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Differentiation of Histidine Tautomeric States using ^{15}N Selectively Filtered ^{13}C Solid-State NMR Spectroscopy

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Abstract

The histidine imidazole ring in proteins usually contains a mixture of three possible tautomeric states (two neutral - τ and π states and a charged state) at physiological pHs. Differentiating the tautomeric states is critical for understanding how the histidine residue participates in many structurally and functionally important proteins. In this work, one dimensional ^{15}N selectively filtered ^{13}C solid-state NMR spectroscopy is proposed to differentiate histidine tautomeric states and to identify all ^{13}C resonances of the individual imidazole rings in a mixture of tautomeric states. When ^{15}N selective 180° pulses are applied to the protonated or non-protonated nitrogen region, the ^{13}C sites that are bonded to the non-protonated or protonated nitrogen sites can be identified, respectively. A sample of ^{13}C , ^{15}N labeled histidine powder lyophilized from a solution at pH 6.3 has been used to illustrate the usefulness of this scheme by uniquely assigning resonances of the neutral τ and charged states from the mixture.

Keywords

Histidine tautomeric states; solid-state MAS NMR; REDOR/TEDOR; selective polarization

1. Introduction

Histidine is an essential residue in many proteins [1, 2]. With its imidazole ring serving as a general base when it is neutral and serving as a general acid when in its charged form, it participates in enzyme catalyzed reactions, assists in stabilizing protein structures, mediates proton transfer and so on. The imidazole side chain has a pKa of approximately 6, which means it plays active roles at physiologically relevant pHs, where a small shift in pH will affect protonation of the imidazole ring. When it acts as a proton shuttle, the basic nitrogen on its imidazole ring can take up a proton to form a charged intermediate, then give up a

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proton to a solvent molecule before returning to the uncharged state, potentially shuttling a proton from one side of a membrane to the other. For instance, the set of four histidine side chains in the pore of the tetrameric M2 protein from *Influenza A* virus is the heart of H^+ conductance responsible for H^+ selectivity, pH activation and interactions with the W41 gate through the protonation and deprotonation of the H37 imidazole rings [3, 4]. Therefore, differentiating the tautomeric states is critical for understanding how the histidine residue plays its structurally and functionally important roles in proteins.

There are three possible tautomeric states for histidine (Scheme 1). At higher pH values, two neutral tautomers can exist: the τ tautomer with non-protonated $N_{\delta 1}$ and protonated $N_{\epsilon 2}$, and the π tautomer with protonated $N_{\delta 1}$ and non-protonated $N_{\epsilon 2}$. While at lower pH values, the imidazole ring becomes charged with both $N_{\delta 1}$ and $N_{\epsilon 2}$ protonated. At physiological pH values different tautomeric states often coexist providing an important indication of the biological processes that are taking place in a system. NMR spectroscopy is an effective approach for detecting different tautomeric states of histidine. However, in biological systems, the situation is complex -both ^{13}C and ^{15}N chemical shifts of the histidine residue are very sensitive to its tautomeric states, hydrogen bond lengths, the chemical environment of the imidazole rings and the backbone structure of the protein. Indeed, the ^{13}C chemical shift range tabulated in the BioMagRes Databank has a range of 17 ppm for the C_{γ} site, 48 ppm for the $C_{\delta 2}$ site and 40 ppm for the $C_{\epsilon 1}$ site. Moreover, the resonance linewidths are highly dependent on the dynamic and proton exchange properties of the imidazole rings and consequently signals from different tautomers can be severely overlapped making it almost impossible to identify different tautomeric states based on the known benchmarks from the histidine amino acid [5-7]. Here, we propose ^{15}N selectively filtered ^{13}C NMR spectroscopy to uniquely differentiate histidine tautomeric states in a uniformly ^{13}C and ^{15}N labeled histidine sample at pH 6.3.

Since the neutral states have their unique non-protonated nitrogen sites ($N_{\delta 1}$ for τ tautomer and $N_{\epsilon 2}$ for π tautomer), whose chemical shifts are different from that of the protonated nitrogen, ^{15}N NMR is a common approach for calculating the relative amount of neutral and charged histidines. However, there are two intrinsic shortcomings associated with the ^{15}N NMR analyses. First, cross polarization (CP) efficiency. It is known that CP [8, 9] is an essential NMR technique in solid state NMR used to enhance the sensitivity of dilute spins like ^{15}N . However, the dipolar coupling between the protonated nitrogen and its bonded proton is much stronger than that between the non-protonated nitrogen and its closest proton, so that the CP dynamics for the protonated and non-protonated nitrogen are rather different. As a result, the magnetization for the protonated nitrogen can be built up very quickly, while the signals for the non-protonated nitrogen require a much longer CP contact time to buildup. In light of short proton spin relaxation time in the rotating frame (i.e. $T_{1\rho}$), which is commonly observed in many proteins, it is rather inefficient to polarize the non-protonated nitrogen signals. Although spin-locking the proton magnetization along the magic angle can lengthen the $T_{1\rho}$ value by a factor of two or three to improve the CP efficiency [10], it is necessary to perform CP variable contact time experiments to analyze the CP dynamics in order to accurately quantitate the protonated and non-protonated nitrogen signals [10]. However, when the population of the neutral tautomeric states is low,

it is difficult to observe the non-protonated nitrogen signals [3] even if the CP contact time is long. Secondly, the resonances may overlap. For the neutral tautomeric states, the protonated nitrogen also exist ($N_{\epsilon 2}$ for τ tautomer and $N_{\delta 1}$ for π tautomer). These protonated nitrogen signals may overlap with the nitrogen signals from the charged tautomer. Therefore, it is necessary to subtract the protonated nitrogen signals contributed from neutral tautomers in order to accurately calculate the total signals from the charged tautomer, which is an impossible task with current one dimensional (1D) spectra. Two-dimensional (2D) heteronuclear ^{15}N - ^{13}C correlation spectra are required, often combined with other correlation spectra such as ^{13}C - ^{13}C correlations, to make resonance assignments. Such experiments are time-consuming. Site specific labeled histidine has to be used to differentiate signals from $N_{\epsilon 2}$ and $N_{\delta 1}$, which is costly.

In this paper, we propose nitrogen selectively filtered ^{13}C NMR spectroscopy to differentiate tautomeric states of histidine. In NMR, ^{13}C is known to be more sensitive than ^{15}N . Therefore, observing ^{13}C instead of ^{15}N provides much needed sensitivity for detecting different tautomers, while protonated or non-protonated nitrogens are used to select different ^{13}C signals. More importantly, the CP dynamics for each carbon site in the histidine tautomers is similar, so that there is no concern about CP efficiency for seeing different tautomeric states. The advantages of this method will be demonstrated by using a histidine powder sample lyophilized from a solution at pH 6.3.

2. Materials and methods

2.1. Preparation of histidine powder sample

Uniformly ^{13}C , ^{15}N -labeled L-Histidine $\cdot\text{HCl}\cdot\text{H}_2\text{O}$, pH 3.3 was purchased from Cambridge Isotope Laboratories, Inc. The crystalline histidine was dissolved in deionized water and titrated with 0.2 M NaOH to reach pH 6.3, then lyophilized in the vacuum pump. The lyophilized histidine powder was packed into a 3.2mm MAS rotor.

2.2. NMR spectroscopy

All MAS NMR spectra were acquired on a Bruker Avance 600.1 MHz NMR spectrometer using a NHMFL 3.2 mm Low-E triple-resonance biosolids MAS probe [11, 12]. The sample spinning rate was controlled within $13.5\text{kHz}\pm 3\text{Hz}$ by a Bruker pneumatic MAS unit. The ^{13}C magnetization was enhanced through CP from protons with a contact time of 1 ms, during which a ^1H spin-lock field of 50 kHz was used and the ^{13}C B_1 field was ramped from 38 to 56 kHz [13]. The ^{13}C and ^{15}N 180° pulse lengths were experimentally calibrated to be 6.6 and 7.9 μs , respectively. The ^{15}N selective 180° pulse was achieved using an 1-side lobe Sinc shape with a pulse-length of 280 μs . A SPINAL64 decoupling sequence [14] with a ^1H B_1 field of 78 kHz was used for proton decoupling. The ^{13}C chemical shifts were referenced to the carbonyl carbon resonance of glycine at 178.4 ppm relative to 4,4-dimethyl-4-silapentanesulfonate sodium (DSS), and the ^{15}N chemical shifts reference was calculated by the known relative frequency ratios between DSS (^{13}C) and liquid ammonia (^{15}N) [15, 16].

2.3. Experimental strategy

Table 1 lists the observable ^{13}C resonances of different histidine tautomeric states that are expected under various 1D experiments to be performed. We will explain in detail in the next section how the ^{13}C resonances from each tautomer can be differentiated based on these 1D experiments.

As shown in Scheme 1, each tautomer exhibits three carbons (C_γ , $\text{C}_{\delta 2}$, and $\text{C}_{\epsilon 1}$) and two nitrogens ($\text{N}_{\delta 1}$ and $\text{N}_{\epsilon 2}$). Since C_γ is the quaternary carbon that does not have a directly bonded proton, its dipolar interaction with its closest proton is considerably weaker compared to the tertiary carbons ($\text{C}_{\delta 2}$, and $\text{C}_{\epsilon 1}$) that have directly bonded protons. Thus a simple ^{13}C - ^1H dipolar dephasing experiment [17] can be performed to differentiate C_γ from $\text{C}_{\delta 2}$ and $\text{C}_{\epsilon 1}$. Since the traditional dipolar dephasing scheme becomes less efficient in terms of suppressing the protonated carbon signals under fast spinning rate, an efficient rotational-echo double resonance (REDOR) [18] based dipolar dephasing scheme is shown in Fig. 1a, while the double-quantum filtering (DQF) [19, 20] experiment can be used to select for directly bonded ^{13}C - ^{13}C pairs identified using a short filtering time. Consequently, C_γ and $\text{C}_{\delta 2}$ resonances for all tautomers are identified.

Fig. 1b shows the pulse sequence for ^{15}N dephased ^{13}C NMR experiments. Without the ^{15}N selective 180° pulse, the ^{13}C - ^{15}N dipolar couplings averaged by MAS will be reintroduced by a train of ^{15}N 180° hard pulses synchronized with MAS, resulting in dephasing of ^{13}C signals, as explained in REDOR [18]. However, when the ^{15}N selective pulse is applied to the nonprotonated ^{15}N (or $^{15}\text{N}^{\text{nonH}}$), the dipolar couplings between ^{13}C and $^{15}\text{N}^{\text{nonH}}$ will *not* be reintroduced because of the additional 180° flip of the ^{15}N magnetization, so that the ^{13}C signals will only be dephased by the protonated ^{15}N (or $^{15}\text{N}^{\text{H}}$), *not* by $^{15}\text{N}^{\text{nonH}}$. Similarly, when the ^{15}N selective pulse is applied to $^{15}\text{N}^{\text{H}}$, the ^{13}C signals will be dephased by $^{15}\text{N}^{\text{nonH}}$, *not* by $^{15}\text{N}^{\text{H}}$.

The pulse sequence used for ^{15}N selective filtering in ^{13}C MAS NMR experiments is shown in Fig. 1c. Without the ^{15}N selective 180° pulses, this sequence (Fig. 1c) is the 1D version of the z-filtered transferred-echo double resonance (TEDOR) sequence for $^{15}\text{N}^{\text{ALL}}$ -filtered ^{13}C spectra [21]. In the histidine imidazole ring, the bond length between the bonded C-N pairs is about 1.4 Å, while the next nearest C-N distance (i.e. $\text{N}_{\delta 1}$ - $\text{C}_{\delta 2}$ and $\text{N}_{\epsilon 2}$ - C_γ) is about 2.2 Å. As indicated in the z-filtered TEDOR spectra [21], at ~ 1 ms TEDOR mixing time the covalently bonded NC cross peaks have maximum signals while the $\text{N}_{\delta 1}$ - $\text{C}_{\delta 2}$ and $\text{N}_{\epsilon 2}$ - C_γ cross peaks generate weak signals because of their longer distances. Therefore, by using ~ 1 ms mixing time, we can effectively select for the directly ^{15}N bonded ^{13}C resonances without polarizing the carbons opposite to the nitrogen sites in the imidazole ring.

When the selective 180° ^{15}N pulses are applied to the non-protonated nitrogen region (~ 250 ppm), the magnetization for the non-protonated nitrogen will be additionally inverted so that any dipolar interactions between the non-protonated nitrogen and carbons will be refocused, leading to diminished signals for these carbons. The protonated nitrogens (in the region of ~ 180 ppm) are not affected by these selective pulses and hence the signals for the carbons directly bonded to the protonated nitrogens remain. Similarly, the selective 180° ^{15}N pulses

applied to the protonated nitrogen region will suppress the protonated nitrogen bonded carbons while allowing the non-protonated bonded carbon resonances to be observed.

3. Results and discussion

Fig. 2 shows the aromatic regions of ^{13}C MAS NMR spectra of a histidine powder sample lyophilized from a pH 6.3 solution. For each histidine tautomeric state, three ^{13}C resonances are expected. Six ^{13}C peaks (139.5, 138.2, 137.3, 130.2, 121.2, and 115.4 ppm) observed in the traditional CPMAS spectrum (c.f. Fig. 2a) suggest that there exist in this preparation two different histidine tautomers at pH 6.3. The spectrum with a 1 ms mixing time shows relatively uniform spectral intensity for the protonated and non-protonated ^{13}C sites. While in the ^{15}N solid-state CPMAS NMR spectrum shown in Fig. 3a, the non-protonated ^{15}N peak at 248.7 ppm is much smaller than the other protonated resonances between 160 and 190 ppm at this given CP contact time (3 ms) owing to different CP efficiencies. Due to its high gyromagnetic ratio, ^{13}C has a much better CP transfer from protons, as compared with ^{15}N , even for carbon sites that are not directly proton-bonded. Fig. 2b shows the REDOR based dipolar dephasing ^{13}C spectrum using the pulse sequence in Fig. 1a. Two peaks at 139.5 and 130.2 ppm remain while the other peaks are completely dephased. Thus the two peaks at 139.5 and 130.2 ppm are the quaternary C_γ carbons. The DQF spectrum in Fig. 2c shows an additional two resonances at 121.2 and 115.4 ppm besides the C_γ resonances. These two new peaks can be assigned to the $\text{C}_{\delta 2}$ carbons which are bonded to C_γ , while $\text{C}_{\epsilon 1}$ is bonded to $\text{N}_{\delta 1}$ and $\text{N}_{\epsilon 2}$. However, none of the carbon resonances have yet to be assigned to specific histidine tautomeric states.

Fig. 3b shows the REDOR based ^{15}N - ^{13}C dipolar dephasing ^{13}C spectra using the pulse sequence in Fig. 1b without the ^{15}N selective 180° pulse. Due to the dipolar recoupling, all the ^{13}C resonances are effectively dephased by near-by ^{15}N . However, when the ^{15}N selective 180° pulse is applied to the protonated region at 180 ppm, the protonated ^{15}N magnetization experiences an additional 180° flip in the z-axis so that the dipolar coupling generated by the REDOR sequence is refocused. As a result, there is no dephasing for those carbons that are bonded to the protonated ^{15}N . Since the ^{15}N selective 180° pulse does not affect the non-protonated ^{15}N sites, the recoupled dipolar interaction between the carbons and non-protonated nitrogens by REDOR remains so that these ^{13}C ($-\text{N}^{\text{nonH}}$) sites continue to be effectively dephased. Thus by varying the power level of the shaped pulse, we can maximize the signals from the carbons that are bonded with the protonated nitrogen so as to precisely determine the ^{15}N selective 180° pulse. As shown in Fig. 3c, the resonances at 139.5 and 137.3 ppm are greatly dephased, while the resonances at 138.2, 130.2, 121.2, and 115.4 ppm remain almost intact. Therefore, by applying the ^{15}N selective 180° pulse, we should be able to differentiate the ^{13}C ($-\text{N}^{\text{nonH}}$) from the ^{13}C ($-\text{NH}$) sites.

The key to the success for this type of selective filtering experiments is ^{15}N selectivity. Fig. 4 shows an array of ^{13}C spectra in the region between 142 and 135 ppm as a function of offset for the ^{15}N selective 180° pulse. The resonance at 138.2 ppm belongs to a carbon bonded with a protonated ^{15}N ($\text{C}_{\epsilon 1}\text{-NH}$) while the peaks at 139.5 ($\text{C}_\gamma\text{-N}_{\delta 1}^{\text{nonH}}$) correspond to carbons bonded with a non-protonated ^{15}N . The offset at 0 Hz was centered on the protonated ^{15}N region (180 ppm), while the negative offsets are shifted towards the non-

protonated nitrogen frequencies. With the ^{15}N selective 180° pulse width of $280\ \mu\text{s}$, the signal intensity at $138.2\ \text{ppm}$ is about 90% and 60% when the ^{15}N offset is ± 1000 and $\pm 2000\ \text{Hz}$, respectively, as compared to that at the offset center. On the other hand, the ^{15}N dipolar dephased ^{13}C peak at $139.5\ \text{ppm}$ decreases in intensity as the ^{15}N offset is shifted further away from the non-protonated ^{15}N region. Therefore, the bandwidth for the ^{15}N selective 180° pulse is sufficient to cover the protonated chemical shift range, which is about $40\ \text{ppm}$, corresponding to $2400\ \text{Hz}$ on the $600\ \text{MHz}$ NMR spectrometer, while it has little effect on the non-protonated ^{15}N , which is more than $60\ \text{ppm}$ (i.e. $> 3600\ \text{Hz}$ on the $600\ \text{MHz}$) away from the protonated ^{15}N region.

Fig. 5a shows the $^{15}\text{N}^{\text{ALL}}$ -filtered ^{13}C spectrum for the aromatic region of the histidine powder sample using the pulse sequence diagramed in Fig. 1c without the ^{15}N selective 180° pulses. Since all of the covalently linked ^{15}N - ^{13}C pairs are recoupled, the $^{15}\text{N}^{\text{all}}$ filtered ^{13}C spectrum has a similar resonance pattern to that of the CPMAS spectrum in Fig. 2a, with all six ^{13}C resonances observed from the two different histidine tautomers present in this sample. When the ^{15}N selective 180° pulses are applied to the non-protonated region at $250\ \text{ppm}$, the sequence generates the $^{15}\text{N}^{\text{H}}$ -filtered ^{13}C spectrum as shown in Fig. 5b. According to Table 1, for the $^{15}\text{N}^{\text{H}}$ filtered ^{13}C spectrum, it is expected to have two resonances from each of the neutral states and three resonances from the charged state. The fact that there are five ^{13}C resonances in Fig. 5b suggests that there is a charged tautomer in this histidine sample.

When the selective 180° pulses are applied to the protonated ^{15}N region at $180\ \text{ppm}$, the sequence produces the $^{15}\text{N}^{\text{nonH}}$ -filtered ^{13}C spectrum as shown in Fig. 5c. In this spectrum, no resonances from a charged state should appear. Fig. 5c shows two resonances, one of which represents C_γ at $139.5\ \text{ppm}$, as indicated in the dipolar dephased ^{13}C spectrum in Fig. 2b. Therefore, this signal at $139.5\ \text{ppm}$ can be unambiguously assigned to C_γ of the τ tautomer, while the other resonance at $137.3\ \text{ppm}$ must be $\text{C}_{\epsilon 1}$ of the τ tautomer. By comparing Fig. 2b and Fig. 5b, the other C_γ peak at 130.2 is assigned to the charged tautomer. Since the two peaks at 121.2 and $115.4\ \text{ppm}$ are assigned to $\text{C}_{\delta 2}$ (c.f. Fig. 2), the signal at $138.2\ \text{ppm}$ in Fig. 5b can be assigned to $\text{C}_{\epsilon 1}$ of the charged tautomer.

So far we have assigned C_γ and $\text{C}_{\epsilon 1}$ to their respective τ and charged tautomers. However, we have not yet assigned the two $\text{C}_{\delta 2}$ resonances to their specific tautomers. In the Fig. 1c pulse sequence, there are two z-filters. When the second z-filter is set to a longer time such as τ' to $4\ \text{ms}$, an extra transfer between ^{13}C 's will take place either through ^{13}C - ^{13}C spin diffusion under the dipolar assisted rotational resonance (DARR) condition [22, 23] or ^{13}C - ^{13}C chemical exchange. Fig. 5d shows the $^{15}\text{N}^{\text{nonH}}$ -filtered ^{13}C spectrum using $\tau' = 4\ \text{ms}$. Clearly, the signal at $115.4\ \text{ppm}$ starts to appear, indicating that this resonance must be $\text{C}_{\delta 2}$ of the τ tautomer, since no carbon signal from the charged histidine is expected to go through the $^{15}\text{N}^{\text{nonH}}$ -filter.

At high pH, the two neutral τ and π states may coexist. Differentiating their resonances is straightforward and unambiguous by a simple subtraction of the $^{15}\text{N}^{\text{H}}$ - from $^{15}\text{N}^{\text{nonH}}$ -filtered ^{13}C spectra, provided that their respective resonances from the neutral τ and π states do not overlap. As indicated in Table 1, $\text{C}_{\delta 2}$ and $\text{C}_{\epsilon 1}$ of the τ tautomer and C_γ and $\text{C}_{\epsilon 1}$ of the

π tautomer are expected to appear in the $^{15}\text{N}^{\text{H}}$ -filtered ^{13}C spectrum, while C_{γ} and $\text{C}_{\varepsilon 1}$ of the τ tautomer and $\text{C}_{\delta 2}$ and $\text{C}_{\varepsilon 1}$ of the π tautomer are observable in the $^{15}\text{N}_{\text{nonH}}$ -filtered ^{13}C spectrum. As the $\text{C}_{\varepsilon 1}$ signals derive from one bond $\text{C}_{\varepsilon 1}-\text{N}_{\delta 1}$ or $\text{C}_{\varepsilon 1}-\text{N}_{\varepsilon 2}$ in both the $^{15}\text{N}^{\text{H}}$ - and $^{15}\text{N}_{\text{nonH}}$ -filtering experiments, the difference between the $^{15}\text{N}^{\text{H}}$ - and $^{15}\text{N}_{\text{nonH}}$ -filtered ^{13}C spectra will completely suppress the $\text{C}_{\varepsilon 1}$ resonances from the τ and π states. This gives rise to a positive $\text{C}_{\delta 2}$ resonance and a negative C_{γ} peak for the τ tautomer, and results in a negative $\text{C}_{\delta 2}$ resonance and a positive C_{γ} peak for the π tautomer.

4. Conclusion

It has been demonstrated experimentally that one-dimensional ^{15}N selective filtered ^{13}C NMR spectroscopy provides an efficient way to differentiate histidine neutral and charged states by identifying the ^{13}C sites bonding with the protonated or non-protonated nitrogen in the imidazole ring of histidine. Unlike the spectral editing methods [24] that were designed to assign the ^{13}C and ^{15}N resonances of histidine imidazole ring in a single tautomeric state, the new ^{15}N selective filtering techniques proposed here, along with dipolar dephasing and DQF experiments, are able to identify all ^{13}C resonances of individual histidine imidazole ring from mixed tautomeric states. Therefore, this proposed methodology opens up a new way of investigating histidine pH titration processes in functionally important biological systems.

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Highlights

- 1D ^{15}N filtered ^{13}C ssNMR spectroscopy is proposed to differentiate histidine tautomers
- ^{15}N selective pulses are used to choose non-protonated and protonated bonded ^{13}C sites
- All ^{13}C resonances of individual imidazole rings from mixed tautomers can be identified
- This may open up a new way of investigating histidine pH titration processes in proteins

Fig. 1. Pulse sequences used in our experiments. (a) REDOR based dipolar dephasing sequence; (b) REDOR based nitrogen selective dephasing sequence; (c) ^{15}N selective filtering ^{13}C spectroscopy. Δ and Δ' are set to one rotor period during which the ^1H rf amplitude of ω_r is applied for effective z-filtering. When Δ' is long, the ^{13}C - ^{13}C transfers are facilitated via spin diffusion under the dipolar assisted rotational resonance (DARR) condition. In all experiments, T_r represents the spinning period, while τ_{mix} is the total mixing time for TEDOR. The phase cycles were as follows: (a) $\phi_1 = 02$, $\phi_2 = 0022\ 1133$, $\phi_3 = 0$, $\phi_{\text{rec}} = 0220\ 3113$; (b) $\phi_1 = 02$, $\phi_2 = 0022\ 1133$, $\phi_3 = 8 \times (0)\ 8 \times (1)\ 8 \times (2)\ 8 \times (3)$, $\phi_4 = 02$, $\phi_{\text{rec}} = 0220\ 3113\ 2002\ 1331$; (c) $\phi_1 = 16 \times (0)\ 16 \times (2)$, $\phi_2 = 0$, $\phi_3 = 0$, $\phi_4 = 0213\ 2031$, $\phi_5 = 2$, $\phi_6 = 0$, $\phi_7 = 02$, $\phi_8 = 1133$, $\phi_9 = 4 \times (0)\ 4 \times (1)\ 4 \times (2)\ 4 \times (3)$, $\phi_{\text{rec}} = 3113\ 0220\ 1331\ 2002\ 1331\ 2002\ 3113\ 0220$, where $0 = x$, $1 = y$, $2 = -x$, and $3 = -y$. All 180° pulses during REDOR/TEDOR were phase cycled using xy-4.

Fig. 2. ^{13}C NMR spectra in the aromatic region of the histidine powder lyophilized from a pH 6.3 solution. (a) CPMAS with 8 scans; (b) REDOR based dipolar dephasing spectrum with 8 scans and $m=2$; (c) DQF spectrum with 32 scans.

Fig. 3.

(a) ^{15}N CPMAS NMR spectrum of the histidine powder lyophilized from a pH 6.3 solution. 3 ms CP contact time was used, during which the ^1H B_1 field of 50 kHz was used and the ^{15}N B_1 field was ramped from 44 to 63 kHz. 16 scans were used to accumulate the signals. REDOR based ^{15}N dipolar dephasing ^{13}C spectrum (b) without and (c) with a ^{15}N selective 180° pulse applied to the protonated ^{15}N region at 180 ppm with $m=1$ and $\tau_{\text{mix}} = 2.08$ ms. 8 scans were used to accumulate the signals.

Fig. 4.

Experimental ^{15}N selective dipolar dephasing ^{13}C spectra (showing only the region between 142 and 135 ppm) as a function of ^{15}N offset. In this plot, the ^{15}N offset at 0 Hz corresponds to the middle of the protonated ^{15}N region (180 ppm), while the negative offset is shifted towards the non-protonated region (250 ppm). The ^{13}C resonances at 138.2 ppm ($\sim$ at the center of each spectrum) and at 139.5 ppm, which have a small and large dephasing effect, respectively, by the ^{15}N selective 180° pulse, are used to demonstrate the selectivity of the given selective 180° pulse.

Fig. 5.

^{15}N filtered ^{13}C spectra of the imidazole rings for the histidine powder at pH 6.3 using the pulse sequence in Fig. 1c. (a) $^{15}\text{N}^{\text{ALL}}$ -filtered ^{13}C spectrum with no ^{15}N selective 180° pulses applied. (b) $^{15}\text{N}^{\text{H}}$ -filtered ^{13}C spectrum with the ^{15}N selective 180° pulses applied to the non-protonated ^{15}N region at 250 ppm. (c) $^{15}\text{N}^{\text{nonH}}$ -filtered ^{13}C spectrum with the ^{15}N selective 180° pulses applied to the protonated ^{15}N region at 180 ppm. (d) The same as in (c) but having a long τ' ($=4$ ms) to permit ^{13}C - ^{13}C transfer. In all experiments, $\tau_{\text{mix}}=1.185$ ms, $m=1$, $\tau=74.07$ μs , and 32 scans were used to accumulate the signals.

Scheme 1.

Table 1
 ^{13}C Peaks for Histidine Tautomers Observed under Various Experiments

Experiment being performed	(τ tautomer)	(π tautomer)	(charged state)
^{13}C - ^1H dipolar dephasing	C_γ	C_γ	C_γ
$^{15}\text{N}^{\text{ALL}}$ -filtering	$\text{C}_\gamma, \text{C}_{\delta 2}, \text{C}_{\epsilon 1}$	$\text{C}_\gamma, \text{C}_{\delta 2}, \text{C}_{\epsilon 1}$	$\text{C}_\gamma, \text{C}_{\delta 2}, \text{C}_{\epsilon 1}$
$^{15}\text{N}^{\text{H}}$ -filtering	$\text{C}_{\delta 2}, \text{C}_{\epsilon 1}$	$\text{C}_\gamma, \text{C}_{\epsilon 1}$	$\text{C}_\gamma, \text{C}_{\delta 2}, \text{C}_{\epsilon 1}$
$^{15}\text{N}^{\text{nonH}}$ -filtering	$\text{C}_\gamma, \text{C}_{\epsilon 1}$	$\text{C}_{\delta 2}, \text{C}_{\epsilon 1}$	N/A
Double Quantum Filtering	$\text{C}_\gamma, \text{C}_{\delta 2}$	$\text{C}_\gamma, \text{C}_{\delta 2}$	$\text{C}_\gamma, \text{C}_{\delta 2}$
$^{15}\text{N}^{\text{nonH}}$ -filtering ^{13}C - ^{13}C	$\text{C}_\gamma, \text{C}_{\epsilon 1}, \{\text{C}_{\delta 2}\}$	$\text{C}_{\delta 2}, \text{C}_{\epsilon 1}, \{\text{C}_\gamma\}$	N/A