Quantitative Measurement of the Solvent Accessibility of Histidine Imidazole Groups in Proteins

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S *Supporting Information*

ABSTRACT: We report a method for expressing the solvent accessibility of histidine imidazole groups in proteins. The method is based on measuring the rate of the hydrogen exchange (HX) reaction of the imidazole $C^{\varepsilon 1}$-hydrogen. The rate profile of the HX reaction as a function of pH gives a sigmoidal curve, which reaches the maximal rate constant (k^{max}) on the alkaline side of the sigmoidal curve. To quantitatively describe the solvent accessibility of imidazole groups in proteins, it is necessary to compare the k^{max} of the imidazole groups with their intrinsic k^{max} ($^{i}k^{max}$), the maximal rate constants

for the given imidazole groups when they are fully exposed to the bulk solvent. However, the mechanism of the HX reaction suggests that the $^{i}k^{max}$ of an imidazole group differs depending on its pK_a, and no systematic study has been conducted to clarify how the $^{i}k^{max}$ is affected by pK_a. We therefore investigated the relationship between $^{i}k^{max}$ and pK_a using four imidazole derivatives at three different temperatures. The experimentally determined pK_a-specific $^{i}k^{max}$ values allowed us to derive a general formula to estimate the $^{i}k^{max}$ value of any given imidazole group exhibiting a specific pK_a at a specific temperature. Using the formula, the protection factors (PF), the ratio of $^{i}k^{max}$ to k^{max}, of five imidazole groups in dihydrofolate reductase were obtained and used to express the magnitude of their solvent accessibility. In this definition, the smaller the PF value, the higher the solvent accessibility, and a value of 1 indicates full exposure to the bulk solvent. The solvent accessibility expressed by the PF values agreed well with the solvent accessible surface areas obtained from the X-ray diffraction data.
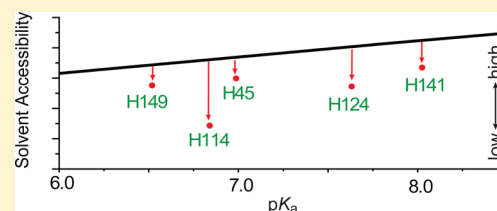
It was found more than four decades ago that the imidazole $C^{\varepsilon 1}$-proton can be exchanged with a deuteron by incubation in deuterium oxide (D_2O).[1] Scheme 1 shows the reaction mechanism of the hydrogen exchange (HX) reaction. The HX reaction has been shown to follow pseudo-first-order kinetics, in which the abstraction of the $C^{\varepsilon 1}$-proton from the cationic imidazolium by OD^- to form a ylide or a carbene intermediate is the rate-determining step.[2−4] The rate profile of the HX reaction as a function of pD yields a sigmoidal curve, which exhibits a progression from the acidic side that accelerates and approaches the plateau on the alkaline side of the sigmoidal curve (Figure 1).[2−6] The rate profile provides two useful parameters that indicate the local environment of the given imidazole group in a protein. The one is the pK_a of the imidazole NH group, which coincides with the inflection point of the sigmoidal curve, because the rate of the reaction depends on both the concentrations of the conjugate acid of imidazole and OD^- as can be seen below in the rate equation for this reaction (eq 1). The other useful parameter is the maximal pseudo-first-order rate constant, k^{max}, which corresponds to the upper plateau of the sigmoidal curve.

Because of the importance of imidazole groups in proteins involved in enzyme catalysis and pH-dependent structural changes, there has been considerable interest in HX of imidazole groups in proteins.[7] Until recently, proton nuclear magnetic resonance (^{1}H NMR) spectroscopy was the

technique of choice for monitoring HX reactions of imidazole groups in proteins.[8] However, mass spectrometry (MS) has gained in popularity in recent years, because MS provides higher sensitivity and straightforward signal assignment and is capable of analyzing membrane proteins and complex protein mixtures.[7,9−12] The MS method has been termed histidine hydrogen−deuterium exchange mass spectrometry (His-HDX-MS)[9−11] to distinguish it from amide-HDX-MS.[13]

Because the pK_a value of an imidazole group is sensitive to neighboring charged groups, experimentally obtained pK_a values are useful indicators of the electrostatic environment of imidazole groups in the proteins.[11] On the other hand, k^{max} values have been used to indicate the solvent accessibilities of imidazole groups,[7,9,11,14,15] because the HX rates at individual imidazole groups are affected significantly by the extent of accessibilities of these groups to the bulk solvent. However, the relationship between the HX rate and solvent accessibility is not straightforward. According to the kinetic studies of the HX reaction,[2−4,6] the reaction rate is expressed by eq 1:
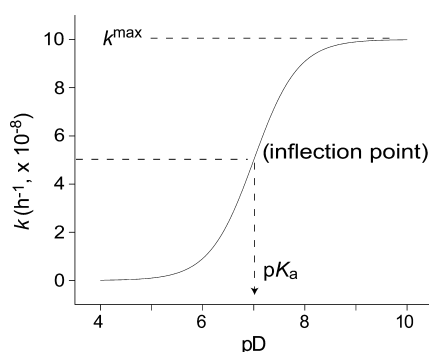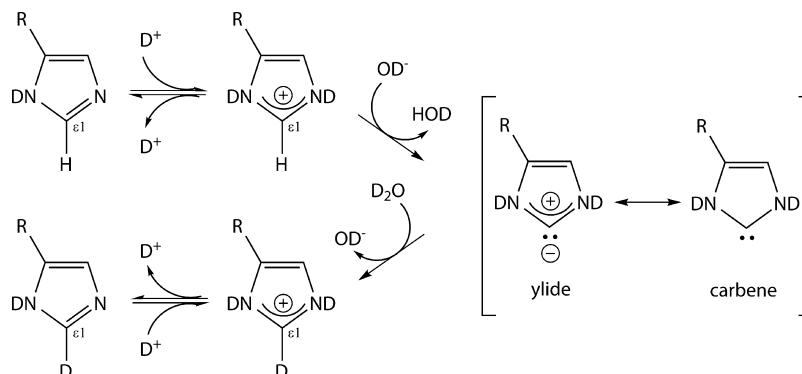
$$\text{rate} = k[\text{Im}_{\text{total}}] = k_2[\text{Im}^+][\text{OD}^-] \qquad (1)$$

Scheme 1. Mechanism of the HX Reaction at the Imidazole $C^{\varepsilon 1}$-Hydrogen of Histidine

Figure 1. Hypothetical rate profile of the HX reaction of the imidazole $C^{\varepsilon 1}$-hydrogen, plotting the pseudo-first-order rate constant (k) vs pD. The rate profile was produced by computing the k vs varying pD using the equation $k = (k_2 K_W)/(K_a + [D^+])$, where k_2 is the second-order rate constant for the rate-determining step of the HX reaction, K_W is the ion product of heavy water, and K_a is the dissociation constant of the imidazolium cation. k_2, K_W, and K_a were set to be 1 mol^{-1} h^{-1}, 10^{-14} mol^2/L, and 10^{-7} mol/L, respectively, in this simulation. The pK_a of the imidazole group can be obtained from the inflection point of the sigmoidal curve, and the maximal pseudo-first-order rate constant, k^{max}, from the upper plateau of the sigmoidal curve.

where k is the pseudo-first-order rate constant, k_2 is the second-order rate constant for the rate-determining step of the HX reaction, $[Im_{total}]$ and $[Im^+]$ represent the concentrations of total imidazole and the charged form of imidazole, respectively, and $[OD^-]$ is the concentration of deuteroxyl ions. Because

$$[Im^+] = \frac{[Im_{total}][D^+]}{K_a + [D^+]}$$

the expression of k can be simplified to eq 2.

$$k = \frac{k_2 K_W}{K_a + [D^+]} \tag{2}$$

where K_a is the dissociation constant of the imidazolium cation and K_W is the ion product of heavy water ($K_W = [D^+][OD^-] = 10^{-14.95}$ at 25 °C).[16] The equation indicates that the magnitude of the k value is influenced by the K_a of a given imidazole group and the pD at which the HX reaction takes place. At high pD values, however, $[D^+]$ is negligibly smaller than K_a; therefore, eq 2 reduces to eq 3.

$$k^{max} = \frac{k_2 K_W}{K_a} \tag{3}$$

where k^{max} is the maximal k obtained from the upper plateau of the sigmoidal curve in Figure 1. As eq 3 implies, k^{max} is independent of pD and is therefore a more straightforward indicator of solvent accessibility than k. The value of k^{max} is proportional to k_2 and inversely proportional to K_a; therefore, it increases 10-fold with a pK_a increase of 1 pH unit, if the k_2 value remains the same (which is not actually the case). This means that we cannot simply assume that a high k^{max} value is due to greater accessibility of an imidazole group in the protein, because it may be simply due to a high pK_a value. Therefore, to quantitatively express the solvent accessibility of a given imidazole group in a protein, it is necessary to know its intrinsic k^{max} ($^i k^{max}$) value, which is the k^{max} value when it is fully exposed to the bulk solvent, and the value is specific to its pK_a value. Such an $^i k^{max}$–pK_a relationship for the HX method has not been investigated in a systematic manner, although some studies of the closely related hydrogen–tritium exchange (HTX) method have been conducted.[17]

We report here the relationship between $^i k^{max}$ and pK_a obtained with four imidazole derivatives. On the basis of the study, we derived a general formula that allows us to estimate the $^i k^{max}$ value of an imidazole group in a protein exhibiting a specific pK_a. The formula was used to obtain the protection factors (PF), which is defined as the ratio of $^i k^{max}$ to k^{max} of a given imidazole group, to express the solvent accessibility of imidazole groups in a protein.

■ EXPERIMENTAL PROCEDURES

Materials. Deuterium oxide (D$_2$O, 99%) was purchased from Cambridge Isotope laboratories (Andover, MA). Histamine, N^α-acetyl-DL-histidine (Ac-His-OH), and 1H-imidazole-5-propanoic acid (IPA) were from Sigma-Aldrich (St. Louis, MO). N^α-Acetyl-L-histidine methylamide (Ac-His-NHMe) was from Bachem (Torrance, CA). All other chemicals and materials used were reagent grade or of the highest quality commercially available.

Buffer Solutions in D$_2$O. Buffers in D$_2$O contained 100 mM pyridine (pH* 4.82–6.51) and 100 mM N-ethylmorpholine (pH* 6.62–9.39). The pH* of the buffer was adjusted with diluted monodeuteroacetic acid (CH$_3$COOD, 99%) at 25 °C with a Solution Analyzer model 4603 instrument (Amber Science, Eugene, OR) equipped with a glass AgCl electrode (model 476086, Nova Analytics, Woburn, MA). The reported pH* values are direct pH meter readings of the D$_2$O buffer solutions calibrated with standard buffer solutions made with H$_2$O and are uncorrected for the isotope effect at the glass electrode. The pH* values of the D$_2$O buffers at different

temperatures were estimated as described in the Supporting Information.

Deuteration of Imidazole Derivatives. One nanomole of histamine, Ac-His-NHMe, Ac-His-OH, or IPA was incubated separately in 50 μL of buffer in D_2O with different pH* values (4.82−9.39) at 25, 31, and 37 °C for 192, 106, and 50 h, respectively (see the Supporting Information for the structures of these four model compounds). The reaction was stopped by the addition of 5 μL of formic acid, and the samples were dried in a Speed Vac. Note that the reaction is negligibly slow at the acidic pH* (see Figure 1). Prior to MS analysis, the dried samples were redissolved in 50 μL of a 0.1% formic acid/50% methanol mixture in H_2O, which promotes deuterium to proton exchange at rapidly exchangeable sites (the amino, amide NH, and carboxyl groups). The samples are then subjected to flow injection MS analysis as described below.

Mass Spectrometry. The samples prepared above were analyzed using a QStar Elite quadrupole/time-of-flight mass spectrometer equipped with a TurboIonSpray ion source (AB Sciex, Framingham, MA). A 10 μL aliquot of each sample was injected into a flowing carrier stream consisting of a 0.1% formic acid/50% methanol mixture at 40 μL/min, which was directly introduced into the ion source of the mass spectrometer. A Shimadzu (Columbia, MD) LC-20AD pump was used to deliver the carrier solvent. Mass spectra (from m/z 100 to 300) were acquired in the time-of-flight mass analyzer with an accumulation time of 1 s. Analyst QS (version 2.0, AB Sciex) was used for instrument control, data acquisition, and data processing.

Measurement of the HX Rate Constant (k) and pK_a. The pseudo-first-order rate constant (k) of the HX reaction was determined by monitoring changes in the ratios of the M + 1/M isotopic peak of a given imidazole derivative before (time zero) and after (time t) the HX reaction as described previously.[11] The pK_a values of imidazole derivatives were obtained from the sigmoid titration curve of k versus pH* using Origin Graphing (version 8.5, OriginLab, Northampton, MA) using the following equation.

$$y = A_2 + \frac{A_1 - A_2}{1 + e^{(x-x_0)/\delta_x}}$$

where A_1 is the minimal rate constant at the lowest pH*, A_2 is the maximal rate constant at the highest pH*, x_0 is the point of inflection, and δ_x is the change in x corresponding to the most significant change in y values.

■ RESULTS AND DISCUSSION

Relationship between $^ik^{max}$ and pK_a. Figure 2a shows the rate profiles of the HX reactions of histamine, Ac-His-NHMe, Ac-His-OH, and IPA at 25 °C as a function of pH*. The pK_a values of these four model compounds obtained from the inflection point of the sigmoidal curves were 5.42 for histamine, 6.35 for Ac-His-NHMe, 7.38 for Ac-His-OH, and 7.77 for IPA. This suggests that a neighboring amino group lowers the pK_a of the imidazole group as in the case of histamine, whereas a neighboring carboxyl group increases the pK_a as in the case of Ac-His-OH and IPA. This result is consistent with the well-established fact that neighboring electron-withdrawing groups (e.g., RNH_3^+, GdnH$^+$, and ImH$^+$) lower the pK_a of the imidazole group, whereas neighboring electron-donating groups (e.g., $RCOO^-$, RO^-, and RS^-) increase it.[18] The pK_a of Ac-His-NHMe is considered
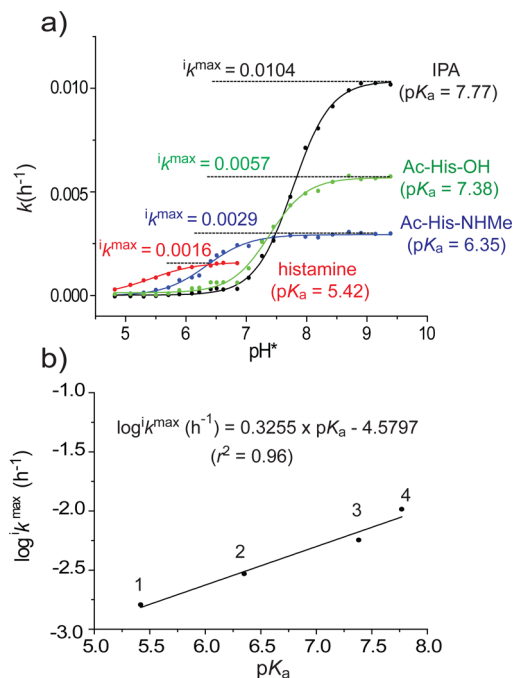
Figure 2. pH* and pK_a dependence of the HX reaction at the $C^{\varepsilon 1}$ position of imidazole groups. (a) pH* dependence of k for HX at the imidazole group in histamine (red), Ac-His-NHMe (blue), Ac-His-OH (green), and IPA (black). The experimentally obtained pK_a and the intrinsic k^{max} ($^ik^{max}$) for the four imidazole derivatives are shown. (b) Log $^ik^{max}$–pK_a plot for the HX reaction at the $C^{\varepsilon 1}$ position of imidazole at 25 °C in (1) histamine, (2) Ac-His-NHMe, (3) Ac-His-OH, and (4) IPA. The equation and the correlation coefficient (r^2) of the linear regression line are presented.

to be the intrinsic pK_a of the imidazole group in proteins that have no adjacent dissociable groups.

The rate profiles in Figure 2a also provide the maximal k values (k^{max}) for the imidazole derivatives. The obtained values were 0.0016, 0.0029, 0.0057, and 0.0104 h^{-1} for histamine, Ac-His-NHMe, Ac-His-OH, and IPA, respectively. As expected from the reaction mechanism,[2−4,6] k^{max} increased with an increasing pK_a in these four model compounds. Because the imidazole groups of these compounds are fully exposed to the bulk solvent, these k^{max} values can be considered to be their intrinsic k^{max} ($^ik^{max}$) values. In the base-catalyzed reaction, the plot of the common logarithm of the reaction rate constant, log k, versus the pK_a, gives a straight line with slope β and intercept log C, which is known as the Brønsted relation. A plot of the log $^ik^{max}$ values of these four model compounds against their pK_a values at 25 °C yielded a straight line with a slope of 0.3255 and an intercept of −4.5797, as shown in Figure 2b. It is, therefore, confirmed that the HX reaction of imidazole $C^{\varepsilon 1}$-hydrogen fulfills the Brønsted catalysis equation.

General Formula for Calculating the pK_a-Specific $^ik^{max}$ Values of Imidazole Groups at a Specific Temperature. Although the equation obtained above can be used to find the pK_a-specific log $^ik^{max}$ values of imidazole groups at 25 °C, the HX reaction may be conducted at a different temperature. To obtain a general formula for calculating the pK_a-specific log $^ik^{max}$ values of imidazole groups at any specific temperature, we determined the $^ik^{max}$ values for the four model compounds at 31 and 37 °C, as well (Table 1). The $^ik^{max}$ values of the model compounds at each temperature were converted to log $^ik^{max}$ and plotted against their pK_a values (Figure 3a). The magnitude

Table 1. $^{i}k^{max}$ Values of Imidazole Derivatives at Different Temperatures

	$pK_a{}^a$	$^{i}k^{max}$ (h^{-1})		
		25 °C	31 °C	37 °C
histamine	5.42 ± 0.11	0.0016 ± 0.0001	0.0036 ± 0.0002	0.0082 ± 0.0009
Ac-His-NHMe	6.35 ± 0.03	0.0029 ± 0.0001	0.0065 ± 0.0002	0.0153 ± 0.0003
Ac-His-OH	7.38 ± 0.02	0.0057 ± 0.0001	0.0124 ± 0.0003	0.0278 ± 0.0005
IPA	7.77 ± 0.01	0.0104 ± 0.0001	0.0208 ± 0.0003	0.0483 ± 0.0011

^{a}The pK_a values were obtained at 25 °C from the pH* dependence of the measured exchange rate.
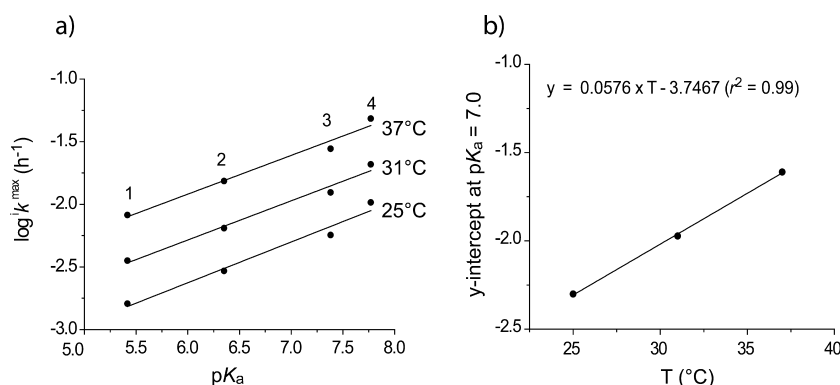
Figure 3. Log $^{i}k^{max}$–pK_a plots at different temperatures for the HX reaction at the $C^{\varepsilon 1}$ position of imidazole. (a) (1) Histamine, (2) Ac-His-NHMe, (3) Ac-His-OH, and (4) IPA. The linear regression analyses for 25, 31, and 37 °C plots yielded slopes of 0.3255, 0.3129, and 0.3094 and y-intercepts (pK_a = 7.0) of −2.3001, −1.9723, and −1.6097, respectively. (b) Relationship between the y-intercept and temperature. The y-intercepts at a pK_a of 7.0 were obtained from Figure 3a. The equation and the correlation coefficient (r^2) of the linear regression line are presented.

of the log $^{i}k^{max}$ values increased as the temperature increased; however, as can be seen, the slopes of the three linear regression lines remained unchanged. We therefore averaged the three slope values, yielding a value of 0.3159, and used this in the general formula given below. In addition to the common slope value, the general formula requires the temperature-specific y-intercept values. The relationship between the y-intercept and temperature was obtained by plotting the y-intercept values at a pK_a of 7.0 from the three linear regression lines against the temperature, as shown in Figure 3b. Note that the y-intercept values at a pK_a of 7.0 were used, because they are much closer to the pK_a values of typical imidazole groups in proteins than those at a pK_a of 0 and are thus expected to provide more accurate log $^{i}k^{max}$ values around typical pK_a values of imidazole groups. From the plot, the relationship between the y-intercept at a pK_a of 7.0 and temperature (T) was found to be

$$y = 0.0576T - 3.7467$$

where y is the y-intercept value at a pK_a of 7.0 and T is the temperature in degrees Celsius. Thus, the general formula for calculating the pK_a-specific log $^{i}k^{max}$ value of an imidazole group at a specific temperature was derived to be

$$\log {}^{i}k^{max} = 0.3159(pK_a - 7) + (0.0576T - 3.7467)$$

and inverting the logarithm function gives us the $^{i}k^{max}$ value.

Quantitative Analysis of Solvent Accessibilities of Five Histidine Imidazole Groups in DHFR. We recently reported the pK_a and k^{max} of five histidine imidazole groups in dihydrofolate reductase (DHFR).[11] The data were obtained at 37 °C. The experimentally determined pK_a and k^{max} values of the five imidazole groups in apo-DHFR and their $^{i}k^{max}$ values at 37 °C calculated using the general formula are listed in Table 2. On the basis of solely the k^{max} values, the most reactive residue

Table 2. Solvent Accessibilities of Five Imidazole Groups in Apo-DHFR and the DHFR−Folate−NADP$^+$ Complex

	pK_a	k^{max} (h^{-1})^a	$^{i}k^{max}$ (h^{-1})^b	PF^c	ASA^d
		Apo-DHFR			
His45	6.99	0.0131	0.0241	1.8	46.0
His114	6.84	0.0017	0.0216	12.4	16.4
His124	7.60	0.0083	0.0375	4.5	25.1
His141	8.04	0.0178	0.0516	2.9	38.7
His149	6.51	0.0087	0.0170	1.9	16.1
		DHFR−Folate−NADP$^+$ Complex			
His45	7.30	0.0265	0.0301	1.1	51.5
His114	7.17	0.0009	0.0274	31.5	17.0
His124	7.61	0.0047	0.0378	8.1	26.2
His141	8.14	0.0203	0.0555	2.7	35.7
His149	6.84	0.0111	0.0216	1.9	35.1

^{a}Calculated from the reported $t_{1/2}$ (days) values using the equation $k^{max} = \ln 2/t_{1/2}/24$. ^{b}Calculated using the general formula given in the text. ^{c}Calculated using the formula PF = $^{i}k^{max}/k^{max}$. ^{d}Solvent accessible surface area. The structural data of apo-DHFR (Protein Data Bank entry 5DFR) and the DHFR−folate−NADP$^+$ complex (Protein Data Bank entry 1RX2) were used to obtain the ASA for the $C^{\varepsilon 1}$ atoms of the five histidine residues.

is His141 and the reactivity decreases in the following order: His141 > His45 > His149 > His124 > His114. However, we cannot simply assume that the decreasing order of reactivity is same as the decreasing order of solvent accessibility, because the magnitudes of their intrinsic reactivities (rate constants), $^{i}k^{max}$, are influenced significantly by their pK_a values. Therefore, to accurately describe the solvent accessibilities of the histidine imidazole groups, we propose to use the protection factors (PF) defined as the ratio of the HX rate of a given histidine imidazole group when it is in a fully solvent-exposed state ($^{i}k^{max}$) to the HX rate in the protein (k^{max}) as shown below.

$$PF = \frac{i^{max}}{k^{max}}$$

In this definition, the smaller the value, the higher the solvent accessibility, and a value of 1 indicates full exposure to the bulk solvent. Now it is evident in Table 2 that the true solvent accessibility decreases in the following order: His45 ≈ His149 > His141 > His124 > His114 [which is different from the order obtained solely on the basis of the reactivity of the residues (log k^{max} values) described above]. Analogous PF concepts are used in amide HX, especially in NMR studies.[19]

The straight line in Figure 4 shows the relationship between log $^i k^{max}$ and pK_a (log $^i k^{max}$–pK_a plot) at 37 °C produced using
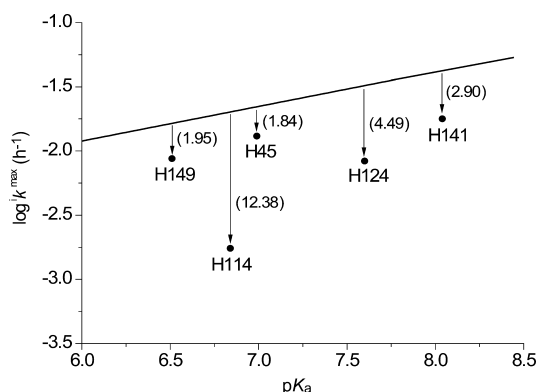
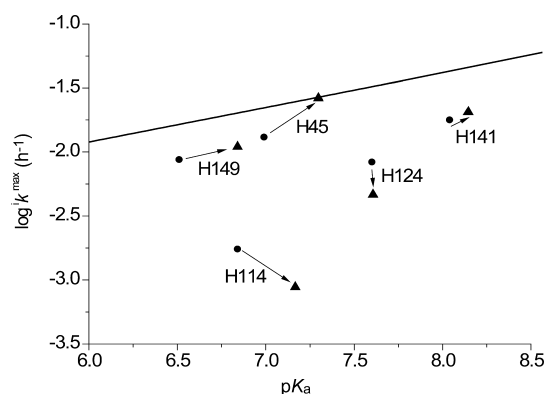**Figure 4.** Log k^{max} values of five histidine imidazole groups in apo-DHFR, on the log $^i k^{max}$–pK_a plot at 37 °C. The straight line in the figure was produced using the formula log $^i k^{max}$ = 0.3159(pK_a − 7) + (0.0576 × 37 − 3.7467). The black circles are log k^{max} values of five histidine imidazole groups in apo-DHFR. The numbers in parentheses are PF values for the imidazole groups.

the general formula. In the figure, the log k^{max} values of the five imidazole groups in DHFR are plotted. The solvent accessibilities of the five imidazole groups are indicated by the distance from the straight line of the plot to the plotted individual k^{max} values. On the plot, the shorter the distance, the higher the solvent accessibility, and the presence of any plotted point on the straight line indicates that the corresponding imidazole group is fully exposed to the bulk solvent. For the sake of easy understanding, the PF values for these histidine residues from Table 2 are also shown. We found that this type of plot with the PF values is convenient for visually indicating the solvent accessibility of imidazole groups in proteins.

Figure 5 shows how the solvent accessibilities of five imidazole groups in apo-DHFR (●) change upon binding of folate and NADP⁺. The experimentally determined pK_a and k^{max} values of the five imidazole groups in the DHFR–folate–NADP⁺ complex are listed in Table 2. It is clearly seen in the plot that the solvent accessibilities of His114 and His124 decrease upon ligand binding, whereas the solvent accessibility of His45 increases, accompanied by an increased pK_a. Thus, the log $^i k^{max}$–pK_a plot is useful for visualizing the changes in solvent accessibility and pK_a of imidazole groups in proteins.

It is worth mentioning that one can conduct an experiment measuring the $^i k^{max}$ values of imidazole groups in a completely unfolded protein and compare the values with the k^{max} values in the folded protein to obtain the PF values. Such approaches are effective in estimating the intrinsic HX rates for backbone amide NH groups,[20] which are significantly influenced by the two adjacent side chains even in the absence of folded

Figure 5. Changes in solvent accessibilities of five histidine imidazole groups in apo-DHFR upon binding of folate and NADP⁺. The log k^{max} values of five histidine imidazole groups in apo-DHFR (●) and the DHFR–folate–NADP⁺ complex (▲) are shown on the log $^i k^{max}$–pK_a plot at 37 °C. The straight line in the figure was produced using the formula log $^i k^{max}$ = 0.3159(pK_a − 7) + (0.0576 × 37 − 3.7467).

structure.[21] This is because the two adjacent side chains have different degrees of steric hindrance and inductive effects with respect to the amide NH group between them. However, in the case of His-HX, the values obtained by such experiments do not accurately reflect the $^i k^{max}$ values of these imidazole groups for the following two reasons. First, the pK_a values of imidazole groups in the unfolded protein are different from those in the folded protein because of the loss of the electrostatic effect by the neighboring side chains and other structural elements in the three-dimensional geometry, which exists only in the folded state. Second, in contrast to amide HX, no steric hindrance by the adjacent side chains with respect to the imidazole groups in unfolded structure is expected, though they may elicit an inductive effect. Such an inductive effect, however, should alter the pK_a of the imidazole ring and therefore is taken into account in the pK_a-specific $^i k^{max}$ values. Thus, the $^i k^{max}$ values of imidazole groups need to be determined by the model compounds with different pK_a values whose imidazole groups are fully solvent exposed.

Correlation of the PF Values with Solvent Accessible Surface Areas Obtained from X-ray Diffraction Data. The solvent accessible surface areas of the $C^{\varepsilon 1}$ atoms of five histidine imidazole groups in apo-DHFR and the DHFR–folate–NADP⁺ complex are listed in Table 2. Note that the smaller the PF values, the higher the solvent accessibility; while the larger the ASA values, the higher the solvent accessibility. On the basis of the PF values for the histidine residues in apo-DHFR, the solvent accessibility decreases in the following order: His45 ≈ His149 > His141 > His124 > His114. ASA data suggest the following order: His45 > His141 > His124 > His114 ≈ His149. The only difference is His149, for which the PF value indicates that the residue is quite accessible to the bulk solvent, yet the ASA data indicate that His149 is the least solvent accessible residue. This discrepancy may be reflecting the difference in protein structures in the solution and crystal. We performed a two-sample Wilcoxon rank-sum test[22] to assess whether the solvent accessibility ranked by the PF and ASA are statistically different. The analysis for the two rankings gave a Pearson's correlation coefficient of 0.53 with a p value for the Wilcoxon rank-sum test of 0.84 (the null hypothesis was that the distributions of PF and ASA are the same), indicating that the difference between the two rankings is not significantly

different. Likewise, the solvent accessibility ranking of these histidine residues by PF and ASA in the DHFR–folate–$NADP^+$ complex appeared to be quite similar to each other as can be seen in Table 2 in the following order: His45 > His141 ≈ His149 > His124 > His114. The statistical analysis for the two rankings gave a Pearson's correlation coefficient of 0.93 with a p value for the Wilcoxon rank-sum test of 0.15. Thus, the solvent accessibilities indicated by the PF values appear to agree well with ASA data. It should be noted, however, that the ASA values we used were for only the $C^{\varepsilon 1}$ atom of the imidazole group. When we used the ASA values for the entire imidazole ring, we did not find good correlation with the FA values. The finding suggests that it is not necessary for an imidazole group to be entirely exposed to the bulk water for HX to occur. Further accumulation of His-HX data will clarify this point.

Even though the size of the pool of data is small, the decent correlation between the PF and SAS value suggests that the PF value reflects the solvent accessibility of imidazole groups in proteins. This is contrary to the findings for amide HX, in which the critical event that allows exchange is hydrogen bond separation and exposure, not solvent access to the amide NH group.[23] More data on PF will be needed to confirm the positive correlation between PF and SAS.

Temperature Dependence of $^ik^{max}$. The $^ik^{max}$ values for the four model compounds at different temperatures listed in Table 1 were used to generate Arrhenius plots (Figure 6). From
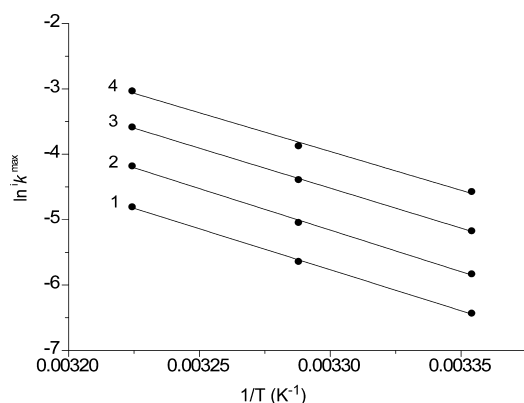
Figure 6. Arrhenius plots of the HX reaction at the $C^{\varepsilon 1}$ position of the imidazole groups of (1) histamine, (2) Ac-His-NHMe, (3) Ac-His-OH, and (4) IPA.

the plots, the activation energies of the HX reaction for histamine, Ac-His-NHMe, Ac-His-OH, and IPA were estimated to be 104, 106, 102, and 99 kJ/mol, respectively. These values agree well with the value of 104 kJ/mol obtained with L-histidine at pH* 9,[6] at which the obtained rate constant can be considered to be $^ik^{max}$.

In summary, we have described a method for measuring the solvent accessibility of histidine imidazole groups in proteins. The key assumption underlying the analysis is that the structure and solvent accessibility do not change over the pH range investigated. It can easily be tested whether the assumption holds by comparing the experimentally obtained titration curves with the theoretical curves. For example, the titration curves of the four model compounds can be superimposed perfectly on their theoretical curves, suggesting no cooperative conformational rearrangement occurred for these compounds over the pH range investigated. However, it may not be unusual to find imidazole groups in proteins whose titration curves are

not superimposed on the theoretical ones. Such nonsuperimposable titration curves are indicative of the pH-dependent changes in the local electrostatic or hydrophobic environment, or both, of the given imidazole group. Apparently, the PF values cannot be given to the buried nontitratable imidazole groups. However, on the basis of the analytical limit of the measurement, the data can provide information about "how slow the HX rates are" by providing the half-lives ($t_{1/2}$) of their HX reactions.[9,11] The measure of solvent accessibility expressed as the PF value is an independent value and can be compared with the values of other imidazole groups in the same protein or in other proteins. This method is intended to be used for the data obtained by mass spectrometry; however, it could also be used for the HX data obtained by ^{1}H NMR spectroscopy. The log $^ik^{max}$–pK_a plot is useful for showing visually the solvent accessibilities of imidazole groups in proteins.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Effect of temperature on the pH* of D_2O buffers and structures of histamine, Ac-His-NHMe, Ac-His-OH, and IPA. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

Ac-His-OH, N^α-acetyl-DL-histidine; Ac-His-NHMe, N^α-acetyl-L-histidine methylamide; ASA, solvent accessible surface area; DHFR, dihydrofolate reductase; HX, hydrogen exchange; IPA, 1H-imidazole-5-propanoic acid; MS, mass spectrometry; $NADP^+$, nicotinamide adenine dinucleotide phosphate; NMR, nuclear magnetic resonance; PF, protection factor.

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