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Studying noncovalent protein complexes in aqueous solution with laser desorption mass spectrometry

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Abstract

The study of noncovalent aggregation with mass spectrometry has been largely the domain of electrospray ionization mass spectrometry (ESI-MS). In contrast, matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) has been applied to this field to a much lesser extent. The main drawback of MALDI-MS is that the sample preparation requires a crystalline matrix. This disrupts the solution environment and often leads to dissociation of noncovalent complexes. A new laser desorption method, developed in our group, promises to circumvent this shortcoming. It is called laser induced liquid beam ionization/desorption mass spectrometry (LILBID-MS). The major advantage of this new method is the use of a liquid beam in vacuum for sample preparation and as target. The beam is directly injected into the mass spectrometer, using the solvent (mostly water) as the natural matrix substance, thus allowing for a softer probe preparation and desorption. In this article we present examples for the application of this new desorption method for detecting noncovalent aggregates of proteins in aqueous solutions. Using ribonuclease S, calmodulin/melittin, and bovine pancreatic trypsin inhibitor as model systems, evidence is given that LILBID-MS is capable of desorbing intact noncovalent complexes into the gas phase. Even water bound into cavities of a protein structure can be detected. In addition, it will be shown that solution parameters (e.g. pH, temperature) have a decisive influence on the mass spectra obtained, thus confirming earlier observations that the ions detected by LILBID-MS are formed in the solution phase and are not gas phase artifacts produced by the detection process. (Int J Mass Spectrom 203 (2000) 49–57) © 2000 Elsevier Science B.V.

Keywords: Laser desorption; Liquid beams; Proteins; Noncovalent interactions

1. Introduction

Noncovalent interactions of proteins are essential for life on earth. In enzyme-substrate complexes, noncovalent interactions ensure that the modified substrate can leave the reactive site, so that the enzyme can work as a catalyst. In signal transduction, noncovalent interactions ensure that information is only transported when it is necessary. Furthermore, noncovalent interactions allow nature to use a given protein as a part of many different complexes, thus providing maximum reusability. All these noncovalent complexes are held together by weak intermolecular forces, allowing them to form and to dissociate very easily. Monitoring noncovalent interactions of proteins in solution is therefore a very demanding

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analytical task. First, the complexes must not be dissociated during preparation. This is a necessary condition because most analytical methods cannot be carried out *in vivo*. Some sample preparation steps are needed, such as the removal of the matrix or the concentration of the sample. Having solved this problem, it is still necessary to use an analytical method that itself does not dissociate the weakly bound complexes in the detection process. In mass spectrometry the realization of both exigencies may be a problem. Electrospray ionization mass spectrometry (ESI-MS), for example, requires very pure samples, because salt impurities decrease the quality of the spectra dramatically. Matrix assisted laser desorption ionization, MALDI (which tolerates much higher salt concentrations) requires a crystalline sample, which is a highly artificial environment for a protein. To detect complexes in the gas phase it is necessary to remove a great part of the surrounding solvent or matrix molecules that are bound by noncovalent interactions. At the same time, the complex must not be destroyed. These various exigencies are hard to fulfill and cannot always be met.

To meet this challenge, we recently developed a new desorption method called laser induced liquid beam ionization/desorption mass spectrometry (LILBID-MS) [1–4]. With this method it is possible to desorb solvated ions directly from a liquid. The latter is injected as a microscopic liquid beam directly into the high-vacuum of a mass spectrometer. In the region of laminar flow the beam is intersected by a pulsed infrared laser beam. The wavelength of the latter is tuned to a vibrational absorption band of the solvent. The ions in the solution are laser desorbed and detected in a time-of-flight (TOF) mass analyzer. It is possible to use pure aqueous solutions, making the sample preparation very easy. Using an infrared desorption laser ensures a soft desorption of the proteins and of their complexes. This method has been applied to the detection of noncovalent complexes such as diglycyl-histidine-copper [5] or human hemoglobin [6] from aqueous solution and gramicidin and oligonucleotide dimers from methanol. A very different application was the study of the nucleation of titanium dioxide in liquid phase under various conditions [7].

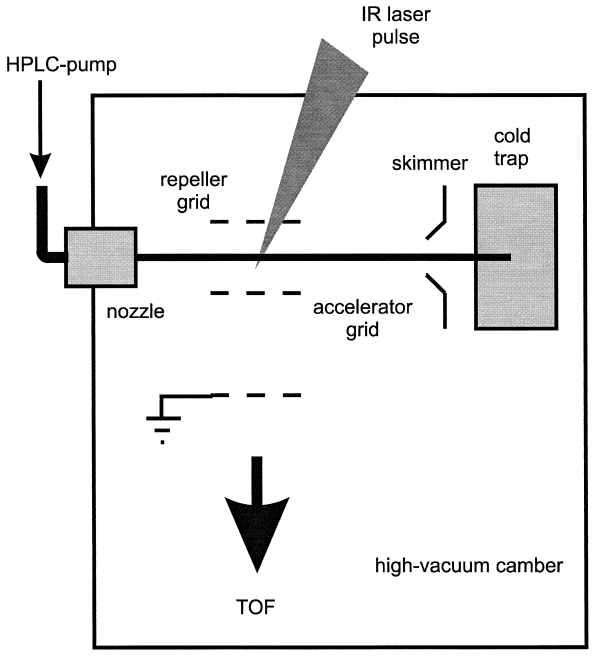

Fig. 1. Schematic view of the LILBID ion source. The liquid beam is introduced into the vacuum chamber through a nozzle with a 10 μm aperture and a backing pressure of 30 bar. The focused infrared laser pulse is intersected with the liquid beam in order to desorb the solvated ions. These ions are then accelerated into the time-of-flight mass spectrometer.

In this article, we report new results on the detection of noncovalently bound protein complexes. Instead of an in-depth analysis of one system, we present different examples where LILBID-MS was applied to analyze biomolecules and their complexes in aqueous solution.

2. Experimental

2.1. Mass spectrometer

Because the experimental set-up of the mass spectrometer used is described in detail elsewhere [2,6], only a brief description will be given here. A diagram of the ion source is given in Fig. 1. The analyte solution is directly injected into the high vacuum of

the mass spectrometer through a 10 μm nozzle by means of a high-performance liquid chromatography (HPLC) high pressure pump at a backing pressure of 30 bar. After about 10 cm free flight the liquid beam is frozen out on a liquid nitrogen cool trap in order to maintain a pressure of 10^{-6} mbar in the desorption chamber. In the region of free flight, the liquid beam is intersected with a pulsed infrared laser beam of which the wavelength is tuned to an infrared absorption band of the liquid. For an infrared laser source we used a home-built pulsed optical parametric oscillator (OPO) pumped at 10 Hz by a Nd:YAG laser. Typical energies per pulse are 10 mJ. The ions desorbed into vacuum are accelerated into a home-built time-of-flight mass analyzer using a two-field extraction region with a total acceleration voltage of 1500 V. After passing through an ion mirror, the ions are post-accelerated and detected on a home-built Daly-type ion detector. The data were stored and averaged in a digital storage oscilloscope (LeCroy, Model 9410). All spectra shown are averages of 1000 laser shots. The typical mass resolution in the high-mass region was up to 50.

2.2. Chemicals

Ribonuclease S, calmodulin, melittin, and bovine pancreatic trypsin inhibitor were obtained from SIGMA (Sigma-Aldrich, Deisenhofen, Germany) and used without further purification. The proteins were dissolved in water or in the buffers as described in the appropriate sections. The solutions were filtered prior to measurement.

2.3. Reduction and alkylation

The bovine pancreatic trypsin inhibitor (BPTI) was dissolved in a 10^{-2} M ammonium-acetate buffer (pH 8) to give a final protein concentration of 10^{-4} M. The disulfide bonds were reduced using an 80-fold excess of dithiothreitol (DTT, obtained from SIGMA). The reaction was carried out for 1 h at 45 °C. The solution was then treated with a 2.5 molar excess of 4-vinylpyridine (relative to DTT). The solution was allowed to react for another half hour at 37 °C whereupon some precipitation occurred. The reaction was stopped by adding a few drops of acetic acid that lead to a resolubilisation of the precipitate. This solution containing the reduced and alkylated BPTI was analyzed directly without further purification.

3. Results

3.1. Ribonuclease S

Ribonuclease S (RNase S) is a noncovalent protein-peptide complex that is produced by a limited proteolysis of ribonuclease A. It still retains the activity of its precursor, but is quite susceptible to the temperature [8,9] and the pH value [10,11] of the solution. Its optimum stability occurs at 273 K and neutral pH value. This lability of the complex makes RNase S a very interesting object for mass spectrometric studies. The RNase S complex has been studied both by ESI-MS [12,13] and MALDI-MS [14]. With ESI-MS, it was possible to detect the intact complex quantitatively and the specificity of the intermolecular binding was shown by competitive binding experiments with mutant peptides. Also, the temperature dependence of the complex stability could be reproduced. The UV-MALDI experiments were undertaken using the “soft” matrix ATT (3-azathreothymidine). Only a small portion of the complex could be detected; most of the RNase S was dissociated. Still, the specificity of the complex was shown by a competitive binding experiment with Angiotensin II. The LILBID-MS studies of RNase S were carried out using a 10^{-5} M RNase-S solution in an aqueous 10^{-2} M ammonium-acetate buffer at pH 6. The temperature of the solution injected into vacuum was regulated by a cooling device.

First, the LILBID-MS spectra were recorded as a function of the temperature of the solution [Fig. 2(a)–(c)]. It was varied from -15 to $+25$ °C while the laser power was held slightly above the desorption threshold of the ions. From the spectra it is evident that with a rising temperature of the solution the complex gets more and more dissociated. In the most favorable case [Fig. 2(c)] only about 50% of the

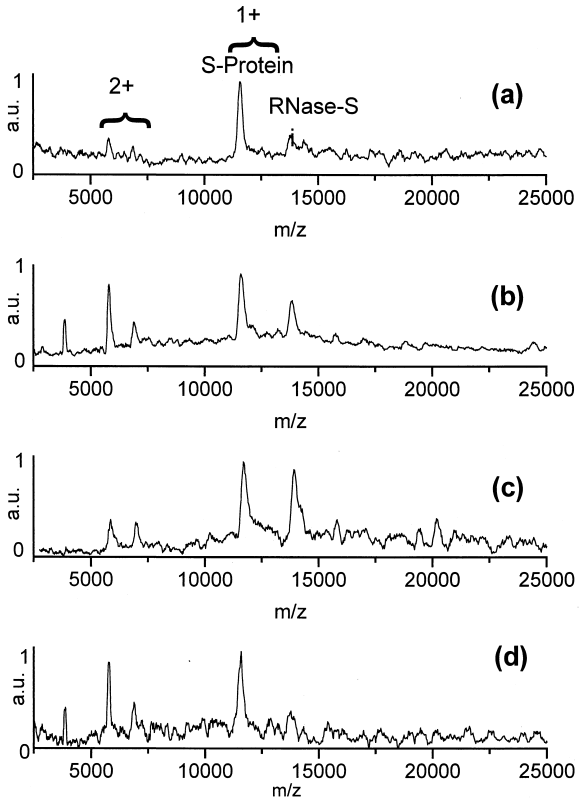

Fig. 2. Positive ion LILBID mass spectrum of a 10-5 M Rnase S solution in a 10-2 M aqueous ammonium acetate buffer, pH 7. The solution temperature and the desorption laser energy were varied as follows: (a) +25 °C, 10 mJ/pulse, (b) −5 °C, 14 mJ/pulse, (c) −15 °C, 16 mJ/pulse, (d) −5 °C, 35 mJ/pulse.

complex is detected unfragmented. Also, the energy needed to desorb the complex is higher at lower temperatures.

In the detection process of this molecule there is a certain extent of laser induced complex dissociation involved. This is shown in Fig. 2(d), where the laser energy was increased at a constant temperature of the solution, leading to an enhanced complex dissociation. To show the specificity of the RNase S complex, two experiments were carried out. First, the solution was acidified with HCl. At a pH value of 2 in the condensed phase the complex should be totally dissociated. Accordingly, the LILBID mass spectrum taken from such a solution no longer show the complex peak [Fig. 3(a)]. Under otherwise identical

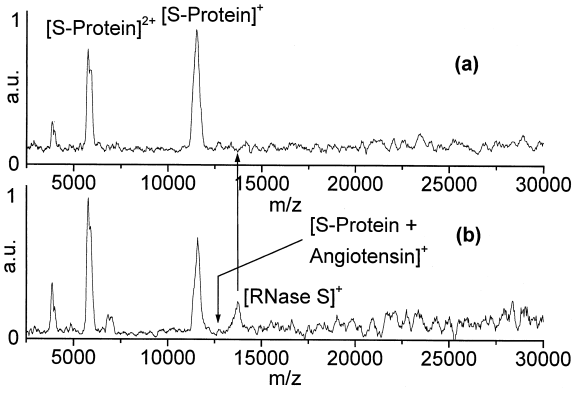

Fig. 3. Positive ion LILBID mass spectrum of a 10-5 M Rnase S solution in a 10-2 M aqueous ammonium acetate buffer with a solution temperature of −15 °C and a desorption laser energy of 20 mJ/pulse. (a) The pH value of the solution was 2. (b) The pH value of the solution was 7 and 10-5 M Angiotensin II was added.

conditions, as in Fig. 2(c), only the free protein can be identified. This shows that the solution conditions have decisive influence on the mass spectra obtained with this method.

Secondly, Angiotensin II, an unrelated peptide, was added to a solution of RNase S at neutral pH value. It is clear from the LILBID mass spectrum shown in Fig. 3(b) that only the S-Protein/S-Peptide complex is formed. No unspecific binding of Angiotensin II can be detected. Even though a major part of the noncovalent complex is dissociated during the desorption process, unambiguous evidence of the specificity of the remaining complex signal could still be given.

3.2. Calmodulin

Calmodulin is a protein that regulates calcium dependent processes in various organisms [15]. It can bind up to four calcium ions with a binding constant of 10^{-5}–10^{-6} M^{-1}. These ions induce a dumbbell shaped conformation of the protein with two globular domains connected by a helix. Each globular domain has two calcium binding sites, with a helix–turn–helix motif. The calcium is coordinated by the oxygen atoms of the backbone, the side-chains, and one water molecule. These binding sites are highly specific, e.g.

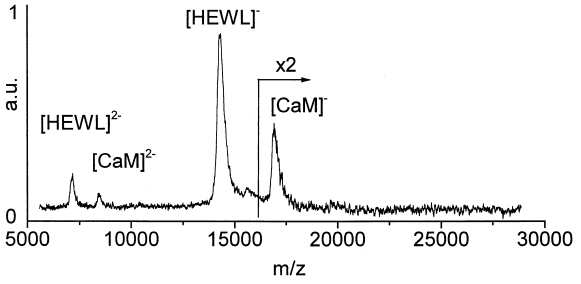

Fig. 4. Negative ion LILBID mass spectrum of 10-5 M calmodulin (CaM) in water. Hen egg-white lysozyme (HEWL) was added as calibration standard.

magnesium ions are not bound and cannot induce this conformational change in the condensed phase.

Only the calcium induced dumbbell conformation is capable of binding small amphiphatic peptides such as melittin. Upon binding the peptide, a second conformational change is induced. Now the long helical region of the calmodulin collapses and the globular domains bind to the peptide. This binding is very strong with a dissociation constant in the nanomolar range [16]. The calmodulin/Ca [17–19] and the calmodulin/Ca/peptide [20,21] complexes have also been investigated by ESI-MS. With these experiments it could be confirmed that calmodulin possesses four calcium binding sites that are all occupied when binding a peptide. No peptide binding could be detected when using magnesium ions.

The metal-induced peptide binding of calmodulin made it a very interesting benchmark system for a study with LILBID-MS. Because calmodulin is a very acidic protein, all spectra have been recorded in the anion mode. In Fig. 4 a negative ion LILBID mass spectrum of calmodulin is shown. Hen egg-white lysozyme (HEWL) (m = 14 305 Da) was used as an internal calibration standard. Singly charged ions dominate the mass spectrum, as in the case of the cation spectrum of RNase S above. LILBID-MS detects ions with a low charge state regardless of their polarity. The mass of the calmodulin is found to be 16 900 ± 50 Da, ~110 Da higher than the theoretical mass. This mass shift presumably is due to the high number of counter ions (23 acidic sites) that are required to neutralize the charged side chains in order

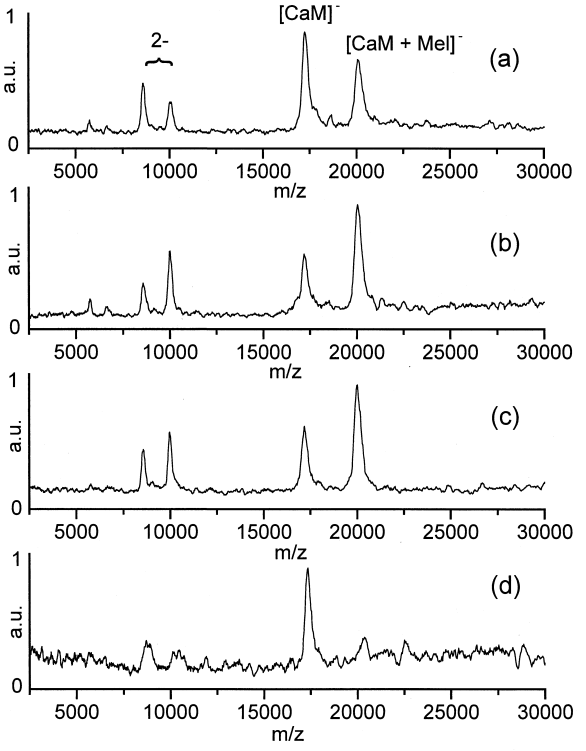

Fig. 5. Negative ion LILBID mass spectrum of 10-5 M calmodulin (CaM) and 10-5 M melittin in 10-2 M ammonium acetate buffer, pH 7, and 10-2 M calcium-acetate. The solution temperature and the desorption laser energy were varied as follows: (a) 0 °C, 18 mJ/pulse, (b) +25 °C, 18 mJ/pulse, (c) +25 °C, 26 mJ/pulse. In (d) the calcium acetate was substituted with magnesium bromide.

to produce the singly charged ion. The heterogeneity of these counter ions leads to a shift and a broadening of the calmodulin peak in the LILBID spectra.

Next, the binding of melittin to calcium-saturated calmodulin was investigated. Therefore, an equimolar solution of calmodulin and melittin was treated with a large excess of calcium acetate. In the LILBID mass spectrum a new peak arises that can be assigned to the newly formed protein-peptide complex. Unfortunately, a part of the complex is dissociated upon desorption [Fig. 5(a)]. Rising the temperature of the injected solution quite unexpectedly leads to less dissociation in the mass spectrum [Fig. 5(b)]. Interestingly enough, the stability of the complex is independent of the laser energy used for desorption [Fig. 5(c)]. This is in contrast to the temperature and laser

energy dependence found in the case of RNase S shown above.

The specificity of the complex observed is demonstrated in a control experiment: the protein-peptide solution was prepared as above, but now magnesium ions are added in great molar excess. The LILBID mass spectrum in Fig. 5(d) shows no formation of the calmodulin/melittin complex, as expected from the condensed phase behavior. This clearly shows that the complex formation reflects properties of the solution, i.e. the specific role of the co-factor ions. Hence, LILBID-MS clearly monitors the solution phase binding behavior instead of a gas phase chemistry because in the latter case specificity would be strongly reduced.

3.3. BPTI

The results for laser induced desorption of bovine pancreatic trypsin inhibitor (BPTI) differ in one central aspect from the systems presented above. In this case, it was not our aim to investigate protein–protein complexes with LILBID-MS. Instead, this protein was chosen because of its ability to bind water molecules within its protein structure. Internal water molecules are a common feature in many protein structures. Unfortunately, ESI-MS and MALDI are not capable of detecting these specific water molecules because all proteins and complexes are usually detected completely dehydrated [22–24]. With ESI-MS it has been possible to observe hydrated ions in a few cases [25], but only broad distributions of bound water molecules were detected. These water molecules could also arise from recondensation of water vapor in the source region at reduced pressure. Only one very recent article has shown evidence of specific water binding [26], but here also the origin of the water molecules remains unclear.

The BPTI protein has four water molecules completely embedded in its structure and one more water molecule with a solvent accessible surface of under 10% [27]. Using ESI-MS one could not detect any water molecules bound to BPTI, even under conditions when the BPTI-trypsin complex was detected [28].

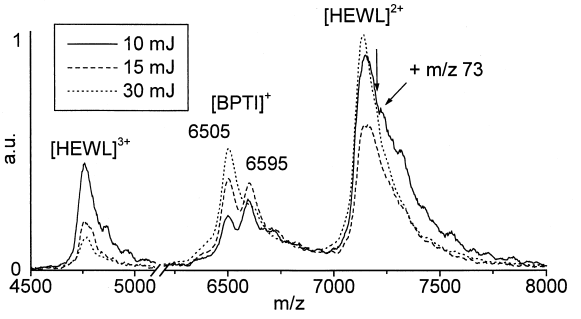

Fig. 6. Positive ion LILBID mass spectrum of 10-4 M BPTI in water. 10-5 M HEWL was added as calibration standard. The solution temperature was 0 °C; the desorption laser energy per pulse is given in the figure.

In Fig. 6 the LILBID mass spectrum of BPTI in an aqueous solution is shown at an energy of 30 mJ/pulse for the desorption laser. The HEWL was added as an internal calibration standard in the mass spectra. As can be seen clearly, the completely desolvated BPTI species (*m/z* 6505 Da/e) dominates the LILBID mass spectrum. Lowering the desorption laser energy gives rise to a peak at higher masses. At the threshold energy, this new peak is even more intense than the signal of the desolvated BPTI. Evaluating 32 individual mass spectra at low desorption laser energy, the mass difference between the two peaks could be determined as 95 ± 5 Da. Because the calibration peaks tend to get broader at lower desorption energy due to less efficient desolvation of the HEWL ions, the exact calibration of the spectra gets increasingly worse. This is the main reason for this rather big uncertainty in mass determination.

A corresponding mass shift cannot be detected for the HEWL peaks. This shows that the new peak is indeed a BPTI-dependent phenomenon. To further elaborate this point, BPTI was reduced and alkylated according to the prescription described in Sec. 2. This reaction destroys the tertiary structure of the protein and at the same time increases the molecular weight by 630 Da. Thus, it is possible to ensure the complete alkylation and, consequently, the denaturation of this protein by mass spectrometry.

The LILBID mass spectra of the denatured BPTI depicted in Fig. 7 show no dependence on the energy

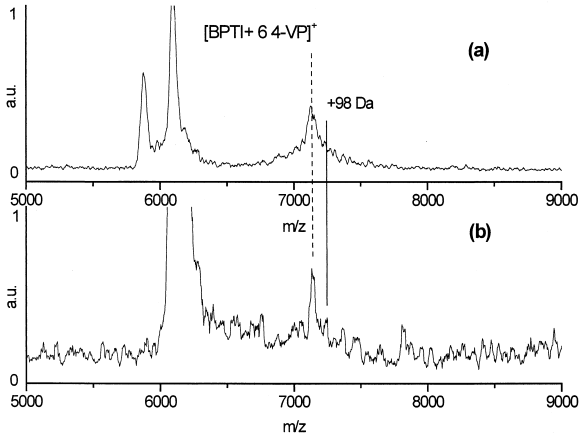

Fig. 7. Positive ion LILBID mass spectrum of 10-4 M reduced and alkylated BPTI in water. The solution was not purified after chemical modification. Cytochrome C was added as calibration standard. The desorption laser energy was (a) 15 mJ/pulse and (b) 10 mJ/pulse.

of the desorption laser. The BPTI peak is now shifted to higher masses as predicted, but no second peak appears on the high mass side. This result clearly gives evidence that the higher mass peak of the native BPTI that arises at low laser energy is related to the structure of BPTI. To explain the mass shift one must assume that five water molecules are bound to the protein. Furthermore, because the new peak is clearly separated from the BPTI ion, the internal water molecules seem to be caged in the molecule throughout the desorption process.

Of course, at the present status of the LILBID experiment, it is not possible to be completely sure about the nature of the 95-Da shifted ion signal. Phosphate or sulfate ions also lie well within this mass range. Even though there is no evidence in the literature for a structure dependent phosphate/sulfate binding of BPTI, this possibility—though from our experience very unlikely—cannot be ruled out.

As can be seen from the LILBID mass spectra presented, the current mass spectrometer was taken to its limits regarding sample consumption, precision, and resolution. The ultimate control experiment would be the usage of ^{18}O-water as solvent. In this case the mass shift should be enlarged by 10 Da compared to the present experiments shown. In order to obtain clear-cut results, the limitations shown above have to be removed. Currently, a redesign of the mass spectrometer is being undertaken to reach this goal.

4. Discussion

The examples presented in this article show that LILBID-MS is capable of desorbing proteins from the liquid phase with either mild or zero fragmentation. The proteins can be analyzed in water or an aqueous buffer without further additives. LILBID-MS even preserves the noncovalent interactions between the biomolecules throughout the desorption and mass analysis process. This makes LILBID-MS a very “soft” desorption method. The control experiments show that LILBID mass spectra reflect the properties of the initial liquid phase rather than gas phase artifacts.

This “softness” of the desorption method has direct consequences for the mass spectra observed. Most striking is the rather poor mass resolution ($R = \sim 100$ for protein ions). In the LILBID process the ions are desorbed from the liquid phase by means of an infrared laser and retain some of their solvation sphere [26]. Because the ions are vibrationally excited after desorption, these bound solvent molecules continue to dissociate from the protein as they are accelerated into the mass spectrometer. This leads to a dramatically reduced mass resolution in the LILBID process.

Even though an absolute quantitative correlation of the data with the solution properties is not yet possible, the solution conditions are reflected in the LILBID mass spectra. For example, it is possible to establish the relative binding strengths of ions or ligands using competitive binding experiments. Still, there are some limitations that hinder a direct correlation of the mass spectra to the solution properties.

There are two main deviations from the solution properties that can be found in the LILBID mass spectra: first, the low charge state of the ions detected and second, the partial dissociation of the complexes throughout the LILBID process. The ions desorbed

from the liquid beam are mainly in a low charge state that, of course, does not reflect the solution phase property. In solution at neutral pH, the amino and carboxylic groups of the proteins are completely (de-)protonated. The high dielectric constant of water shields the charged groups very well and allows for the high charge state. Through the desorption process, the solvating water is removed or depolarizes, leading to strong ion recombination and thus charge neutralization. Only low charged ions appear in the gas phase. This neutralization depends on the dielectric constant ϵ of the solvent. The higher ϵ, the higher the average charge of the ions. This makes the charge distribution of the mass spectra similar to that observed in MALDI-MS. Although this change in charge state deviates from the solution behavior, it makes the mass spectra very suitable for interpretation.

Beside the change in charge state distribution, the other striking difference between the solution phase properties and the LILBID mass spectra is the partial dissociation of the solution based complexes by desorption (even though in another case, the noncovalent hemoglobin complex could be detected undissociated) [13]. The main parameters that affect the complex stability in the LILBID desorption process are the initial solution temperature, the intensity of the desorption laser radiation, and the nature of the binding forces in the noncovalent complex. Upon desorption of the noncovalent complex into the gas phase the dominating binding forces that hold the subunits together change dramatically. In solution, it is mainly hydrophobic interactions that stabilize noncovalent complexes. H bonds and other polar interactions are weakened due to the effect of the surrounding solvent. Upon desorption into the gas phase, the surrounding solvent has to be removed. This leads to an enhancement of the polar interactions between subunits, and the hydrophobic interactions cease to play the dominant role. Consequently, as the binding forces change, the structure of most gas phase complexes will also change. This still allows for the detection of solution based noncovalent complexes as long as the complexes are not dissociated and do not undergo a gas phase ion chemistry. Because the gas phase stability is governed by polar interactions, complexes with metal ion binding involved in complex formation will be very stable and easy to detect in the mass spectrometer. On the other hand, complexes that are mainly stabilized by hydrophobic interactions in solution will dissociate more easily. This effect can be seen clearly when comparing RNase S and BPTI with the calmodulin/melittin example. Looking at the effect that the desorption laser energy has on the complex stability in the LILBID mass spectra it is clear that the calmodulin/melittin complex is not prone to laser induced dissociation during the desorption process. In contrast, both Rnase S and BPTI water can be easily dissociated by increasing the desorption laser energy. In the former complexes, H bonds and hydrophobic interactions play a major stabilizing role. In the latter case calcium ions are involved in complex formation.

The increase in laser energy leads to an enhanced deposit of excess energy into the liquid during desorption. Thus, the partially solvated ions are thermally excited when expelled into the gas phase. Depending on the gas phase stability of these noncovalent complexes, i.e. on the nature of the binding forces, this thermal excitation can lead to dissociation into the subunits. Accordingly, the complexes Rnase S and the BPTI-water complex dissociate under enhanced laser energy and the very stable calcium-calmodulin/melittin complex does not. Still, it was not possible to detect the calmodulin/melittin complex without some dissociation. Because gas phase dissociation of the complex can be ruled out, as shown above, some of the complex must already be dissociated in the liquid beam prior to desorption. This is also supported by the fact that normally the initial temperature of the solution has a strong effect on the mass spectra acquired. At lower temperatures the relative intensity of the complex increased. The temperature of the solution before its injection into vacuum was also varied in the case of Rnase S and calmodulin/melittin. Whereas the complex of the former tended to dissociate to a greater extent at higher temperature, that of the latter showed the opposite behavior. On the other hand, the temperature of the solution in the liquid beam in vacuum is also affected by evaporative

cooling. It can be estimated that this cooling takes place on the ms timescale. It will amount to a temperature reduction of less than 40–50 K so that the liquid is super-cooled at the moment of desorption. This could lead to a partial denaturation of the proteins and their complexes in the liquid beam. Depending on the nature of the complex under study, these different parameters will affect the LILBID mass spectrum differently. In spite of this aspect, which has to be studied in greater detail at much higher mass resolution, the examples confirm that it is possible to study the solution behavior of proteins and their complexes with our method. Of course, suitable control experiments must be carried out as shown in this article.

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