ions for the protein dimer, and (b) the 4:1 gene V monomer:16-mer DNA (4.9 kDa DNA) complex in 10 mM ammonium acetate (pH 7). Spectra were acquired with a triple quadrupole mass spectrometer and FTMS, respectively. Reprinted with permission from (Cheng et al., 1996d). Copyright 1996 by the National Academy of Sciences, U.S.A.

VI. CONCLUSIONS

It is clear that there is considerable interest in the application of MS for studying noncovalently bound macromolecules (or so-called ''supramolecular'' systems (Przybylski & Glocker, 1996)). The field of biochemistry would benefit greatly from methodologies that can provide information in a rapid and sensitive manner about how molecules interact. Although this review focused specifically on the application of ESI, matrix-assisted laser desorption/ionization (MALDI) may also be applied for the study of noncovalent complexes, as suggested by recent examples of anionic compounds binding to polybasic peptides (Juhasz & Biemann, 1994; Juhasz & Biemann, 1995), DNA duplexes (Lecchi & Pannell, 1995), and protein–protein complexes and protein quaternary structures (Glocker et al., 1996; Moniatte et al., 1996; Rosinke et al., 1995). However, it is premature at this time to make any conclusions regarding the general applicability of MALDI-MS for such studies. The rapidly expanding literature of ESI-MS studies suggests that the ESI method has benefit for many types of biochemical systems. As the field matures, new applications of MS will be presented and tested.

New compounds are continuously being synthesized through combinatorial chemistries. Such synthetic methods have greatly increased the diversity for which new structures can be screened as possible new therapeutics in the pharmaceutical industry. Approaches involving MS identification may be developed for the affinity selection and identification of novel protein–ligand interactions for drug discovery. Possible candidates derived from mixtures from combinatorial libraries may be initially screened by MS, as suggested by the ''bioaffinity characterization mass spectrometry (BACMS)'' method (Cheng et al., 1995a; Gao et al., 1996).

Going back to the question discussed earlier, ''Why use *mass spectrometry* to study noncovalent complexes,'' a possible reply could be, ''Why not?'' A scientist may have only a microgram or less of protein in possession. The protein is suspected to form a specific aggregation state, or maybe it interacts with a particular small molecule. ''What's the stoichiometry?'' ''Is the interaction covalent or noncovalent in nature?'' NMR and crystallography are well-established techniques for providing detailed three-dimensional structures of biomolecules. They are ''nondestructive'' methods, but milligram quantities are required to produce the images. With much less material, MS can provide the answers to some of these important questions. A workshop on ''New Methods for the Study of Molecular Aggregates'' was held in Alberta, Canada

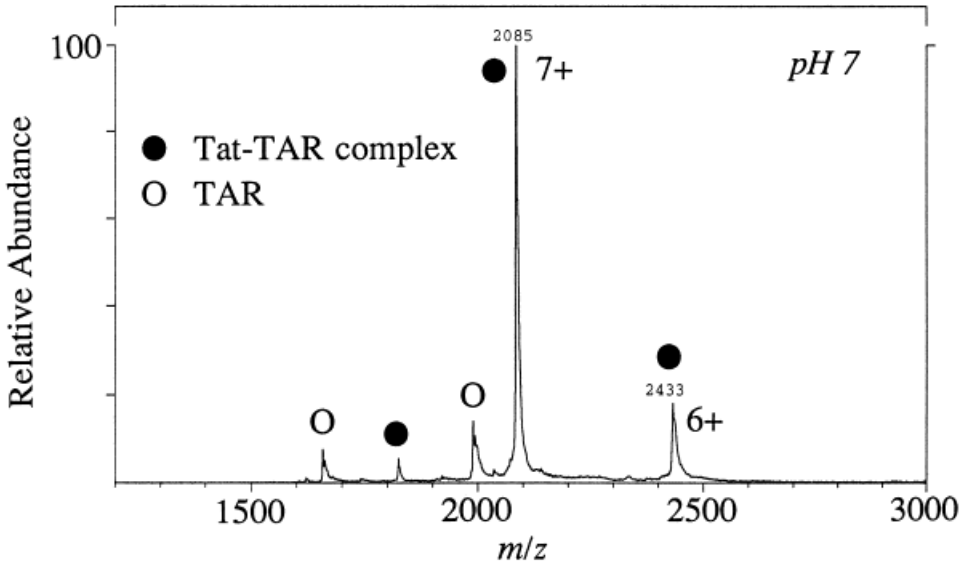

FIGURE 8. Positive ion ESI mass spectra of the 1:1 Tat peptide (40 residues, M_r 4644) – TAR RNA (31-mer, M_r 9941) complex. Tat peptide was added to a solution containing previously annealed TAR RNA in 5 mM ammonium bicarbonate buffer (pH 7). The solid (●) symbols represent peaks for the multiply charged 14.6 kDa peptide-RNA complex, and the open (○) label the ions for uncomplexed TAR RNA.

in June 1996 (Standing, 1996). Although the main focus of the meeting was the investigation of noncovalent complexes by MS, other more-established techniques such as crystallography and NMR were considered. It was evident from the discussions that the role MS plays in biomolecular research is entirely complementary to other techniques.

The results of the ESI-MS experiment are only as good as the judicious planning prior to the experiment. It is prudent to be cautious. Positive and negative control experiments, as many as can be performed, are necessary. Over 100 reports have been published on the subject of MS of noncovalent complexes; yet, it is probably safe to say that each biomolecular system has its unique experimental features. For example, the experience obtained from studying zinc-binding proteins by ESI-MS may not be the proper preparation for investigating the calcium-binding properties of proteins. On the other hand, if the goal is to study the aggregation of streptavidin, then using a test sample of avidin to establish the necessary experimental conditions is wise.

Is there any utility in studying the structure of the *gas-phase* protein complex? Most ESI-MS experiments do not really address the gas-phase structure of the unhydrated noncovalent complex. For many, there is a certain fascination on the possible folded (or unfolded) structure of a biomolecular ion in vacuum. The possibility of finding or creating a ''gas-phase enzyme'' for applications such as gas-phase sequencing of a gas-phase protein might be considered as science fiction. However, for the present time, the end result of these gas-phase experiments, the mass spectrum, appears to be faithful to the known solution-phase binding characteristics of many systems. In 1948, prior to the first protein X-ray crystal structure, Linus Pauling stated (Pauling, 1948), ''Even though the general picture of some important biological processes is becoming clear, our present knowledge of the detailed structure of the complex substances of biological importance is vague. We may expect that as more precise information about the structure of these molecules is obtained in the future, a more penetrating understanding of biological reactions will develop, and that this understanding will lead to great progress in the fields of biology and medicine.'' (Fittingly, for the purpose of this review, Pauling's article was entitled, ''Nature of Forces Between Large Molecules of Biological Interest.'') Although much progress has occurred in the last 50 years, Pauling's statement still applies. Perhaps MS can play a vital role towards answering some of these important questions.

ABBREVIATIONS

ADH alcohol dehydrogenase
API atmospheric pressure ionization
BACMS bioaffinity characterization mass spectrometry
BPTI bovine pancreatic trypsin inhibitor
CAD collisionally activated dissociation
CaM calmodulin

CoA coenzyme A
DNA deoxyribonucleic acid
ESI electrospray ionization
FTMS Fourier transform mass spectrometry
HIV human immunodefficiency virus
LTR long terminal repeat
MALDI matrix-assisted laser desorption/ionization
MS mass spectrometry
MS/MS mass spectrometry/mass spectrometry or tandem MS
m/*z* mass-to-charge
NCp7 nucleocapsid protein
NMR nuclear magnetic resonance
4OT 4-oxalocrotonate tautomerase
PECAM platelet endothelial cell adhesion molecule
RNA ribonucleic acid
RNase S ribonuclease S
SBP spermine-binding peptide
SH2 Src homology 2
SPR surface plasmon resonance
TAR trans-activation responsive element
TOF time-of-flight

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