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Determination of pK_a Values of Individual Histidine Residues in Proteins Using Mass Spectrometry

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We developed a mass spectrometric method to determine the pK_a values of individual histidine residues in proteins. The method is based on the fact that the imidazole C_2-proton undergoes pH-dependent hydrogen−deuterium exchange reaction, of which the rate constant (k_φ) reflects the pK_a for the ionization of imidazole to imidazolium. The experimental procedure consists of the following: (1) protein incubation in D_2O solvent at various pH values, (2) protein digestion by proteolytic enzyme(s), during which all the rapidly exchanging deuterons such as those in amide and hydroxyl groups are back-exchanged for protons, and (3) measurement of the mass spectrum of each histidine-containing peptide by LC/ESI-MS. The k_φ of the H−D exchange reaction is obtained from the mass spectrum reflecting the extent of deuterium incorporation. The pK_a value is then determined from a plot of k_φ versus pH, which gives a typical sigmoidal curve. Unambiguous assignment of the pK_a values to individual histidine residues can be achieved simultaneously based on the observed molecular mass of the peptide. The pK_a values of three of four histidine residues (His12, -105, and -119) in RNase A were successfully determined by this method and were in good agreement with those determined by ^{1}H NMR and hydrogen−tritium exchange methods. The method uses subnanomole quantities of protein, allowing measurement at a much lower concentration than that of 1 mM required for the conventional NMR approach that is currently almost exclusively the method of choice.

The imidazole group of histidine residues in proteins is distinguished from other ionizable side-chain groups by its characteristic pK_a value near 7 for ionization of the corresponding imidazolium cation, allowing the involvement of histidine residues in the catalytic processes of enzyme reactions that use general acid−base catalysis. The pK_a value of a histidine residue can vary depending on the interaction with other ionizable group(s) in proteins and thus provides useful information on the microenvironment of this residue. For example, the pK_a value of a catalytic histidine residue is closely associated with the catalytic mechanism of the enzyme. To date, the most useful technique for determining the pK_a values of histidine residues in proteins has been pH titration of imidazole C_2-proton signals by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy, because the C_2-proton signals appearing at ~8 ppm sensitively reflect the ionization states of individual imidazole N−H groups and are usually well resolved from each other and separated from other aromatic protons.[1,2] However, this method suffers from the difficulty in assigning ^{1}H NMR signals to individual histidine residues, even though the development of multidimensional NMR spectroscopy has greatly reduced the ambiguity of assignment.

Hydrogen atoms that are covalently bonded to nitrogen, oxygen, or sulfur atoms exchange rapidly with deuterium atoms in heavy water (D_2O). In addition to such labile hydrogen atoms, the C_2-hydrogen of the imidazole ring of histidine residues also exchanges with deuterium atoms but at a much slower rate, with a half-life in the order of days.[3] The hydrogen−deuterium (H−D) exchange reaction at the imidazole C_2-position has been shown to follow pseudo-first-order kinetics, in which the abstraction of the C_2-proton from the cationic imidazolium by OD^- to form a ylide or a carbene intermediate is the rate-determining step

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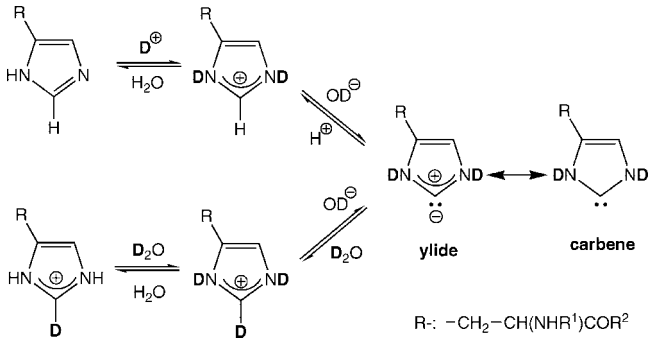
Scheme 1. Mechanism of the H−D Exchange Reaction at the Imidazole C_2-Position of Histidine[a]



[a] The rate-determining step of this reaction lies on the abstraction of the protonated imidazolium C_2-proton to form the ylide or formally neutral carbenic intermediate.

(Scheme 1).[4–6] Intriguingly, the dependency of the apparent H−D exchange rate of the imidazole C_2-hydrogen on pH reflects the pK_a value of the imidazole N−H group, but not that of the rate-determining imidazole-ylide (carbene) equilibrium that has a pK_a value of 23.8.[6] Accordingly the pK_a value of the imidazole N−H group can be determined by the measurement of H−D exchange rates at the imidazole C_2-position at various pH (pD) values in the manner of ordinary acid−base titration (pH versus H−D exchange rate). Because the H−D exchange reaction at the C_2-proton of histidine residues in proteins is accessible by ^{1}H NMR spectroscopy, this kinetic method has proved very useful for the unambiguous assignment of histidine NMR signals.[2,7] However, severe limitations remain with respect to the applicability of NMR spectroscopy to large proteins and a small amount of sample.

Adopting this principle to the hydrogen−tritium (H−T) exchange reaction, Matsuo and co-workers developed a method to determine the pK_a values of histidine residues in proteins, enabling analysis of the microenvironment of histidine residues with additional information on the H−T exchange reaction rate.[8,9] Although the practicality of the method can be enhanced significantly by employing high-performance liquid chromatography (HPLC) for the separation of peptides and modern mass spectrometry for the assignment of histidine residues, the use of radioisotope remains a major drawback.

Prior to the development of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) techniques, Ohe and Kajita reported successful determination of the pK_a values of individual histidine residues in proteins using gas chromatography/mass spectrometry.[10,11] In this mass spectrometric method, deuterated protein was proteolytically digested, the generated peptides were separated by two-dimensional paper chromatography−electrophoresis, and the extent of deuterium incorporation into individual histidine residues was measured by gas chromatography/mass spectrometry at the amino acid level after acid hydrolysis of the deuterated peptides. The assignment of individual histidine residues was performed separately by amino acid sequence analysis of the deuterated peptides using the Edman degradation. They demonstrated the applicability of the technique with hemoglobin,[11] which has extremely difficult accessibility for ^{1}H NMR due to the large size of the molecule (67 kDa) and which contains a large number of histidine residues (human hemoglobin contains 10 residues in the α-chain and 9 in the β-chain). Since then, however, this mass spectrometric technique has remained largely ignored, probably because it has an unfortunate reputation of involving tedious and laborious experiments, while extending the H−T exchange method to which the description "the assignments of pK_a are unambiguous" was applied.[12]

We predicted that the major difficulties associated with the original mass spectrometric method could be removed by implementing ESI or MALDI MS, which can ionize peptides with high efficiency,[13] allowing us to achieve both measurement of H−D exchange rate and assignment of deuterium incorporated histidine residues simultaneously at the peptide level. Therefore, we have resurrected the H−D exchange technique to develop an improved method using ESI-MS. The experimental procedure consists of the following: (1) protein incubation in D_2O solvent at various pH values, (2) protein digestion by proteolytic enzyme(s), during which all the rapidly exchanging deuterons such as those in amide and hydroxyl groups are back-exchanged for protons, and (3) measurement of mass spectra of histidine-containing peptides by LC/ESI-MS. The method was applied to determine the pK_a values of four histidine residues in bovine pancreatic ribonuclease A (RNase A). The results demonstrate that assignment of pK_a values to individual histidine residues is straightforward and the corresponding rate constant of H−D exchange at each histidine residue can be determined at a sub-nanomole level of sample protein. We also discuss the applicability of the present method to the study of higher-order protein structure and dynamics by exploiting histidine residues as probes to provide the H−D exchange rate constant as one of good measures of solvent accessibility.

EXPERIMENTAL SECTION

Materials. Deuterium oxide (D_2O, 99.9%) was purchased from Cambridge Isotope Laboratories (Andover, MA), and deuterium chloride (DCl) and sodium deuteroxide (NaOD) were from Sigma-Aldrich (St. Louis, MO). [Val,^{4} Ile^7]-Angiotensin III (Arg-Val-Tyr-Val-His-Pro-Ile) and bovine pancreatic RNase A were obtained from Sigma-Aldrich and used without further purification. Immobilized trypsin and immobilized chymotrypsin were from Princeton Separations (Adelphia, NJ). All other chemicals and materials were either reagent grade or were of the highest quality that was commercially available.

Buffer solutions in D_2O. Buffers in D_2O contained 50 mM sodium acetate (pH 3.5−4.5), 50 mM MES (pH 5.0−7.5), and 50 mM HEPES (pH 8.0−9.0). The pH of the buffers was adjusted with diluted DCl or NaOD and measured with a Solution Analyzer model 4603 (Amber Science, Eugene, OR) equipped with a glass/

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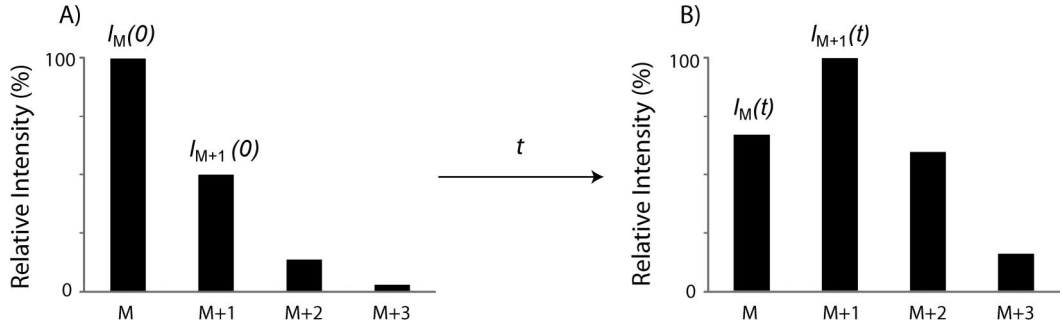

Figure 1. Hypothetical mass spectra of a peptide before and after H−D exchange reaction. Before the H−D exchange reaction takes place, a peptide with a molecular mass of M that contains one histidine residue gives multiple isotopic peaks (M, M + 1, M + 2, M + 3) reflecting the natural abundances of heavy isotopes such as ^{13}C (1.1%) and ^{15}N (0.4%) (A). Incubation of the peptide in D_2O solvent results in partial deuterium incorporation at the imidazole C_2-position of the histidine residue, and the relative abundance of isotopic peaks changes as shown in (B). Only peaks M and M + 1 were used for the calculation of k_φ.

AgCl electrode (model 476086, Nova Analytics, Woburn, MA). The reported pH values are direct pH meter readings of the D_2O buffer solutions calibrated with standard buffer solutions made with H_2O and are uncorrected for the isotope effect at the glass electrode. The ionic strength of the buffers was standardized with pH 4.5 buffer consisting of 50 mM MES and 50 mM NaCl, which had a conductivity value of 4.5 mmho/cm. The conductivity of all other buffer solutions was adjusted to 4.5 mmho/cm with NaCl.

Deuteration of Angiotensin III. Angiotensin III (3 nmol) was dissolved in 300 μL of pH 7.0 buffer in D_2O and incubated at 37 °C to investigate the time-dependent H−D exchange reaction at the imidazole C_2-position of the histidine residue. Aliquots (50 μL) were withdrawn at different intervals (0, 8, 24, 48, and 72 h), mixed with 5 μL of formic acid to stop the H−D exchange reaction, and dried in a Speed Vac. Angiotensin III (1 nmol) was also incubated in 100 μL of buffers in D_2O with different pH (pH 4.5−9.0) at 37 °C for 20 h to allow pH-dependent H−D exchange to occur. The reaction was stopped by mixing with 10 μL of formic acid and dried in a Speed Vac. Prior to mass spectrometry analysis, the dried peptide samples were redissolved in 50 μL of ammonium bicarbonate in H_2O and incubated at room temperature for 10 min to promote deuterium to hydrogen exchange at rapidly exchangeable sites of the peptide (the amino, imino, and amide NH and OH groups). The resulting peptide was dried again, redissolved in 100 μL of 0.1% formic acid, and then desalted using a $ZipTip_{C18}$ (Millipore, Bedford, MA) according to the manufacturer's instructions. The eluted peptides were dissolved in 100 μL of 50% acetonitrile/0.1% formic acid (v/v) and subjected to mass spectrometric analysis as described below.

Deuteration, Performic Acid Oxidation, and Digestion of RNase A. RNase A (500 pmol) was incubated in 100 μL of buffer with different pH values (pH 3.5−9.0) at 37 °C for 48 h. The reaction was stopped by mixing with 10 μL of formic acid, and the protein was freed from the buffer salts using an Ultra Micro Spin C18 column (Nest Group, Southboro, MA) according to the manufacturer's instructions and dried in a Speed Vac. The protein was redissolved in 50 μL of formic acid/methanol (5/1, v/v) and oxidized with performic acid at 4 °C for 2.5 h.[14] After the reaction, 950 μL of H_2O was added to the solution and dried in a Speed Vac. The protein was then redissolved in 20 μL of 0.1 M ammonium bicarbonate and digested with 1 μg of immobilized trypsin for 1 h followed by digestion with 1 μg of immobilized chymotrypsin for 1 h so that each peptide fragment could contain only one histidine residue. The resulting digest solution was diluted 10-fold with 0.1% formic acid and analyzed as described below.

Mass Spectrometry. All mass spectrometry analyses were conducted using a QStar quadrupole/time-of-flight mass spectrometer (Applied Biosystem-MDS Sciex, Foster City, CA) equipped with a nanoelectrospray ion source. Deuterated angiotensin III samples were directly introduced into the mass spectrometer using a syringe pump (Harvard Apparatus, Holliston, MA) as a solvent delivery system at a flow rate of 5 μL/min. Mass spectra were acquired for 1 min with an acquisition time of 5 s for each scan (12 scans/min) in the mass range of m/z 400−900. The reported mass spectra are the average over 12 scans. RNase A digests were analyzed by liquid chromatography/mass spectrometry (LC/MS) using an Agilent 1100 nano HPLC system (Agilent Technologies, Santa Clara, CA) interfaced to the mass spectrometer as described previously.[15] Briefly, the peptide mixture (5 μL, ~12.5 pmol) was injected directly into a reversed-phase analytical column (75 μm × 15 cm, Dionex, Sunnyvale, CA), washed with 2% acetonitrile/0.1% formic acid (v/v) in water for 15 min at a flow rate of 350 nL/min. The peptides were eluted with a 40-min linear gradient of 2% acetonitrile/0.1% formic acid (v/v) in water to 22% acetonitrile/0.1% formic acid (v/v) in water at a flow rate of 350 nL/min. The column effluent was directed online to the nanoelectrospray ion source. The total ion current was obtained in the mass range of m/z 400−2000 in the positive ion mode with an acquisition time of 5 s for each scan.

Measurement of Rate Constant (k_φ) and Half-Life ($t_{1/2}$) of H−D Exchange Reaction. A peptide with a single histidine residue that incorporated deuterium partially at the imidazole C_2-position of this residue gives a mass spectrum similar to that shown in Figure 1. The spectrum at time $t = 0$ reflects the isotope distribution due to the natural abundance of heavy isotopes such as ^{13}C (1.1%) and ^{15}N (0.4%) and that at time $t > 0$ is a mixture of the natural isotope patterns from nondeuterated and deuterated peptide species. We determined the k_φ and $t_{1/2}$ by monitoring the changes in relative abundance between the M and M + 1 isotopic peak from a given peptide after the H−D exchange reaction. In Figure 1 the intensities of the M and M + 1 peaks at time $t = 0$ are represented as $I_M(0)$ and $I_{M+1}(0)$, respectively. Similarly, the intensities of the same peaks at time t are represented as $I_M(t)$

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and $I_{M+1}(t)$, respectively. Since the H−D exchange reaction follows pseudo-first-order kinetics, the intensities of $I_M(t)$ and $I_{M+1}(t)$ can be expressed by eqs 1 and 2, respectively.

$$I_M(t) = I_M(0)\ \exp(-k_\varphi t) \quad (1)$$

$$I_{M+1}(t) = I_{M+1}(0)\ \exp(-k_\varphi t) + I_M(0)[1 - \exp(-k_\varphi t)] \quad (2)$$

Because it is impractical to use directly the intensities of the same isotopic peaks in different spectra [$I_M(0)$ and $I_M(t)$, $I_{M+1}(0)$ and $I_{M+1}(t)$] to calculate k_φ using eq 1 or 2, we need to compare the relative abundance between the relevant isotopic peaks in different spectra. Therefore, taking the ratio $R(t) = I_{M+1}(t)/I_M(t)$, we obtain a simple relationship as expressed by eq 3, where $R(0) = I_{M+1}(0)/I_M(0)$. The ratio $R(0)$ may either be determined from the corresponding peak intensities measured at $t = 0$ or predicted by calculation from the chemical formula of a given peptide.

$$R(t) = \frac{I_{M+1}(t)}{I_M(t)} = \frac{I_{M+1}(0)}{I_M(0)} + \frac{1}{\exp(-k_\varphi t)} - 1 = R(0) + \exp(k_\varphi t) - 1 \quad (3)$$

From this equation, k_φ is related to the experimental data straightforwardly as:

$$k_\varphi = \ln[1 + R(t) - R(0)]/t \quad (4)$$

According to kinetic studies of the H−D exchange reaction of the imidazole C_2-proton,[4,5] the explicit representation of first-order rate constant k_φ as a function of second-order rate constant (k_2) for the rate-determining step (see Scheme 1), is given by eq 5, where K_a is the dissociation constant of imidazolium cation and K_W is the ion product of heavy water ($K_W = [D^+][OD^-] = 10^{-14.87}$).[16]

$$k_\varphi = \frac{k_2 K_W}{[D^+] + K_a} \quad (5)$$

By introducing the maximal pseudo-first-order rate constant:

$$k_\varphi^{max} = \frac{k_2 K_W}{K_a} \quad (6)$$

which corresponds to k_φ in an alkaline condition where D^+ concentration is negligible by the relationship $[D^+] \ll K_a$, the simple Henderson−Hasselbalch equation to determine the pK_a value from the titration curve is derived as follows:[8,9]

$$\log\left(\frac{k_\varphi^{max} - k_\varphi}{k_\varphi}\right) = pK_a - pH \quad (7)$$

The value of k_φ^{max} is independent of the variation of pH and can be obtained from the plateau to the alkaline side of the sigmoidal titration curve plotted k_φ versus pH. We compared the k_φ^{max} value of individual histidine residues with respect to the half-life ($t_{1/2}$) of the exchange reaction

$$t_{1/2} = \frac{\ln 2}{k_\varphi^{max}} \quad (8)$$

The experimental data were analyzed using Origin Graphing software (Ver. 7.5, OriginLab, Northampton, MA) to obtain pK_a values.

RESULTS AND DISCUSSION

Deuteration of Angiotensin III. To test whether the H−D exchange reaction at the imidazole C_2-position of a histidine residue in a peptide can be followed by mass spectrometry, a model peptide, angiotensin III (Arg-Val-Tyr-Val-His-Pro-Ile), was incubated in D_2O buffered with MES (pH 7.0) at 37 °C for various times and the resulting peptide was analyzed by ESI-MS. As shown in Figure 2, increasing the incubation time led to a steady increase in the peak intensity at m/z 442.8 ($z = 2$) relative to that at m/z 442.3 ($z = 2$). A linear relationship between $\ln[1 + R(t) - R(0)]$ and the incubation time (t) was obtained by using the values of $R(0)$ and $R(t)$ calculated from the relevant peak intensities (Figure 3), demonstrating that the H−D exchange reaction could be monitored quantitatively by ESI-MS. The pseudo-first-order rate constant k_φ of the H−D exchange reaction corresponds to the slope of the plot of $\ln[1 + R(t) - R(0)]$ versus t as described in eq 4. Figure 4 shows the pH (pD) dependency of k_φ. From the plot, the pK_a value (the midpoint of the single sigmoidal curve) of the histidine residue in angiotensin III was determined to be 6.50 ± 0.03. This value was reasonably close to that previously determined for histidine residues in small peptides, such as 6.9 in H-Gly-His-Ala-OH (H−T exchange reaction)[8] and 6.5 in angiotensin II (H−T exchange reaction and ^{1}H NMR titration).[17] According to the k_φ^{max} value for the H−D exchange reaction of this histidine residue, the half-life was estimated to be 1.89 ± 0.04 days, which could be regarded as the standard time characteristic of a histidine residue freely accessible to the bulk water. Tandem mass spectrum of deuterated angiotensin III is provided (Figure S-1, Supporting Information) to confirm that the histidine residue in angiotensin III is the sole site of deuterium incorporation.

pK_a Values of Histidine Residues in RNase A. The present method was applied to RNase A to test the practicality of the method. We chose this enzyme because it is well characterized with respect to the ionizing behavior of all four histidine residues by other techniques, including ^{1}H NMR spectroscopy[1,2,18] and H−T exchange reaction.[9] The H−D exchange reaction of RNase A was performed essentially as reported by Ohe and co-workers,[9] but using a greatly reduced quantity of sample protein (~0.5 nmol compared with the preceding experiment using 289 nmol of protein). This reduction in sample quantity, of nearly 3 orders of magnitude, was enabled mainly by the use of nano-HPLC instead of two-dimensional paper chromatography and electrophoresis for the separation of peptides, in addition to the simultaneous detection, identification, and measurement of individual histidine-

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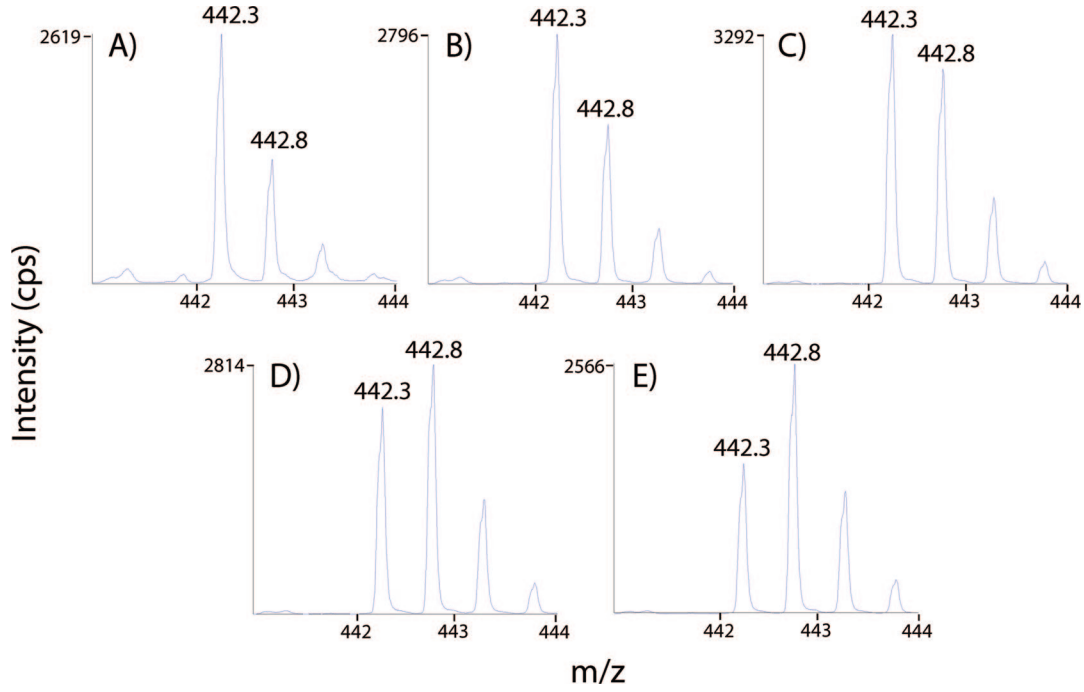

Figure 2. Mass spectra of angiotensin III incubated in D_2O at pH 7.0 for various times. Angiotensin III was incubated in D_2O buffered with MES (pH 7.0) at 37 °C for various durations, and the resulting peptides were analyzed by ESI-MS. Incubation times: (A) 0, (B) 8, (C) 24, (D) 48, and (E) 72 h.

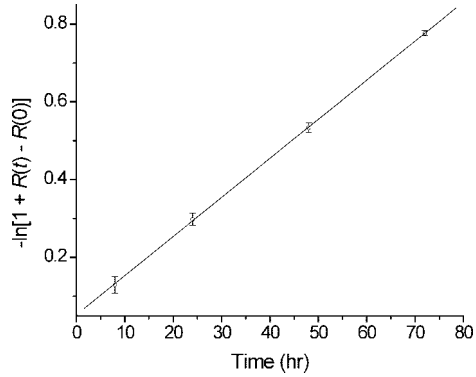

Figure 3. First-order plot of the H−D exchange data for the imidazole C_2-position of the histidine residue in angiotensin III. $R(t) = I_{M+1}(t)/I_M(t)$ and $R(0) = I_{M+1}(0)/I_M(0)$. Data are means ± standard deviation from triplicate reactions.

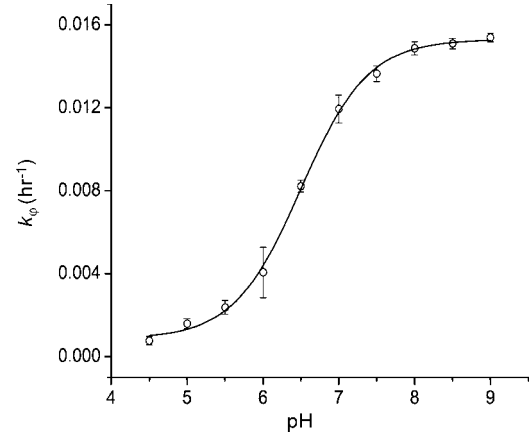

Figure 4. pH dependence of the pseudo-first-order rate constants for H−D exchange at the C_2-position of the histidine residue in angiotensin III. Angiotensin III was incubated in D_2O at different pH values (pH 4.5−9.0) at 37 °C for 20 h, and the resulting peptide was analyzed by MS. The rate constant k_φ was calculated from the obtained mass spectrum as described in the Experimental Section. Data are means ± standard deviation from triplicate reactions.

containing peptides by LC/MS. Particular care was taken not to cause back-exchange of the imidazole C_2-D with H by unnecessarily subjecting the deuterated peptides to reactions in alkaline conditions. For this purpose, we employed performic acid oxidation of disulfide bonds, since the rate of H−D exchange at the C_2-position is negligibly slow below pH 4,[3] and used immobilized trypsin and chymotrypsin in relatively high concentrations so that the time required for proteolytic digestion at pH 8 can be kept minimal.

Four histidine-containing peptides, each containing one histidine residue, were detected by the LC/MS analysis (Figure S-2, Supporting Information) and identified by matching the observed molecular mass of the peptide with the amino acid sequence of RNase A. As shown in Table 1, we referred to these peptides as His12-, His48-, His105-, and His119-peptides, indicating the position of the relevant histidine residue in the amino acid sequence. The identity of each peptide was also independently confirmed by tandem mass spectrometry (Figure S-3, Supporting Information).

The pseudo-first-order rate constants (k_φ) for the imidazole C_2-deuteration of the four histidine residues were calculated from the mass spectra of the four histidine-containing peptides and plotted as a function of pH in Figure 5. Due to the very slow H−D exchange rate, the pK_a value for His48 could not be determined. We predict that a longer H−D exchange reaction (more than 10 days) is required to obtain an interpretable sigmoidal curve for His48. The pK_a values for His12, -105, and -119 determined are shown in Table 2. These values are in good agreement with those determined by ^{1}H NMR spectroscopy and H−T exchange methods, demonstrating that the present mass spectrometric method

Table 1. Four Histidine-Containing Peptides Observed

peptide	position	amino acid sequence	molecular mass[a]	ions used for calculating the rate of H−D exchange, m/z
His12	Q11−Y25	QHJDSSTSAASSSNY[b]	1603.6	802.8 and 803.3
His48	V47−K60	VHESLADVQAVBSQK[c]	1660.8	831.4 and 831.9
His105	H105−Y115	HIIVABEGNPY[c]	1262.6	1263.6 and 1264.6
His119	V116−F120	VPVHF	597.3	598.4 and 599.4

[a] Monoisotopic masses of nondeuterated forms of the peptides. [b] J denotes methioninesulfone. [c] B denotes cysteic acid.

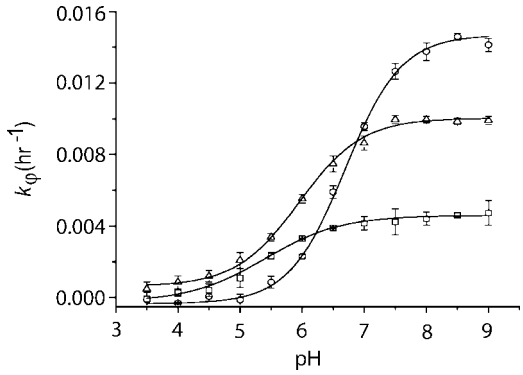

Figure 5. pH dependence of the pseudo-first-order rate constants for H−D exchange at the C_2-position of the histidine residues in His12-, His105-, and His119-peptides derived from RNase A. RNase A was incubated in D_2O with different pH values (pH 3.5−9.0) at 37 °C for 48 h. After the H−D exchange reaction, the protein was digested sequentially by trypsin and chymotrypsin, and the resulting peptides (12.5 pmol) were analyzed by LC/MS. The rate constant k_φ was calculated from the mass spectrum of His12-, His105-, and His119-peptide as described in the Experimental Section. (□) His12-peptide, (○) His105-peptide, and (Δ) His119-peptide. Data are means ± standard deviation from triplicate reactions.

Table 2. pK_a Values of Histidine Residues in RNase A[c]

position	pK_a present method	pK_a NMR spectroscopy[a]	pK_a H−T exchange method[b]
His12	5.48 ± 0.06	5.79 ± 0.07	5.8
His48	nd[c]	nd	6.0
His105	6.67 ± 0.02	6.72 ± 0.02	6.4
His119	5.95 ± 0.04	6.19 ± 0.04	5.6

[a] Taken from Table 3 of Markley.[2] [b] Taken from Table 1 of Ohe et al.[9] [c] nd, not determined.

provides unambiguous assignments of pK_a values to individual histidine residues in proteins.

The titration curve also provides the k_φ^{max} value as the maximal H−D exchange rate constant, which has been proved to be a useful index of solvent accessibilities of individual histidine residues in proteins.[9] To compare the present data with those obtained by other methods, we took the half-life $t_{1/2}$ derived from k_φ^{max} by eq 8. The order of $t_{1/2}$ for H−D exchange of the four histidine residues was His105 < His119 < His12 < His48 (Table 3), indicating that His105 ($t_{1/2}$ = 2.00 day) has solvent accessibility as high as the histidine residue in a small peptide, angiotensin III ($t_{1/2}$ = 1.89 day), while His48 ($t_{1/2}$ = 46 ± 7 day) has very little solvent accessibility. Although the order of these $t_{1/2}$ values is consistent with the general trends observed by ^{1}H NMR spectroscopy[2] and H−T exchange[9] methods, the actual values appear

Table 3. Half-Life of H−D Exchange at the C_2-Position of Histidine Residues in RNase A

position	$t_{1/2}$ (day) present method	$t_{1/2}$ (day) NMR spectroscopy[a]	$t_{1/2}$ (day) T−H exchange method[b]
His12	6.54 ± 0.80	4.55 ± 0.07	9.3
His48	46 ± 7[c]	43 ± 8	57.7
His105	2.00 ± 0.04	1.43 ± 0.03	3.0
His119	2.88 ± 0.02	2.02 ± 0.07	4.9

[a] Taken from Table 1 of Markley.[2] [b] Taken from Table 2 of Ohe et al.[9] [c] Calculated from k_φ at pH 8.0 instead of using k_φ^{max}, because k_φ^{max} could not be obtained.

to involve considerable systematic errors, which could occur due to an unexpected H−D exchange associated with the experimental procedures of sample preparation and analysis. The values of $t_{1/2}$ tend to be shorter in ^{1}H NMR analysis and longer in H−T exchange kinetics, compared with the data obtained in this study (Table 3). The deviation to lower $t_{1/2}$ values in the ^{1}H NMR method may be associated with the pre-equilibration of protein in D_2O solvent prior to the ^{1}H NMR measurement. Extensive deuteration of labile hydrogen atoms and significant replacement of H_2O molecules in the protein structure with D_2O molecules could occur during the pre-equilibration period. These preexisting deuterium atoms could turn into readily available deuterion sources for the C_2-positions of imidazole rings, therefore increasing the apparent rate of H−D exchange reaction. Indeed, such diffusion often takes a few days, according to a neutron diffraction study showing that some amide N−H groups in the interior of folded RNase A molecule undergo very slow H−D exchange.[19] A controversy exists as to whether the water reaches the exchanging group by penetrating the folded protein or through space opened by a transient unfolding.[20] Although several mass spectrometric methods have been proposed to address the problem of solvent accessibility by mapping the surface structure of proteins and analyzing protein dynamics,[21,22] the parameter $t_{1/2}$ obtained by the present experiment should provide information not only about solvent accessibility but also about protein dynamics such as how water diffuses into the interior of a protein molecule. The opposite effect of increased $t_{1/2}$ values in the H−T exchange approach could arise from the back-exchange reaction during proteolytic digestion at an alkaline pH and subsequent lengthy chromatographic operations using two-dimensional paper chromatography−electrophoresis.

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In the present method, special precautions were taken to minimize the possibility of back-exchange reaction by submitting the deuterated samples to mass analysis with the shortest delay possible. Interestingly, our data listed in Table 3 are approximately the average of those reported in preceding studies with ^{1}H NMR and H−T exchange, suggesting that our protocol has successfully suppressed undesirable H−D and D−H exchange reactions to a minimal level. Notably, the problem of back-exchange at the imidazole C_2-position is much less significant compared with back-exchange at the amide N−H groups, imposing severe limitations on mass spectrometric approaches to analyze protein structures dynamically by monitoring the exchange rate, which is excessively fast.

Advantages and Limitations of the Present Method. Generally, measurement of pK_a values of histidine residues require either a relatively large amount or a high concentration of protein. In NMR spectroscopy, ~1 mM is claimed to be the lower limit of protein concentration for both natural-abundance ^{1}H NMR and multidimensional heteronuclear NMR of isotopically labeled protein, despite many efforts to enhance the sensitivity of the method.[23] For a 100-kDa protein, the lower limit of 1 mM concentration amounts to a 10% (w/w) solution, posing a severe problem in solubilizing the protein in addition to line broadening due to rapid transverse spin relaxation. Therefore, unambiguous assignment of all the histidine residues in such a large molecule is difficult even if a large number of histidine C_2-proton signals are well resolved. Although it is possible to determine pK_a values by pH titration of biosynthetically enriched ^{13}C or ^{14}N signals of histidine residues,[24] the problem of sensitivity for large protein molecules remains unsolved. Using a radioisotope can alleviate the problem of sensitivity, as exemplified in the H−T exchange method; however, it is still difficult to comply with the requirement that every peptide containing a single tritiated histidine residue is separated and analyzed within a reasonably short running time to suppress the effect of back-exchange. In contrast, the present experiment was conducted using a 5 μM solution of RNase A (14 kDa) for the H−D exchange reaction and ~12.5 pmol (at 2.5 μM) of peptide for analysis with nano-LC/MS, improving the sensitivity by a factor of 2−3 orders of magnitude with respect to protein concentration. This suggests that the present method is applicable to proteins with molecular mass up to 1−10 MDa without extensive optimization.

After the H−D exchange reaction, a series of experiments can be completed within 1 day, performic acid oxidation and proteolytic digestion of RNase A required less than 6 h in total, and analysis with nano-HPLC linked to ESI-MS took ~1 h per sample. Because each peptide separated by HPLC could readily be identified by ESI-MS based on the known amino acid sequence of RNase A, identification and kinetic analysis were conducted simultaneously. This represents one of the most advantageous features of the present method compared with ^{1}H NMR, in which unambiguous assignment of all the histidine signals could scarcely be achieved within two weeks for large proteins above 30 kDa.

One limitation of the present method is that only one histidine residue is allowed in a peptide in order to obtain the simple pH−k_φ profile that is necessary to determine the pK_a and $t_{1/2}$ values. This limitation could possibly be overcome by applying tandem mass spectrometry to a peptide that contains more than two histidine residues, because fragment ions that contain only a single histidine residue can be produced and analyzed independently. Furthermore, using tandem mass spectrometry, we could omit some enzymatic or chemical procedures, allowing the method to be conducted even faster and more easily; this approach is being pursued in our laboratory and will be reported elsewhere. The use of a different protease, or multiple proteases in combination, could also help solve this problem.

CONCLUSIONS

We developed a novel method to determine the pK_a values of individual histidine residues in proteins using nano-HPLC/ESI-MS. The most significant feature of the present method is that it allows simple and unambiguous assignments of pK_a values to individual histidine residues in proteins based on the observed molecular masses of the histidine-containing peptides. The peptide amount analyzed by LC/MS in this study was ~12.5 pmol, which is significantly lower than the amount required by the NMR spectroscopy method. This high sensitivity is another significant advantage of the present method, which we predict will be especially useful for proteins available in very small quantities, large proteins, and membrane proteins, which are difficult to analyze by NMR spectroscopy. We have also demonstrated that the use of tandem mass spectrometry is effective to verify the assignment of each peptide containing a single histidine residue. This suggests that a peptide containing more than one histidine residue could be analyzed by a slight improvement of the method without compromising the simplicity and efficiency.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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