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Biophysical Chemistry 79 (1999) 63–79

Biophysical Chemistry

Review

Electrospray ionization mass spectrometry in the study of biomolecular non-covalent interactions

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Received 2 July 1998; received in revised form 17 March 1999; accepted 17 March 1999

Abstract

In the past mass spectrometry has been limited to the study of small, stable molecules, however, with the emergence of electrospray ionization mass spectrometry (ESI-MS) large biomolecules as well as non-covalent biomolecular complexes can be studied. ESI-MS has been used to study non-covalent interactions involving proteins with metals, ligands, peptides, oligonucleotides, as well as other proteins. Although complementary to other well-established techniques such as circular dichroism and fluorescence spectroscopy, ESI-MS offers some advantages in speed, sensitivity, and directness particularly in the determination of the stoichiometry of the complex. One major advantage is the ability of ESI-MS to provide multiple signals each arising from a distinct population within the sample. In this review I will discuss some of the different types of non-covalent biomolecular interactions that have been studied using ESI-MS, highlighting examples which show the efficacy of using ESI-MS to probe the structure of biomolecular complexes.

Keywords: Electrospray ionization mass spectrometry; Protein/metal interactions; Protein/ligand interactions; Protein/protein interactions; Protein/DNA interactions; Gas-phase protein conformation

1. Introduction

The function of a biomolecule usually depends on its specific, non-covalent interactions with another molecule. For example, proteins interact with other proteins, peptides, small molecules, metal ions, lipids, polysaccharides, nucleic acids and oligonucleotides. These interactions drive critically important cellular processes such as cell division, cell signaling, ion transport, homeostasis, gene transcription, and translation. Several well-established spectroscopic techniques are used to study biomolecular interactions including circular dichroism (CD), fluorescence, infra-red (IR) and ultraviolet (UV) spectroscopy, nuclear magnetic resonance, and X-ray crystallography. One technique that is emerging as a new and exciting

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0301-4622/99/$ - see front matter
PII: S0301-4622(99)00037-X

method by which to study of biomolecular non-covalent interactions is electrospray ionization mass spectrometry (ESI-MS) [1]. Although routinely used in the determination of protein and peptide molecular mass as well as the sequencing and identification of proteins, ESI-MS is finding an increasing use in the study of non-covalent interactions. The results provided by ESI-MS are complementary to those provided by CD, fluorescence, IR, and UV spectroscopy, as well as one-dimensional NMR. Since ESI-MS provides an accurate measure of the molecular mass of the complex being studied, the primary piece of information obtained is the stoichiometry of the components which form the complex. There are, however, reports which suggest ESI-MS is able to detect conformational changes [2,3], measure relative dissociation constants [4], and discriminate strongly cooperative vs. sequential metal-binding to metalloproteins [5]. The structural information obtained using ESI-MS cannot be compared to that obtained by multi-dimensional NMR and X-ray crystallography, which provide high resolution three-dimensional structures of biomolecular interactions. ESI-MS can, however, provide important stoichiometric information about a complex, and it requires significantly less material as well as data analysis time than multi-dimensional NMR and X-ray crystallography.

ESI-MS produces singly or multiply charged ions directly from solution by creating a fine spray of highly charged droplets in the presence of a strong electric field [1]. ESI-MS is a gentle ionization technique that produces gas phase ions < 1 eV above their ground state energy [6]. The gentleness of this process does not cause any unwanted molecular fragmentation and allows intact weakly bound non-covalent interactions to survive the electrospray process. The sample solution is typically sprayed from the tip of a metal needle to which voltage is applied. To cause to solvent to evaporate, heat or a dry gas is applied to the charged droplets. As the evaporation process causes the droplets to decrease in size, the surface charge density within the droplets increases. The charged ions are transferred to the gas phase as a result of their repulsion from the droplet and focused into the mass analyzer through a series of lenses [7]. The data are presented as a relative

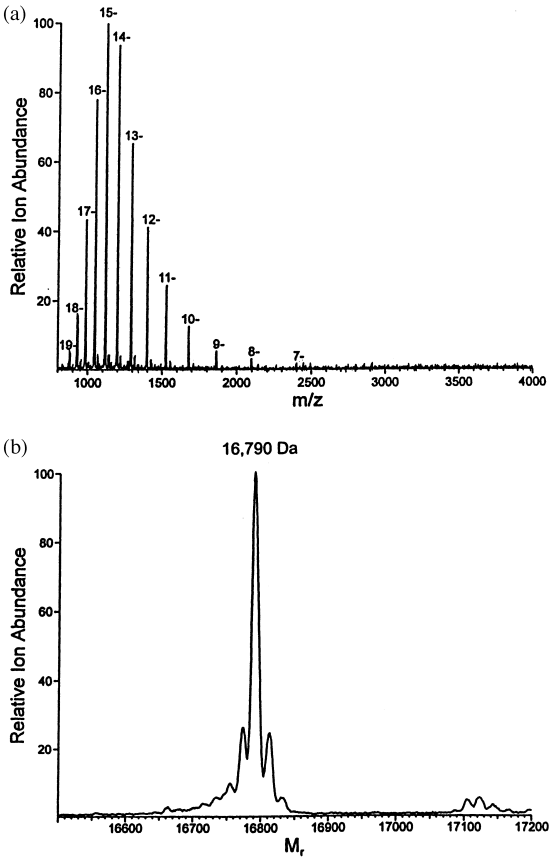

Fig. 1. (a) Multiply charged and (b) deconvoluted ESI-MS spectra of apo-calmodulin. Reprinted with permission from Veenstra et al. [12]. Copyright 1997 by IM Publications.

population of the various charge states of the molecule separated according to their mass-to-charge ratio (m/z) (Fig. 1a), not their molecular mass. This spectrum is commonly referred to as the multiply charged spectrum. The molecular mass of the molecule is calculated from these multiply charged peaks by software algorithms included within the instrument software (Fig. 1b). This ESI-MS spectrum is referred to as the deconvoluted or transformed spectrum. Typically the average mass, rather than the monoisotopic, is used as a measure of the molecular mass of the biomolecular species present.

Although a relative newcomer to the study of biomolecular interactions, ESI-MS is proving itself to be an exciting new technique in this field. The ability of ESI-MS to study specific non-cova-

lent complexes was first suggested based on the observation of a non-covalent interaction between myoglobin and its heme group [8] as well as a receptor–ligand complex [9]. Since these initial observations, ESI-MS has been used to study several complexes including protein/protein, protein/metal, protein/ligand, and protein/oligonucleotide interactions [10]. The primary advantage of ESI-MS over other spectroscopic techniques lies in the direct nature of the results obtained. The detection of a non-covalent interaction relies solely on the observation of a mass shift. Although this seems simplistic at first thought, the mass of a biomolecule is one of its most distinctive characteristics. In addition, the accuracy of the mass measurement afforded by ESI-MS enables an exact identification and stoichiometric determination of the components that comprise the complex. For example, the non-covalent adduction of metal ions, such as calcium (Ca^{2+}) or zinc (Zn^{2+}), to a metalloprotein is detected by the presence of a signal, which is some multiple of the mass of the ion, greater than the mass of the apo-protein. The ability to accurately determine the stoichiometry of a metal bound to a protein will depend on the mass of the protein and the resolution of the mass spectrometer, since the mass shift observed upon metal-binding may be quite small compared to the mass of the protein. When studying oligomeric protein complexes the inherent mass accuracy afforded by ESI-MS makes the ability to distinguish between, for example, trimer and tetramer forms trivial. While one-dimensional NMR, CD, fluorescence, IR, and UV spectroscopies provide a single signal which is the weighted average of the species present within the system, ESI-MS displays multiple signals arising from individual species of differing mass. For example, in the study of the Ca^{2+}-binding protein calmodulin [5,11,12], ESI-MS provides signals showing the relative populations of the protein containing various numbers of metal ions bound to it (Fig. 2). Other spectroscopic techniques would only provide a single signal corresponding to the weighted average of the discrete signals arising from the various metal-complexed protein species.

Unfortunately, the typical solvent conditions

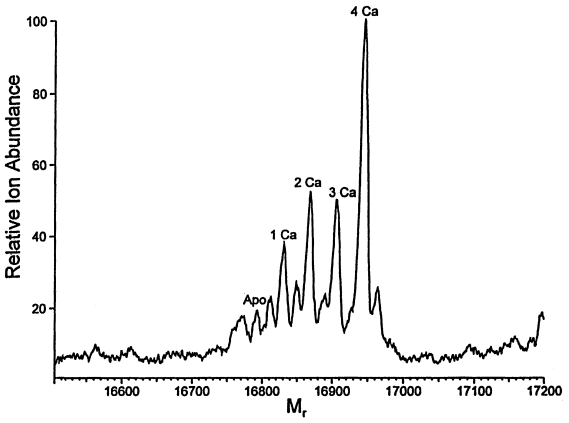

Fig. 2. Deconvoluted ESI-MS spectrum of calmodulin showing the relative populations of the protein bound to various numbers of Ca^{2+} ions. Reprinted with permission from Veenstra et al. [12]. Copyright 1997 by IM Publications.

used in ESI-MS to achieve maximum sensitivity, are not always optimal for maintaining an intact biomolecular complex. Many biomolecular non-covalent interactions are highly dependent on many conditions, including the pH and ionic strength of the solution. Unlike most solution-phase spectroscopic techniques, ESI-MS is intolerant to the presence of high salt and non-volatile buffer concentrations. Therefore a large majority of the biomolecular non-covalent complexes studied by ESI-MS are done in the absence of salts. Also, solutions of pH 2–4 and 8–10 are often used for positive and negative ion modes, respectively. Organic solvents, such as methanol or acetonitrile may be added to the solution to obtain maximum sensitivity and signal stability. Obtaining ESI-MS data under more physiological conditions would make this technique more attractive to the biochemical community. Indeed, there are an increasing number of studies showing non-covalent complexes observed from pure aqueous solutions without the addition of organic solvents [10,13,14]. For example, Standings group was able to observe the *trp* apo-repressor DNA-binding protein bound to its specific operator DNA and as well as its ligand, tryptophan, in 5 mM ammonium acetate aqueous buffer [15]. The advent of low-flow ESI-MS, commonly referred to as nanospray [16,17], is making the analysis of

aqueous solutions at physiological pH more routine and has shown the capability of tolerating much greater salt and buffer concentrations than conventional ESI. A recent study compared mass spectra of bovine insulin (10 μmol) at various sodium chloride (NaCl) concentrations obtained via a conventional electrospray source (flowrate = 3 μl/min) to those obtained using a nanospray source (flowrate = 20 nl/min) [18]. The results showed that, although the mass spectrum was noisy and dominated by salt clusters, multiply charged insulin peaks could be distinguished from those due to NaCl clusters using a nanospray ion source at salt concentrations as high as 0.1 M. At 0.01 M NaCl, the nanospray source was able to provide spectra in which insulin peaks clearly dominated. In contrast, insulin peaks could not even be distinguished at a NaCl concentration of 0.01 M using the ionspray source. Only when the NaCl concentration was lowered to 1 mM could insulin peaks be discerned from those of the NaCl clusters. Although salt and/or buffer concentrations this high have not been routinely used when studying non-covalent interactions, this study shows that the nanospray source is a major development towards using ESI-MS to study biomolecular complexes under more physiologically relevant conditions.

Although a destructive technique, in that the sample cannot be reclaimed after analysis, ESI-MS makes up for this in its sensitivity. Typical experiments examining a non-covalent interaction by ESI-MS require < 100 μl of sample in the low micromolar range. Recent advances in ESI and mass spectrometry technology have shown the ability to study complexes at low picomolar and femtomolar levels [13,19]. The advent of low-flow nanospray systems [16,17] allows samples to be introduced into the mass spectrometer at rate on the scale of nanoliters per minute, minimizing the total amount of sample required.

In this review some of the results obtained in the study of biomolecular interactions by ESI-MSll are highlight. Although mass spectrometrists have been aware of the ability of ESI-MS to study non-covalent interactions for some time, it is something that is still not commonly known within the biochemistry community. One of the major reasons for this is that many of these studies are published in journals directed towards the mass spectrometry community, but are not widely read within the larger biochemistry community. Therefore this review is directed towards physical biochemists and in it, I will show a variety of different types of non-covalent biomolecular interactions observed by ESI-MS. Examples are selected based on their ability to highlight advantages in using this technique in this field of study. For a comprehensive list of non-covalent complexes studies by ESI-MS, the reader is directed to an excellent review by Joseph Loo [10]. Since the ability of biomolecules to form specific non-covalent interactions depends on their conformation and biomolecules studied by ESI-MS are analyzed in the gas phase the obvious question arises; 'How can a gas-phase biomolecule possess the same structure as it has in solution?' To address this concern a section of this review will be devoted to discussing direct and indirect evidence which suggests similarities in the conformation of biomolecules in the gas and solution phase.

2. Non-covalent biomolecular complexes studied by ESI-MS

Since the first reports showing the ability to observe intact non-covalent biomolecular complexes using ESI-MS [8,9], numerous different types of interactions have now been described using this technique [10]. The different types of interactions observed include protein/metal [5,11,12,20], protein/protein [21,22], protein/peptide [23,24], and protein/oligonucleotide [14,15,25,26]. With the accumulating number of reports detailing non-covalent interactions involving biomolecules, it is becoming increasingly evident that the complex observed using ESI-MS behaves, at least to some extent, similar to the complex in solution.

2.1. Protein / metal interactions

ESI-MS has shown particular promise in the study of non-covalent protein/metal interactions. ESI-MS provides a direct and accurate measure of the metal-binding stoichiometry of a protein,

based on an observed shift in the mass of the protein arising from the non-covalent binding of the metal ion. The signal or signals observed can be translated into stoichiometry based on their mass difference from the apo-protein; the change in mass is some multiple of the mass of the metal ion of interest. The direct results provided by ESI-MS are not influenced by inaccurate measurements in metal or protein concentrations. Since the signal(s) generated by ESI-MS are not dependent on a change in conformation occurring within the protein, the change in signal generated is the same for each ion of the same type that binds to the protein. For example, the adduction of a Ca^{2+} ion to an EF-hand calcium-binding protein will always result in a signal showing the mass addition of 38 Da to the protein, regardless of the binding affinity. Although Ca^{2+} has a mass of 40 Da, a mass increase of 38 Da is observed upon its binding to a protein, since the non-covalent binding of the Ca^{2+} ion results in the loss of two protons (2 Da) by the protein.

An example where ESI-MS accurately determined the metal-binding stoichiometry of a protein in which other techniques were not as successful is the Ca^{2+}-binding protein, calbindin D_{28K} [20]. Calbindin D_{28K} is composed of six EF-hand Ca^{2+}-binding domains. Traditional methods have yielded discordant results with respect to the Ca^{2+}-binding stoichiometry of the protein suggesting anywhere from three to six Ca^{2+}-binding sites [27–30]. In addition several of the studies were unable to determine an exact integral value of the binding stoichiometry [27–29]. A recent study using ESI-MS conclusively showed that calbindin D_{28K} binds 4 mol of Ca^{2+} per mole of protein (Fig. 3), suggesting that two of the six EF-hand domains do not bind Ca^{2+} [20]. This conclusion was based on a mass difference of 151 Da between the protein in the apo-state and the protein saturated with Ca^{2+}. This mass difference of 151 Da is nearly equal to the addition of four Ca^{2+} ions (4×38 Da = 152 Da) to the protein molecule. The efficacy of using ESI-MS to rapidly measure metal-binding stoichiometries of metalloproteins was shown in a recent study examining the binding of Ca^{2+} to wild-type parvalbumin and nine mutants of the

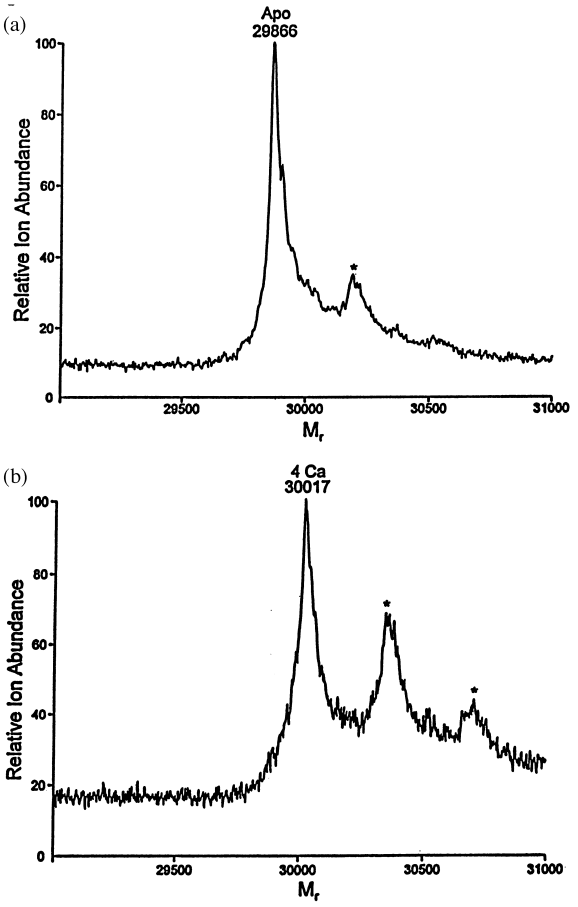

Fig. 3. Deconvoluted spectra of calbindin D_{28K}: (a) without added calcium acetate and (b) in the presence of 1 mM calcium acetate. The protein concentration was 60 μM and the solvent was 4 mM NH_4HCO_3, 15% CH_3OH, pH 8.0. The peaks marked by asterisks (*) correspond to sodium/EDTA adducts bound non-specifically to the protein. Reprinted with permission from Veenstra et al. [20]. Copyright 1997 by the American Chemical Society.

protein [31]. The ESI-MS results confirmed that wild-type parvalbumin bound two Ca^{2+} ions while the mutants studied bound either zero or one Ca^{2+} ion. All of these results fully agree with previously reported results using flow dialysis experiments, however, obtaining the results by ESI-MS offers many advantages such as speed and the direct observation of the metal-binding stoichiometry.

The direct results obtained by ESI-MS are also invaluable when studying metalloproteins which

possess two classes of metal-binding sites. For example, in the ESI-MS study of the matrix metalloproteinase, matrilysin, the protein was shown to possess two Zn^{2+} and two Ca^{2+}-binding sites at physiological pH [11]. Since Zn^{2+} (atomic mass 65.4 Da) and Ca^{2+} (atomic mass 40.1 Da) have significantly different masses, the individual binding stoichiometry of each is easily discerned. It is unlikely that other comparable spectroscopic techniques could so easily distinguish the binding stoichiometry of the two ions in a single experiment.

The capability of ESI-MS to provide multiple, discreet signals corresponding to the relative populations of metal-bound protein species co-existing within solution provides detailed information about the metal-ion partitioning and sequence of metal-binding within metalloproteins. For example, a study examined the Ca^{2+}- and cadmium (Cd^{2+})-binding to calbindin D_{9K}, a small protein containing two EF-hands [32]. The titration of calbindin D_{9K} with Ca^{2+} revealed a low abundance of the protein bound to a single Ca^{2+} ion, even in cases when a large population of the protein bound to 0 and 2 Ca^{2+} ions was observed (Fig. 4a). In contrast, when calbindin D_{9K} was titrated with Cd^{2+}, a large population of the protein bound to a single Cd^{2+} ion could be observed (Fig. 4D). In the case of Ca^{2+}-binding to calbindin D_{9K}, the results suggest that the binding of Ca^{2+} to the first EF-hand increases the Ca^{2+}-affinity of the second EF-hand to an extent that Ca^{2+} binds to this form of the protein preferentially over the apo-form of the protein. Therefore the ESI-MS data clearly shows that calbindin D_{9K} binds Ca^{2+} cooperatively. In the case of Cd^{2+}-binding to calbindin D_{9K}, the binding of the first Cd^{2+} ion has no effect on the metal-ion affinity of the second EF-hand, therefore the protein binds this metal sequentially. These results agreed with solution-phase studies of the metal-binding characteristics of calbindin D_{9K} [33] and provide further evidence that the distribution of gas-phase ions can reflect solution properties.

The information provided by ESI-MS is not limited to determining the metal-binding stoichiometry or sequence, but can also record

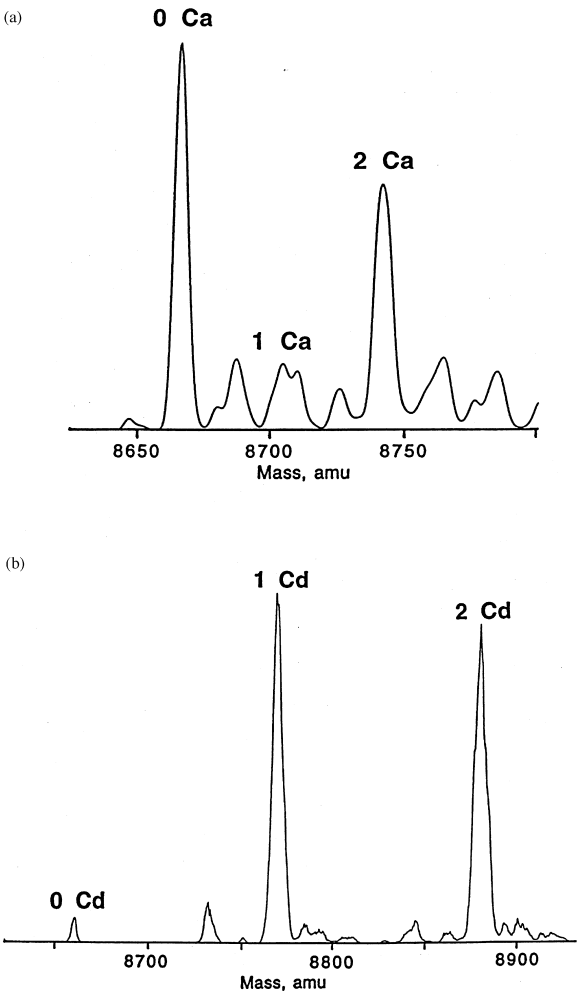

Fig. 4. ESI-MS deconvoluted spectra of calbindin D_{9K} in the presence of a (a) fivefold molar excess of Ca^{2+}, and (b) a 10-fold molar excess of Cd^{2+}. Reprinted with permission from Chazin and Veenstra [32]. Copyright 1999 by John Wiley and Sons, Ltd.

metal-induced conformational changes within a protein. Conformational changes are assessed by changes observed in the multiply charged ESI-MS spectrum (Fig. 5). The electrospray process produces a series of multiply charged protein ions [1], in which the distribution of charge states is related to the number of basic or acidic amino acid side chains present at or near the protein surface [6]. The solvent accessibility and pK_a values, which are dependent on the conformation of the protein, are important factors related to the

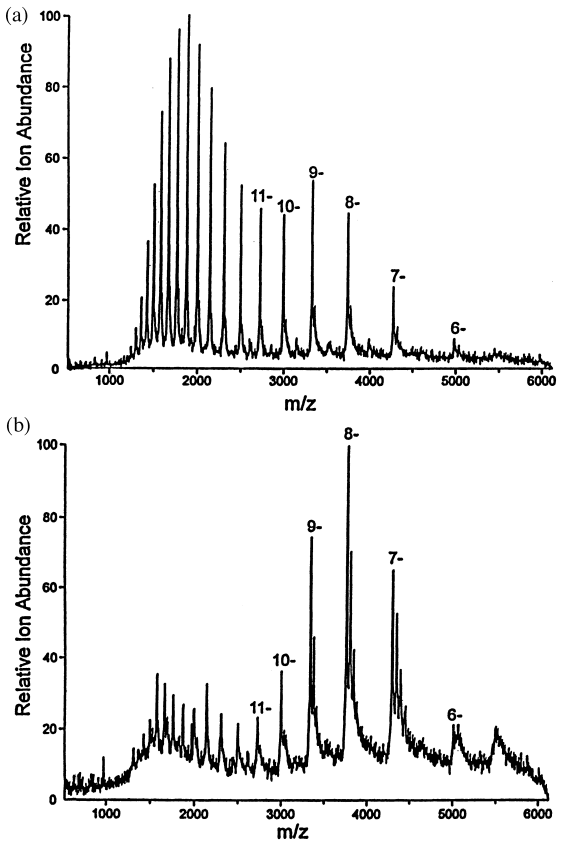

Fig. 5. Multiply charged ESI-MS spectra of calbindin D_{28K} (a) without added calcium acetate and (b) in the presence of 1 mM calcium acetate. Reprinted with permission from Veenstra et al. [20]. Copyright 1997 by the American Chemical Society.

propensity of these amino acid residues to be protonated or deprotonated. This reasoning suggests that the multiply charged ESI spectrum may provide information related to conformational differences in a protein. Previous studies have observed differences in the multiply charged spectra of proteins under denaturing conditions [34–36]. These changes in the multiply charged spectra were concluded to be a result of the change in the proteins conformation based on hydrogen/deuterium exchange experiments as well as results obtained by CD [34].

Changes in the multiply charged spectra of proteins have also been previously observed upon metal binding to metalloproteins [11,20,35]. Two recently published studies compared changes within the ESI-MS multiply charge spectrum of metal-binding proteins upon metal-binding with changes in the protein's spectra acquired using CD and fluorescence spectroscopy [2,3]. The two proteins were the DNA-binding domain of the vitamin D receptor (VDR DBD), which binds Zn^{2+}, and the Ca^{2+}-binding proteins calbindin D_{28K}, calbindin Δ2, and calbindin Δ2,6 [29,36]. Changes within the structure of the VDR DBD upon the addition of Zn^{2+} were monitored by ESI-MS and CD [2]. The CD results show that very little change occurs in the structure of the VDR DBD at a Zn^{2+} to protein molar ratio of one, however, a substantial change occurs upon increasing this molar ratio to two. Smaller changes within the CD spectrum of the protein occur upon further addition of Zn^{2+}. Similar to the results obtained by CD, the ESI-MS multiply charged spectrum of the VDR DBD undergoes little change upon the uptake of the first Zn^{2+}, however, a dramatic change in the spectrum occurs as the second Zn^{2+} is bound. Again smaller changes in the multiply charged spectrum are observed upon further uptake of Zn^{2+} by the protein. The results showed a positive correlation between changes in the ESI-MS and far-UV CD spectra of the protein upon Zn^{2+} uptake. The metal-dependent conformational change can be unambiguously assigned to a specific metal-binding event, since the ESI deconvoluted spectrum provides a direct measurement of the metal-bound species within the solution. This deconvoluted spectrum can be correlated to changes within its corresponding multiply charged spectrum. A correlation between changes within the ESI-MS, fluorescence, and near- and far-UV CD spectra of the calbindins was also seen upon the binding of Ca^{2+} to these proteins, strongly suggesting that changes in the multiply charged spectrum are indicative of changes in metalloprotein conformation [3].

2.2. Protein / ligand (or substrate) interactions

Two of the earliest studies which showed the ability to observe intact non-covalent interactions between biologically important molecules using

ESI-MS involved protein/ligand interactions. One of these studies reported the observation of the intact heme-myoglobin complex [8] while the other study reported an intact non-covalent complex FK binding protein (FKBP), an immunosuppressive binding protein, to the immunosuppressive agents FK506 and rapamycin [9]. These examples showing the ability of ESI-MS to detect intact protein/ligand complexes pioneered the use of this technique in the study of other types of non-covalent interactions between biomolecules.

Since ESI-MS measures the molecular mass of the protein complex it is able to accurately identify two closely related ligands which bind to a common site within a protein. A good example of this is the ESI-MS study of *ras* protein that is bound by GTP and GDP [37,38]. *ras* Protein is a G protein involved in the regulation of cell growth and differentiation. When the *ras* protein is bound to GTP it becomes biologically active promoting growth and differentiation, however, the protein is inactive when bound by GDP. Therefore, in the study of uncontrolled cell growth it is important to be able to differentiate between GDP-bound *ras* protein and GTP-bound *ras*-protein. GDP-bound and GTP-bound *ras* proteins are easily distinguished using ESI-MS due to the differing molecular mass between the two non-covalent complexes (M_r GDP/*ras* = 19 295 Da; M_r GTP/*ras* 19 375 Da). The direct measurement afforded by ESI-MS leaves no ambiguity to the identity of the ligand bound within the active site of the *ras* protein.

An excellent example of the ability of ESI-MS to provide information beyond the identity of the components that comprise a biomolecular complex is the study of the enzymatic reaction of hen egg-white lysozyme (HEWL) with various oligosaccharides of *N*-acetylglucosamine (NAG). Ganem et al. [39] used a continuous infusion system to record real time ESI-MS spectra of the hydrolysis of NAG_6 by HEWL to NAG_2 and NAG_4. Immediately after mixing solutions of HEWL and NAG_6, peaks which could be assigned to the +7 and +8 charged states of substrate-free HEWL and the +8 charge state of HEWL bound to NAG_6 were detected (Fig. 6a). At a later time point, the peak corresponding to the HEWL/NAG_6 complex decreased in size and a new peak corresponding to HEWL bound to NAG_4 was observed and continued to increase in size as time passed (Fig. 6b). Since the binding of NAG_2 to HEWL is approximately 100 times weaker than the binding of NAG_4, this species was not detected by ESI-MS. This method was also able to detect the slow hydrolysis of NAG_4 to NAG_3 and NAG by the appearance of a peak corresponding to the HEWL/NAG3 complex (Fig. 6b,c).

2.3. Protein / protein interactions

In the past investigators have measured the formation of a protein/protein non-covalent association using spectroscopic techniques such as fluorescence, CD, and UV. The basis of these techniques rely on a change in the signal originating from one of the proteins as it is titrated with the other protein. The stoichiometry of binding is then determined by the molar concentration ratio at which no further change in the signal is observed. In order to achieve accurate, integral results these techniques require meticulous concentration measurements. Since ESI-MS provides a direct, unambiguous result showing the formation of the protein/protein complex based on the mass of the newly formed complex, it is not influenced by errors in concentration measurements. ESI-MS has been used to study several protein oligomeric complexes [10].

ESI-MS has a significant advantage compared to other spectroscopic techniques when examining co-factor dependent protein/protein non-covalent interactions. By using ESI-MS, the stoichiometry of the protein/protein, and co-factor stoichiometry is readily determined within a single experiment since the observed mass of the non-covalent complex includes all of these entities. Using other spectroscopic techniques it would require a minimum of two separate experiments to obtain the same amount of information. These experiments include determining the protein/protein stoichiometry in the presence of an excess of co-factor, followed by another experiment to determine the co-factor/protein stoichiometry in the presence of an excess of the second protein.

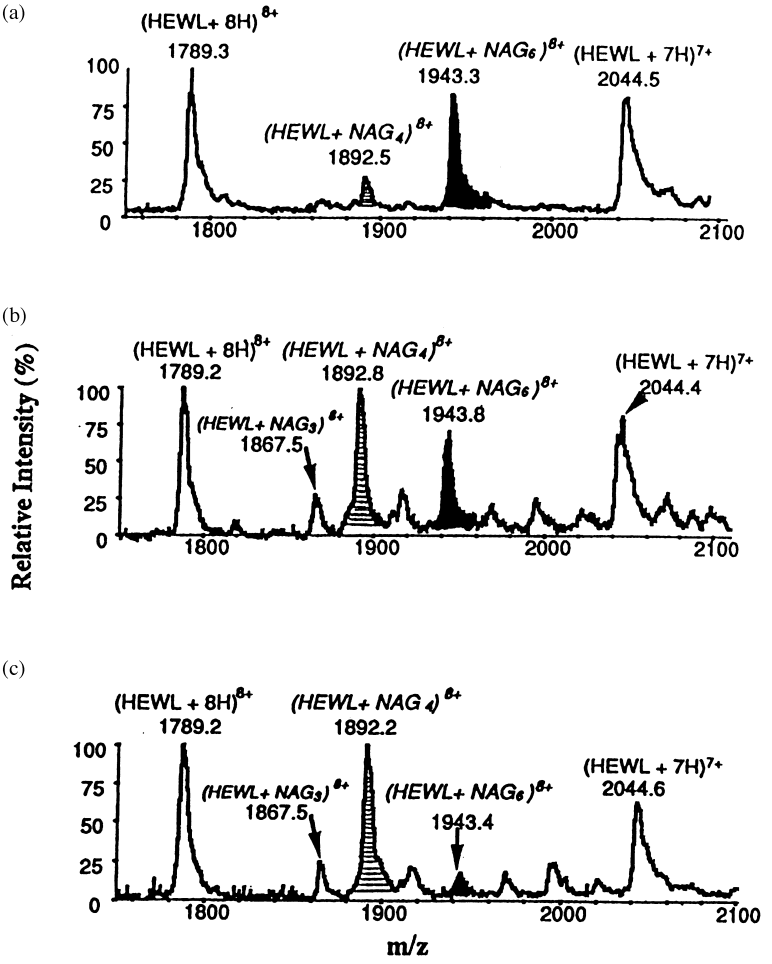

Fig. 6. ESI-MS time-course study of the hydrolysis of a hexamer of *N*-acetylglucosamine (NAG_6) by hen egg white lysozyme (HEWL). Reprinted with permission from Ganem et al. [39]. Copyright 1991 by the American Chemical Society.

The efficacy of using ESI-MS to study protein complexes, which are dependent on the presence of a co-factor, is illustrated by two studies examining the binding of peptides to calmodulin. Calmodulin is a well-characterized Ca^{2+}-signaling protein which binds peptides in a Ca^{2+}-dependent manner [40]. Two studies used ESI-MS to examine the Ca^{2+}-dependent non-covalent complex formed between calmodulin and mellitin [23] and the calmodulin-binding peptide from calmodulin-dependent protein kinase II (CK-II) [24]. Details of the study examining the binding of calmodulin to CK-II are highlighting below and in Fig. 7. The ESI-MS multiply charged spectrum of apo-calmodulin dissolved in H_2O is shown in Fig. 7a. A single major peak at M_r 16 790 Da representing apo-calmodulin is observed. Addition of an equimolar amount of CK-II resulted in no change in the spectrum. The addition of a two-fold molar excess of Ca^{2+} to the protein/peptide solution, however, resulted in the addition of a second major response (M_r 19 216 Da) in the deconvoluted spectrum, which was assigned to the formation of a non-covalent complex between CK-II and calmodulin (Fig. 7b). The measured molecular mass of the complex showed that calmodulin was bound by four Ca^{2+}, consistent with the reported Ca^{2+}-dependent binding of CK-II by

calmodulin (M_r calmodulin (16 790 Da + M_r 4 Ca^{2+} (152 Da) + M_r CK-II (2274 Da) = 19 216 Da). Similar results were observed in the study examining the binding of calmodulin to mellitin in that the binding of Ca^{2+} by calmodulin was necessary for the formation of the protein/peptide complex [23].

A recent study examined the hexameric structure of insulin [41]. The active form of insulin is a monomer, however, insulin is stored in the β-cells of the pancreas as a hexamer bound to two Zn^{2+} ions. Extensive spectroscopic studies have shown that the hexameric form of insulin is an allosteric protein capable of adopting three distinct conformations designated T_6, T_3R_3, and R_6. The conformation is dependent on the coordination of the two Zn^{2+} ions within the complex; in the T_6 state, the Zn^{2+} assume an octahedral coordination bound to three histidine residues (His) and three H_2O molecules, while in the R_6 state the Zn^{2+} ions are in a tetrahedral coordination bound by three His and one phenolic ligand. In the T_3R_3 conformation, the two Zn^{2+} ions adopt different coordinations, one being octahedral and the other tetrahedral. The ESI-MS study looked at the hexameric insulin non-covalent complex in the absence and presence of Zn^{2+}, as well as the Zn^{2+}-bound complex in the presence of different amounts of phenol. In the absence of Zn^{2+}, insulin was observed as a monomer, tetramer, hexamer, and dodecamer (Fig. 8a). Incubation of the insulin solution in the presence of Zn^{2+}, not only reduced the amount of monomeric protein observed, but displayed charged states assignable to hexameric insulin molecules bound to two Zn^{2+} ions and six H_2O molecules ($Ins_6Zn_2(H_2O)_6$) (Fig. 8b). This result would predict the coordination of the Zn^{2+} ions to be octahedral (three His and three H_2O), suggesting the conformation of the insulin hexamer to be in the T_6 state. The ESI-MS spectra of insulin was then recorded in the presence of Zn^{2+} and a 1:50 and 1:200 molar ratio of phenol. In the presence of Zn^{2+} and 1:50 molar ratio of phenol (Fig. 8c), charge states corresponding to the insulin hexamer bound to two Zn^{2+} ions, one phenol ligand, and three H_2O molecules were observed ($Ins_6Zn_2phenol(H_2O)_3$), suggesting one of the Zn^{2+} ions had a

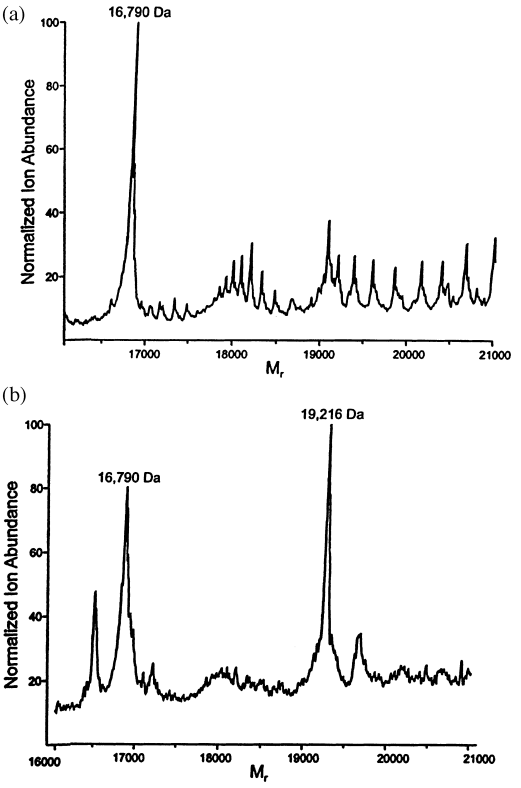

Fig. 7. (a) Deconvoluted spectrum of apo-calmodulin. (b) Deconvoluted spectrum of calmodulin (50 μM) in the presence of Cam-KII (50 μM) and 100 μM calcium acetate. Reprinted from Veenstra et al. [24] with permission from Elsevier Science Inc. Copyright 1998 by the American Society for Mass Spectrometry.

octahedral coordination (three His and three H_2O) while the other had a tetrahedral coordination (three His and one phenol). Therefore, based on previous studies, this insulin complex was most likely in the T_3R_3 conformation. Finally at a 1:200 molar ratio of phenol (Fig. 8d), the observed charge states corresponded to the insulin hexamer bound to two Zn^{2+} ions and two phenol ligands ($Ins_6Zn_2phenol_2$), suggesting both Zn^{2+} ions adopted a tetrahedral coordination (three His and one phenol) and that insulin was now in the R_6 conformation. This study of the non-covalent hexameric insulin complex in the presence of Zn^{2+} and phenol suggests ESI-MS has a rapid method with which to characterize the metal- and ligand-dependent allosteric conformation of multimeric proteins.

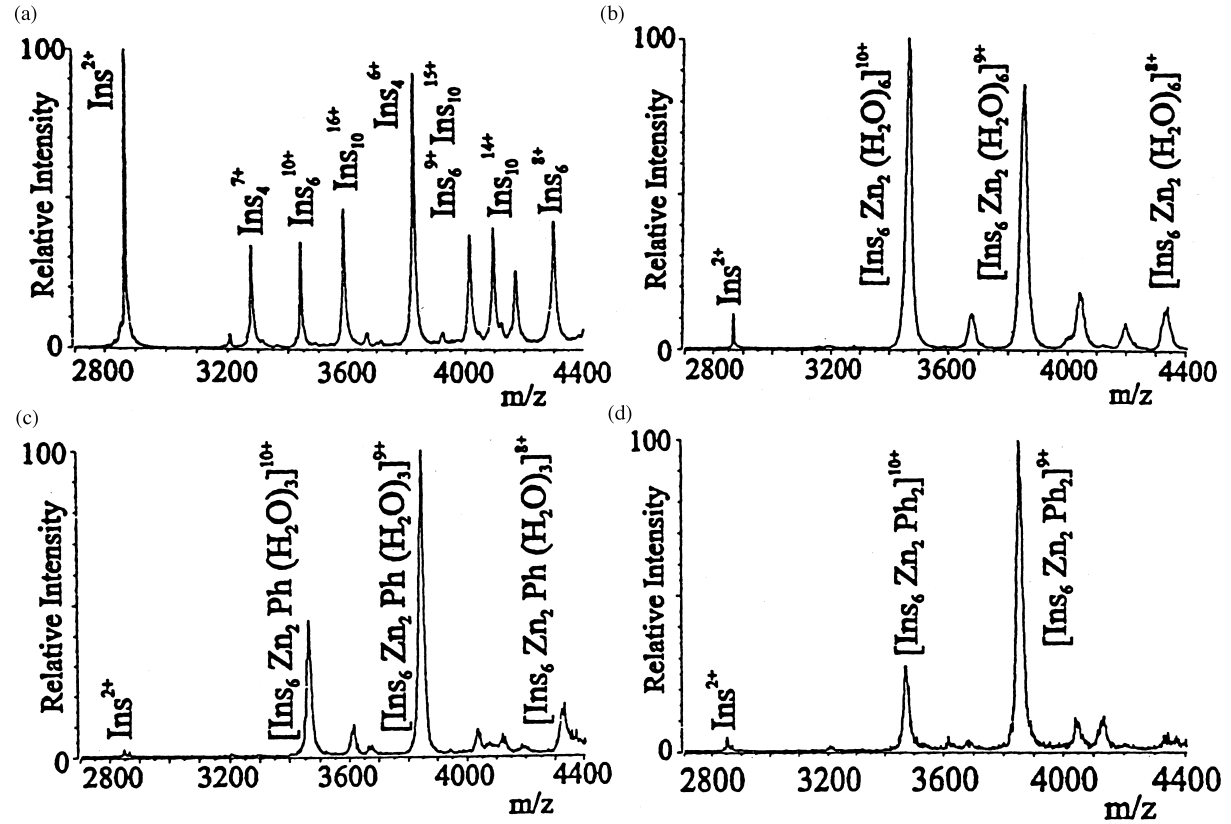

Fig. 8. ESI-MS multiply charged spectra of insulin (a) with no added ligand, (b) treated with $ZnCl_2$, (c) treated with $ZnCl_2$ and a 50-fold molar excess of phenol, and (d) treated with $ZnCl_2$ and a 200-fold molar excess of phenol. Reprinted with permission from Fabris and Fenselau [41]. Copyright 1999 by the American Chemical Society.

2.4. Protein / DNA interactions

One of the most intense areas of biochemical research deals with the regulation of gene transcription, in which protein transcription factors bind to specific DNA sequences ultimately resulting in the expression of the protein product of that gene. The most common techniques used to examine protein/DNA interactions is the electrophoretic gel mobility shift assay [42,43]. This technique typically requires radioactively labeling a DNA probe which is incubated with the protein of interest. The mixture is then separated on a polyacrylamide gel which is exposed to a radiographic film. The presence of a positive protein/DNA interaction is visualized by the presence of a radiographic band on the film. In situations where the protein sample is heterogeneous, a super-shifted assay is performed using an antibody directed against the protein of interest to confirm its interaction with the target DNA. The ability to detect specific protein/DNA interactions by ESI-MS provides many advantages over this technique. ESI-MS provides an accurate record of the identity and stoichiometry of the complex which is not provided by an electrophoretic gel mobility shift assay. In cases where protein/DNA associations require a co-factor, as illustrated below, ESI-MS analysis can measure the stoichiometry of the co-factor in the complex. Whereas specific protein/DNA complex formation can be determined within a few hours by ESI-MS, the electrophoretic gel mobility shift assay requires several days to achieve a comparable

result. Since ESI-MS provides direct evidence of the formation of a complex based on the expected molecular mass, no radioactive labeling, or any other type of modification to aid in detection, is required.

The ability of ESI-MS to detect specific non-covalent complexes between DNA and protein was first illustrated in the lab of Richard Smith [25,26]. This group studied the complex formation between the gene V protein and different oligonucleotides. Gene V protein exists as a homodimer in solution and binds single-stranded DNA molecules. They were able to show that the gene V protein homodimer bound a single molecule of $d(pT)_{13}$, consistent with results obtained by NMR and gel shift analysis [25]. They further demonstrated that increasing the oligonucleotide length (i.e. $d(pT)_{16}$) resulted in the binding of a second homodimer pair to the oligonucleotide, consistent with previous results showing that each gene V protein homodimer binds eight DNA bases. This group was also able to show a complex formation between the PU.1 transcription factor and a double-stranded DNA containing the protein's target sequence [26]. The specificity of the interaction was proven by experiments showing that mutations within the DNA target sequence severely affected the binding of PU.1 to the DNA as measured by ESI-MS.

An example of the ability of ESI-MS to provide information not readily detectable using an electrophoretic gel mobility shift assay is illustrated by the ESI-MS study of the binding of the DNA-binding domain of the vitamin D receptor (VDR DBD) to a vitamin D response element (VDRE) from the mouse osteopontin (mOP) gene [14]. The VDR is a nuclear hormone receptor, which is made up of a DNA-binding domain and a ligand binding domain. The VDR DBD is located within the first 110 residues of the protein and is composed of two zinc-finger metal-binding domains [36]. Previous studies of the glucocorticoid receptor (GR) and the human transcription factor SP1 by electrophoretic gel mobility shift analysis have shown that both of these proteins required zinc for binding to their respective target DNA sequences [44,45]. These studies also showed that the protein/DNA complex dissociated at elevated Zn^{2+} concentrations, however, no definite reason could be determined from the available data.

A study using ESI-MS to probe the relationship between Zn^{2+} concentration and protein/DNA complex stability has shed some light on the reason. The study compared the formation of the VDR/mOP gene complex in the presence of various concentrations of Zn^{2+} [14]. The VDR has been shown by CD, inductively coupled plasma MS, and ESI-MS to contain two-high affinity and at least three low-affinity Zn^{2+}-binding sites [36]. The importance of the Zn^{2+} concentration in maintaining a stable VDR DBD/mOP gene complex was studied by ESI-MS. Addition of 100 μM EDTA to a solution containing the VDR DBD and mOP gene did not show the presence of any complex formation suggesting the requirement for Zn^{2+} to maintain a stable protein/DNA non-covalent interaction. The negative ion ESI multiply charged spectrum of the mOP gene in the presence of 20 μM VDR DBD and 100 μM Zn^{2+} is shown in Fig. 9a. The ESI-MS spectrum of this target DNA in the presence of the VDR DBD showed two multiply charged ion series between m/z 1700–2500 and 2400–3500 representing the protein bound to the DNA as a monomer and dimer, respectively. Addition of Zn^{2+} to 200 μM final concentration, however, severely reduced the amount of protein/target DNA complex detected (Fig. 9c). The same pattern of Zn^{2+}-dependent complex association and dissociation was observed using Cd^{2+} in place of Zn^{2+}. Cadmium, which has $\approx 10^2$ higher affinity for zinc-finger domains than Zn^{2+}, is often used as an isomorphous replacement for Zn^{2+} and when substituted for Zn^{2+} still permits DNA-binding by zinc-finger proteins [44]. ESI-MS spectra of the protein in solution with a dsDNA containing a randomized VDRE as well as separate solutions of the VDR DBD mixed with each of the single stranded oligonucleotides used to prepare the mouse OP VDRE target DNA were also acquired. The addition of Mg^{2+} or Ca^{2+} to a final concentration of 300 μM was unable to cause the VDR DBD to dissociate from its target DNA, suggesting the effect of the Zn^{2+} was not

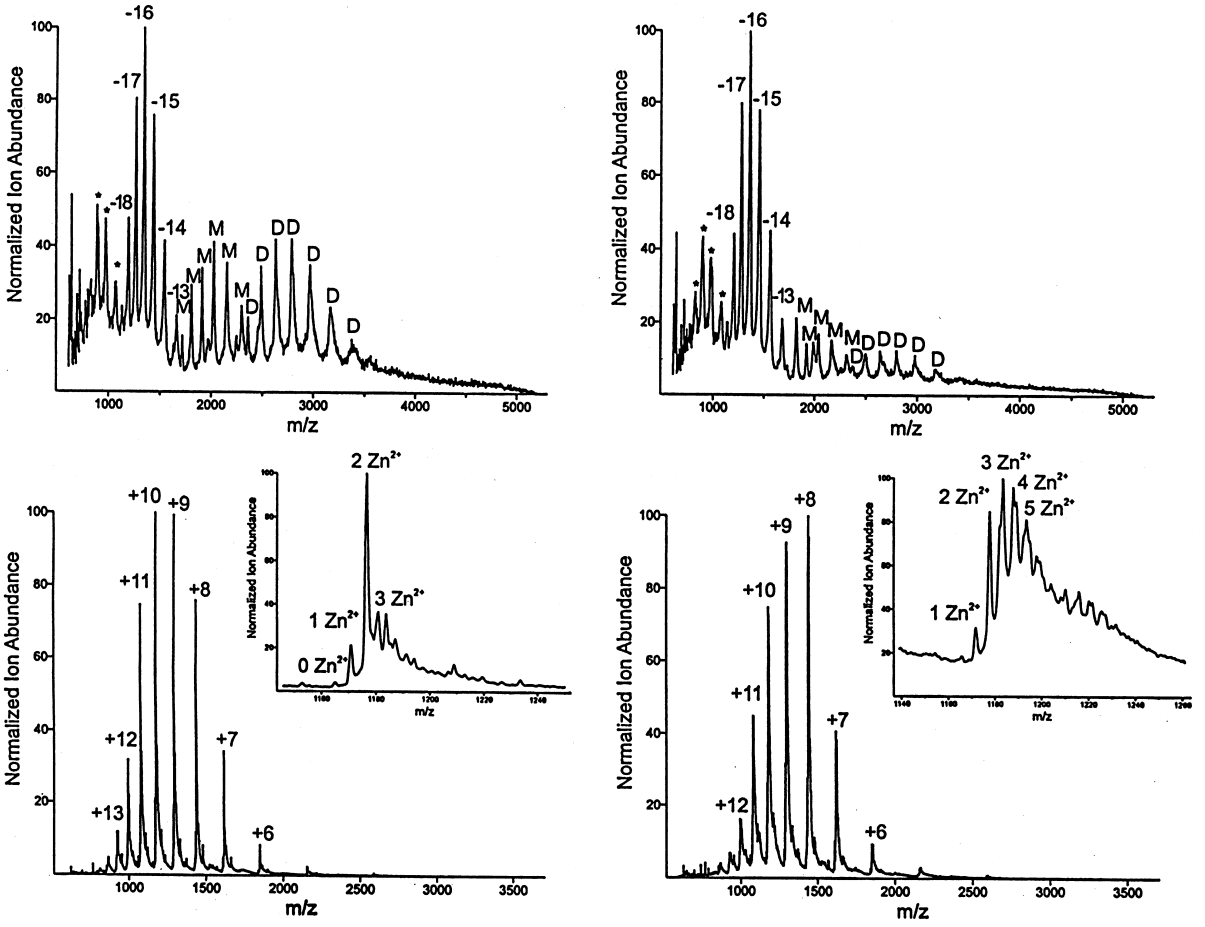

Fig. 9. Dependency of the Zn^{2+} concentration upon the binding of the VDR DBD to VDRE sequences. (a) Negative ion ESI spectrum of the VDR DBD in solution with the dsDNA containing the VDRE from the mOP gene in the presence of 100 μM EDTA. (b) Negative ion spectrum of the VDR DBD/mOP gene VDRE complex in the presence of 100 μM Zn^{2+}. Inset: +11 charge state showing the number of Zn^{2+} ions bound to the protein. (c) Negative ion spectrum of the VDR DBD/mOP gene VDRE complex in the presence of 200 μM Zn^{2+}. (d) Positive ion multiply charged spectrum of the VDR DBD in the presence of 200 μM Zn^{2+}. Inset: +11 charge state showing the number of Zn^{2+} ions bound to the protein. VDR DBD/dsDNA mOP gene VDRE complexes with the protein bound as a monomer or dimer are labeled with an M or D, respectively. Reprinted with permission from Veenstra et al. [14]. Copyright 1998 by Nature America Inc.

simply due to a change in the ionic strength of the solution.

To investigate this metal-dependent effect on the association and dissociation of the protein/DNA complex, ESI-MS spectra of the VDR DBD were recorded at the same metal ion concentrations used to examine the complex. The results showed that in the presence of 100 μM Zn^{2+} (the level at which the greatest amount of stable protein/DNA complex was observed), the VDR DBD was predominantly bound to two Zn^{2+} ions (Fig. 9b). In the presence of 200 μM Zn^{2+} (the level at which caused a significant amount of the complex to dissociate), the VDR DBD had predominantly three and four Zn^{2+} ions bound to it (Fig. 9d). Consistent with the results obtained using Zn^{2+}, the uptake of three or more Cd^{2+} by the VDR DBD was responsible for the dissociation of the complex. The similarity in the ESI-MS results in the Zn^{2+}- and Cd^{2+}-dependent pro-

tein/DNA complex formation suggests that the binding at the first two sites, presumable within the zinc-finger domains, is required for DNA-binding by the VDR DBD. Binding to the additional lower-affinity metal binding sites, however, is responsible for dissociation of the protein/DNA complex. These results show the ability of ESI-MS to monitor subtle changes that occur within a complex which add to the stability or instability of a non-covalent interaction.

3. Matters of controversy

The obvious matter of controversy when discussing the observation of a biomolecular complex by ESI-MS is how can a gas phase complex reflect the behavior of the complex in solution. It has not been conclusively demonstrated that a gas-phase complex is identical to its counterpart in solution with respect to activity, binding affinities, or conformation. There are, however, several different pieces of indirect evidence, which strongly suggests a positive correlation between a complex's behavior in the gas and solution-phase. The most fundamental piece of evidence lies in the specificity of the non-covalent interactions observed by ESI-MS. In a majority of the cases reported the observed stoichiometry is consistent with that observed using solution-phase spectroscopic techniques. For example, calmodulin has consistently been shown to bind 4 mol of Ca^{2+} per mole of protein [5,11,12] in agreement with the well-established Ca^{2+}-binding stoichiometry of this protein [40]. A positive correlation between the tertiary structure of proteins in the gas-phase and solution has also been indirectly observed. ESI-MS studies examining the denaturation of proteins as a function of pH, temperature, or solvent conditions have shown the ability to differentiate between proteins in their native and denatured states [34,36]. This result suggests that the conformation of the protein in solution is conserved through the electrospray process. As mentioned previously, a positive correlation between changes in the ESI-MS multiply charged spectra and spectra obtained by CD and fluorescence spectroscopy of proteins upon metal binding has also been observed [2,3].

Many ESI-MS studies have also shown a positive correlation between the stability of a complex when compared to their solution-phase dissociation constants. Although the ability of ESI-MS to provide absolute dissociation constants has not yet been realized, this technique does appear able to provide a measure of the relative stability of a complex. A study examining the binding of ribonuclease S protein to the first 15 residues of the S peptide (S15) and a mutant form of the S-peptide (M13G) showed much less binding of M13G to the S protein [46]. These results are consistent with the relative dissociation constants measured for each system in solution [47]. Robinson et al. [48] performed a series of experiments measuring the binding of acyl CoA derivatives to acyl CoA binding protein (ACBP) as well as a mutant in which tyrosine (Y) residues 28, 31, and 37 were individually mutated to asparagine (N) residues. In a series of competition experiments, they showed that the relative affinity of the acyl CoA derivatives was typically 25-fold greater for the wild-type protein than any of the mutants. The same study, however, was unable to show a correlation between the dissociation constants of the binding of acyl CoA ligands of increasing chain lengths to the wild-type protein and the amount of non-covalent complex observed by ESI-MS.

The previous observation brings up a key point as to the role of solvent molecules in the stability of biomolecular non-covalent interactions. It has been suggested that the absence of water from complexes studied by ESI-MS favors the stability of those complexes in which electrostatic interactions play a large role in stabilizing the complex, such as protein/metal, protein/DNA, and DNA/DNA interactions [48]. Interactions that are primarily hydrophobic in nature (including the binding of acyl CoA ligands to ACBP) are more likely to dissociated in the ESI process due to the absence of water.

Although the three-dimensional structure of a large biomolecule in the gas-phase has not been determined, a method for measuring the cross-section of gas-phase protein ions has been developed [49]. A study showed that the gas-phase ions of cytochrome *c* in aqueous solution had a subs-

tantially small cross-sectional area than ions generated from a denaturing solution of 50% acetonitrile. Assuming denatured cytochrome *c* has a less compact structure than native cytochrome *c*, these results suggest that the protein may retain at least some of its solution conformation. A comparison of other protein ions by this method showed a positive correlation between the measured cross-sectional area of the gas-phase protein with that calculated from its three-dimensional structure [49].

The question as to whether the ESI-MS spectrum observed accurately reflects what is observed in solution is always a matter which must be addressed when studying any non-covalent biomolecular interaction using this technique. In some studies on non-covalent complexes by ESI, non-specific aggregates have been observed [48,50,51]. The presence of unexpected oligomers and other complexes observed in mass spectra have been shown, in some cases, to depend on the concentration of the protein and ligand [52]. It should be noted, however, that higher order oligomers can be observed at high analyte concentrations in solution as well. In ESI-MS the formation of unexpected oligomers are believed to be a result of both solution conditions and artifacts of the electrospray process. Studies which have examined ion–ion and ion–neutral reactions within the gas-phase have generally shown that the formation of complexes in the gas-phase is zero or very small [53]. It has also been shown that ion–ion and ion–neutral reactions in the gas-phase are important only at very high concentrations of analytes, typically greater than can be sprayed using conventional electrospray sources [54]. Nonetheless higher order oligomers are sometimes observed at high analyte concentrations and these observation necessitate careful control experiments to prove that the biomolecular complex observed in the gas-phase is specific and reflective of solution conditions. A good example of a simple experiment which may be performed to rule out the formation on non-specific aggregates which are due to concentration effects is shown in the study of the ras-GDP complex [55]. This group examined changes in the ESI-MS spectra of the ras-GDP complex over a concentration range of 0.625–20 μM. They observed a strong signal for the complex at all concentrations, strongly suggesting that the non-covalent complex was not a concentration effect. This type of experiment is not unlike a dilution experiment routinely performed during CD experiments to confirm the oligomeric state of a protein under study. Instrumental conditions may also be varied to see if they contribute any factor to the stoichiometry of the complex. Ligands which can be dissociated by raising the electrospray potential or orifice voltage may be non-specifically bound to the protein.

Control experiments to prove that the non-covalent complexed observed in the gas-phase is reflective of that observed in solution must be performed during ESI-MS studies, as with other spectroscopic techniques. One of the simplest control experiments involves altering the conditions of the solution in which the complex is maintained. Variations in pH, temperature, or adding organic solvents can dissociate many specific associations in solution. Any complex disruption observed in solution should be reflected as a corresponding change in the ESI mass spectrum. Probably the most convincing piece of evidence which can be used to prove the specificity of a gas-phase non-covalent complex is by modifying one of the complex components. For example, in the study of the Zn^{2+}-dependent binding of the VDR DBD with its target DNA, the specificity of the observed complex was proven by showing that the protein did not bind to a DNA oligonucleotide in which the bases in the binding site were randomized [14]. The same study also showed that the addition of the non-specific divalent cations, Ca^{2+} and Mg^{2+}, did not produce the same effects on complex formation as Zn^{2+} or Cd^{2+}.

4. Conclusions

The potential of ESI-MS in the study of non-covalent interactions involving biologically important molecules is only beginning to be realized. Being a relatively new technique in this field, ESI-MS has had to establish itself as a viable method which provides accurate results. Corre-

spondingly, many of the ESI-MS complex studies to this point have provided results which confirm those obtained by other more established methods. There are, however, a growing number of studies which have used the ability of ESI-MS to answer important biological questions which could not be sufficiently answered using other techniques. As with any other type of spectroscopy used to study non-covalent interactions, ESI-MS studies require the proper control experiments to confirm the specificity of the non-covalent interactions being observed. Although not discussed here, ESI-MS is widely used in the sequencing and identification of proteins. Of current interest is the use of ESI-MS in the proteome project whose goal, analogous to the genome project, is the sequencing and identification of all the proteins within the human proteome, including the types and positions of post-translational modifications.

As mentioned previously, non-covalent biomolecular interactions dictate the activity and function of a cell. Over the next few years there will be an increase in research studying large supramolecular complexes. This research trend provides a unique opportunity to use ESI-MS to determine the components and stoichiometries within these complexes. In addition to studying samples prepared by titrating isolated proteins with their prospective binding partner, ESI-MS will be used to study in vivo complexes derived from cell cultures or tissue preparations. This will place an emphasis on sample preparation, since the solution conditions used in the isolation of biomolecules from cells and tissues are not usually compatible with ESI-MS analysis. At this time there is an impass in exploiting ESI-MS to study non-covalent biomolecular complexes to the fullest. Most mass spectrometrists know the ability of ESI-MS to detect non-covalent complexes involving biomolecules, however, they lack knowledge in the field of biochemistry. Biochemists know the importance of the study of non-covalent complexes, however, they lack knowledge of the ability of ESI-MS to study non-covalent complexes. As the abilities of ESI-MS become better known in the scientific community, the collaboration between mass spectrometrists and biochemists will help develop more creative and unique opportunities in the study of biomolecular complexes by ESI-MS.

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