Review
Temperature-Resolved Proton Transfer Reactions of Biomolecular Ions

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Abstract
Temperature-resolved proton transfer reactions of multiply-protonated angiotensin I, disulfide-intact and -reduced lysozyme, and ubiquitin ions to primary, secondary and aromatic amines were examined in the gas phase. Absolute reaction rate constants for proton transfer were determined from intensities of parent and product ions in the mass spectra. Remarkable change was observed for distribution of product ions and reaction rate constants. In particular, the rate constants for disulfide-intact lysozyme ions changed more drastically with change of charge states and temperature than those for disulfide-reduced ions. Proton transfer reaction was enhanced or suppressed by complex formation of the ions with gaseous molecules, which relates to their conformation changes of with change of temperature.

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INTRODUCTION
The advantage of gas-phase studies is that ideas can be tested on simple model systems. Substantial progress has been made in the past 40 years toward achieving this understanding for simple reactions of small molecular systems through experimental developments.1,2) In particular, proton transfer reactions between a small molecule and a small molecular ion are known as typical elementary processes for ion-molecule reactions. Development of electrospray ionization (ESI) mass spectrometry have made possible to place ions of large biomolecules into the gas phase without any destruction.3,4) Access to biomolecules in the gas phase provides an ideal opportunity to study them in isolated states. Protein folding is so complex that the connection between the sequence and three-dimensional structure of a protein, and the mechanism of the folding process itself, are
not understood despite many years of study. Levinthal has proposed a thought experiment, constituting a self-reference about protein folding. If a protein were to attain its correctly folded configuration by sequentially sampling all the possible conformations, it would require a time longer than the age of the universe to arrive at its correct native conformation, while proteins fold spontaneously on less than a millisecond time scale. Anfinsen et al. have studied unfolding and refolding kinetics of ribonuclease and has concluded that its native structure is determined only by the protein's amino acid sequence, and that the native structure is a unique, stable and kinetically accessible minimum of the free energy. Wolynes has supposed that the spontaneous protein folding would proceed along a specific funnel on the free energy landscape.

A lot of water molecules and the other biomolecules, which would influence their native configurations, surrounds proteins in living systems. Moreover, interactions with surrounding molecules make more complex to understand protein folding. By introduction a biological molecule into the gas phase, it is possible to separate its hydration interactions and intramolecular interactions and examine them independently, even though the gas phase is an unusual environment for investigation of biological molecular systems. Structures and reactions of gas-phase biomolecules have been studied extensively using various mass spectrometric methods. Protein conformations and dynamics have been studied extensively by mass spectrometry-based approaches to characterize kinetic intermediates in refolding experiments. A new experimental approach is expected to determine exact biomolecular structures in the gas phase.

Angiotensin I is a relatively small peptide, which is composed of 10 residues of amino acids and contains four basic residues. The peptide is known to act as a precursor to angiotensin II in biological system for blood pressure regulation. Understanding their structures bound to the receptor are important for determining the ligand-receptor interaction that is necessary for receptor activation. Their native states have been qualitatively characterized by NMR. There are a lot of gas-phase mass-spectrometric studies for angiotensin I or II ions. Lysozyme is a relatively small protein which has four intramolecular disulfide bonds at cysteine residues. This protein has represented one of the most popular and important model systems for understanding the complexity of protein structure and function. Lysozyme and its derivative of disulfide linkages in solution phase has been investigated by a wide range of complementary biophysical techniques. The role of disulfide bridges in the formation and maintenance of the three-dimensional fold of lysozyme has been studied. Mass spectrometric studies have been shown that conformations in the gas phase are highly dependent on the presence of disulfide bonds. Conformations of the disulfide-intact protein ions are a highly folded,
whereas disulfide-reduced protein ions favor highly diffuse, unfolded forms of the protein, since there are no disulfide bonds to impose structural restrictions. Ubiquitin is a small protein, which is composed of 76 residues and contains 13 basic residues. The function of ubiquitin is to tag target proteins via covalent attachment for proteasomal degradation. Ion mobility mass spectrometric studies have provided evidence for the preservation of native state ubiquitin conformations in the gas phase.\(^{10}\)

In this review, proton transfer reactions of angiotensin I, disulfide-intact and -reduced lysozyme, and ubiquitin ions for multiply-protonated states were described.\(^{15-17}\) Temperature dependence of absolute reaction rate constant for proton transfer and distribution of product ions were examined for those peptide and protein ions in the gas phase. An issue that is attracting considerable attention is their gas-phase conformations might resemble structural evolution that originated from temperature in the gas phase. Based on experimental observations, we discussed conformation change of those ions with change of temperature. In addition, relationship between gas-phase conformation, self-solvation to protons by hydrophilic residues in polypeptide chains, and delocalization of charges with self-solvation were discussed.

**EXPERIMENTAL**

Details of the experimental apparatus used in the present study have been described elsewhere.\(^ {15-17}\) Briefly, the system consists of four vacuum chambers with an ESI source, a tandem mass spectrometer, and a gas cell equipped with an octapole ion trap. Multiply-protonated peptide and protein ions were produced by ESI. A specific charge state of ions was selected by a quadrupole mass spectrometer (QMASS). The charge-selected ions emerging from QMASS were admitted into the gas cell equipped with an octapole ion trap. The gas cell was filled with He including gaseous target molecules (TM). As TM, primary amines, secondary amines, and aromatic amines were used to collide with ions and to induce proton transfer reactions. Temperature dependence of reaction rate constants and branching fractions for proton transfer from the charge-selected ions to TM was measured, by changing temperature of the gas cell in the range of 280 - 460 K. Parent and product ions were extracted from the gas cell, and were mass-analyzed by a time-of-flight mass spectrometer equipped with reflectron.

**RESULTS AND DISCUSSION**

1. **Angiotensin I**

Proton transfer reactions of angiotensin I ions for 2+ charge state, [Ang + 2H]\(^{2+}\) were studied.\(^ {15}\) In Fig. 1 (a), time-of-flight mass spectra of [Ang + 2H]\(^{2+}\) reacted with 1-
butylamine (1-Bu) are presented as a function of mass-to-charge ratio (m/z). In (A) of Fig. 1 (a), an ion of specific charge states, [Ang + 2H]^{2+}, was selected with QMASS as a reactant. In (B) ~ (H), mass spectra of [Ang + 2H]^{2+} reacted with 1-Bu at various temperatures are presented. Temperature of gas cell was (B) 448, (C) 377, (D) 317, (E) 305, (F) 295, (G) 285, and (H) 282 K, respectively. As seeing the figures, a product ion, [Ang + H]^+, was observed. Not shown in the figure, a protonated molecular ion, 1-Bu-H^+, and a protonated dimer ion, (1-Bu)_2-H^+, were also detected at lower mass range. Therefore, proton transfer reaction from [M + zH]^{z+} to TM proceeds in the gas cell;

\[ [M + zH]^{z+} + TM \rightarrow [M + (z-1)H]^{(z-1)+} + TM-H^+. \]  

In Fig. 1 (b), branching ratios of parent and product ions are plotted as a function of temperature in the gas cell. Abundance of parent ion, [Ang + 2H]^{2+}, increased gradually with decrease of temperature from 470 to 330 K, whereas it decreased sharply below 330 K. Complex ions, [Ang + 2H]^{2+}-(1-Bu)\_n (n = 1 - 3), appeared at 330 K, and abundances of them increased suddenly below 330 K. Those of [Ang + H]^+ gradually decreased with decrease of temperature from 470 to 330 K, then it increased rapidly from 330 to 300 K, whereas it decreased suddenly below 300 K. Enhancement and suppression of proton transfer at 330 and 300 K are seemed to be related to appearance of [Ang + 2H]^{2+}-(1-Bu)\_n.

Following equation (1), absolute reaction rate constants, k, of proton transfer from [Ang + 2H]^{2+} to gaseous target molecules (TM) were determined. Details of scheme to determine absolute values of the rate constants have been described elsewhere.\textsuperscript{16,17} In Fig. 2, Reaction rate constants of proton transfer from [Ang + 2H]^{2+}, to 1-Bu, dipropylamine (Dpr), 3,5-dimethylpyridine\textsuperscript{30} (35Dmpy) and 2,6-dimethylpyridine (26Dmpy) are plotted as a function of temperature in the gas cell. The rate constant for [Ang + 2H]^{2+} reacted with 1-Bu gradually decreased with decrease of temperature from 470 to 330 K, then it increased suddenly from 330 to 300 K, whereas it dropped rapidly below 300 K. Sudden jump of the rate constant below 330 K corresponds to complex formation, as shown in Fig. 1(b). Similar features were obtained for the reactions with 1-pentylamine and tert-butylamine. The reaction would proceed via a two-steps process by way of complex formation as reaction intermediate. Enhancement of proton transfer is due to favorable formation of the complex. Suppression of proton transfer is due to stabilization of the complex. The rate constant for [Ang + 2H]^{2+} reacted with Dpr gradually decreased with decrease of temperature from 470 to 330 K, and it decreased sharply below 330 K. Similar features were obtained for the reactions with diethylamine. Stabilization of the complex

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suppresses the reaction. The rate constant for \([\text{Ang} + 2\text{H}]^{2+}\) reacted with 35Dmpy slightly decreased with decrease of temperature from 470 to 330 K, whereas it increased rapidly below 330 K. Similar features were obtained for the reactions with pyridine, 2-methylpyridine. However, the rate constant with 26Dmpy slightly decreased with decrease of temperature from 470 to 360 K, it increased slightly from 360 to 330 K, whereas it decreased gradually below 330 K, which was completely different from that of 35Dmpy. With decrease of temperature below 330 K, structure of the peptide ions changes from elongated to compact conformations. There would be two factors to enhance or suppress the proton transfer reaction, which are influenced to conformation change related temperature. One factor is steric hindrance. Protons attached on the peptide ions of compact conformations would locate inside sites. TM are blocked to approach the protons and the reaction is suppressed. Another factor is charge delocalization. Hydrophilic groups bind the protons. Proton charge are delocalized by those hydrogen bonds, so called self-solvation. Proton hopping occurs along hydrogen bonds with hydrophilic groups on the polypeptide chain. It enhances the reactions. These two factors are closely related to geometric structures and proton affinities (PA) of TM. In secondary amines such as Dpr, which has two alkyl chains, steric hindrance is more effective than charge delocalization. Therefore, the reaction with Dpr is suppressed in low temperature range. In protonated aromatic amine ions such as 35Dmpy, the charge is delocalized into the conjugated \(\pi\) electrons system, which interacts with lone-pair electrons at a nitrogen atom. There is no steric hindrance by the two methyl groups on 35Dmpy. And suppression by steric hindrance in the peptide ion is less effective than enhancement by charge delocalization. Therefore, the reaction is enhanced in low temperature range. On the other hand, on 26Dmpy, not like 35Dmpy, there exists steric hindrance by the two methyl groups close to the nitrogen atom. And effects of enhancement and suppression are compensated. In primary amines such as 1-Bu, there is one alkyl chain bound with the nitrogen atom. It is little steric hindrance. PA of 1-Bu is relatively small, comparing with those of Dpr, 35Dmpy, and 26Dmpy. Therefore, the reaction is enhanced by charge delocalization with decrease of temperature below 330 K, whereas it is suppressed by steric hindrance with below 300 K.

2. Disulfide-intact and -reduced lysozyme

Proton transfer reactions of disulfide-intact and -reduced lysozyme ions, \([\text{Lys}_i + z\text{H}]^{z+}\) and \([\text{Lys}_r + z\text{H}]^{z+}\) respectively, (7+ through 14+) to 26Dmpy were examined.\(^{16}\) The disulfide-reduced form of lysozyme was obtained by boiling a lysozyme in 0.01 M dithiothreitol (DTT) aqueous solution for 30 min.\(^{16}\) Time-of-flight mass spectra of

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disulfide-intact lysozyme ions for 11+ charge state, [Lys_i + 11H]^{11+} reacted with 26Dmpy at various temperature are shown in Fig. 3. In Fig. 3(A), a mass spectrum of multiply-protonated lysozyme ions, [Lys_i + zH]^z+ (z = 6 - 12), is presented. In Fig. 3(B), ions of specific charge states, [Lys_i + 11H]^{11+}, was selected as a reactant with QMASS. In Fig. 3(C) ~ (G), mass spectra of [Lys_i + 11H]^{11+} reacted with 26Dmpy at various temperature, are presented. As a target molecule, 26Dmpy was introduced into the gas cell, as well as He. Temperature of gas cell was (C) 460, (D) 410, (E) 360, (F) 310, and (G) 290 K, respectively. As shown in the figures, drastic changes of intensities for product ions, [Lys_i + z'H]^z'+ (z' = 7 - 10), with decrease of temperature were observed. Not shown in these figures, a protonated molecular ion, 26Dmpy-H+, and a protonated dimer ion, (26Dmpy)_2-H+, were also detected. In Fig. 4, the rate constants for [Lys_i + zH]^z+ and [Lys_r + zH]^z+ are plotted as a function of temperature, for comparing the same charge states. The rate constants for 7+, 8+, 9+, 10+, 11+, and 12+ charge states are presented in Fig. 4(a), (b), (c), (d), (e), and (f), respectively. In the figures, letters represented “I” indicate the rate constants of [Lys_i + zH]^z+, whereas letters represented “R” indicate those of [Lys_r + zH]^z+. As shown in the figures, for 9+, 10+, 11+, and 12+ charge states, the rate constants of [Lys_i + zH]^z+ were larger than those of [Lys_r + zH]^z+. At high temperature range, the rate constant of [Lys_i + 7H]^7+ was equal to that of [Lys_r + 7H]^7+, while it was smaller than that at low temperature range. On the other hand, the rate constant of [Lys_i + 8H]^8+ was much smaller than that of [Lys_r + 8H]^8+. For 9+ and 10+ charge states, there were humps in line shapes of [Lys_i + zH]^z+, whereas line shapes of [Lys_r + zH]^z+ were smoothly curved lines. As a whole, the rate constants for [Lys_i + zH]^z+ changed more drastically than those for [Lys_i + zH]^z+ with change of temperature and of charge state. For disulfide-intact ions, [Lys_i + zH]^z+, they increased more rapidly with increase of charge states than those for disulfide-reduced ions, [Lys_r + zH]^z+. Exceptionally, the reaction rate constant of [Lys_i + 7H]^7+ was much larger than that of [Lys_i + 8H]^8+ over all temperature range. With change of temperature, the rate constants for [Lys_i + zH]^z+ changed more drastically than those for [Lys_r + zH]^z+. Drastic changes of the rate constants in [Lys_i + zH]^z+ relate to restriction of protein conformation by disulfide bonds.

3. Ubiquitin

Proton transfer from multiply-protonated ubiquitin ions, [Ubi + zH]^z+ (z = 6 - 12), to 1-Bu was studied in the gas phase.\(^{(17)}\) Absolute reaction rate constant for proton transfer was determined from intensities of parent and product ions in the mass spectra. In Fig. 5, the reaction rate constants for [Ubi + zH]^{z+} (z = 6 - 12), are plotted as a function of temperature in the gas cell. Generally for multiply-protonated protein ions, reaction rate
constant of proton transfer grows larger with increase of charger states, $z$. However, exceptionally, the reaction rate constant of $[\text{Ubi} + 6\text{H}]^{6+}$ is much larger than that of $[\text{Ubi} + 7\text{H}]^{7+}$ in $T = 350 - 450$ K. For $[\text{Ubi} + 6\text{H}]^{6+}$, it suddenly increases with decrease of temperature from 450 to 380 K, and decreases from 370 to 300 K. Gas-phase conformations of ubiquitin ions have been determined by means of ion mobility measurements. Those on lower charged states, $z = 6, 7$, are compact in low temperature range, whereas they have more unfolded and elongated conformations with increase of temperature. Remarkable changes of the reactivities for the ions on the lower charge states relate to structural transitions from elongated to compact conformations with decrease of temperature. The reaction is enhanced by charge delocalization and is suppressed by steric hindrance. Similar feature was observed to those in the reaction of $[\text{Ang} + 2\text{H}]^{2+}$ with 1-Bu, which was described in previous section.

**CONCLUSION**

Proton transfer reactions of multiply-protonated angiotensin I, disulfide-intact and -reduced lysozyme, and ubiquitin ions to primary, secondary and aromatic amines were examined. By changing temperature of the gas cell, temperature dependence of absolute reaction rate constants for proton transfer was measured. The reaction of $[\text{Ang} + 2\text{H}]^{2+}$ was enhanced and reduced by complex formation. The rate constants for $[\text{Lys}_i + z\text{H}]^{z+}$ changed more drastically with change of charge states and temperature than those for$[\text{Lys}_i + z\text{H}]^{z+}$, which are due to the protein conformations stabilized by disulfide bonds. Drastic changes of the rate constants for $[\text{Ubi} + z\text{H}]^{z+}$ in lower charge states relate to conformation transitions from elongated to compact states. For the ions on lower charge states at lower temperature range, which have compact conformations, the two factors compete to enhance or to suppress the reaction. Delocalization of proton charge enhances the reactions, whereas steric hindrance for blocking TM to access to protons suppresses them.

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**Figure Captions**

**Fig. 1.** (a) Time-of-flight mass spectra of [Ang + 2H]$^{2+}$ reacted with 1-Bu at various temperatures. (A) Ions of specific charge state, [Ang + 2H]$^{2+}$, is selected with QMASS as a reactant. (B) ~ (H) Mass spectra of [Ang + 2H]$^{2+}$ reacted with 1-Bu at various temperatures. (b) In the reaction of [Ang + 2H]$^{2+}$ with 1-Bu, branching ratios of parent and product ions are plotted as a function of temperature in the gas cell.

**Fig. 2.** Reaction rate constants of proton transfer from [Ang + 2H]$^{2+}$, to 1-Bu, Dpr, 35Dmpy and 26Dmpy are plotted as a function of temperature in the gas cell.

**Fig. 3.** Time-of-flight mass spectra of disulfide-intact lysozyme ions for 11+ charge state, [Lys$i$ + 11H]$^{11+}$ reacted with 26Dmpy at various temperature. (A) Mass spectrum of all ions produced with ESI, where DC voltage in QMASS was put off. (B) Ions of a specific charge state, [Lys$i$ + 11H]$^{11+}$, was selected with QMASS as a reactant. (C) ~ (G) Mass spectra of [Lys$i$ + 11H]$^{11+}$ reacted with 26Dmpy at various temperatures. Temperature of gas cell was (C) 460, (D) 410, (E) 360, (F) 310, and (G) 290 K, respectively.

**Fig. 4.** The reaction rate constants for disulfide-intact and -reduced lysozyme ions, [Lys$i$ + zH]$^{z+}$ and [Lys$r$ + zH]$^{z+}$ respectively, are plotted as a function of temperature in the gas cell, for comparing the same charge states. The rate constants for 7+, 8+, 9+, 10+, 11+, and 12+ charge states are presented in (a), (b), (c), (d), (e), and (f), respectively. In the figures, letters represented “I” indicate the rate constants of [Lys$i$ + zH]$^{z+}$, whereas letters represented “R” indicate those of [Lys$r$ + zH]$^{z+}$.

**Fig. 5.** Absolute reaction rate constants of proton transfer for [Ubi + zH]$^{z+}$ ($z = 6 - 12$) are plotted as a function of temperature in the gas cell.
Figures

(A) QMASS off
(B) QMASS on, 1-Bu off
(C) 1-Bu on, 448 K
(D) 1-Bu on, 377 K
(E) 1-Bu on, 317 K
(F) 1-Bu on, 305 K
(G) 1-Bu on, 295 K
(H) 1-Bu on, 282 K

[Ang+2H]+
[Ang+H]+
[Ang+2H]+(1-Bu)
[Ang+H]+(1-Bu)

Temperature (K)

Branching Ratio

[Ang+2H]+-1-Bu
[Ang+2H]+(1-Bu)₂
[Ang+2H]+(1-Bu)₃
[Ang+H]⁺-1-Bu

Fig. 1
Fig. 2
Fig. 3
\begin{align*}
\text{(a)} & \quad \frac{k(\times 10^{-12} \text{ mole}^{-1} \text{ cm}^3 \text{ s}^{-1})}{(\pi K)} = 7 \\
\text{(b)} & \quad \frac{k(\times 10^{-12} \text{ mole}^{-1} \text{ cm}^3 \text{ s}^{-1})}{(\pi K)} = 8 \\
\text{(c)} & \quad \frac{k(\times 10^{-12} \text{ mole}^{-1} \text{ cm}^3 \text{ s}^{-1})}{(\pi K)} = 9 \\
\text{(d)} & \quad \frac{k(\times 10^{-12} \text{ mole}^{-1} \text{ cm}^3 \text{ s}^{-1})}{(\pi K)} = 10 \\
\text{(e)} & \quad \frac{k(\times 10^{-12} \text{ mole}^{-1} \text{ cm}^3 \text{ s}^{-1})}{(\pi K)} = 11 \\
\text{(f)} & \quad \frac{k(\times 10^{-12} \text{ mole}^{-1} \text{ cm}^3 \text{ s}^{-1})}{(\pi K)} = 12
\end{align*}

Fig. 4
Fig. 5