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Exploring Deprotonation Reactions on Peptides and Proteins at Atmospheric Pressure by Electro-Sonic Spray Ionization-Mass Spectrometry (ESSI-MS)

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Abstract: Electrospray ionization mass spectrometry (ESI-MS) rapidly became established as the method of choice for the production of large biomolecular ions in the gas phase. For peptides and proteins, ESI leads to the formation of multiply charged ions, in both the positive and negative ion mode. The charge-state distribution is directly related to the conformation of the macromolecular ions and to proton transfer reactions in the gas phase. Deprotonation reactions of multiply charged peptide and protein ions were studied by introducing volatile reference bases at atmospheric pressure between an electro-sonic spray ionization (ESSI) source and the inlet of a mass spectrometer. This new set-up offers the unique possibility to measure the apparent gas-phase basicity GB_{app} of multiply charged ions by a bracketing approach. The proof of principle was made using bradykinin derivatives, substance P and insulin chain B. We obtained values in excellent agreement with known GB_{app} values obtained at low pressure. These experiments were extended to seven model proteins showing that the thermodynamical properties of protein ions are directly correlated to the amino-acid sequence and the conformation in the gas phase. We also demonstrated that salt bridges between ionized basic and acidic sites still exist in the gas phase, confirming that electro-sonic spray is a very soft ionization technique.

Keywords: Charge residue model · Electro-sonic spray ionization · Gas-phase basicity · Multiply charged ions · Proton transfer reactions

Using mass spectrometry (MS) the mass-to-charge ratio of ions in the gas phase can be measured. In a first step, ions must be produced in the gas phase, which can then be analyzed using a mass analyser. The first and easiest way to produce ions in the gas phase is to study the gases themselves, as described for the first time by Thomson (Nobel Prize in Physics 1906).[1] In the middle of the 20th century ion sources based on electron ionization and chemical ionization of chemical compounds were developed.[2] Unfortunately these two techniques were partly destructive and numerous compounds of biological interest, like intact proteins or DNA, were impossible to analyze. In order to overcome this problem, two techniques were developed in the late 80s: Matrix-Assisted Laser Desorption/Ionization (MALDI)[3] and Electrospray (ESI).[4] MALDI-MS was simultaneously discovered by Tanaka (Nobel Prize in Chemistry 2002) in Japan, and Hillenkamp and Karas in Germany, whereas ESI-MS was first developed by the group of John Fenn (Nobel Prize 2002) in the USA. Both techniques are now widely used for *e.g.* trace analysis, metabolomics and proteomics. MALDI-MS consists of using a mixture of the dissolved analytes with the matrix, which will cocrystallize when drying. The crystals are then irradiated by a UV or IR laser and ions are ejected from the surface.[5] For ESI a liquid sample is delivered through a small (some hundreds micrometers diameter) capillary held at a high voltage. The electric field between the end of the capillary and the sampling cone of the mass spectrometer leads to the formation of small charged droplets (up to tens of micrometers diameter).[6] A nebulizing gas is usually used in order to favour the desolvation process. In the evolution of the spray process, the size of the charged droplets decreases due to solvent evaporation until the surface tension is smaller than the Coulomb repulsion between the ions in solution.[7] At this point, a droplet fission ('Coulomb explosion') occurs, leading to smaller charged droplets, which will decrease again due to solvent evaporation. This dynamic process will eventually form a partially or completely desolvated ion at atmospheric pressure that is introduced into the first part of the mass spectrometer ($\sim 10^{-2}$ mbar). This process of solvent evaporation and droplet fission has been studied in detail by Kebarle.[8]

For peptides and proteins, ESI leads to the formation of multiply charged ions in the gas phase, both in the positive and negative ion modes. The charge-state distribu-

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tion is directly related to the conformation of the macromolecular ions and to proton transfer reactions in the gas phase.[7,8] Thermodynamic data, such as the gas-phase basicity (GB), are essential to the understanding of the ion generation by ESI. The GB plays a similar role as the pKa in solution. In fact, pKa values are directly linked to the strength of the intramolecular interactions, depending on the conformation of the molecule. The main difference is the absence of intermolecular interactions with solvent molecules in mass spectrometry.

The intrinsic GB of a $[M+(n-1)H]^{(n-1)+}$ ion, where M is the analyte and n the number of protons, is defined as the free enthalpy change ΔG^0 of the reaction:

$$[M+nH]^{n+} \rightarrow [M+(n-1)H]^{(n-1)+} + H^+ \quad (1)$$

Since this reaction proceeds with a reverse activation barrier, due to Coulomb repulsion between the two ions $[M+(n-1)H]^{(n-1)+}$ and H^+, the term 'apparent gas-phase basicity' (GB_{app}) is more appropriate and is equal to the ΔG^0 of reaction (1) corrected by the energy of the activation barrier.[9]

For more than fifteen years, researchers have tried to measure the GB_{app} of peptides and proteins by studying deprotonation reactions between reference bases and the ions of interest. The first historical set-up designed to do this was a Y-shaped capillary interface/flow reactor connected to a quadrupole mass spectrometer.[10] The first inlet was connected to an ESI source whereas the second one was fitted with a gas reservoir of the reference base. Nowadays, GB_{app} values are measured by trapping the ions in a Fourier-Transform Ion Cyclotronic Resonance (FT-ICR) cell.[9,11] The base is then introduced by a leak and reacts with the multiply charged ions. Unfortunately, poor ionization efficiency in non-denaturing buffers, low ion transmission or limitations in the maximal detected *m/z* range in an FT-ICR cell were the main limitations of previous measurements. Thus only few quantitative values were published until now with these techniques. An alternative method is to directly mix the reference base with the analyte in the solution, as described for the first time by Laprévote and co-workers in 2001.[12] Triethylammonium bicarbonate (TEAB) buffer was used to reduce the charge state of the proteins and to stabilize non-covalent protein complexes.

At the same time, models for predicting the GB_{app} values of globular protein ions were developed. In 1995, Williams described for the first time a simple electrostatic model, which allows the calculation of the apparent and intrinsic GB for each charge state of a protein.[9] However, *ab initio* calculations showed that the equation for the two-proton system used before[9] predicts GB_{app} values that are too low.[13]

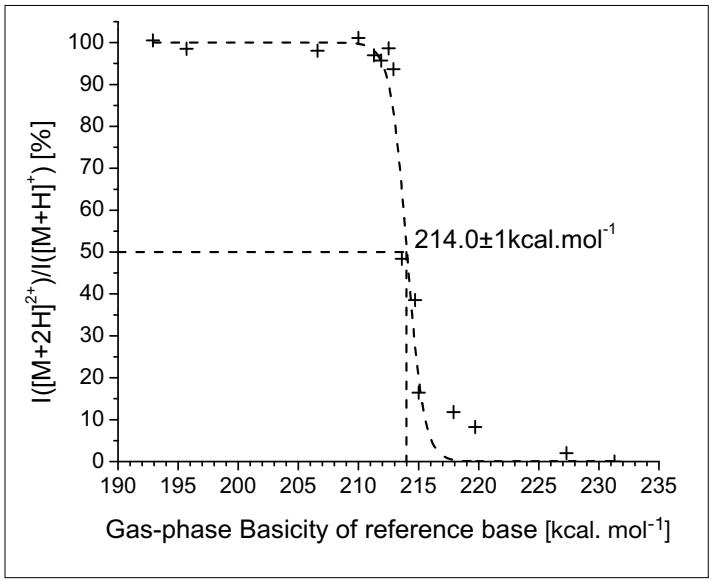

Fig. 1. Normalized ratio between the signal intensity of $[M+2H]^{2+}$ ions and the signal intensity of the $[M+H]^+$ ions for des-Arg^9-bradykinin, *versus* gas-phase basicity of the volatile reference bases. The GB_{app} values were determined for a deprotonation rate of the $[M+2H]^{2+}$ ion of 50%.

In 2002, Kebarle and co-workers developed a new electrostatic model giving access to calculated GB_{app} of folded proteins.[8] This calculation is based on a correction of the intrinsic GB of each basic site (arginine, lysine and histidine) by taking into account the crystal structure, the Coulomb repulsion energy between two positively charged sites, the charge/dipole interaction energy and some entropic terms. The idea of Laprévote and co-workers[12] was thus taken up and fully rationalized by Verkerk and Kebarle in 2003 to demonstrate the validity of their model on three different proteins.[8] Good agreement between experiments and calculations were shown for lysozyme and ubiquitin, but not for cytochrome c due to experimental problems. Unfortunately, all of these experiments were made using different inorganic or organic salts in the solvent, which made it impossible to determine the relative influence of solution and gas-phase reactions, especially during the droplet fission and evaporation processes. Thus, a new experimental set-up had to be developed in order to overcome all the experimental problems linked to the GB_{app} measurements of peptides and proteins.

Electro-Sonic Spray Ionization (ESSI) was first described by Cooks and co-workers in 2004.[14] A supersonic nebulizing gas at a high pressure (P~15 bar) is used instead of one at low pressure (P~3 bar), leading to a linear velocity of about 350 $m.s^{-1}$. With ESSI, the desolvation process before the entrance of the mass spectrometer is more complete than with ESI. More 'naked' ions are thus formed at atmospheric pressure. Narrower charge-state distributions and peak widths, and very good sensitivity were shown in comparison with traditional ESI. We decided to take advantage of this in order to develop a new set-up for GB_{app} measurements of biomolecular ions.[15] The idea is quite simple: vapor of reference bases (typically volatile amines and alcohols) is allowed to react with analyte ions generated by ESSI at atmospheric pressure, before they are sampled by the mass spectrometer. A high collision rate is ensured by the high reference base pressure, close to the saturation pressure, and a sufficient reaction time, estimated to be a few hundreds of µs. A short acquisition time is thus needed to acquire a representative mass spectrum for measuring the efficiency of the proton transfer between the multiply charged ions and the reference base. The GB_{app} of an individual charge state *n* can be measured by monitoring the intensity ratio between two successive charge states $[M+nH]^{n+}$ and $[M+(n-1)H]^{(n-1)+}$. The GB_{app} values were determined for a reduction of the $[M+nH]^{n+}$ ion intensity by 50% (Fig. 1). The precision on the measurement was always better than 2%.

In order to validate the method, bradykinin and derivatives (des-Arg^1-bradykinin and des-Arg^9-bradykinin) $[M+2H]^{2+}$ ions were used as reference compounds, as GB_{app} values had already been determined before.[11] Excellent agreement was found between the GB_{app} values obtained with ESSI-MS for des-Arg^1-bradykinin and des-Arg^9-bradykinin (221.7±5 $kcal.mol^{-1}$ (Fig. 1) and 214±1 $kcal.mol^{-1}$, respectively) and the values in the literature. Bradykinin $[M+2H]^{2+}$ ions showed a GB_{app} value at 229.3±2 $kcal.mol^{-1}$, which is between the values obtained by the deprotonation reaction method (225.8±4.2 $kcal.mol^{-1}$) and by the kinetic method (236.1±1.7 $kcal.mol^{-1}$).[11]

Table 1. Apparent gas-phase basicity (GB_{app}) for some peptide ions. All values are expressed in kcal. mol^{-1}.

Peptide	GB_{app} (kcal.mol^{-1}, from the literature)	GB_{app} (kcal.mol^{-1},ESSI at atmospheric pressure)
Des-Arg^1-bradykinin ($[M+2H]^{2+}$)	222.8±4.6	221.7±5
Des-Arg^9-bradykinin ($[M+2H]^{2+}$)	214.9±2.3	214±1
Bradykinin ($[M+2H]^{2+}$)	225.8±4.2	229.3±2
Substance P ($[M+2H]^{2+}$)	226.4±3.6	227.8±0.5
Insulin, Chain B ($[M+5H]^{5+}$)	198.2±5.6	194.3±0.2
Insulin, Chain B ($[M+4H]^{4+}$)	203.4±5.7	201.2±5.4
Insulin, Chain B ($[M+3H]^{3+}$)	212.2±6.8	217.4±2.4
Insulin ($[M+7H]^{7+}$ and $[M+6H]^{6+}$)	_	194.3±0.2
Insulin ($[M+5H]^{5+}$)	_	201.2±5.4
Insulin ($[M+4H]^{4+}$)	_	217.4±2.4
Insulin ($[M+3H]^{3+}$)	_	227.1±1.0

In fact, bradykinin ions in the gas phase can adopt different conformations depending on the experimental conditions (solvent, ion source, mass analyzer).[11] Experiments made with substance P (charge state 2) and insulin chain B (charge state 3, 4 and 5) also led to an excellent agreement with the literature values (Table 1). As only two basic residues (Arg^1 and Lys^3) are present in the sequence of substance P and as lysine is less basic than arginine in the gas phase, we can conclude that the first charge is located on Arg^1 and the second one on Lys^3. These experiments confirmed that ESSI-MS can efficiently be used to determine GB_{app} values of peptides at atmospheric pressure. It must be noted that each experiment, with a complete set of reference bases, was performed in less than 30 minutes, compared to hours of measurement time necessary using an FT-ICR. Thus, the precision of the ESSI measurement was consistently better than the one obtained by FT-ICR measurements because of the use of more volatile reference bases.

For the first time, GB_{app} measurements on the insulin chain B and on complete insulin were carried out.[15] When increasing the GB of the volatile reference base, charge states 6 to 3 of complete insulin disappeared successively. It must be noted that the GB_{app} value of charge state *n* of insulin is equal to the one of charge *n*–1 of insulin chain B, for *n* = 4 to 6. In fact, insulin chain A contributes only one charge of multiply charged complete insulin ions because only the N-terminal NH_2 can be considered as basic.

These experiments were then extended to proteins.[16] A first set of experiments was made on lysozyme (MW = 14,313 Da, 11 arginines, 1 histidine, 6 lysines), ribonuclease A (MW = 13690 Da, 4 arginines, 4 histidines, 10 lysines) and α-lactalbumin (MW = 14186 Da, 1 arginine, 3 hisitidines, 12 lysines) in a non-denaturing buffer (10 mM ammonium bicarbonate buffer at pH 7.2). It is assumed that these three proteins conserve their tertiary and quaternary structure, even in the gas phase, when sprayed by ESSI. These proteins were chosen because they have similar molecular masses, around 14 kDa, but have totally different basic amino acid compositions. First, it must be noted that the charge states disappeared successively, as expected, with increasing basicity of the reference volatile base (Fig. 2). GB_{app} values were obtained and compared for different charge states for each of the three proteins (Table 2). Second, it is clear that for a specific charge state, the GB_{app} values obtained for each protein are quite different. Thus, the thermodynamical properties of a protein in the gas phase are not dependent on the size of the protein, but on the amino acid sequence.

For lysozyme, experiments were also conducted for non-reduced and reduced (addition of dithiotreitol during 30 min at 40 ºC) lysozyme using a denaturing buffer (water/methanol/acetic acid (50/50/1, V/V/V)). As usually observed, denaturation of the protein leads to the observation of higher charge states compared to non-denatured samples. Reducing the disulfide bridges or using a denaturing buffer changes the conformation of the protein in solution. As ESSI-MS is a very soft ionization technique, this change of conformation can be conserved in the gas phase. By perturbing the tertiary structure of the protein, the basic amino acids on the protein surface are more exposed to the solvent and their intrinsic GB values are considerably modified. For lysozyme, this effect on the GB_{app} values measured for different charge states is obvious (Table 2). Thus the 3D structure of

Table 2. Values of GB_{app} in kcal.mol^{-1} obtained by deprotonation reaction of intact lysozyme in denaturing and non-denaturing buffers, disulfide reduced lysozyme in a denaturing buffer, ribunoclease A and α-lactalbumin in a non-denaturing buffer. The first column correspond to the GB_{app} values for lysozyme calculated by Kebarle and co-workers[8] and refined by taking into account the salt bridges.

Charge state	corrected GB_{app} from the model developed by the group of Kebarle	intact lysozyme in non-denaturing buffer	intact lysozyme in denaturing buffer	disulfide-reduced lysozyme in denaturing buffer	ribonuclease A in non-denaturing buffer	α-lactalbumin in non-denaturing buffer
4	240.9	>231.3	>231.3	>231.3	>231.3	>231.3
5	233	>231.3	>231.3	>231.3	225.8	>231.3
6	227.1	220.4	219.2	225.1	216.7	225.8
7	223.9	214.2	202.5	217.5	214.2	220.4
8	206.0	202.5	195.7	202.5	202.5	<195.7
9	196.0	<195.7	<195.7	<195.7		
10	188.1		<195.7	<195.7		
11			<195.7	<195.7		
12				<195.7		
13				<195.7		

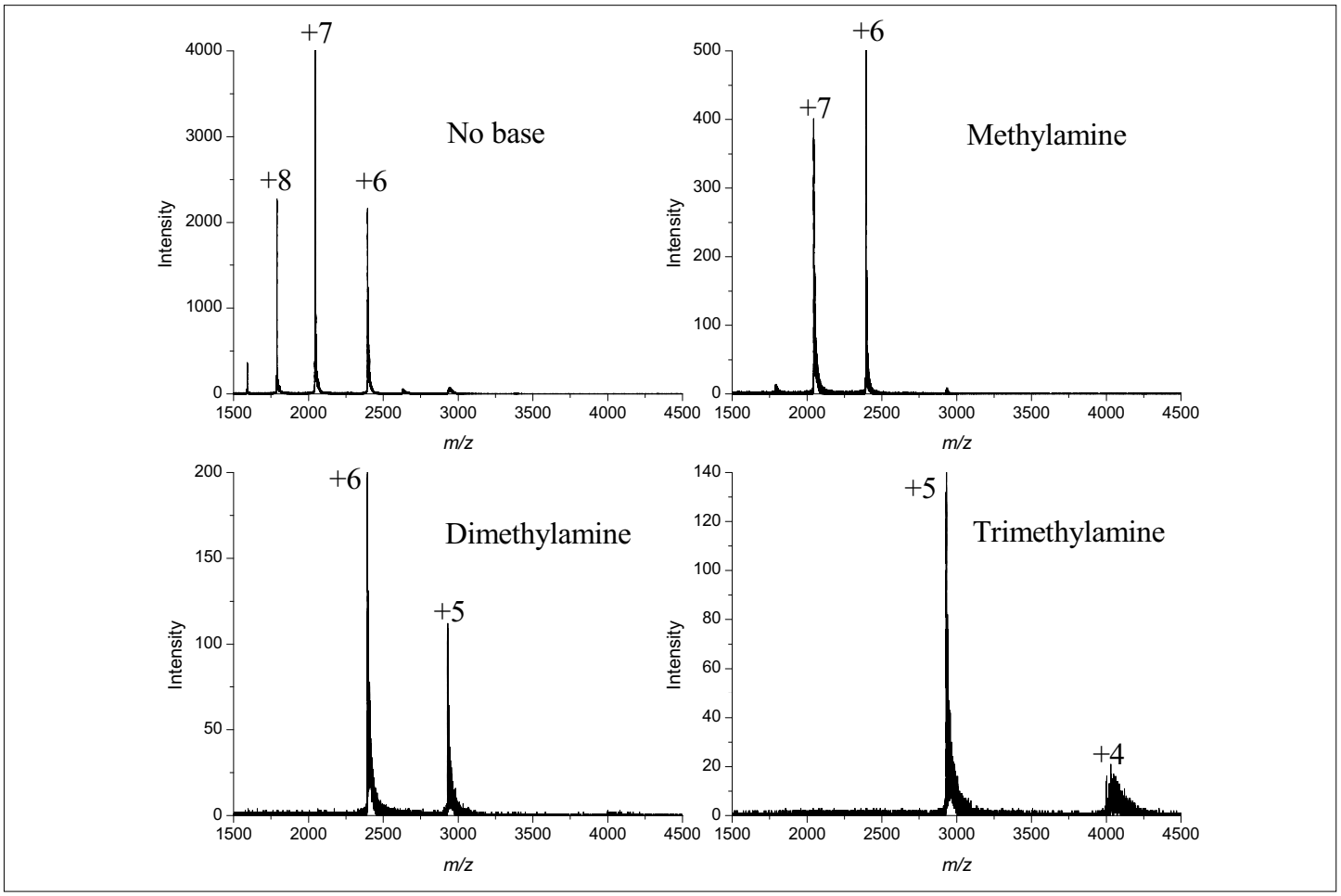

Fig. 2. Representative mass spectra obtained by reacting α-lactalbumin ions sprayed by ESSI from a non-denaturing buffer and reacting with different neutral volatile reference bases.

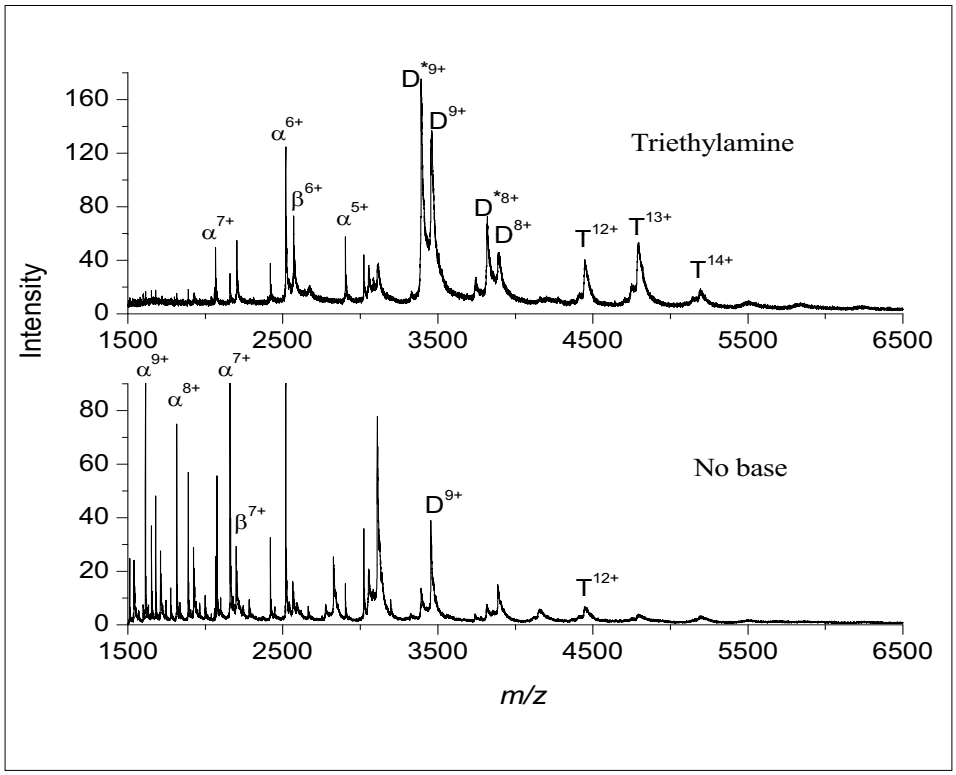

Fig. 3. Representative mass spectra obtained by reacting a mixture of hemoglobin chains α and β with or without triethylamine as volatile base. D and T refer to the dimer αβ and tetramer $\alpha_2\beta_2$. The * corresponds to the complexes without the heme.

a protein plays a great role in the ionization efficiency and the charge state distribution observed by ESSI-MS.

As stated in the introduction, GB_{app} values were also calculated by Kebarle and co-workers for different charge states of lysozyme, ubiquitin and cytochrome c.[8] Our measurements were directly compared to the calculated values. A poor agreement was found. In order to overcome this problem, we decided to correct the model by taking into account all the salt bridges between ionized basic and acidic sites close on the protein surface. In fact, Prakash and Mazumdar showed in 2005 that ESI-MS is soft enough to maintain all hydrogen bounds and salt bridges already present in solution.[17] Three salt bridges (arginine61/aspartic acid48, arginine125/aspartic acid119, lysine1/glutamic acid7) were identified on the crystal structure of lysozyme (PDB: 1DPX) leading to a new scale of estimated GB_{app} values (Table 2). The same was done for ubiquitin and cytochrome c.[16] In each case, much better agreement between experimental values obtained by ESSI-MS and calculated values with the corrected model was achieved. Our experiments confirm that ESSI-MS is so soft that hydrogen bonds and salt bridges

can survive to the complex desolvation and ionization process.

Finally we took advantage of the deprotonation reaction by volatile bases to decrease the charge state of non-covalent complexes. Hemoglobin was used as it forms a heterotetramer ($\alpha_2\beta_2$) when a non-denaturing buffer is used. It was difficult to determine the GB_{app} of different charge states for each structure, but we confirmed qualitatively that the tetramer could be stabilized by using triethylamine as demonstrated by Laprévote and co-workers with triethylammonium bicarbonate buffer (Fig. 3).[12] The major part of the stabilization is probably due to a decrease of the repulsive electrostatic interactions when reducing the charge state of the ions. Thus deprotonation reactions at atmospheric pressure could be very useful when studying large macromolecular assemblies in order to reduce the Coulomb repulsions and to maintain the gaseous non-covalent complexes in a folded conformation, like in solution.

ESSI-MS offers the unique possibility to measure the GB_{app} values of peptides and proteins at atmospheric pressure with good sensitivity (for concentrations less than 10 μM in denaturing or non-denaturing buffer), good precision (less than 2%) and in a short time (less than 30 minutes to screen up to 23 volatile reference bases). We clearly demonstrated that:

i) GB_{app} values are dependent on the basic amino acids sequence and not merely on the size of the proteins.
ii) GB_{app} values are directly correlated to the conformation of the protein ions in the gas phase.
iii) ESSI-MS is sufficiently soft to transfer non-covalent complexes in the gas phase in a way such hydrogen bonds and salt bridges remain intact.
iv) Deprotonation reaction at atmospheric pressure could be useful tool to stabilize fragile non-covalent complexes in the gas phase.

Our work will be extended to the study of DNA and RNA. In fact, few studies on oligonucleotides involved analysis by electrospray in the positive ion mode, due to low sensitivity compared to negative ion modes. The knowledge of the GB_{app} values of such species could be of interest in order to optimize the ionization conditions and significantly increase the sensitivity.

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