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ARTICLE *in* THE JOURNAL OF PHYSICAL CHEMISTRY B · NOVEMBER 2007

Impact Factor: 3.3 · DOI: 10.1021/jp075127q · Source: PubMed

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2007, *111,* 11629−11631
Published on Web 09/19/2007

Rapid and Precise Measurements of Gas-Phase Basicity of Peptides and Proteins at Atmospheric Pressure by Electrosonic Spray Ionization-Mass Spectrometry

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Received: July 2, 2007; In Final Form: August 23, 2007

Deprotonation reactions of peptide and protein ions have been studied by introducing volatile reference bases at atmospheric pressure between an electrosonic spray ionization (ESSI) source and the inlet of a hybrid quadrupole time-of-flight mass spectrometer. This new setup offers the unique possibility to measure the apparent gas-phase basicity GB_{app} of multiply charged ions by a bracketing approach. A very good agreement has been obtained with reference values obtained by Fourier transform-ion cyclotronic resonance (FT-ICR), validating our approach. The measurements were then extended to larger biomolecules such as insulin and myoglobin in native and denaturing buffers. The main advantages of this methodology are measurements at atmospheric pressure with good sensitivity (for concentrations less than 10 μM in denaturing or nondenaturing buffer), very good precision (less than 2%), and in a short time (less than 30 min to screen up to 23 volatile reference bases).

Introduction

The study of biomolecules by electrospray ionization mass spectrometry (ESI-MS)[1] is currently widely used in biochemistry, especially in metabolomics and in proteomics. For small molecules, singly charged ions are mostly generated. For ionization by protonation, the efficiency is directly linked to the gas-phase basicities (GB) of the analytes. Thus, many research groups have been involved in GB measurements of amino acids,[2−4] which are the building blocks of peptides and proteins, and small model peptides.[5] For the larger biological compounds, ESI leads to the formation of multiply charged ions. The average charge state (ACS) and the width of the charge-state distribution depend on a number of parameters: the ionization source, the solvent used, the amino acid composition, the gas-phase conformation of the protein, and the relative GB of analyte and buffer.[6−9] The GB is predicted to have a dominant influence on ESI ion formation if it indeed occurs by the charged residue model.[6] Two different electrostatic models were developed to calculate the GB for peptides and proteins, but few comparisons between experimental and calculated values are available.[8−10] Williams and co-workers were the first to attempt to measure the GB of some denatured proteins or, more precisely, the apparent GB (GB_{app}), which is larger than the true thermodynamic GB by a value approximately equal to the reverse activation barrier. This high-energy barrier is due to the intramolecular Coulomb repulsion from the different charges present on the ions. These authors studied deprotonation reactions between protein ions and volatile bases and measured GB_{app} values by using the bracketing method in a Fourier transform-ion cyclotron resonance (FT-ICR) instrument.[10−14] The ions must be stored for a sufficiently long time (several tens of seconds) to interact with the reactive gas introduced by a leak. This leads to experimental difficulties in measuring and controlling the pressure and the temperature in the cell. High-pressure mass spectrometry (HPMS) was also used for qualitative studies of the ACS of proteins. In this case, a reaction time of only a few milliseconds is necessary to attain equilibrium for a pressure of few kPa.[15]

Electrosonic spray ionization mass spectrometry (ESSI-MS) was developed by the group of Cooks in 2004.[16,17] This technique consists of a traditional micro-ESI source with a supersonic nebulizing gas, leading to a narrow charge-state distribution, which can be due to the production of ions that are either completely desolvated or nearly so before they enter into the mass spectrometer. A travel time of a few hundreds of microseconds for ions between the spray tip and the entrance of the mass spectrometer inlet was estimated due to a linear velocity of the nebulizing gas of 350 $m \cdot s^{-1}$.[16] The protein or peptide ions were completely thermalized within tens of microseconds after leaving the ESSI source. ESSI data have also been interpreted in the sense that the species being studied represent a single conformer of the protonated molecule,[16,17] although a rigorous proof of this interpretation, for example, by ion mobility spectrometry, is still missing.

Experimental Setup And Data Analysis

Here, we report a fast and sensitive mass spectrometric measurement of the GB_{app} of peptides and proteins based on ESSI-MS at atmospheric pressure. Vapor of reference volatile bases (see Table 2 in Supporting Information) was introduced to react with the protein ions in the atmospheric pressure region before the MS inlet. The vapor pressure was close to the saturation pressure, ensuring a high collision rate with the protein ions and efficient deprotonation reactions at room temperature (298 ± 2 K). An acquisition time of about 1 min (accumulation

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10.1021/jp075127q CCC: $37.00

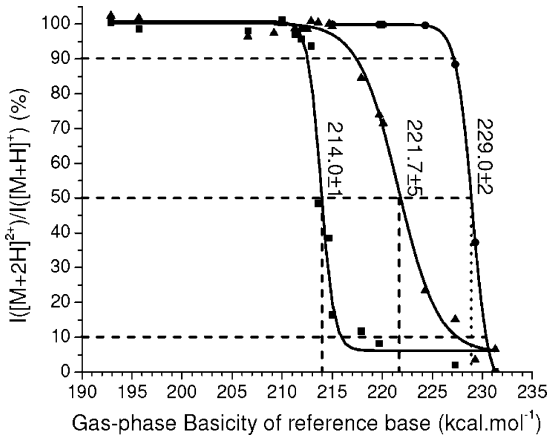

Figure 1. Relative intensity of the $[M + 2H]^{2+}$ ions (● for bradykinin, ▲ for des-Arg^1-bradykinin, ■ for des-Arg^9-bradykinin), calculated as the normalized ratio between the signal intensity of $[M + 2H]^{2+}$ ions and the signal intensity of the $[M + H]^+$ ions, versus the gas-phase basicity of the volatile reference bases. The GB_{app}'s were determined for a deprotonation rate of the $[M + 2H]^{2+}$ ion of 50%. The deviation is calculated for deprotonation rates of 10 and 90%.

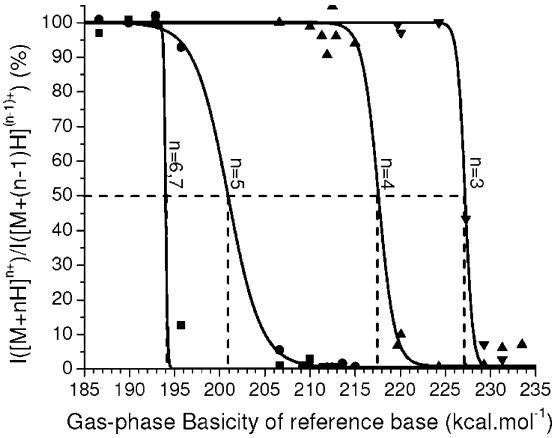

Figure 2. Relative intensity of the $[M + nH]^{n+}$ ions, calculated as the normalized intensity ratio between two successive charge states $[M + nH]^{n+}$ and $[M + (n - 1)H]^{(n-1)+}$ of complete insulin, versus the gas-phase basicity of the volatile reference bases.

of 60 scans) was necessary to obtain a representative mass spectrum of the deprotonation reaction. When the volatile base was removed, the system went back to the initial state in less than 10 s, depending on the volatility of the base. We attempted to conduct the same experiments with ESI or nanoESI, but a charge reduction was never observed. By producing partially or totally desolvated ions at atmospheric pressure, ESSI apparently facilitated the deprotonation reaction between the protein ions and the bases. 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 $[M + (n - 1)H]^{(n-1)+}$ (Figures 1 and 2). The lines used in Figures 1 and 2 are here to guide the eye; the GB_{app}'s were determined for a deprotonation reaction rate of the $[M + nH]^{n+}$ ion of 50%. Ideally, the GB_{app} should be assigned at the point where the deprotonation rate increases sharply. This break point is easily observed for small molecules, while the transition between the slow and fast deprotonation reaction is more gradual for protein ions. In this case, the break point can be chosen for a deprotonation rate of 10%, as described by the group of Cassady,[18] 20% as described by Bouchoux et al.,[19] between 20 and 50% as described by Wu and Lebrilla,[20,21] or at the first distinct change of rate as described by Williams and co-workers.[10] Gronert concluded that a deprotonation rate of 50% gave the best agreement between experimental and calculated values for the dication 1,7-diaminoheptane.[22] Here, we decided to use this last criterion to evaluate the GB_{app}'s with a deviation calculated for deprotonation rates of 10 and 90%. The average charge state (ACS) is given by $ACS = \Sigma(N_i \times I_i) / \Sigma I_i$, where N_i is the number of charges on each peak representing the protein and I_i is the absolute intensity of each peak.

Results and Discussion

In order to validate the method, GB_{app} measurements were performed using bradykinin and derivative (des-Arg^1-bradykinin and des-Arg^9-bradykinin) $[M + 2H]^{2+}$ ions (Figure 1). The values obtained for des-Arg^1-bradykinin and des-Arg^9-bradykinin, 221.7 ± 5 and 214 ± 1 kcal·mol^{-1}, respectively, are in excellent agreement with literature values from the bracketing method[18] or the kinetic method.[23,24] Moreover, the precision for ESSI-based GB_{app} measurements, determined as 10−90% of the rate of the deprotonation reaction, and for other methods is comparable. The GB_{app} value obtained for bradykinin $[M + 2H]^{2+}$ in this study (229.0 ± 2 kcal·mol^{-1}) 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}).[18] This can be explained by different conformations of bradykinin ions in the gas phase, which depend on experimental conditions (solvent, ion source, mass analyzer).[18] Our experiments confirmed the possibility to access GB_{app} of peptides by ESSI-MS experiments at atmospheric pressure. It must be emphasized that each experiment, with the complete set of reference bases, was performed in less than 30 min, compared to hours of measurement times necessary using a FT-ICR.

Experiments to determine GB_{app}'s were extended to substance P (charge state 2) and insulin chain B (charge states 3, 4, and 5). The GB_{app} values were in excellent agreement with the literature values (Table 1).[25,26] 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. Substance P has only two basic residues (Arg^1 and Lys^3). Lysine is less basic than arginine, that is, we can assume that the GB_{app} of Lys^3 in substance P is equal to 227.8 $\pm$ 0.5 kcal·mol^{-1}. Insulin chain B presents five basic residues

TABLE 1: Apparent Gas-Phase Basicity for Some Peptides Ions; All Values Are Expressed in kcal·mol^{-1}

peptide	GB_{app} (deprotonation reaction method)	GB_{app} (ESSI at atmospheric pressure)
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 ($[M + 5H]^{4+}$)	203.4 ± 5.7	201.2 ± 5.4
insulin chain B ($[M + 5H]^{3+}$)	212.2 ± 6.8	217.4 ± 2.4
insulin ($[M + 5H]^{7+}$ and $[M + 6H]^{6+}$)		194.3 ± 0.2
insulin ($[M + 5H]^{5+}$)		201.2 ± 5.4
insulin ($[M + 5H]^{4+}$)		217.4 ± 2.4
insulin ($[M + 5H]^{3+}$)		227.1 ± 1.0

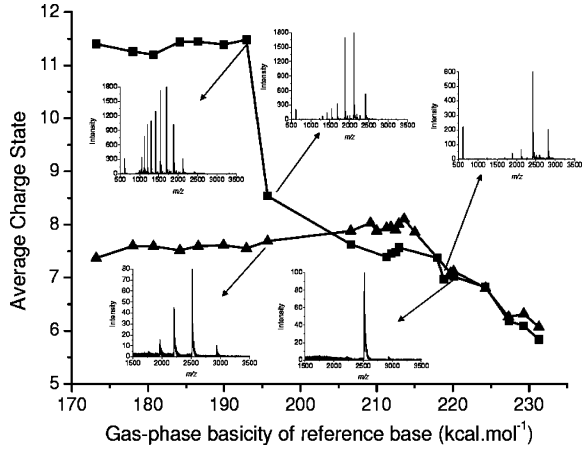

Figure 3. Average charge state (ACS) versus the gas-phase basicity of the volatile reference bases for myoglobin in denaturing (■) and nondenaturing (▲) buffer. Insets: representative mass spectra for some experimental points.

(Lys,[29] His,[5] His,[10] Arg[22], and N-terminal Phe[1]). Charge state 5 was observed but with a very low intensity compared to the other charge states; the corresponding GB_{app} was estimated to be 194.3 ± 0.2 kcal·mol^{-1}. Thus, the GB_{app} of $[M + 5H]^{5+}$ can be attributed to the N-terminal residue of the chain. Lys and His have the same intrinsic GB,[3] that is, 237 kcal·mol^{-1}; calculations could be very useful for a complete assignment in this case. For insulin chain A, only the GB_{app} of charge state 2 was accessible and evaluated to be 194.3 ± 0.2 kcal·mol^{-1} (N-terminal NH_2).

When increasing the GB of the volatile reference base, charge states 6 to 3 of complete insulin disappeared successively (Figure 2). For the first time, the GB_{app}'s for complete insulin (charge states 6, 5, 4, and 3) were measured (Table 1). The G_{app} of charge states 6 and 7 can be assigned to the N-termini of chains A and B. It is quite interesting to notice 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 $n = 6$ to 4. This means that the GB_{app}'s of the basic residues of insulin chain B are not influenced by the presence of two interchain disulfide bonds and by the chain A.

Finally, the ESSI source offers the possibility to analyze proteins in many different buffers, with good sensitivity (protein concentrations in the low μM range). For the first time, we report deprotonation reactions at atmospheric pressure with ESSI-MS of multiply charged myoglobin ions in nondenaturing (ammonium bicarbonate, 10 mM, pH 7.5) and in denaturing (water/methanol/acetic acid, 50/50/1) buffer (Figure 3). The ACS with denaturing buffer is higher than that in nondenaturing buffer until a GB of the reference base of 192.9 kcal·mol^{-1} (anisole).[27] This can be attributed to a higher exposure of the basic residues to the deprotonation agent when the protein is completely unfolded in the gas phase. With ammonia as the volatile reference base, a sudden decrease of the ACS of myoglobin in denaturing buffer occurs due to the simultaneous disappearance of the charge states 9−17. The GB_{app}'s of charge states 9−17 are thus all close to the GB of NH_3 (195.7 kcal·mol^{-1}).[27] For volatile reference bases with GBs higher than 195.7 kcal·mol^{-1}, the two curves are very close. This means that the basic residues that are protonated for low charge states (5−8) are neither dependent on the buffer nor dependent on the protein conformation. These experiments could be very useful to refine the calculations made by Kebarle and co-workers and to better understand the parameters controlling the observed charge states of the proteins.[7,8]

Conclusions

In conclusion, ESSI-MS offers the unique possibility to measure the apparent gas-phase basicity GB_{app} of peptides and proteins at atmospheric pressure with good sensitivity (for concentrations less than 10 μM in denaturing or nondenaturing buffer), very good precision (less than 2%), and in a short time (less than 30 min to screen up to 23 volatile bases). In the near future, this capability will be exploited to measure the GB_{app} of model proteins, such as ubiqitin, lysozyme, or cytochrome c, in order to compare experimental values to the electrostatic models developed by the group of Williams[10−14] or Kebarle.[7−9] This setup could be also useful to control the charge state of the proteins before the analysis by the mass spectrometer, especially in the case of noncovalent complexes, where the electrostatic repulsion can play a great role in the gas-phase stability of such complexes.

Acknowledgment. We thank the Novartis Institutes for BioMedical Research for financial support.

Supporting Information Available: Table 2: GB values of the reference volatile bases.[27] This material is available free of charge via the Internet at http://pubs.acs.org.

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