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Manipulation of charge states of biopolymer ions by atmospheric pressure ion/molecule reactions implemented in an extractive electrospray ionization source

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A home-made extractive electrospray ionization source is coupled to an linear quadrupole ion trap mass spectrometer to investigate ion/molecule reactions of biopolymers at ambient pressure. Multiply charged biopolymers such as peptides and proteins generated in an electrospray are easily reduced to a low charge state by the atmospheric pressure ion/molecule reactions occurring between the multiply charged ions and a strong basic reagent sprayed in neutral form into the electrospray plume. The charge state of the biopolymer ions can be manipulated by controlling the amount of the basic reagent. The production of biopolymer ions with low charge states results in a substantial improvement of sensitivity and reduced spectral congestion in ESI-MS. This is of importance for biopolymer mixture analysis and could have promising applications in proteomics.

Keywords: atmospheric pressure ion/molecule reaction, charge state manipulation, biopolymer ions, spectral congestion, proton stripping

Introduction

Electrospray ionization mass spectrometry (ESI-MS) is routinely used in biochemical studies since it facilitates mass measurement of high molecular weight species by generating multiply charged ions of most biopolymers that fall into the low *m/z* range of the spectrum (*ca* 500–2500).[1,2] However, the "squeezed" *m/z* range also results in serious peak overlap, creating extra difficulties in spectral interpretation, especially for protein mixture analysis.[3–5]

One way to avoid overlap of peaks is to work with a high mass resolving power, for example, by employing high performance instruments [for example, a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer,[6] an orbitrap mass analyzer,[7] etc.]. Alternatively, spectral congestion can be moderated by reducing the charge, *z*, of the protein ions so that the peaks of multiple charges can spread out along the *m/z* scale. Additionally, studies of biopolymer ions with different charge states, especially singly charged ions in the gas phase, might help to better understand the physico–chemical and/or biological properties (for example, conformation[8]) of the molecules. It might also be helpful to conserve a high bioactivity in soft landing experiments[9,10] when singly charged ions rather than

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multiply charged ions are used. Intrinsically, charge reduction is compatible with any type of mass analyzer, subject only to mass range limitations. In principle, charge reduction can be done by either ion–ion reactions or ion/molecule reactions. Ion–ion reactions[3,4,11] have been implemented in a variety of mass analyzers coupled with different ionization sources (for example, a custom-designed corona discharge source[5]), providing an effective way to reduce the charge state of biopolymer ions generated in ESI. However, ion–ion reactions usually occur in high vacuum,[3,4,11] which requires custom designed instruments and results in an overall low efficiency. Unlike ion–ion reactions occurring between two species charged with opposite electric polarity, positive ions of biopolymers could be reduced to low charge states by exposure of the electrospray-generated aerosol to a neutralizing gas contaning a high concentration of bipolar (i.e. both positively- and negatively charged) ions.[12] However, a substantial decrease of signal intensity was observed in the charge-reduced spectra due to the unique charge reducing process.[12]

Ion/molecule reactions, well known in mass spectrometry for charge reduction, usually occur at a relatively low pressure (for example, 1–100 torr).[13,14] An general trend in modern mass spectrometry is to create analyte ions outside the vacuum system using atmospheric pressure ionization methods[15–20] which renders samples more easily accessible and amendable for high throughput analysis. Here, a new and convenient method for ion/molecule reactions at atmospheric pressure is proposed to reduce the charge states of vaious biopolymer ions. This method, as reported here, allows charge manipulation with easy access, higher efficiency and simple instrumentation and facilitates fast measurement of the gas-phase basicity of individual charge states.[21]

Experimental section

Experimental set-up

All the experiments were carried out with a home-made extractive electrospray ionization (EESI) source, which was coupled to a commercially available linear quadrupole ion trap LTQ-XL mass spectrometer (Finnigan, San Jose, CA, USA). The EESI source was initially developed for real time direct mass spectrometric analysis of liquid samples with dirty matrices without sample pre-treatment. The set-up details have been described elsewhere.[22] The flexibility of the EESI source allows ion/molecule reactions at atmosheric pressure so that more frequent collisions can be expected compared to ion/molecule reactions occurring in vacuum.

In the current study, the EESI is used directly for charge reduction of biopolymer ions by ion/molecule reactions in open air. Figure 1 shows the schematic diagram of the EESI soure for this purpose. The angle formed between channel 1 and channel 2 is 90°, the distances between the various parts are shown in Figure 1. The analyte solution is infused (2 μL min^{-1}) directly for electrospray (channel 1) to generate ions with multiple charges. Solutions of basic reagents such as ammonia are nebulized by using another gas spray source (channel 2). The pressure of the nebulization gas is set to be about 50274 PSI so that the basic reagent can be sprayed into the electrospray beam via channel 2, allowing ion/molecule reactions to occur in the region formed between the two sprays. Ions that have undergone ion/molecule reactions are introduced into the ion trap mass analyzer throught the ion sampling orifice of the LTQ mass spectrometer. The electrospray voltage was set to +3 kV, the temperature of the heating capillary was 150°C. For collision-induced dissociation, the isolation window width was 2 Da and the collision energy

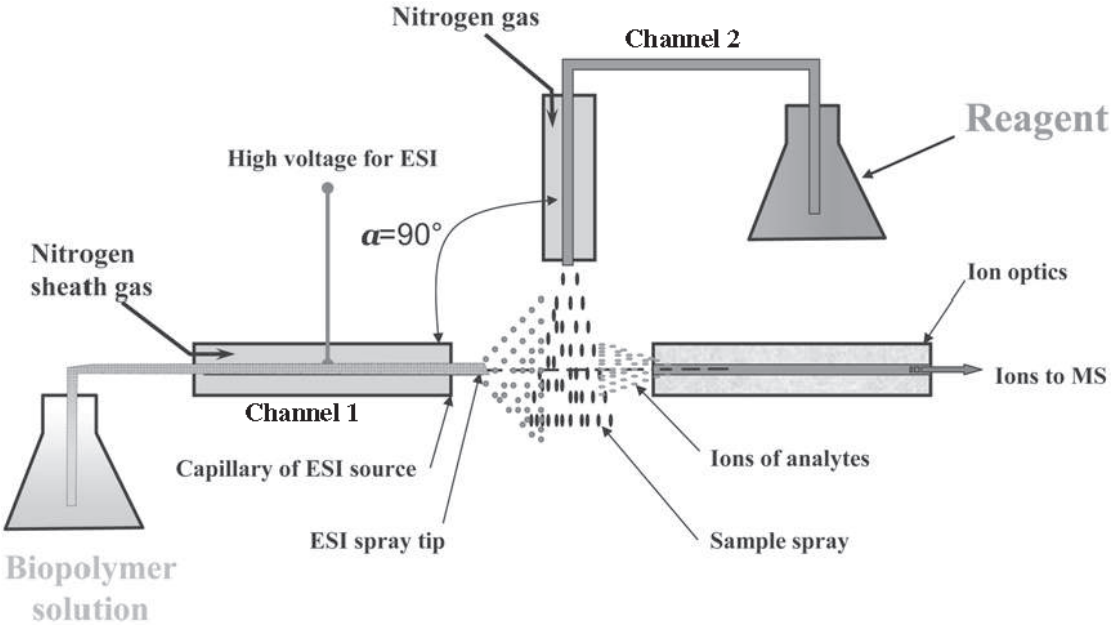

Figure 1. Schematic diagram of the EESI source used for ion/molecule reactions at ambient conditions.

was about 20–25%. The instrument was set for positive ion detection mode and the other parameters were the default values of the LTQ instrument.

Apparent gas-phase basicities (GB_{app}) of protein ions were determined using electrosonic spray ionization (ESSI) MS as described by Touboul *et al.*[21] The idea is to introduce the vapor of a volatile reference base to react with the protein ions in the atmospheric pressure region, before the MS inlet. High vapor pressure, close to the saturation pressure and room temperature ensure a high collision rate between protein ions and the base. A deprotonation reaction of the protein ions can occur when the *GB* of the volatile base is higher than the *GB* of the protein ions. GB_{app}, which corresponds to the true thermodynamic *GB* corrected by approximatively the reverse activation barrier energy, can be determined by monitoring the intensity ratio between two successive charge states $[M+nH]^{n+}$ and $[M+(n-1)H]^{(n-1)+}$. We found that a deprotonation reaction rate of the $[M+nH]^{n+}$ ion of 50% is a good criterion to determine GB_{app}.

Reagents

All chemical reagents were purchased from Sigma–Adrich (Sigma–Aldrich Chemie GmbH, Buchs, SG, Switzerland) at the highest purity available and were used directly without further pre-treatment. Peptides and proteins were dissolved in methanol/water/acetic acid solution (50:50:5, v/v) to form a working solution with a concentration of 1×10^{-6} M. The water used was deionized.

The biopolymer solution was infused with an infusion rate at 2 μL min^{-1}. The basic reagents such as ammonia were dissolved in water to create a series of concentrations (pH 8–14) and then infused to channel 2, which served to spray the solution directly into the ESI beam generated from channel 1 (Figure 1). Basic liquid reagents such as 1,5-diazabicyclo[4,3,0]-non-5-ene was directly infused to generate the reagent spray. The infusion rate in channel 2 was varied from 0.1–2 μL min^{-1}.

For GB_{app} determination, nine volatile reference bases were used: cyclohexylamine (*GB* = 899.6 kJ mol^{-1}), diethylamine (*GB* = 919.4 kJ mol^{-1}), piperidine (*GB* = 921.0 kJ mol^{-1}), dipropylamine (*GB* = 929.3 kJ mol^{-1}), diisopropylamine (*GB* = 938.6 kJ mol^{-1}), triethylamine (*GB* = 951.0 kJ mol^{-1}), tripropylamine (*GB* = 960.1 kJ mol^{-1}), tributylamine (*GB* = 967.6 kJ mol^{-1}), *N*,*N*,*N*′,*N*′-Tetramethyl-1,3-propanediamine (985.4 kJ mol^{-1}), where all the *GB* values were taken from the NIST database.

Results and discussion

Ion/molecule reaction with small peptides

Atmospheric ion/molecule reactions occurred efficiently in this EESI source installed on an LTQ mass spectrometer. The mass spectra of peptide neurotensin (MW 1673) generated by ESI were recorded before [Figure 2(a)] and after [Figure 2(b)] the ion/molecule reactions with an aqueous ammonia solution (1 M) sprayed into open air. The ESI mass spectrum was dominated by doubly and triply protonated peaks [Figure 2(a)]. Compared to the intensities of high charge states, the singly charged ion (*m/z* 1674) was almost undetectable in the ESI mass spectrum [Figure 2(a)]. In contrast, the peak at *m/z* 558.9, the highest charge state (three

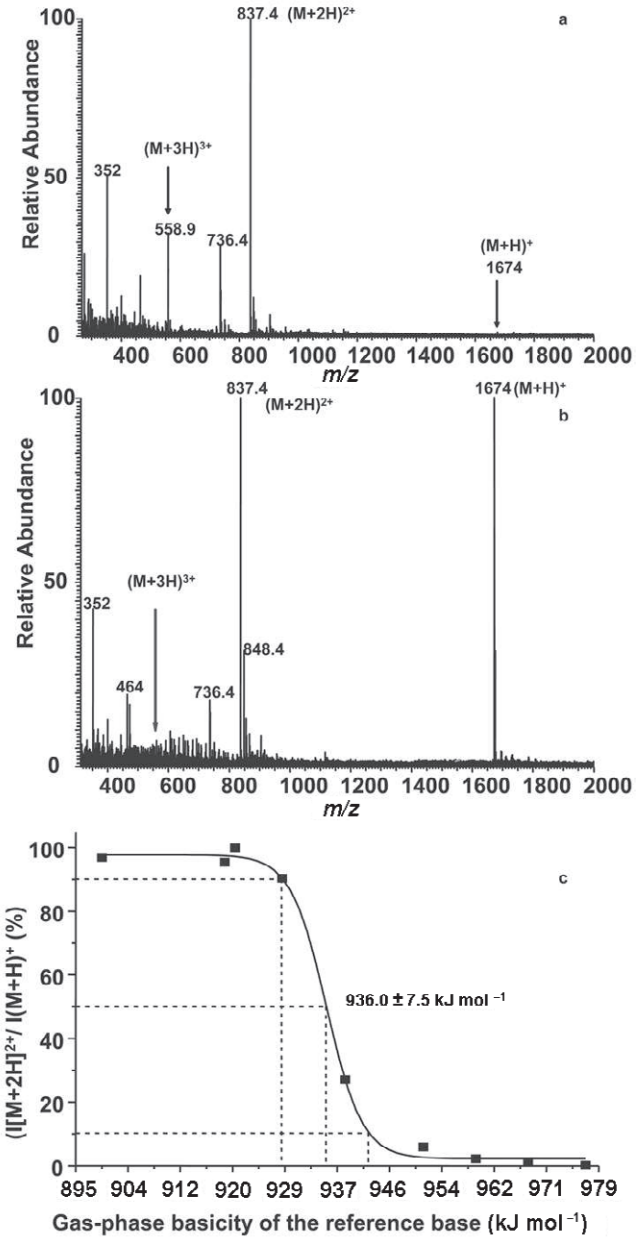

Figure 2. Ion/molecule reactions to reduce charge state of neurotensin (MW 1673) in open air. (a) Predominant multiply charged neurotensin ions are generated in normal electrospray; (b) predominant singly charged neurotensin ions are observed after reaction with sprayed aqueous NH_3 solution (1 M). (c) Relative intensity of the protonated neurotensin molecules versus gas-phase basicity of the volatile base references. The GB_{pp}s were determined for a deprotonation rate of the $(M+2H)^{2+}$ ions of 50%. The deviation is calculated for deprotonation rate of 10% and 90%. See Reference 21 for details.

charges) generated in the electrospray, disappeared, while singly charged neurotensin (*m/z* 1674) became the most abundant peak in the spectrum recorded in the presence of ammonia [Figure 2(b)]. Experimentally, it was found that intensities of other originally singly charged ions (for example, *m/z* 352, 464) remain constant before and after the ion/molecule reactions, probably because ions of higher charge states are reduced much faster than those of lower charge states.[3] This feature allows differentiation of peptides, which are usually multiply charged in ESI, from singly charged components such as amino acids, simplifying data interpretation in biological mixture analysis.

Apparent gas-phase basicities (GB_{app}) for charge states 3 and 2 of neurotensin were determined using electrosonic spray ionization (ESSI) MS as described by Touboul *et al*.[21] The GB_{app} of charge state 3 was close to the *GB* of ammonia (819.0 kJ mol^{-1}) and corresponds to the protonation of the lysine residue. Figure 2(c) shows the rate of the deprotonation reaction of charge state 2 against the *GB* of the reference bases. We found a GB_{app} of 936.0 ± 7.5 kJ mol^{-1} which is linked to the protonation of one of the two adjacent arginines. These results confirm that charge state 3 can be deprotonated with ammonia using EESI, but a stronger base is recommended for the deprotonation of charge state 2.

Ion/molecule reaction with large peptides

It is highly desirable to convert all multiply charged ions to singly charged ions so that spectral congestion can be reduced and the sensitivity can be enhanced. Figure 2 shows the mass spectra of melittin (MW 2846) recorded by ESI before and after ion/molecule reactions with different amounts of 1,5-diazabicyclo[4,3,0]-non-5-ene (MW 124). In ESI, melittin gives predominant peaks at *m/z* 569.8, 712.2 and *m/z* 949.4 corresponding to melittin ions with 5, 4 and 3 charges, respectively [Figure 3(a)]. Note that the highest peak was at *m/z* 569.8 (3.39×10^5) and no peak corresponding to a charge state lower than three was detected. When a small amount of the basic liquid reagent was sprayed with an infusion rate of 0.1 µL min^{-1} into the peptide electrospray beam, the spectral pattern of the melittin changed instantly: the intensity of the previously abundant peak (*m/z* 569.8) decreased significantly and a doubly charged peak (*m/z* 1423.8) became the most predominant one in the spectrum after ion/molecule reactions [Figure 3(b)]. Note that the intensity of the peak at *m/z* 1423.8 is 8.82×10^6, which is about 26 times higher than that of the highest peak (*m/z* 569.8) before the ion/molecule reactions. Further increase of the reagent infusion rate to 0.6 µL min^{-1} caused most ions with more than two charges to be reduced to the +2 charge state, yielding a predominant peak in the spectrum with an increased intensity of 5.38×10^7 [Figure 3(c)]. Note that there is a tiny peak at *m/z* 2847, which is the singly charged melittin. In a typical electrospray mass spectrum recorded with acidified solution, the singly protonated melittin cannot be observed directly. However, it can be produced easily by increasing the infusion rate of the basic reagent to be 2 µL min^{-1}, then the singly protonated melittin (*m/z* 2847) shows up with a further increased intensity of 8.83×10^7 [Figure 3(d)]. Compared to the highest peak (*m/z* 569.8) of

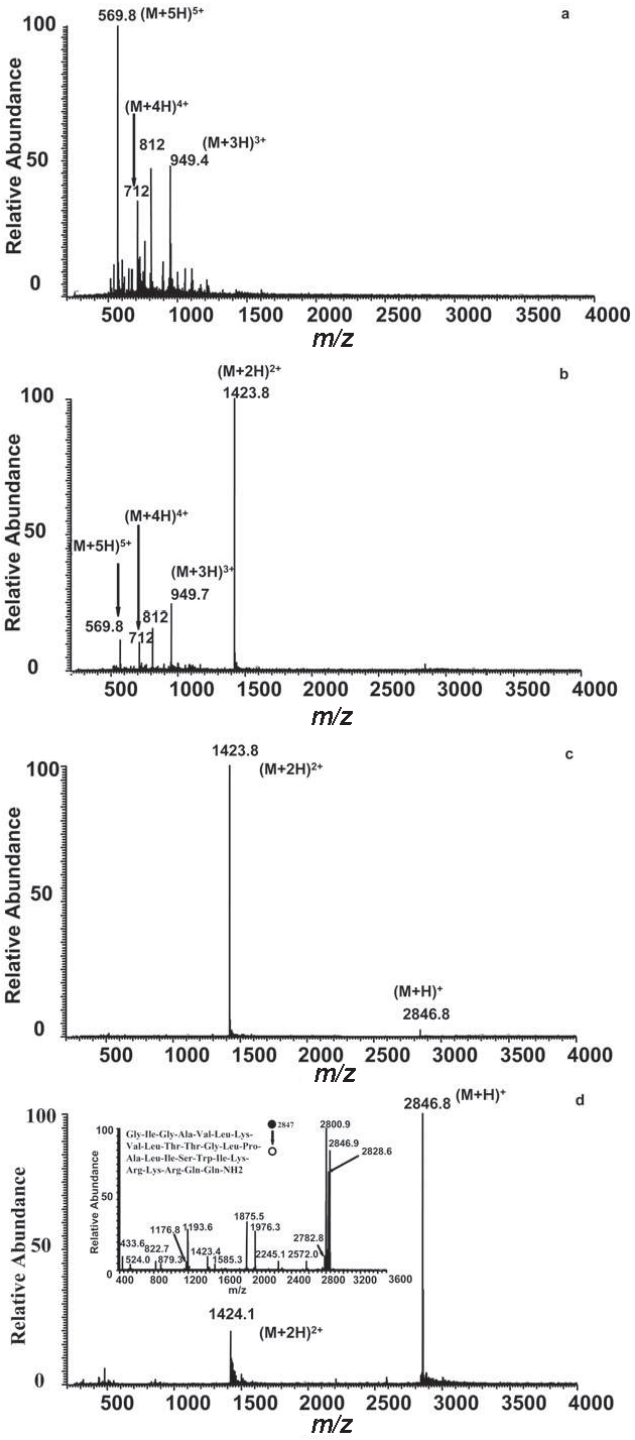

Figure 3. Dynamic charge states of melittin; (a) high charge states generated in ESI; (b) intensity of multiply charged ions deceased by spraying a small amount of neutral base into the electrospray beam; (c) doubly charged ions generated exclusively by the ion/molecule reactions; (d) singly charged ions generated by the ion/molecule reactions in open air.

melittin with five charges [Figure (a)], the singly charged ions detected were enhanced by 260. The very large apparent signal enhancement for melittin is because peak heights are reported, not total (=integrated) signal. In such a case, the singly charged ions could be easily isolated for collision-induced dissociation, which provided intense fragmentation and confirmed that the ions observed at *m/z* 2847 were singly charged, since there was no fragment at an *m/z* above that of the parent ions [inset in Figure 3(d)]. Obviously, this data shows that the detection limit can be improved significanly if singly charged ions of biopolymers can be produced.

To test whether singly charged biopolymers can be produced using an analyte solution containing a base, ammonia and 1,5-diazabicyclo[4,3,0]-non-5-ene were added directly into solutions of melittin and insulin for ESI. Only multiply charged ions were observed, with decreased intensities, for either melittin (i.e. +3, +4) or insulin (i.e. +3, +4, +5) when ammonia was used at various concentrations (pH 8~13). Further increase of the concentration of ammonia significantly decreased the signal, but no singly charged ion was detected. The 1,5-diazabicyclo[4,3,0]-non-5-ene suppressed the ESI signal of the melittin/insulin much more seriously than ammonia and produced a strong peak at *m/z* 125, which was ascribed to the protonated base. Therefore, for charge reduction purposes, the base should not be added directly into the solution for electrospray, especially for trace analysis where a high sensitivity is required.

GB_{app}s of melittin measured by ESSI-MS for charge state 5 were close to the *GB* of ammonia (819.0 kJ mol^{-1}), for charge state 4 close to the *GB* of methylamine (864.5 kJ mol^{-1}) and 945.9 ± 5.4 kJ mol^{-1} for charge state 3, as shown in Figure 4. The charge states 2 and 1 exhibit GB_{app}s higher than the *GB* of the *N*,*N*,*N*′,*N*′-Tetramethyl-1,3-propanediamine (985.4 kJ mol^{-1}) and correspond to the protonation of the two arginines, which are the most basic residues on the peptide. These residues could be deprotonated using EESI with a high amount of 1,5-diazabicyclo[4,3,0]-non-5-ene (*GB* of 1006.3 kJ mol^{-1}) as shown in Figure 3(d).

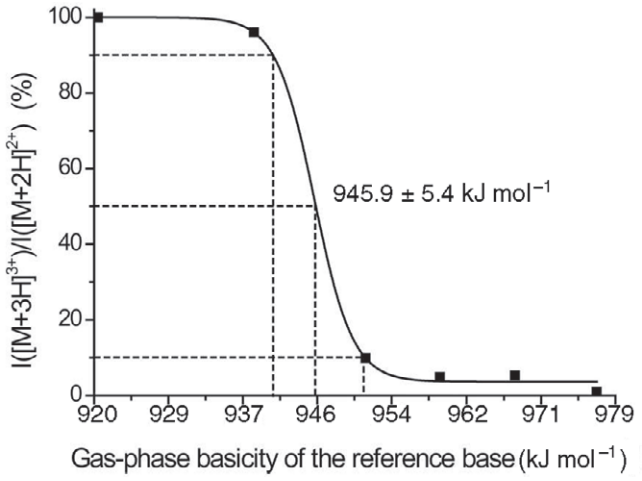

Figure 4. Relative intensity of the melittin ions versus gas-phase basicity of the volatile reference bases. The GB_{pp}s were determined for a deprotonation rate of the $(M + 3H)^{2+}$ ions of 50%. The error is calculated for a deprotonation rate of 10% and 90%. See Reference 21 for details.

Ion/molecule reaction with protein

Myoglobin (MW 17600), was used to further validate this method. Before the ion/molecule reactions, myoglobin presents a spectral pattern dominated by peaks of high charge states ($26 \geq z \geq 10$) [Figure 5(a)], where the peak at *m/z* 1211.6 (14 charges) is the highest signal (4.46×10^6). All the peaks are located in a narrow *m/z* range (*m/z* 652~1695), showing the typical spectral congestion in ESI of biopolymers. The charge state distribution shifts from the

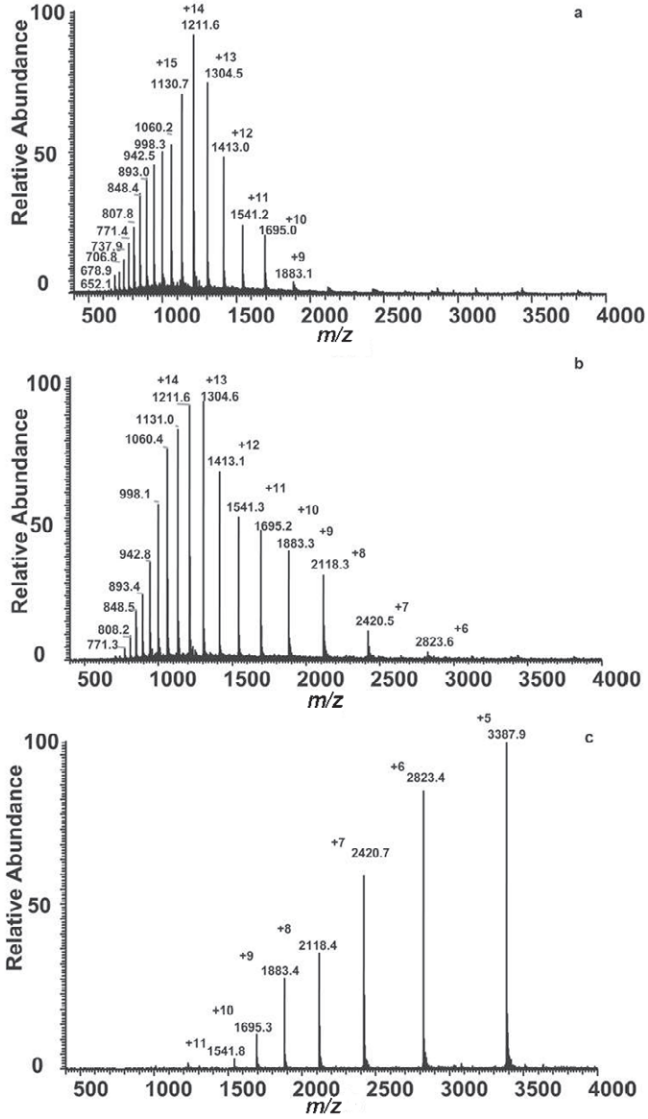

Figure 5. Significant charge state shift of protonated myoglobin; (a) Before and after the ion/molecule reactions with (b) a small amount of and (c) a increased amount of base in the open air.

range of $26 \geq z \geq 10$ to the range of $22 \geq z \geq 6$ when the basic reagent, 1,5-diazabicyclo[4,3,0]-non-5-ene, was sprayed into the ESI beam at an infusion rate of 0.1 μL min^{-1}. Note that the highest signals, for the two peaks with 13, or 14 charges, in this spectrum have a count rate of 5.25×10^7, showing about one order of magnitude increase in intensity than that before the ion/molecule reactions [Figure 5(b)]. After adding an increased amount of the base (2 μL min^{-1}), the multiply charged ions are dramatically reduced and the spectrum shows new peaks such as *m/z* 3387.9 (5 charges) with a signal intensity of 6.05×10^7 [Figure 5(c)]. The peaks of protonated myoglobin spread out over a much wider *m/z* range after the ion/molecule reactions, showing a significant charge state shift in the mass spectra (Figure 5). Extra experiments showed that all the multiply charged ions were further shifted towards the high mass range if a higher amount of the base was used, which produced a spectrum with no peak associated with myoglobin in the relatively low mass range (*m/z* 400–4000). The spectral pattern is restored to the one shown in Figure 5(a) once the introduction of the basic reagent is terminated. Unfortunately, the ions of charge states lower than five were not detected due to the limited *m/z* range (*m/z* 20–4000) of the LTQ mass spectrometer. Recently, EESI has been implimented in the ESI interface of a commerically available time-of-flight mass spectrometer.[23] Theoretically, the ion/molecule reactions can easily be performed in this EESI source,[23] providing an easy access to this method without any hardware modification. The present data clearly shows that charge states of multiply charged proteins can be reduced significanly by ion/molecule reactions in the open air, providing an easy way to simplify the complexity of mass spectra for protein mixture analysis.

As observed in the previous work of Touboul *et al*.,[21] the predominant charge state of myoglobin in denaturing buffer is six using triethylamine as the deprotonation agent at atmospheric pressure. This data is consistent with the experiments using ESI with 1,5-diazabicyclo[4,3,0]-non-5-ene where the most intense charge state is 5 using this base directly in solution.[24]

Conclusion

In conclusion, multiply charged biopolymers such as peptides and proteins can easily be reduced to a low charge state by atmospheric pressure ion/molecule reactions occurring between the multiply charged ions and a strong basic reagent sprayed in neutral form into the electrospray beam. The charge state of the biopolymer ions can easily be manipulated by controlling the amounts of the basic reagent. The production of biopolymer ions with low charge states results in a substantial improvement of sensitivity and less spectral congestion in ESI-MS for biopolymer mixture analysis, with promising applications in proteomics.

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