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Identification of Histidine Tautomers in Proteins by 2D $^{1}H/^{13}C^{\delta 2}$ One-Bond Correlated NMR

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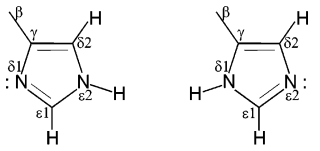
The neutral imidazole side chain of histidine, generally found at high pH, exists in equilibrium between two forms: the $N^{\epsilon 2}-H$ tautomer (**1**) and the $N^{\delta 1}-H$ tautomer (**2**). In proteins, and in smaller His compounds, nature exhibits a distinct preference for **1**, while reserving **2** for special tasks.



An example of tautomer **2** is the catalytic His 57 ($pK_a \approx 7$) in the active site of the serine protease α-lytic protease (α-LP). The existence of **2** in α-LP at high pH was first proven by using direct observation of ^{15}N NMR resonances in ^{15}N His-labeled enzyme.[1,2] In all serine protease active sites, tautomer **2** is stabilized by H-bonding between the $N^{\delta 1}-H$ proton and the carboxylate group of the adjacent "catalytic triad" residue.[3] Other examples of **2** are "structural" His residues, generally H-bonded, shielded from solvent,[4] and pH-invariant (i.e. $pK_a < 3$), such as His 226 in subtilisin BPN′.

We have recorded the 2D $^{1}H/^{13}C$ correlated NMR spectra shown in Figure 1 of various samples containing uniformly $^{13}C,^{15}N$-labeled histidines, utilizing delays optimized for the directly bonded $^{1}J_{CH}$ spin-coupling constants of ∼200 Hz exhibited by His side chains. Panel A is from the spectrum of monomeric [U-$^{13}C,^{15}N$]-L-His (Cambridge Isotope Labs) at pH 12 and −55 °C in 80% d_6-ethanol/20% water. As shown using ^{15}N NMR,[5] under these conditions the rapid equilibrium between the two His tautomers can be frozen, and panel A in Figure 1 displays only the $^{1}H/^{13}C^{\delta 2}$ correlation resonance of tautomer **2**, constituting 26% of the mixture by 1D ^{13}C spectral integrations. The dumbbell-shaped resonances in Figure 1 are caused by the large one-bond $C_\gamma-C_\delta$ spin-coupling constants, $^{1}J_{C\gamma C\delta}$, showing interesting variations (62−92 Hz) compared to monomer (71 Hz), of unknown origin. As illustrated in panel B for His 187 of "TBD", the origin-specific DNA binding domain from simian virus 40 T-antigen (T-ag-$OBD_{131-259}$),[6] these spin-couplings can readily be "tuned out", yielding a single resonance with increased sensitivity by use of a constant time heteronuclear single quantum coherence (ct-HSQC) pulse program.[7]

Of the six His residues in subtilisin BPN′ (MW = 27.5, high-stability mutant N155A),[8] the four $C^{\delta 2}$ nuclei that resonate between 113.3 and 115.7 at pH 10.5 (Table 1S) are all assigned to tautomer **1**. The two remaining His residues in subtilisin BPN′, shown in Figure 1, panel C, exhibit $^{13}C^{\delta 2}$ resonances at 125.0 and 124.2 ppm—the former the nontitrating signal assignable to His 226, the latter the titrating "catalytic triad" active site His 64—both predicted from the X-ray crystal structure[4] to be tautomer **2** and confirmed here in solution. His 64 undergoes a large downfield shift in its $^{13}C^{\delta 2}$ resonance from 118.0 to 124.2 ppm as the pH is increased from 5.5 to 10.5—a difference of 6.2 ppm between cation and tautomer **2**.

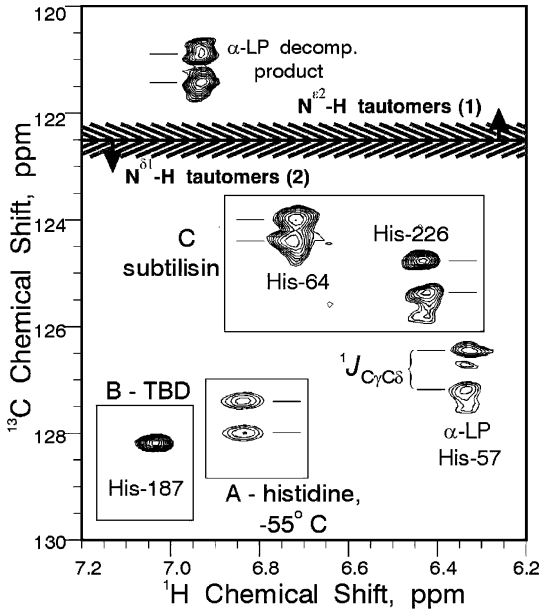


Figure 1. Selected portions of 2D $^{1}H/^{13}C^{\delta 2}$ correlation NMR spectra of four uniformly $^{13}C,^{15}N$-labeled His-containing samples: Underlying is a 500 MHz HMQC spectrum of α-lytic protease grown with [U-$^{13}C,^{15}N$]-L-His, pH 9.0 and 25 °C. (A) 500 MHz HMQC of [U-$^{13}C,^{15}N$]-L-His at pH 12 and −55 °C in 80% d_6-ethanol/20% H_2O; (B) 600 MHz ct-HSQC of U-$^{13}C,^{15}N$-labeled TBD at pH 5.5 and 30 °C (constant delay 13.5 ms). TBD has no tryptophans. (C) 600 MHz HMQC of subtilisin BPN′ (N155A) grown with [U-$^{13}C,^{15}N$]-L-His, pH 10.5, 25 °C. The hatched barrier shows the divide between tautomers **1** and **2**.

The use of $^{13}C^{\delta 2}$ NMR chemical shifts for discrimination between His tautomers was first performed by ^{13}C NMR spectra of small histidine-containing compounds.[9] Using His derivatives 1-methyl- and 3-methyl-L-histidine, these researchers predicted an upfield $^{13}C^{\delta 2}$ deprotonation shift of −2.1 ppm for tautomer **1** and downfield +7.1 ppm for tautomer **2**—a differential of 9.2 ppm. This assumes the methyl group to be a valid proton substitute for ^{13}C chemical shift contributions. Our low-temperature results (Table 2S) for monomeric [U-$^{13}C,^{15}N$]-L-His confirm these trends, showing an even higher tautomer difference of 11.1 ppm. $^{13}C^{\gamma}$ chemical shifts produce about −8 ppm tautomer difference,[10] which has been successfully employed for His tautomer discrimination in proteins by direct ^{13}C observation in very large samples of natural isotopic abundance ribonuclease,[11] but this method lacks general applicability.

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α-Lytic protease (MW = 19.8) contains only one His, the catalytic His 57, whose $^{13}C^{\delta 2}$ resonance undergoes a large downfield shift, from 120.6[3] to the 126.8 ppm shown in Figure 1 (the same increase of 6.2 ppm as in subtilisin BPN′) during a pH increase from 4.0 to 9.0. Thus the presence of $N^{\delta 1}$ tautomer (**2**) is easily distinguished on the basis of $^{13}C^{\delta 2}$ chemical shifts, providing further confirmation of the assignments of the $C^{\delta 2}-^{1}H$ (and indirectly the intensity-paired $C^{\epsilon 1}-^{1}H$) protons of His 57 as the authentic catalytic triad signals.[12] The resonance of the major α-LP autolysis product, which grows steadily with time, is shown in Figure 1 and Table 1S. This $C^{\delta 2}-^{1}H$ proton resonance, ranging from 7.35 ppm at pH 4 to 6.93 ppm at pH 9 and its companion $C^{\epsilon 1}-^{1}H$ proton ranging from 8.65 ppm at pH 4 to 7.70 at pH 9 had been accepted for decades as the authentic His 57 signal (called "fresh"[13]), but from the $^{13}C^{\delta 2}$ chemical shift is shown here to be primarily the wrong tautomer **1**. Correct assignment of the anomalous ^{1}H resonances in α-LP was a vital step in confirming in solution[12] the presence of a $C^{\epsilon 1}-H$-donated H-bond predicted from X-ray crystallographic data,[14] and led to a proposed new reaction-driven imidazole ring flip mechanism in serine protease catalysis.[12]

Of the six His residues in TBD (MW = 15.4), five $C^{\delta 2}$ nuclei resonate between 116.7 and 118.9 ppm at pH 5.5, and the sixth, His 187, the only tautomer **2**, appears at 128.1 ppm (Figure 1, panel B). The only protein in this communication prepared by uniform $^{13}C,^{15}N$-labeling of all residues, TBD contains no tryptophans, otherwise interference from Trp $^{1}H/^{13}C^{\delta 1}$ might occur. Originally the $^{13}C^{\delta 2}$ carbon and $C^{\delta 2}-^{1}H$ proton resonances of His 187 were unassigned,[15,16] in part due to degeneracy caused by the unusual $C^{\epsilon 1}-H$ proton chemical shift (6.97 ppm). Thus, we assumed His 187 to be tautomer **1**, leading to a reversal of the $^{15}N^{\delta 1}$ and $^{15}N^{\epsilon 2}$ assignments.[16] ^{15}N chemical shifts are generally the most informative NMR probe of His protonation, tautomerism, and H-bonding, whether obtained by direct ^{15}N observation[3] or indirect observation using either 2D $^{1}H/^{15}N$ two-bond correlation[17,18] or 3D $^{1}H/^{13}C/^{15}N$ "HCN" sequential one-bond correlation.[16] In the case of TBD His 187, however, the $^{13}C^{\delta 2}$ carbon chemical shift elucidated the tautomeric equilibrium, which in turn corrected the ^{15}N assignments.

Tautomer identification by direct observation of histidine ^{15}N requires large samples, ≥1.5 mL of ∼2 mM protein, but has the advantage that line widths of 100 Hz or more due to high molecular weight, aggregation, or acid−base fast-exchange broadening[19] can be tolerated.[1] The indirect ^{15}N observation methods offer the advantage of higher ^{1}H sensitivity and multiple correlation of intraresidue ^{1}H, ^{15}N, and for HCN, ^{13}C resonances. A disadvantage is their reliance upon relatively small spin-coupling constants, i.e., $^{2}J_{NH} \leq |11\ Hz|$ and, for HCN, $^{1}J_{CN} \leq |16\ Hz|$, which limits the tolerable line widths.

In summary, if the $^{13}C^{\delta 2}$ chemical shift of neutral ("high pH") His is >122 ppm, a predominance of $N^{\delta 1}-H$ tautomer (**2**) is indicated; if <122 ppm, mostly $N^{\epsilon 2}-H$ tautomer (**1**) is indicated. The method is easy to implement, requiring only bioincorporation of [U-^{13}C] (or the more readily available [U-^{13}C, ^{15}N])-histidine. Standard HMQC or HSQC NMR pulse programs then yield the chemical shifts of His $^{13}C^{\delta 2}$ with the benefit of high ^{1}H sensitivity. Because of the large one-bond spin-coupling ($^{1}J_{CH} \approx 200$ Hz), the method should extend to proteins having large ^{1}H and ^{13}C line widths, including very high molecular weights. A TROSY HSQC pulse program specifically for aromatic $^{1}H/^{13}C$ correlation[20] is available for very large proteins and higher magnetic fields, although it was not necessary for the proteins up to 27.5 kD at 600 MHz in this work.

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Supporting Information Available: Table 1S lists ^{1}H and $^{13}C^{\delta 2}$ chemical shifts, pK_a values, and other data on the His residues of the three proteins presented here; Table 2S lists all NMR parameters of monomeric [U-^{13}C,^{15}N]-His in 80% ethanol (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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