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International Journal of Mass Spectrometry 239 (2004) 33–41

International Journal of Mass Spectrometry

www.elsevier.com/locate/ijms

Review

The study of protein–ligand interactions by mass spectrometry—a personal view

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Received 3 August 2004; accepted 13 September 2004

Abstract

Mass spectrometry is an evolving technique for the study of protein–ligand interactions, and is unique in its ability to probe desolvated as well as solvated protein systems. This personal view highlights its potential to answer some fundamental questions on structure and energetics of protein–ligand interactions.
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Keywords: Mass spectrometry; Structure; Energetics; Protein–ligand interactions

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1. Introduction

Earlier this year, when I was travelling from my hometown Innsbruck in Austria to Ithaca, NY, with the purpose of conducting experiments in the laboratory of my collaborator Professor McLafferty at Cornell University, I came to immigration in Philadelphia. The immigration officer studied my visa and wanted to know what exactly my research was about. My rather self-conscious answer was “I am interested in what happens to biomolecules when you remove their native environment, water”. He looked at me and said “What is that good for?” I told him that “When you remove the water you can learn something about the intrinsic properties of biomolecules, such as proteins, without interference from the native environment, which is an extrinsic factor”. He smiled, stamped my forms, and said, “That sounds reasonable.” When

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doi:10.1016/j.ijms.2004.09.004

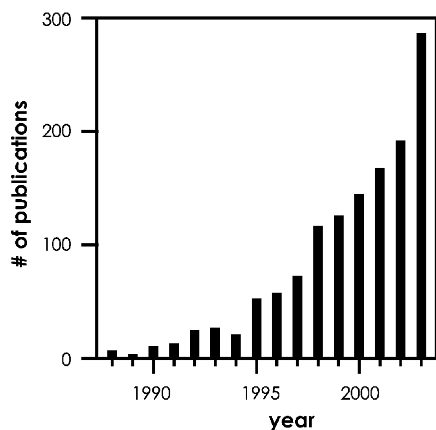

Fig. 1. The number of publications found in a literature search (SciFinder) on "mass spectrometry", "protein", and "ligand", for the years 1988–2003.

I started to leave, he suddenly waved me to stop and asked "So what happens when you remove the water?" I still owe him the answer.

This is a personal perspective on the study of protein–ligand interactions by mass spectrometry (MS), and is meant to make a case for the importance of studying desolvated as well as solvated protein systems, despite the occasionally intricate efforts involved. The steeply growing number of publications (Fig. 1) ever since the introduction of matrix-assisted laser desorption/ionization (MALDI) [1–3] and electrospray ionization (ESI) [4] mirrors the increasing importance of MS in the study of protein–ligand systems [5], a topic which has already been reviewed extensively [6–17]. Among the large variety of protein–ligand interactions are protein–protein, protein–RNA, protein–DNA, protein–cofactor, and protein–drug interactions, but here I want to discuss more the general aspects of protein interactions rather than individual systems. Understanding protein–ligand interactions as they affect biological function requires knowledge of the protein–ligand complex structures as well as the energetics (kinetics and thermodynamics) of binding. While NMR or X-ray structural data can be visualized in graphic representations, it is usually less straightforward to picture energetics. In particular, the interpretation of the energetics of protein folding and interactions is nontrivial. For solution studies, Cooper pointed out that "the measurement of the thermodynamics of biomolecular interactions is now relatively easy. Interpretation of these thermodynamics in simple molecular terms is not" [18]. In like manner, Clarke and Schreiber state that for protein–ligand binding, "...the underlying physics and chemistry of these processes are not yet completely understood" [19]. Considering the large variety of experimental techniques and strategies offered by mass spectrometry based approaches, along with its unique advantages such as speed and sensitivity, the contributions of MS to the understanding of protein–ligand interactions are likely to gain even more importance in the near future. Above all, the possibility to study completely desolvated yet thermally equilibrated protein systems in MS instruments with ion trapping capabilities will very likely help to answer some fundamental questions on structure and energetics of protein–ligand interactions.

In the following discussion of MS based techniques for the study of conformation and energetics of protein systems, a distinction is made between "solution experiments with MS detection" and "MS gas phase experiments". The latter refer to experiments in the low-pressure region of the mass spectrometer, and typically involve fully desolvated species from ESI. In "solution experiments with MS detection", the structural probing reaction involves covalent modifications of the protein in solution, and MS is merely a way of detecting the products.

1.1. Structural information from solution experiments with MS detection

Among the solution-based methods for structural probing by MS are hydrogen/deuterium (H/D) exchange of protein backbone amide hydrogens [13,16,20–29], oxidative radical reactions [30–32], and crosslinking experiments [33,34]. Each of the above reactions results in covalent modifications at accessible protein sites, and the corresponding changes in mass are easily determined by mass spectrometry given a mass spectrometer with sufficiently high mass resolving power [35]. For site-specific structural information with up to single-residue resolution, the chemically modified protein is typically digested and its peptide fragments analyzed by MS (the bottom-up approach), although top-down strategies with protein purification and fragmentation inside the mass spectrometer can be a valuable alternative [36–41]. A particularly promising top-down fragmentation method is electron capture dissociation [42–47], as it provides extensive sequence coverage and, unlike conventional dissociation methods, is not based on thermal or collisional ion heating [48] which can bring about deuterium scrambling [49–51].

Backbone amide hydrogens in proteins can be found next to every amino acid except on the N-terminal side of proline and the C-terminus. Their solution exchange by deuterium primarily depends on their involvement in hydrogen bonded structure and exposure to solvent [26], and the influence of neighboring residues via inductive and steric blocking effects can be calculated from values for model peptides [52]. Depending on experimental conditions, backbone amide H/D exchange reflects transient protein structures (EX1 regime) or equilibrium dynamics (EX2 regime). The kinetics of H/D exchange can be monitored by MS at nearly every inter-residue site, and data analysis can be automated for high-throughput applications [53]. Unwanted back-exchange after quenching of the exchange reaction and during sample handling prior to MS detection can be a problem, but is minimized by use of columns packed with immobilized pepsin [28], or in the top-down approach that does not require a digestion step at all [38]. More stable covalent protein modifications can be realized with chemical reagents such as acetic anhydride or *N*-hydroxysuccinimidyl acetate [39,54,55], but care has to be

taken that the reagents do not perturb protein structure, especially when high concentrations are used. From this standpoint, H/D exchange is clearly superior because any solvent can simply be replaced by its deuterated counterpart (e.g. H_2O by D_2O, CH_3OH by CD_3OD, etc.). Another limitation of aminoacetylation is that only the N-terminus and the lysine residues react, so that the extent of structural information critically depends on protein sequence. However, this approach could prove very useful in cases where extensive purification is required or protein solubility is an issue, for example with membrane proteins.

Hydroxyl radical reactions to probe the exposed surfaces of proteins or protein complexes are usually irreversible and result in stable oxidation products [30]. Moreover, no potentially interfering solution additives are needed because hydroxyl radicals can be generated by interaction of X-rays from a synchrotron source with H_2O, the main constituent of aqueous protein solutions. So far, twelve different amino acid residues have been identified as useful probes for the structural characterization of protein systems via hydroxyl radical reactions [32]. A unique aspect of radiolysis is that the hydroxyl radicals can be formed with high efficiency and on a relatively short time scale from just any H_2O molecules in the protein solution, including those in the first hydration layer. Thus the speed of chemical labeling via hydroxyl radical reactions is not generally limited by diffusion of the reactant through the protein solution, which allows the study of fast conformational changes [13,30]. As an alternative to synchrotron radiation, electrical discharge within an atmospheric pressure ESI source for the generation of hydroxyl radicals from gaseous O_2 and H_2O has also been reported. However, here the chemical probing reaction appears to occur at the interface of solution and gas phase [30,56], where protein structure may be disturbed [57].

In summary, both H/D exchange of backbone amide hydrogens and side-chain modifications provide time-resolved, site-specific information on the exposure of individual residues to solvent, which can be used for the identification of ligand binding sites and the determination of changes in protein conformational flexibility upon ligand binding [15,16,21,23–25,58–60]. However, none of these methods provides direct information on the three-dimensional structure of protein systems. This is where yet another approach comes in, chemical crosslinking for the determination of through-space distance constraints. MS analysis of chemical crosslinking products in combination with molecular modeling leads to low-resolution three-dimensional structures of proteins and protein complexes [33,34], which can potentially be refined by H/D exchange data.

1.2. Energetic information from solution experiments with MS detection

For a bimolecular protein–ligand complex, the Gibbs free energy of dissociation, ΔG_d, is related to the equilibrium dissociation constant, K_d, by $\Delta G_d = -RT\ln(K_d/c_0)$, where c_0 is

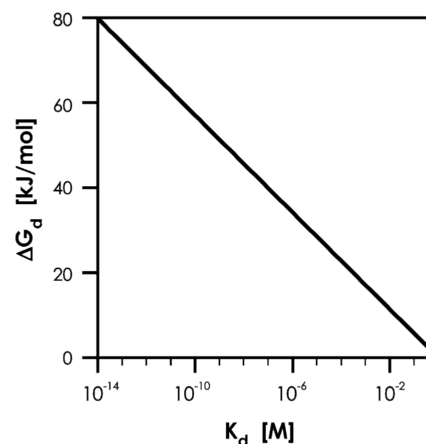

Fig. 2. The free energy change, ΔG_d, versus equilibrium constant, K_d, for protein–ligand dissociation reactions at 298 K, illustrating the relatively narrow energy range of biologically relevant protein–ligand interactions.

the standard state concentration (1 M); Fig. 2 illustrates this relation for equilibrium constants of up to 10^{-14} M at 298 K. The below mass spectrometry based approaches for the determination of protein–ligand binding constants in solution either monitor changes in protein molecular weight, or use MS for the quantification of small ligand molecules in the binding assay.

Besides site-specific structural information, solution H/D exchange of protein backbone amide hydrogens can also yield thermodynamic information, i.e. equilibrium constants for protein–ligand dissociation, K_d, or association, $K_a = 1/K_d$. Two such methods have been developed and discussed recently [61], SUPREX (*S*tability of *U*npurified *P*roteins from *R*ates of H/D *EX*change) [62–66] and PLIMSTEX (*P*rotein–*L*igand *I*nteractions in solution by *MS*, *T*itration, and H/D *EX*change) [67,68]. Briefly, the SUPREX approach relies on a difference in backbone amide H/D exchange for the protein by itself and the protein–ligand complex when changing the solution conditions from native to denaturing. For two solutions, one containing only protein and the other the protein–ligand complex, protein mass increases due to H/D exchange after specific exchange times are plotted versus denaturant concentration. The transitions from lower to higher protein mass values reflect the two-state transitions from folded to unfolded protein or protein–ligand complex (SUPREX requires EX2 exchange), as with increasing denaturant concentration more amide hydrogens become accessible for H/D exchange. Indicative of binding-induced stabilization, the denaturant concentration in the transition midpoint is higher for protein–ligand complex than for the protein by itself. Protein stabilities in the absence and presence of ligand obtained from transition midpoint values at various exchange times (between a few minutes and several hours) are then used for the calculation of equilibrium binding constants [63,64,66]. For the PLIMSTEX analysis, the protein is titrated with ligand, and the increase in mass due to backbone amide H/D exchange is plotted versus

ligand concentration [67]. Typically, the increase in protein mass is largest in the absence of ligand, and gradually decreases with increasing ligand concentration as a result of an increased protection of backbone amide protons on forming the protein–ligand complex. Equilibrium binding constants are obtained from fitting the H/D exchange data with the newly developed "1:*n* protein:ligand sequential binding model" [68]. Both SUPREX and PLIMSTEX have been successfully used for the determination of solution binding constants in protein systems of various stoichiometry, and the partially deuterated proteins can in principle be subjected to further analysis as outlined above.

For very large or membrane-bound proteins, the measurement of H/D exchange by MS may be difficult or even impossible. In such cases, a "competitive MS binding assay" that is similar to competitive radioligand binding assays but uses native markers and MS for quantification [69] may be more useful. Briefly, the competitive binding of the ligand under study to a specific protein-binding site releases a marker molecule (that binds with high affinity and selectivity at this site) from the protein–marker complex. The free marker is separated from the assay and quantified by MS using a calibration curve from another experiment without protein. K_d values are ultimately calculated from the change in equilibrium concentrations of free marker with competing ligand concentration.

1.3. Structural information from MS gas phase experiments

Structural information on desolvated protein ions comes from ion mobility measurements and related techniques [70–82], gas phase H/D exchange [49,83–89], gas phase proton transfer reactions [90–95], electron capture dissociation [89,96,97], and, very recently, infrared photodissociation spectroscopy (IRPDS) [89,98,99]. Ion mobility and related experiments provide only a single parameter, the collision cross section, but this can be followed for individual protein charge states at different temperatures, pressures, initial ion kinetic energies, and in the presence of proton transfer reagents [74]. Moreover, structural transitions can be monitored on time scales ranging from ~1 ms to 30 s in hybrid instruments with ion trapping capabilities [81,82]. The collision cross sections depend directly on conformation and can be correlated with theoretical values for calculated structures [76,100,101]. H/D exchange in the gas phase is consistent with a "relay" mechanism [83,86] and can resolve a multitude of coexisting stable gaseous protein conformers [49,89]. However, data interpretation is more complex than for H/D exchange in solution because the gas phase exchange mechanism has higher structural requirements involving the simultaneous formation of two hydrogen bonds with D_2O [86]. The elevated structural demands of the H/D exchange reaction in the gas phase may also obscure the contribution of the exposed surface area [102]. For example, increased protonation is generally associated with more open structures as a result of Coulombic repulsion (corresponding to decreased pH in solution), yet within a given conformer family this actually decreases gas phase H/D exchange because unlike in solution, protonated basic residues are not exposed to solvent but solvated intramolecularly instead [49,89]. ECD gives site-specific information on whether or not individual residues are involved in tertiary bonding, and can be used to monitor the site-specific unfolding and refolding of gaseous protein ions [89,96,97]. IRPDS is the gas phase variant of classical infrared spectroscopy and provides information on the noncovalent bonding of functional groups such as O–H and N–H [89,98,99]. Although proposed gas phase protein structures are beginning to appear in the literature, it is also evident that the field is still in its infancy.

At present, even less is known about the structure of gaseous protein–ligand systems. Many spectra with signals corresponding to protein–ligand masses have been published during the last decade, and these are often referred to as the "intact complex" signals. The term is misleading, because it implies that the solution structure of the complex is retained in the gas phase if only the complex partners are not separated during transfer into the gas phase and an "intact complex" signal is observed. However, such reasoning ignores possible conformational rearrangements during transfer into the gas phase that can substantially alter the complex structure, even without separation of the complex partners. Put another way, structural rearrangements do not necessarily result in dissociation of the complex. Evidence for considerable conformational rearrangements as a result of desolvation comes from native electron capture dissociation (NECD) [103,104], ion mobility studies [81,82], and molecular modeling calculations [105]. The "gentle" desolvation and ion transfer conditions typically used for MS of protein complexes minimize energetic activation, but cannot prevent changes in the stability of higher-order interactions as a result of solvent removal. Dehydration eliminates the competition of water for hydrogen bonds within and between complex partners, whose strength can increase in the gas phase up to a point where a hydrogen-bonded structure is thermally more stable than its covalent bonds [106]. On the other hand, removal of water drastically weakens hydrophobic interactions that may account for the most stable regions in a native structure [103,104], and can disrupt a stabilizing network of hydrogen bonds [107]. Moreover, electrostatic interactions in protein systems cannot remain unchanged when the surrounding water with its rather high dielectric constant ($\varepsilon_r \sim 80$) is replaced by vacuum. Given the altered stability of higher order interactions in a gaseous environment, it can be expected that some bonds are broken on transfer into the gas phase, while new ones may be formed. Thus it is rather unlikely that the original solution structure is preserved in the gas phase, and this may just as well affect the binding interface of a protein–ligand system [108,109].

In cases where the intermolecular bonds present in solution are broken upon desolvation and no new ones formed, the protein–ligand complex will dissociate. Studies on the effect

of inert gas pressure in the ion desolvation region found that the relative abundance of gaseous protein complex versus its dissociation products can actually increase with increasing pressure [110,111]. This is consistent with the rapid formation of new intermolecular bonds in the presence of multiple collisions, which locally transfer momentum and can thereby aid the complex in swiftly exploring the conformational space to adopt more stable gas phase structures. Support for this hypothesis of collisionally assisted structural rearrangements of gaseous protein systems comes from gas phase H/D exchange experiments which identify new stable gaseous protein conformers after exposure to collision gas [49] and ion mobility data [112]. In the absence of collisions, the timescale for protein conformational reorganization may be too long to prevent complex dissociation [96,97]. New noncovalent bond formation during ESI can also lead to the formation of unspecific protein–ligand association products, i.e. complexes that were not originally present in solution, whose kinetic stability in the gas phase can even exceed that of specific complexes [109,113]. Because unspecific complexes can form during ESI, and specific complexes may not be stable in the gas phase, caution is advised with approaches that infer solution characteristics from relative ion abundances in a mass spectrum [113–117]. For example, consider a solution containing 10 μM protein and 10 μM ligand. Given a 1:1 complex stoichiometry and a protein–ligand dissociation constant of 10 nM, the solution concentrations of protein, ligand, and protein–ligand complex are 311, 311 nM, and 9.689 μM, respectively. If only 10% of the protein–ligand complex dissociates upon transfer into the gas phase, the mass spectrum would indicate protein, ligand, and protein–ligand concentrations of 1.280, 1.280, and 8.720 μM, respectively, and an erroneous dissociation constant of 188 nM. However, for larger protein systems, it appears that complex stoichiometry is usually retained in the gas phase [6,10,12,15,17,118,119], possibly because of a sufficiently large binding interface. It was estimated that a binding interface area of about 1500 $Å^2$ that comprises about ten hydrogen bonds is sufficient to ensure dissociation constants as low as 10^{-14} M for protein complexes in solution [120]. Although a corresponding estimate for protein complexes in the gas phase is difficult to make, it is reasonable to assume that larger binding interfaces generally increase stability in the gas phase as well.

More detailed information on the gas phase structures of protein–ligand complexes is currently only available for smaller systems, for which several experimental strategies have been applied, among them ion mobility [121,122], ECD [123], and gas phase ligand-exchange reactions [124]. With the perpetual refinement of mass spectrometry instrumentation and the development of new gas phase probing methods, it is only a question of time until site-specific structural data on larger gaseous protein–ligand systems will appear in the literature. These will then go a long way towards the understanding of the intrinsic factors of protein complex stability, as well as the role of hydration in forming a native structure [74,125–130].

1.4. Energetic information from MS gas phase experiments

Most energetic data on desolvated protein–ligand complexes come from blackbody infrared radiative dissociation (BIRD) experiments in Fourier transform mass spectrometers (FTMS) [131–133]. In a typical BIRD experiment, ions from ESI are transferred into the low-pressure region ($<10^{-9}$ mbar) of the FTMS instrument and trapped in its ion cell for extended periods of time. Energy exchange between the ensemble of protein–ligand complex ions and the ion cell, which is held at a defined temperature between typically 20 and 200 °C, occurs via blackbody infrared radiation [132]; specially designed ion cells which can be operated at temperatures as low as −196 °C can be useful for the study of very weak interactions [134,135]. Arrhenius parameters for the dissociation of protein–ligand complexes are obtained from a fit of dissociation rate constants at different temperatures with the Arrhenius equation, $k_d(T) = A\exp(-E_a/RT)$. Because of their large number of degrees of freedom, most biologically relevant protein systems can safely be assumed to fall into the rapid exchange limit where the measured activation energy equals the activation energy in the high-pressure limit [132,133,136]. BIRD can be used to study the kinetics of protein–ligand complex dissociation in the gas phase, but the reverse reaction is not generally possible. However, complex formation from monomers of opposite charge is a promising new experimental strategy for the study of protein–ligand association reactions in the gas phase [137,138].

As discussed above, the structure of a protein–ligand complex in the gas phase may differ from that in solution, in which case BIRD probes the dissociation energetics of the rearranged gaseous complex. If desolvation did not result in significant structural rearrangements, BIRD probes the dissociation energetics of the original complex, but with its altered strengths of interactions in the gas phase. This is consistent with the observation that the kinetic stabilities of protein complexes in the gas phase do not generally correlate with solution values [14,139,140]. Differences in energetic stability of protein–ligand complexes in solution and in the gas phase can, however, be used to evaluate enthalpy changes associated with complex desolvation: In a recent study based on functional group replacement and BIRD, the effect of protein-carbohydrate complex desolvation on binding enthalpy was quantified for individual interactions in the complex binding interface [141].

It was shown recently that kinetic data from a large number of BIRD experiments on different protein–ligand systems give strongly correlated Arrhenius activation energies, E_a, and preexponential factors, A, with $E_a \sim \ln A$ [142]. A similar phenomenon for biomolecular interactions in solution is the thermodynamic entropy–enthalpy compensation, where $\Delta H \sim \Delta S$. Although both ΔH and ΔS vary strongly with temperature, the changes in free energy, $\Delta G = \Delta H - T\Delta S$, are relatively small and a plot of ΔH versus ΔS is nearly linear [143,144]. The strong temperature dependence of ΔH and

ΔS has been attributed to large changes in heat capacity, ΔC_p, associated with biomolecular interactions [143]. In solution, large changes in heat capacity are the result of changes in solvation and conformational flexibility. The observed correlation of E_a and A for gas phase dissociation reactions initiates a number of questions. How large is the heat capacity change on protein–ligand dissociation in the gas phase, and how strong is the variation of E_a and A with temperature? What is known about the conformational flexibility of protein systems in the gas phase, and can gas phase H/D experiments teach us more? Many proteins have regions of high flexibility that become structured only when bound to ligand, but is this determined by protein sequence or hydration, or both? What is the role of water in protein dynamics and equilibrium fluctuations [145,146]? Do individual water molecules increase [147] or decrease [148] protein flexibility? Or, in general, what is the contribution of hydration to the thermodynamics and kinetics of protein interactions? Experimental approaches in solution replace water by poly(vinyl alcohol), ice, trehalose, sucrose solutions (92%), glycerol/water mixtures [146], water/ethanol mixtures [149], or confine water in porous materials [150]. However, changing the solution composition adds complexity to the protein system instead of reducing it. In contrast, mass spectrometry offers the unique possibility to completely eliminate any solvent and study dehydrated protein systems in gas phase experiments, which can be complemented with molecular dynamics simulations [151,152]. Moreover, individual conformers with distinct charge states (corresponding to pH in solution) can be experimentally selected and isolated based on their m/z values and reactivity or collision cross section [153,154], allowing a much more detailed analysis than is possible in bulk solution.

An intriguing phenomenon observed with the collision-induced or thermal dissociation of gaseous protein multimers was termed “disparate charge separation” or “asymmetric charge partitioning” [155–158]. Although this unusual behavior has been documented for noncovalent protein complexes comprising as many as 33 proteins and two RNA units [118], it is perhaps best illustrated with noncovalent protein homodimer complexes [155,157,158]. Here, the two protein monomers from energetic activation of a positively charged gaseous homodimer complex carry off not half of its charge each, but instead the complex charge is distributed unevenly among the two dissociation products, with asymmetries as high as 11:4 [158]. This charge asymmetry is in fact sufficient to initiate intermolecular electron transfer in gaseous Cytochrome *c* dimer ions, from which detailed tertiary structural information can be obtained [103]. A thorough study on the effect of protein conformational flexibility revealed that asymmetric charge partitioning is caused by the (partial) unfolding of one of the protein monomers prior to dimer dissociation, thereby increasing its basicity [158]. Moreover, it was found that the extent of charge asymmetry increases with increasing energy available for dissociation, with symmetric dissociation being the lowest energy process [158]. Assuming a two-state process for homodimer protein complex dissociation in the gas phase, this means that dissociation must proceed via considerably different transition states depending on the energy deposited, in conflict with a data analysis based on transition state theory.

Currently, a major limitation of gas phase experiments for the study of noncovalent protein–ligand complexes is that a true equilibrium situation cannot be readily established for most systems. This is because for a charged protein and a neutral ligand, the ligand may be absorbed by the vacuum chamber walls or pumped away after protein–ligand dissociation, and can only be replenished if sufficiently volatile. For protein and ligand of the same charge polarity, dissociation is accelerated and association decelerated as a result of Coulombic repulsion between protein and ligand, strongly shifting the equilibrium towards separated products. For protein and ligand of opposite charge, both dissociation and association reactions in ion trap mass spectrometers are possible [137,138], but a dissociation–association equilibrium at uniform temperature has not yet been reported. Thus all current energetic data on larger protein–ligand systems in the gas phase are kinetic data of complex dissociation, which are typically analyzed in terms of transition state theory. However, the application of transition state theory to protein folding in solution has been questioned [159–161], and it is equally questionable if transition state theory is sufficient to describe protein–ligand dissociation as this may involve a rather broad ensemble of transition state conformations, or even intermediate structures [105,162]. Gas phase MS experiments in which discrete conformers of a single charge state are selected and isolated prior to dissociation, and the products analyzed with respect to conformation, for example by use of H/D exchange reactions or spectroscopic methods, may provide invaluable insights in addressing these fundamental questions.

2. Conclusions

In his brilliant 1997 review on the study of noncovalent protein complexes by ESI, Joseph Loo remarks that “mass spectrometrists tend to extend an MS-based methodology to solve virtually every scientific problem” [6]. I love this enthusiasm, which, in my view, comes from the truly interdisciplinary nature of MS-based research that brings together physicists, chemists, pharmacologists, biologists, and natural scientists in general. However, one of the things we are truly good at is that we surely know how to remove solvent from a biomolecular system (any solvent, that is). Personally, I do not consider it an unfortunate situation that protein systems can lose biological function in their desolvated states. Instead, I think of a desolvated protein system as an undisturbed model for investigating protein–ligand interactions, which offers a great opportunity for detailed studies using the wide variety of already established as well as future MS based methods.

Acknowledgements

The author acknowledges generous funding from the Austrian Science Foundation (grant P15767) and the Austrian Science Department BMBWK (grant T229), and discussions with Fred W. McLafferty, Barry K. Carpenter, Rich Knochenmuss, Bernhard Kräutler, Huili Zhai, Cheng Lin, Xuemei Han, Harold Hwang, Robert Konrat, Julia Laskin, Ronald Micura, Claudia Höbartner, Renate Rieder, Christoph Kreutz, Kathrin Lang, and Thomas Müller.

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