Determination of Protonation and Deprotonation Forms and Tautomeric States of Histidine Residues in Large Proteins Using Nitrogen−Carbon *J* Couplings in Imidazole Ring

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It is well-known that histidine residues are frequently responsible for the functions of proteins, mainly due to the chemical versatility of its imidazole ring which includes the protonation and deprotonation forms and the tautomeric states.[1−3] To determine the protonation and deprotonation states and the tautomeric structures of the histidine residues in solution, NMR spectroscopy is extensively used and many successful attempts were made. Along with the recent progress in stable-isotope labeling techniques for proteins, several pulse techniques, based on HMQC,[4−6] HMBC,[7,8] and HSMQC[9,10] experiments, for the investigation of the chemistry of histidine residues have been specially developed. These measurements heavily depend on two- and three-bond remote couplings, $^2J_{N3-H4}$ and $^3J_{N1-H4}$ in the imidazole ring; therefore, a long duration time over 50−100 ms for coherence transfers, which results from small values of the remote couplings, is required. Consequently, it is difficult to apply the strategy to a system of proteins with a large molecular weight (>MW = 30−40 K).

In this paper, we report an experiment for determining the protonation and deprotonation forms and the tautomeric states of histidine residues even in large molecular weight proteins, using relatively large coupling constants between ^{15}N and ^{13}C in the imidazole ring.

One-, two-, and three-bond coupling constants in the imidazole ring for the various chemical states have been extensively studied using model compounds of monomeric histidine[11] and *N*-methylimidazole.[12] As shown in Figure 1, it is indicated that the values of the C−N coupling constants centered at the C2 and C4 carbons depend on the protonation and deprotonation forms and the tautomeric states of the imidazole ring.

Figure 2 depicts the pulse sequences used in the present study for the identification of the chemical states of the imidazole rings, which are closely related to the CT-HSQC type measurements established by Bax et al.[13] In the pulse sequence, adjusting the constant-time 2T, to a multiple of $1/^1J_{C4-C5}$, renders the ^{13}C (C4) magnetization in phase with respect to its directly coupled ^{13}C (C5) nuclei at the end of the period 2T. In the constant period of the pulse sequences, the ^{15}N 180° pulse is inserted at an appropriate position so as to generate or not generate the amplitude modulation by C−N spin coupling.

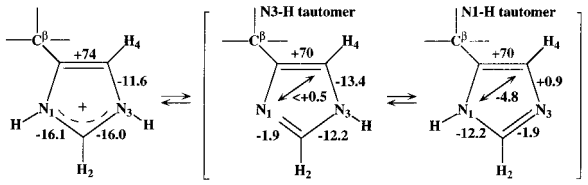

Figure 1. *J* coupling constants in imidazole ring of histidine residue.[11,12,14]

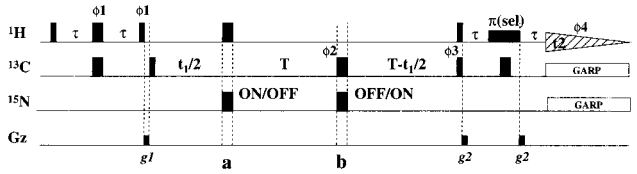

Figure 2. Pulse sequence for ^{1}H−^{13}C CT-HSQC experiments with and without J_{C-N} amplitude modulation. For measuring a spectrum without the modulation, the ^{15}N 180° pulse is applied at position a, whereas for measuring the spectrum with the modulation, the ^{15}N 180° pulse is instead applied at position b. Narrow and wide bars indicate 90° and 180° pulses, respectively, Phase cycling: $\phi_1 = y, -y$; $\phi_2 = 2(y), 2(-y), 2(-x), 2(x)$; $\phi_3 = 8(x), 8(-x)$; $\phi_4 = 2(x, -x), 4(-x, x), 2(x, -x)$. The phases for other pulses are fixed at *x*. For quadrature detection, the t_1 dimension is obtained in the States-TPPI manner. Delay duration: $\tau = 1.2$ ms; gradients (sine bell shaped): $g1 = (600\ \mu s, 20$ G/cm) and $g2 = (600\ \mu s, 15$ G/cm). The WATERGATE water suppression scheme with 3-9-19 refocusing pulse is incorporated into the reverse INEPT step;[26] 384 scans per t_1 (90 complex points, spectral width 4500 Hz) were recorded with 1024 complex points in t_2 (spectral width 9000 Hz).

The intensity ratios of the HSQC cross-peaks with and without the modulation are indicated as a function of J_{C-N}, *T*: I/I_0-(C2−H) = $\cos(2\pi\ ^1J_{C2-N1}T)\cos(2\pi^1J_{C2-N3}T)$, and I/I_0 (C4−H) = $\cos(2\pi\ ^1J_{C4-N3}T)\cos(2\pi\ ^2J_{C4-N1}T)$, where *I* and I_0 are the cross-peak intensities in the ^{1}H−^{13}C CT-HSQC spectra with and without the J_{C-N} amplitude modulation, respectively. In the experiment of the ^{1}H−^{13}C CT-HSQC spectra with modulation, the ^{13}C magnetization at the C2 position for the deprotonation form is mainly modulated by $^1J_{C2-N3}$ and $^1J_{C2-N1}$ which have the similar coupling constant of −12.2 Hz. In contrast, the modulation for the protonation state is generated by the relatively large coupling constants of $^1J_{C2-N1}$ (∼−16.1 Hz) and $^1J_{C2-N3}$ (∼−16.0 Hz). It was also demonstrated that the modulation of the ^{13}C magnetization at the C4 position is effectively dominated by $^1J_{C4-N3}$ (∼−13.4 Hz) for the N3−H tautomer and by $^2J_{C4-N1}$ (∼−4.8 Hz) for the N1−H tautomer. Therefore, the identification of the protonation and deprotonation forms and the tautomeric states of the histidine residues is able to be achieved by using the characteristic coupling constants for each of the chemical states.

The increase in the constant time of 2*T* might be a possible way to effortlessly discriminate between the chemical states of the histidine residues. However, for proteins with a large molecular weight, the long duration period is not necessarily appropriate since ^{13}C T_2 relaxation during the time of 2*T* attenuates the magnetization by exp $(-2T/T_2)$. Therefore, to avoid the loss of the signal intensities due to the T_2 relaxation process and to ensure

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Table 1. Values of I/I_0 for the Histidine Residues in the Fab Fragment

residue	I/I_0 (C2−H)	protonation or deprotonation state	I/I_0 (C4−H)	major tautomeric state
H27d(L)	0.68 ± 0.19	deprotonation state	0.27 ± 0.13	N3−H tautomer
H93(L)	0.46 ± 0.01	deprotonation state	0.52 ± 0.02	N3−H tautomer
H189(L)	0.13 ± 0.03	protonation state		
H198(L)	0.49 ± 0.06	deprotonation state	0.57 ± 0.05	N3−H tautomer
H97(H)	0.23 ± 0.05	equilibrium[a]		
H164(H)	0.008 ± 0.02	protonation state		
H199(H)	0.49 ± 0.01	deprotonation state	0.44 ± 0.03	N3−H tautomer

[a] Equilibrium between protonation and deprotonation states.

the discrimination of the states of the imidazole ring being facile, it is required, in practice, for the value of $2T$ to be set to 28.6 ms ($= 2/{}^1J_{C4-C5}$). Under the conditions related to the constant time described above, the values of the intensity ratios, I/I_0, which are calculated from the C2−H and C4−H resonances, are expected to be I/I_0 (C2−H) = 0.45 ∼ 0.65 and 0.016 for the deprotonation and protonation state, respectively, and I/I_0 (C4−H) = 0.36 and 0.91 for the N3−H and N1−H tautomers, respectively. Therefore, the identification of the protonation form and the tautomeric states can be achieved by using the cross-peaks from C2−H and from C4−H, the assignments of which are possibly established even in the large molecular weight proteins.[14−16]

These methods were applied to an antigen-binding fragment (Fab) of a catalytic antibody, 6D9,[17−19] in the presence of the transition state analogue, chloramphenicol phosphonate. The molecular weight of the complex is 50 K. The mouse hybridoma cell line 6D9 was adapted to a serum-free medium (Nissui NYSF 404) and then grown in a medium containing histidine uniformly labeled with ^{13}C and ^{15}N. The labeled Fab fragment was obtained by using the method which was previously reported.[20] Spectral assignments of C2−H and C4−H protons of histidine residues are established by the recombination experiments for the heavy and light chains[20,21] along with the strategy which was previously reported.[22] Details of the assignments will be published elsewhere.

Figure 3 shows the C2−H regions of the $^1H-^{13}C$ CT-HSQC spectra with and without J amplitude modulation, respectively. By comparing both spectra, it is indicated that significant differences in intensities of the cross-peaks are observed, which are created by the modulation. The intensity ratios, I/I_0, calculated from the spectra, are summarized in Table 1.

On the basis of the obtained values of I/I_0, it is concluded that the chemical forms of His164(H) and His189(L) correspond to the protonation forms, and His27d(L), His93(L), His198(L), and His199(H) to the deprotonation forms. The intermediate value of I/I_0 obtained for His97(H) indicates that His97(H) is in equilibrium between both forms at pH 6.0.

From the results of the pH dependence of the chemical shifts for the histidine residues, it is indicated that the imidazole rings

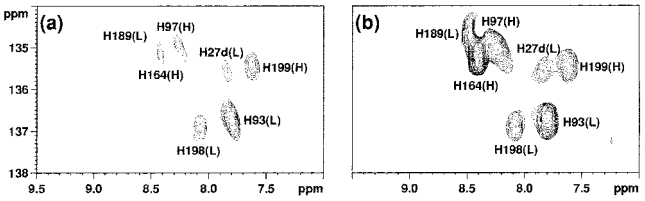

Figure 3. $^1H-^{13}C$ CT-HSQC spectra of a Fab fragment of catalytic antibody 6D9, of which histidine residues were uniformly labeled with ^{13}C and ^{15}N, in the presence of chloramphenicol phosphonate. C2−H regions measured with (a) and without (b) J_{C-N} amplitude modulation. The sample solution was prepared in 90% H_2O/D_2O, at pH 6.0, to avoid the loss of the intensities of C2-proton caused by H−D exchange. The sample concentration was adjusted to 1 mM. The experiments were performed on a Bruker DRX-600 spectrometer at 45 °C. It has been confirmed that the binding activity of the Fab fragment is retained under the condition.

of His164(H) and His189(L) are fully protonated and that of His97(H) is partially protonated at pH 6.0 (manuscript in preparation). These observations are very consistent with the data obtained in the present study. It should be noted that, compared with the traditional pH titration study, the present experiment is useful for the determination of the protonation state of histidine residues for proteins which are not stable over a wide range of pH values.

All values of I/I_0 for the C4−H peaks are also shown in Table 1, except for those of His97(H), His164(H), and His189(L), of which imidazole rings are fully or partially protonated. The intensity ratios for His27d(L), His93(L), His198(L), and His199-(H) indicate that these histidine residues primarily take the N3−H tautomer. It would also be possible to estimate the fraction of each tautomeric state by using the values of the intensity ratio. The intensity ratios for His93(L) and His198(L) indicate that about 80% of these residues exist as the N3−H tautomer. The percentage of the N3−H tautomer obtained here is actually similar to that of the deprotonation form of the imidazole ring of histidine in the free state.[12,23,24] Interestingly, the equilibrium between the tautomers for His27d(L) lies toward the N3−H tautomer significantly. It is suggested that His27d(L), which is largely responsible for the catalytic activity of 6D9, stabilizes the transition state analogue in the complex through a hydrogen bond between the imidazole ring and the transition state analogue.[19,25] The hydrogen bond might also stabilize the N3−H tautomer of His27d(L).

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