# Direct Observation of the Tautomeric Forms of Histidine in $^{15}N$ NMR Spectra at Low Temperatures. Comments on Intramolecular Hydrogen Bonding and on Tautomeric Equilibrium Constants

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**Abstract:** The individual tautomeric forms of histidine have been directly observed in $^{15}N$ NMR spectra at −55 °C in ethanol/water solution. The chemical shifts of the nitrogen atoms in the tautomers agree well with values previously estimated for them from $^{15}N$ NMR spectra of $N^{\delta 1}$ and $N^{\epsilon 2}$ methyl histidines and of histidine in the dry powder state. A comparison of the chemical shift position of $N^{\delta 1}$ in the amphion and anion forms of histidine shows that $N^{\delta 1}$ of the $N^{\epsilon 2}$–H tautomer forms at most only a very weak hydrogen bond with the α-amino group. The existence of this hydrogen bond has been proposed previously to explain the effect ionization of the amino group has on the tautomeric equilibrium constant. The pH dependence of the $^{15}N$ chemical shifts of histidine at 25 °C in the ethanol/water cosolvent closely matches that observed in water alone, showing that the ethanol cosolvent solution used in the low-temperature work has little or no effect on the intrinsic $^{15}N$ shifts of the individual tautomeric forms or on the acid–base and tautomeric equilibria of the imidazole ring. The significance of these results for determining tautomeric equilibrium constants of imidazoles and for interpreting $^{15}N$ chemical shift data from histidyl residues in proteins is discussed.

Recognizing the frequency with which histidyl residues play key functional roles in proteins, early biochemical applications of NMR spectroscopy devoted considerable effort to resolving, assigning, the studying the behavior of NMR signals from these residues in one-dimensional spectra.[1] Observation of the imidazole ring $H^{\epsilon 1}$ proton[2] was the most widely used approach,[3-7] in part because it could be applied to proteins as they are found in nature, i.e., incorporation of isotopic labels was not necessary, and in part because of the sensitivity advantage of $^1H$ NMR spectroscopy. However, the information about histidyl residues extractable from the chemical shift behavior of this proton is somewhat limited. It reveals whether or not the imidazole ring titrates. If it titrates, the pH dependence of the $^1H^{\epsilon 1}$ signal yields a microscopic $pK_a$ value, and if it does not titrate the chemical shift of this signal may reveal whether the ring is protonated or neutral, although for nontitrating imidazole rings the $^1H^{\epsilon 1}$ chemical shift is often ambiguous on this point. One-dimensional $^{13}C$ NMR spectroscopy of $^{13}C^{\epsilon 1}$ histidyl labeled proteins, developed at about the same time as the $H^{\epsilon 1}$ proton NMR approach,[8-11] offers some advantages over the $^1H^{\epsilon 1}$ approach in the resolution and assignment of the signals, but it supplies essentially the same information.

The $^{15}N$ signals of the imidazole ring nitrogen atoms, while more difficult to detect directly in one-dimensional NMR spectra owing to the low gyromagnetic ratio of this nucleus (sensitivity is about 1000-fold less than that of $^1H$ for equal numbers of nuclei), are considerably more informative than are the $^1H^{\epsilon 1}$ or $^{13}C^{\epsilon 1}$ signals.[12-18] The $^{15}N$ chemical shifts of imidazole ring >N–H and >N: type nitrogens differ by about 80 ppm. There is therefore no ambiguity about whether a nontitrating imidazole ring is protonated or neutral, as may be the case with the $^1H^{\epsilon 1}$ and $^{13}C^{\epsilon 1}$ signals. The $^{15}N$ chemical shifts also reveal the tautomeric structure of neutral imidazole rings with striking clarity, again owing to the large chemical shift difference between >N–H and >N: type nitrogens. Such information about tautomeric structure is not directly provided by X-ray crystal or NMR solution structures, and this information can yield important insights into the environment and chemical behavior of a histidyl residue in a protein. The $^{15}N$ shifts also have been shown to be sensitive to hydrogen bonding interactions involving the imidazole ring, with strong hydrogen bonds inducing shift changes of up to 10 ppm.[13,14,16-20] This sensitivity of the $^{15}N$ chemical shifts to

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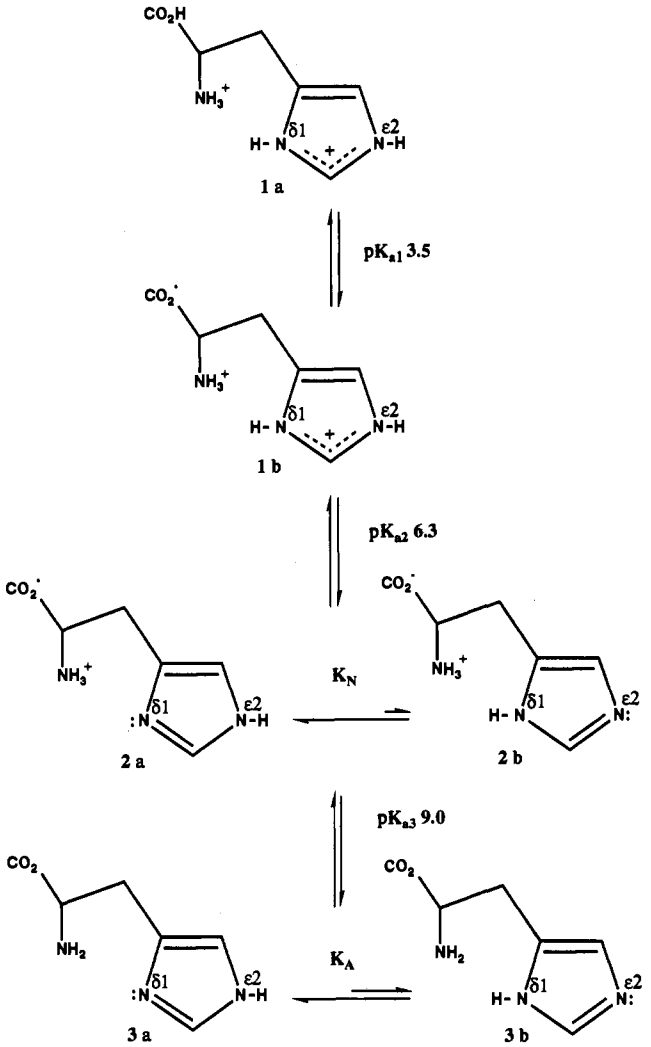
**Scheme I**




hydrogen bonding interactions has been utilized to gain mechanistically important hydrogen bond information in $\alpha$-lytic protease,[13,17-19] in triose phosphate isomerase,[21] and in the phosphocarrier protein, HPr, of the phosphoenolpyruvate dependent sugar transport systems (PTS).[22]

Finally, the nitrogen atoms of histidyl residues are chemically reactive, and functionally important histidyl residues often form covalent bonds with substrates or inhibitors, become phosphorylated, or coordinate metals. The $^{15}N$ chemical shifts can provide clear and unambiguous information about such structures. For example, the $^{15}N$ chemical shifts have recently shown that certain boronic acid inhibitors of $\alpha$-lytic protease, a serine protease of the trypsin family, form complexes involving a covalent bond between $N^{\epsilon 2}$ of $His^{57}$ and the boron atom of the inhibitor.[18,20] Histidine $^{15}N$ chemical shifts have also been used to identify the histidine phosphorylation sites in three different proteins of the bacterial PTS[22-24] and the site of inhibitor alkylation in *Escherichia coli* $\beta$-hydroxydecanoyl thioester dehydrase.[25]

With the advent of two- and three-dimensional NMR methods, NMR studies of biochemical molecules have justifiably emphasized solving solution structures utilizing $^1H$–$^1H$ correlations. However, as outlined above, the $^{15}N$ NMR chemical shifts of histidyl residues can often provide important information about these functionally important residues which is complementary to that provided by such structural NMR spectroscopy, and modern NMR methods can in some cases overcome the sensitivity problems associated with direct $^{15}N$ detection through the use of two-dimensional $^1H$–$^{15}N$ correlation experiments in which the proton magnetization is detected. Thus modern NMR methodologies will expand the number of histidyl residues for which $^{15}N$ shifts will be available or which will be accessible for study by NMR. It should therefore become increasingly important to develop a working framework for interpreting the $^{15}N$ shifts that covers all circumstances. Until now this approach has been limited to histidyl residues in aqueous like environments and for which the H-bonding effects have been demonstrably large.[13,17-22] Developing a more general framework will require, on the most basic level, a more precise knowledge of the $^{15}N$ chemical shifts of the imidazole ring nitrogen in each of the individual tautomeric forms and of how these "canonical" values depend on environmental effects other than protonation and hydrogen bonding.

In aqueous solutions at 25 °C the individual tautomers of histidine rapidly exchange and only a single averaged signal is observed for each ring nitrogen (Scheme I). Values for these shifts have been estimated previously from *N*-methylhistidines[13,15] and from $^{15}N$ spectra of histidine powders where tautomeric exchange is slow.[26] Here we report the direct observation by $^{15}N$ NMR spectroscopy of the tautomeric forms of histidine in solution at low temperature in an ethanol-based cryosolvent and discuss the implications of the results for past estimates of the tautomeric equilibrium constants of the histidine amphion and anion[12,15] for the conclusion that the $\alpha$-amino group of histidine forms an intramolecular hydrogen bond with $N^{\delta 1}$ of the imidazole ring[12] and for the interpretation of $^{15}N$ chemical shift data from histidyl residues in proteins.

## Experimental Section

L-Histidine specifically labeled with $^{15}N$ at $N^{\delta 1}$ (99%) was obtained from ICON (Summit, NJ). D,L-Histidine $^{15}N$ labeled at both ring nitrogen atoms was obtained from ICON or synthesized by the method of Totter and Darby.[27,28] The Stable Isotope Resource of the Los Alamos National Laboratory provided $^{15}NH_3$ (99%) for this synthesis. *N*-Methylated histidines were purchased from Sigma Chemical Co. and *N*-methylimidazole (natural abundance $^{15}N$) was purchased from Aldrich Chemical Co.

NMR samples were prepared by dissolving $^{15}N$-labeled histidine (~0.15 M) in 80% ethanol and 20% $^1H_2O$ or 90% $^1H_2O$ and 10% $^2H_2O$. The lock signal for the 80% ethanol samples was provided by a capillary of acetone-$d_6$. The pH of the samples was adjusted by addition of 0.1 M NaOH or HCl. No correction was made for the measurement of pH in 80% ethanol. For $^{15}N$ spectra of methyl histidines at natural abundance $^{15}N$, samples of 0.7 M were dissolved in 90% $^1H_2O$ and 10% $^2H_2O$, and the pH was then adjusted to 4.0 or 9.0. Low temperatures were achieved and maintained with the Bruker low-temperature accessory. EDTA (~5 $\mu$M) was added to both the aqueous and the 80% alcohol samples to prevent broadening of the signals by adventitious paramagnetic metal ions.

To obtain $^{15}N$ spectra of *N*-methylhistidine at natural abundance $^{15}N$ in organic solvents, $N^{\delta 1}$-methylhistidine was first dissolved in $^1H_2O$ and the pH adjusted to pH 9.0; the sample was then lyophilized and redissolved in DMSO or benzene. Neither $N^{\epsilon 2}$-methylhistidine nor histidine was sufficiently soluble in DMSO, chloroform, methylene chloride, carbon disulfide, or acetone to acquire $^{15}N$ spectra at natural abundance.

$^{15}N$ NMR spectra were recorded at 40.55 MHz on a Bruker AM wide-bore 400-MHz spectrometer using a 10-mm single-frequency $^{15}N$ probe. Spectra were acquired with a 90° pulse (24 $\mu$s), a spectral width of 20 kHz, 8K real data points, and a recycle time of ~10 s for the spectra

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**Table I.** $^{15}N$ Chemical Shifts[a] of Histidine and *N*-Methylimidazole under Various Conditions

| compd | nitrogen atom | imidazolium cation[b] | $\Delta\delta\lvert N^{\delta1}-N^{\epsilon2}\rvert$ cation | neutral imidazole[c] | $\Delta\delta\lvert N^{\delta1}-N^{\epsilon2}\rvert$ neutral |
|---|---|---|---|---|---|
| histidine, $H_2O$, 25 °C[d] | $N^{\delta1}$ | 200.5 | | 144.58 | |
| | $N^{\epsilon2}$ | 202.9 | 2.4 | 197.9 | 53.32 |
| | $N^{\delta1}$ | | | 158.55 | |
| | $N^{\epsilon2}$ | | | 182.22 | 23.67 |
| histidine, $N^{\delta1}$–H tautomer, 80% EtOH, –55 °C, pH 11.2 | $N^{\delta1}$ | 198.8 | | 208.3 | |
| | $N^{\epsilon2}$ | 201.4 | 2.6 | 131.6 | 76.7 |
| histidine, $N^{\epsilon2}$–H tautomer, 80% EtOH, –55 °C, pH 11.2 | $N^{\delta1}$ | 206.4 | 2.6 | 128.5 | |
| | $N^{\epsilon2}$ | 208.4 | | 211.6 | 83.1 |
| $N^{\delta1}$-methylhistidine, $H_2O$, 25 °C | $N^{\delta1}$ | 203.6 | | 210.3 | |
| | $N^{\epsilon2}$ | 204.9 | 1.3 | 134.5 | 75.8 |
| $N^{\epsilon2}$-methylhistidine, $H_2O$, 25 °C | $N^{\delta1}$ | 204.3 | | 129.1 | |
| | $N^{\epsilon2}$ | 200.4 | 3.9 | 211.8 | 82.7 |
| $N^{\delta1}$-methylhistidine, DMSO, 25 °C | $N^{\delta1}$ | 200.4 | | 210.8 | |
| | $N^{\epsilon2}$ | 200.4 | | 116.0 | 94.8 |
| $N^{\delta1}$-methylimidazole,[e] $H_2O$, 25 °C | $N^{\delta1}$ | 204.1 | | 211.5 | |
| | $N^{\epsilon2}$ | 203.6 | 0.5 | 128.5 | 83 |
| $N^{\delta1}$-methylimidazole,[e] MeOH, 25 °C | $N^{\delta1}$ | 202.5 | | 212.5 | |
| | $N^{\epsilon2}$ | 200.7 | 1.8 | 127.8 | 84.7 |
| $N^{\delta1}$-methylimidazole,[e] $CHCl_3$, 25 °C | $N^{\delta1}$ | 200.7 | | 215.1 | |
| | $N^{\epsilon2}$ | | | 119.3 | 95.8 |
| $N^{\delta1}$-methylimidazole,[e] $C_6H_6$, 25 °C | $N^{\delta1}$ | | | 215.7 | |
| | $N^{\epsilon2}$ | | | 111.4 | 104.3 |

[a] Chemical shifts are in ppm from external 1 M $HNO_3$ in $D_2O$. [b] Shifts under conditions of full protonation. [c] Shifts under conditions of no protonation. [d] Shift data from Blomberg et al.[12] and recalculated to 1 M $HNO_3$ standard. The first set of shifts for neutral imidazole is for histidine amphion, and second is for histidine anion. [e] $^{15}N$ shifts from Shuster and Roberts.[14] The methylated nitrogen is arbitrarily labeled $N^{\delta1}$.

of histidine and ~30 s for the spectra of the methyl-substituted histidines. Chemical shifts are reported relative to external 1 M $H^{15}NO_3$ with positive shifts being upfield.

**Analysis of pH Titration Data.** In aqueous solution at room temperature the $^{15}N$ chemical shifts of the imidazole ring nitrogens respond first to the protonation of the α-amino group ($pK_{a3}$ of Scheme I) and then to the protonation of the imidazole ring ($pK_{a2}$, Scheme I) as the pH is lowered from ~12 to ~4.0. This situation can be described by the following equilibrium expression:

$$I + H \overset{K_3}{\rightleftharpoons} IH + H \overset{K_2}{\rightleftharpoons} IH_2. \quad (1)$$

From the mass balance relationship,

$$[I_T] = [I] + [IH] + [IH_2] \quad (2)$$

and the equilibrium relationships (note that we are using association equilibrium constants)

$$[IH] = [I][H]K_3 \quad (3)$$

$$[IH_2] = [I][H]^2K_3K_2 \quad (4)$$

the following relationship can be derived:

$$[I_T] = [I] + [I][H]K_3 + [I][H]^2K_3K_2 \quad (5)$$

Which can be solved for I to give:

$$[I] = \frac{[I_T]}{1 + [H]K_3 + [H]^2K_3K_2} \quad (6)$$

Under the conditions of fast exchange the chemical shift of each imidazole ring nitrogen represents a weighted average of its shift in the three forms as given by:

$$\delta^{15}N_{obs} = \frac{[I]\delta^{15}N_I + [IH]\delta^{15}N_{IH} + [IH_2]\delta^{15}N_{IH_2}}{[I_T]} \quad (7)$$

Substituting eqs 3 through 6 into eq 7 yields

$$\delta^{15}N_{obs} = \frac{\delta^{15}N_I + [H]K_3\delta^{15}N_{IH} + [H]^2K_3K_2\delta^{15}N_{IH_2}}{1 + [H]K_3 + [H]^2K_3K_2} \quad (8)$$

By definition

$$[H] = 10^{-pH} \quad (9)$$

$$K_3 = 10^{pK_{a3}} \quad (10)$$

$$K_2 = 10^{pK_{a2}} \quad (11)$$

Substituting eqs 9, 10, and 11 into eq 8 gives

$$\delta^{15}N_{obs} = \frac{\delta^{15}N_I + 10^{-pH}10^{pK_{a3}}\delta^{15}N_{IH} + (10^{-pH})^2 10^{pK_{a3}}10^{pK_{a2}}\delta^{15}N_{IH_2}}{1 + 10^{-pH}10^{pK_{a3}} + (10^{-pH})^2 10^{pK_{a3}}10^{pK_{a2}}} \quad (12)$$

The pH-dependent $^{15}N$ chemical shift data were fit directly to this equation by nonlinear least squares with use of the BMDP routine AR (BMDP Statistical Software Inc., Los Angeles, CA). The $^{15}N$ chemical shift data form $N^{\delta1}$ and $N^{\epsilon2}$ were fit together to provide estimates for the two $pK_a$'s, the three chemical shifts for $N^{\delta1}$, and the three chemical shifts for $N^{\epsilon2}$. The adjustable parameters and their fitted values are given in Table II, and the data and fitted curves are shown in Figure 3.

## Results

The two tautomeric forms of the histidine amphion, structures **2a** and **2b** of Scheme I, are the predominant species present in aqueous solution at pH 8.2. The chemical shifts of 144.6 ppm for $N^{\delta1}$ and of 197.9 ppm for $N^{\epsilon2}$ observed in room temperature spectra of histidine[12] at this pH in water (Table I) or in an 80% ethanol/water cosolvent (Table I, Figure 3) represent weighted averages of essentially these two forms. As the temperature is lowered these two signals broaden and disappear and at –55 °C (in the 80% ethanol/water cosolvent) two new signals appear at 129.1 and 210.8 ppm as shown in Figure 1A. The spectrum shown in Figure 1A is of a sample at pH 9.22. However, essentially the same spectrum is observed from pH 7.0 to 9.22. Below pH 7.0 the $N^{\delta1}$ signal begins to broaden and move upfield (spectra not shown). The chemical shifts of the signals at 129.1 and 210.8 ppm are consistent with those expected for an individual tautomeric form of histidine. The proton coupled spectrum shown in Figure 1D, by showing clearly resolved one bond $^1H$ splitting ($J$ = 96 Hz) of the signal at 210.8 ppm, confirms that the tautomeric exchange must indeed be slow on the NMR time scale. Spectra of singly $^{15}N^{\delta1}$ labeled histidine exhibit only the signal at 129.1 ppm (spectra not shown), thereby demonstrating that spectra A and D in Figure 1 are of the $N^{\epsilon2}$–H tautomer, **2a** of Scheme I. The absence of any signal in the high-field range, i.e., ~210 ppm in the spectrum of singly $^{15}N^{\delta1}$ labeled histidine, demonstrates that signals from the $N^{\delta1}$–H tautomer are not hidden beneath those of the $N^{\epsilon2}$–H tautomer. Thus, the signals from the

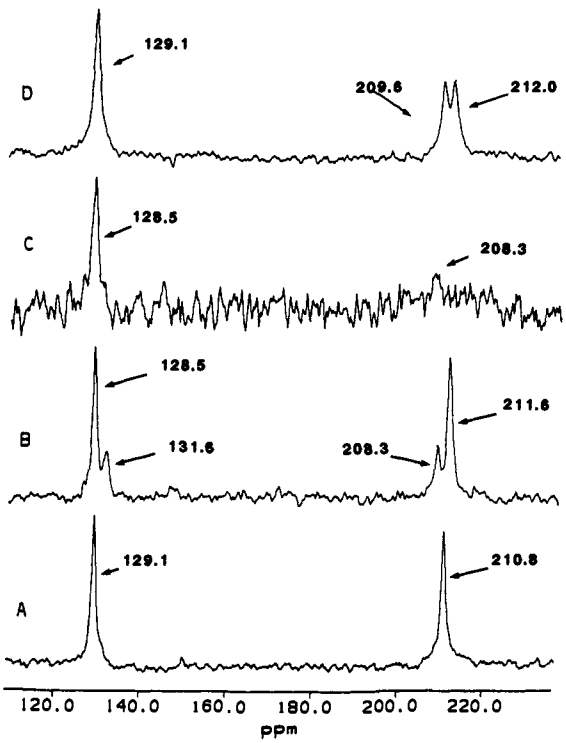


**Figure 1.** $^{15}N$ NMR spectra of $^{15}N$ labeled histidine in 80% EtOH/$H_2O$ at −55 °C under pH conditions where the imidazole ring is fully neutral: (A) $N^{\delta 1}$, $N^{\epsilon 2}$ labeled, pH 9.22, $^1H$ decoupled; (B) $N^{\delta 1}$, $N^{\epsilon 2}$ labeled, pH 11.11, $^1H$ decoupled; (C) $N^{\delta 1}$ labeled, pH 11.34, $^1H$ decoupled; (D) $N^{\delta 1}$, $N^{\epsilon 2}$ labeled, pH 9.2, $^1H$ coupled.

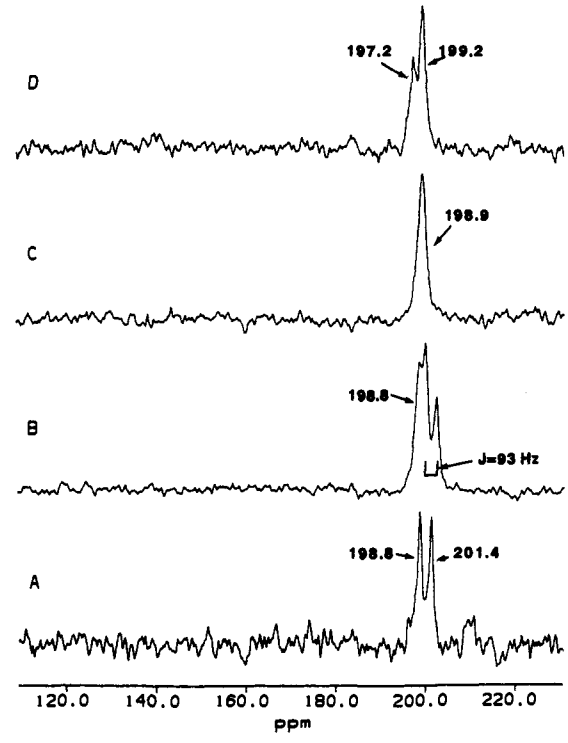


**Figure 2.** $^{15}N$ NMR spectra of $^{15}N$ labeled histidine in 80% EtOH/$H_2O$ at −55 °C under pH conditions where the imidazole ring is fully protonated: (A) $N^{\delta 1}$, $N^{\epsilon 2}$ labeled, pH 3.92, $^1H$ decoupled; (B) $N^{\delta 1}$, $N^{\epsilon 2}$ labeled, pH 3.92, $^1H$ coupled; (C) $N^{\delta 1}$ labeled, pH 4.05, $^1H$ coupled; (D) $N^{\delta 1}$ labeled, pH 2.2, $^1H$ coupled.

$N^{\delta 1}$–H tautomer, **2b**, if present are below the level of detection in spectra A and D in Figure 1. It is known that **2a** is favored over **2b** by a factor of ~10 at room temperature.[12,15] However, a signal 10% as strong as those observed for the $N^{\epsilon 2}$–H tautomer should have been detected. Apparently **2a** is even more favored over **2b** at −55 °C than at room temperature.

Deprotonation of the α-amino group at room temperature is known to shift the tautomeric equilibrium in the direction of the $N^{\delta 1}$–H tautomer.[12] This phenomenon is reflected in the room temperature behavior of the $^{15}N$ chemical shifts of $N^{\delta 1}$ and $N^{\epsilon 2}$ which move from 144.6 and 197.9 ppm, respectively, at pH 8.2 to 158.55 and 182.22 ppm, respectively, at pH 11.2.[12] The net effect is such that at pH 11.2 structure **3a** is still favored over **3b** but not by as much as **2a** is favored over **2b** at pH 8.00. The same phenomenon also occurs at −55 °C as demonstrated by the appearance of both tautomeric forms in the pH 11.11 spectrum (Figure 1B) whereas only the $N^{\epsilon 2}$–H tautomer was observed in spectra from pH 7.0 to 9.22 (Figure 1A). The stronger signals at 128.5 and 211.6 ppm in the spectrum in Figure 1B are from $N^{\delta 1}$ and $N^{\epsilon 2}$, respectively, of the $N^{\epsilon 2}$–H tautomer, **3a**, whereas the weaker signals at 131.6 and 208.3 ppm arise from $N^{\epsilon 2}$ and $N^{\delta 1}$, respectively, of the $N^{\delta 1}$–H tautomer, **3b**. The signals observed at 129.1 and 210.8 ppm in Figure 1A must therefore be of **2a**. Assignments of $N^{\delta 1}$ and $N^{\epsilon 2}$ nitrogens were made through the use of singly $^{15}N^{\delta 1}$ labeled histidine (Figure 1C). The signal at 128.5 ppm is 3.07 times more intense than the signal at 131.6 ppm, and the signal at 211.6 ppm is 2.9 times more intense that the signal at 208.3 ppm. Thus, the ratio of the intensities of the nonprotonated nitrogens (i.e., $N^{\delta 1}$ of **3a**/$N^{\epsilon 2}$ of **3b**) and of the protonated nitrogens ($N^{\epsilon 2}$–H of **3a**/$N^{\delta 1}$–H of **3b**) agrees in indicating a relative tautomeric ratio of about 3/1 in favor of the $N^{\epsilon 2}$–H tautomer, **3a**, over the $N^{\delta 1}$–H tautomer, **3b** at −55 °C and pH 11.11. This agrees well with past estimates of the tautomeric abundance for the histidine anion in aqueous solutions at room temperature based on averaged chemical shifts and coupling constants.[12,15] In summary, Figure 1 shows that we have directly observed in $^{15}N$ NMR spectra at −55 °C three, **2a**, **3a**, and **3b** of Scheme I, of the four possible structural forms of histidine with a neutral imidazole ring. Only **2b** was not directly observed.

Protonation of the imidazole ring at low pH (~4.0) eliminates complications from chemical shift averaging as now only a single structural form of histidine, **1b** of Scheme I, contributes to the observed $^{15}N$ chemical shifts. At room temperature $N^{\delta 1}$ and $N^{\epsilon 2}$ of **1b** resonate at 200.5 and 202.9 ppm, respectively (Table I). Figure 2A shows the spectrum of **1b** at −55 °C in 80% ethanol/water (pH 3.92). Under these conditions, $N^{\delta 1}$ and $N^{\epsilon 2}$ resonate at 198.8 and 201.4 ppm, close to their positions in room temperature spectra. Interestingly, although both nitrogens have a directly bonded proton, only $N^{\epsilon 2}$ exhibits the one-bond proton splitting (Figure 2, spectra B and C) at pH 3.92 and −55 °C. Apparently, the proton on $N^{\delta 1}$ is more exchange labile that the one on $N^{\epsilon 2}$. This fits with the observation that titration preferentially involves the $N^{\delta 1}$ proton. Exchange at $N^{\delta 1}$, however, is slowed sufficiently to allow observation of the $^{15}N^{\delta 1}$–$^1H$ splitting by lowering the pH to 2.2 at −55 °C (Figure 2D). The application of proton decoupling to spectrum D in Figure 1 collapses the doublet into a singlet (decoupled spectrum not shown) thereby confirming that it is a doublet and not two separate species.

The use of the 80% ethanol/water cosolvent in the above experiments raises questions about the effect of ethanol on the $^{15}N$ shifts of the N atoms of the various forms of histidine. We can conclude that ethanol does not substantially affect the $^{15}N$ shifts of the ring nitrogens in the imidazolium cation, structure **1b**, as the $^{15}N$ shifts of **1b** in 80% ethanol and −55 °C are less than 2 ppm from their values in aqueous solution at room temperature as discussed above and shown in Figure 2A. But what about the $^{15}N$ shifts of the ring nitrogens in species **2a**, **2b**, **3a**, and **3b**? The N–H or pyrrole type nitrogens should behave like the protonated nitrogens and show only insignificant solvent effects, but the N: or pyridine type nitrogens might be more strongly affected by solvent. Unfortunately we cannot directly compare the $^{15}N$ spectra of species **2a**, **2b**, **3a**, and **3b** in water

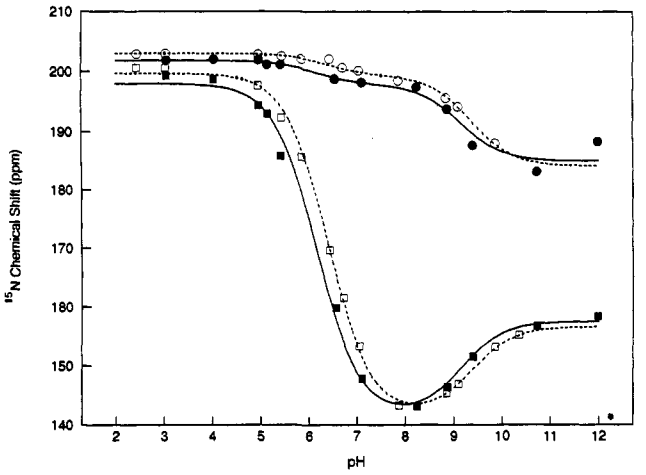


**Figure 3.** Comparison of the pH dependence of the $^{15}$N shifts of histidine in 80% EtOH/$H_2O$, solid symbols, to 100% $H_2O$, open symbols, at room temperature, ~22 °C. Squares are $N^{\delta 1}$ and circles are $N^{\epsilon 2}$. Lines represent best fit of data to eq 1 as described in the Experimental Section.

**Table II.** Summary of Parameters from the Fit to Equation 12[a]

| | $H_2O$ | | EtOH/$H_2O$ | |
|---|---|---|---|---|
| | $N^{\delta 1}$ | $N^{\epsilon 2}$ | $N^{\delta 1}$ | $N^{\epsilon 2}$ |
| $pK_{a1}$ | 9.38(0.10) | | 9.16(0.15) | |
| $pK_{a2}$ | 6.40(0.02) | | 6.16(0.07) | |
| $\delta^{15}N_I$[b] | 156.9(1.5) | 183.7(0.4) | 157.9(1.2) | 185.2(1.1) |
| $\delta^{15}N_{IH}$[b] | 142.1(1.0) | 198.2(0.4) | 142.7(1.6) | 198.6(1.4) |
| $\delta^{15}N_{IH_2}$[b] | 199.6(0.6) | 202.8(0.1) | 198.1(1.0) | 202.0(1.1) |

[a] Numbers in parentheses are standard errors. [b] I corresponds to the anion (average of **3a** and **3b**), IH to the amphion (average of **2a** and **2b**), and $IH_2$ to the catioin (**1b**).

and in ethanol/water solution as was possible for **1b** because these species are only directly observable in ethanol/water. However, Figure 3 compares the room temperature $^{15}$N chemical shift behavior of histidine as a function of pH in 80% ethanol/water to that in 100% water. The titration curves are remarkably similar. The lines show the best fit of the data to eq 12 (see Experimental Section) with the adjustable parameters and their fitted values given in Table II. At most there may be a small effect on $pK_{a1}$ and $pK_{a2}$ (lower in ethanol by ~0.2 $pK_a$ units (Figure 3 and Table II). The observed weighted averaged $^{15}$N chemical shifts for the $N^{\delta 1}$ and $N^{\epsilon 2}$ atoms in the histidine cation ($IH_2$, structure **1b**), the amphion (IH, structures **2a** and **2b**), and the anion (I, structures **3a** and **3b**) are remarkably similar in 80% ethanol/water, as is the apparent tautomeric equilibrium of both the amphion and anion samples. The titration curve observed in ethanol solution could not be so similar to that observed in water if the >N: type nitrogens were substantially affected by the solvent unless the >N–H type nitrogens were affected equally in magnitude but opposite in direction. This is unlikely, on the basis of the $^{15}$N shifts of **1a** in 80% ethanol/water versus water, and we therefore conclude that 80% ethanol/water solution does not substantially affect the $^{15}$N shifts of the nitrogen atoms of the pure tautomers.

Nonaqueous solvents, however, have been shown to affect the $^{15}$N chemical shifts of both the >N–H type and >N: type nitrogens.[14] Using *N*-methylimidazole to model the shifts of the nitrogen atoms of the pure tautomeric forms of histidine, Schuster and Roberts[14] have found that solvents of decreasing dielectric (or decreasing H-bonding ability) tend to induce upfield chemical shift changes in the >N–H type nitrogen (i.e., the $CH_3$–N< nitrogen) and downfield chemical shift changes in the >N: type nitrogen. For example, the methylated nitrogen of *N*-methylimidazole resonates at 211.5 ppm in water, 212.5 ppm in methanol, 215.1 ppm in chloroform, and 215.7 ppm in benzene. The unprotonated or the >N: type nitrogen resonates at 128.5 ppm in water, 127.5 ppm in methanol, 119.3 ppm in chloroform, and 111.4 ppm in benzene. Note that these results agree with the present results with histidine in two important respects. *First*, the $^{15}$N chemical shifts of *N*-methylimidazole in water or in methanol are quite close to those we observe for the individual tautomers of histidine in water or in 80% ethanol. Compare the shift of 211.5 ppm for the $CH_3$–N< type nitrogen of *N*-methylimidazole with those of 211.6 and 208.3 ppm for the N–H type nitrogens of histidine, and the shift of 128 ppm for the >N: type nitrogen of *N*-methylimidazole with those of 128.5 and 131.6 ppm for the >N: type nitrogens of histidine (Table I). This confirms that *N*-methylimidazole is a good model for the shifts of the individual tautomers of histidine. *Second*, Shuster and Roberts' results also show that an alcoholic solvent, i.e., methanol, does not substantially affect the $^{15}$N shifts of either the >N–H type (211.5 ppm for water versus 212.5 ppm for methanol) or the >N: type (128.5 ppm for water versus 127.8 ppm for methanol) nitrogens. Table I compares the shifts of the individual tautomers of histidine observed at low temperature with those of *N*-methylimidazole in various solvents and with those of histidine at room temperature under fast exchange conditions.

## Discussion

**Intramolecular Hydrogen Bonding and Tautomeric Equilibrium.** The $^{15}$N chemical shift data clearly demonstrate that deprotonation of the α-amino group of histidine ($pK_a$ = 9.38) induces a shift in the tautomeric equilibrium in the direction of the $N^{\delta 1}$–H tautomer, i.e., **3a** becomes not so strongly favored over **3b** as **2a** is over **2b**. To explain this phenomenon, Blomberg and coworkers[12] have proposed that the ammonium group of the histidine amphion forms a H bond with the pyridine-like $N^{\delta 1}$ nitrogen of the $N^{\epsilon 2}$–H tautomer, **2a**, thereby stabilizing it with respect to the $N^{\delta 1}$–H tautomer, **2b**. Thus, deprotonation of the α-amino group at high pH to form the histidine anion releases this interaction thereby triggering a shift in the tautomeric equilibrium in the direction of the $N^{\delta 1}$–H tautomer, **3b**. X-ray and neutron crystallographic studies[29,30] support the existence of this H bond by showing the ammonium group to be within hydrogen bonding distance of $N^{\delta 1}$ in crystals of the histidine amphion.

Past $^{15}$N NMR studies have demonstrated that H bonding can induce fairly large changes, as much as ~10 ppm, in the chemical shift of the involved imidazole ring nitrogen atom.[13,14,16-19] Pyridine type ring nitrogen atoms acting as H-bond receptors are shifted upfield, whereas pyrrole type ring nitrogens acting as the H-bond donors are shifted downfield. Figure 1 shows that $N^{\delta 1}$ resonates at 129.1 ppm in **2a** and at 128.5 ppm in **3a**. Thus, no more than a 0.6 ppm upfield shift in the position of $N^{\delta 1}$ can be assigned to the H bond from the ammonium group. The magnitude of the perturbation is small compared to H bond induced perturbations observed previously and therefore indicates that the proposed H bond between the α-amino group and $N^{\delta 1}$ of **2a** is either very weak or absent.

A relatively weak hydrogen bond, however, may be sufficient to account for the increased stabilization of **2a** over **2b** relative to **3a** over **3b**. From past estimates of tautomeric ratios,[12,15] it can be calculated that ionization of the α-amino group changes the tautomeric equilibrium constant by a factor of between 2.8 and 3.6, depending on the method used to estimate the ratio of tautomers. This corresponds to a stabilization energy of 0.6 to 0.8 kcal/mol, which is quite small compared to the 5–7 kcal/mol estimated for normal strength H bonds. Thus, the $^{15}$N NMR chemical shifts and the magnitude of the effect on the tautomeric equilibrium are in agreement in indicating that if an H bond links the α-amino group and $N^{\delta 1}$ of **2a** it must be a relatively weak one.

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An alternative explanation for the change in tautomeric equilibrium on depronation of the α-amino group is that it is simply the result of the difference in the electronic inductive effect of $NH_2$ relative to $NH_3^+$. The tautomeric equilibrium of the histidine anion, where the α-amino group is not able to donate a H bond to $N^{\delta 1}$, still favors the $N^{\epsilon 2}$–H tautomer. Moreover, N-blocked derivatives of histidine, such as Ac-Histidine, Gly-His-Gly, and Bacitracin, and 4-substituted imidazole derivatives (the 4 position corresponds to $C^{\gamma}$ in the crystallographic designation for the histidine imidazole ring system), such as 4-methylimidazole, all of which preclude a stabilizing H bond interaction, also favor the $N^{\epsilon 2}$–H tautomer.[31,32] Thus, stabilization of the $N^{\epsilon 2}$–H tautomer in the histidine anion and in the other derivatives listed above must be the result of the electronic inductive effect of the substitution at $C^4$ ($C^{\gamma}$). Correlation of Hammett σ constants with tautomeric equilibrium constants for 4-substituted imidazoles has led to the conclusion that electron withdrawing substituents favor the $N^{\epsilon 2}$–H tautomer.[33] If the negative inductive effect of the substitution at $C^4$ is responsible for the $N^{\epsilon 2}$–H being the preferred tautomer in the anion, then the inductive effect should be even greater for the amphion where $NH_3^+$ with a σ constant of 0.6 replaces $NH_2$ with a σ constant of 0.1.[33] However, the α-amino group is too far from the imidazole ring for its ionization to have a substantial inductive effect on the ring, and therefore the explanation we favor is that a H bond as proposed by Blomberg and co-workers[12] does exist and that it is strong enough to affect the tautomeric equilibrium but not strong enough to cause a measurable displacement of the $^{15}N$ chemical shift of $N^{\delta 1}$.

$^{15}N$ chemical shift evidence, however, supports the existence of a stronger H bond between the α-amino group and $N^{\delta 1}$ of histidine for histidine in the dry powder state.[26] In dry powders $N^{\delta 1}$ of the amphion resonates at 133.5 ppm whereas the anion $N^{\delta 1}$ resonates at 127.5 ppm. Thus, about a 6-ppm perturbation can be directly attributed to the H bond. The difference in H-bond strength between solutions and dry powders may reflect the influence of water on this interaction. The hydration state has been shown to influence the tautomeric equilibrium as the $N^{\epsilon 2}$–H tautomer is even more favored in the gas phase than in solution.[34] This finding correlates with the solid-state $^{15}N$ NMR results as only signals from the $N^{\epsilon 2}$–H, **2a** and **3a**, were observed in dry powders of histidine.[26] Whether or not histidine in crystals experiences an environment more like that in dry powders or in aqueous solutions is arguable, but the tautomeric equilibrium in crystals clearly resembles that in the powders more closely as again only the $N^{\epsilon 2}$–H tautomer is present.[29,30] Taken together, the results indicate that the H bond between the α-amino group and the $N^{\delta 1}$ nitrogen of **2a** is relatively weak in aqueous solutions, as measured by its effect on both the tautomeric equilibrium and the chemical shift of $N^{\delta 1}$, but that this hydrogen bond becomes stronger in dry powders or in crystals owing to the changed environment in these states.

**Calculation of Tautomeric Equilibrium Constants from $^{15}N$ Chemical Shifts.** The $^{15}N$ chemical shifts of imidazole ring nitrogen atoms are obviously very sensitive to the presence or absence of a directly bonded proton. Therefore, in principle it should be possible to obtain an accurate and reliable determination of the position of the tautomeric equilibrium from the $^{15}N$ chemical shifts. However, using the averaged $^{15}N$ chemical shifts for this purpose requires a knowledge of the $^{15}N$ chemical shifts of the individual tautomeric forms. Although estimates for these values have been available from the shifts of methyl-substituted histidines, the uncertainty about how well these values reflect those of the individual tautomers of histidine has been sufficient to have led to the use of other parameters for estimating tautomeric ratios of histidine and other imidazoles.[12,15] Another obstacle to using the averaged $^{15}N$ chemical shifts to calculate tautomeric ratios of histidine in the past has been the uncertainty regarding how much the putative H bond with the α-amino group affected the averaged chemical shift of $N^{\delta 1}$.

Now that we know this hydrogen bond has at most an insignificant 0.6-ppm effect on the chemical shift of $N^{\delta 1}$ in aqueous solutions and that $^{15}N$ shifts of the individual tautomeric forms of histidine are available, it should prove useful to evaluate the tautomeric equilibrium constant of the amphion and anion forms of histidine from the observed averaged $^{15}N$ chemical shifts and compare the results to those obtained using other parameters. Past estimates of the tautomeric equilibria for histidine have been summarized and discussed by Alei and co-workers.[15] Expressed as mole fraction of $N^{\delta 1}$–H tautomer, the estimates range from 0.11, based on $^2J_{N^{\alpha}H^{\alpha}}$, to 0.24, based on $^3J_{C^{\beta}C^{\alpha}}$, for the histidine amphion and from 0.2, based on $^2J_{N^{\alpha}H^{\alpha}}$, to 0.5, based on $^3J_{C^{\beta}C^{\alpha}}$, for the histidine anion. Alei and co-workers[15] concluded that $J_{C^{\alpha}N^{\alpha}}$ was the parameter best suited for estimating the tautomeric equilibria because it gives the most consistent results. Calculations with this parameter yield 0.21 and 0.43 for the mole fraction of $N^{\delta 1}$–H tautomer in the histidine amphion and anion, respectively.

In solutions of pH ~8.2 where the histidine amphion predominates, $N^{\delta 1}$ and $N^{\epsilon 2}$ resonate at 143.2 and 198.3 ppm, respectively. Because we have not directly observed **2b** in $^{15}N$ NMR spectra, the mole fraction calculations below for both the amphion and the anion equilibria are based on the chemical shifts of the anionic species, **3a** and **3b**. Thus with use of the values of 128.5 and 208.3 ppm for $^{15}N^{\delta 1}$ in **2a** and **2b**, respectively, the averaged $N^{\delta 1}$ shift indicates the mole fraction of the $N^{\delta 1}$–H tautomer in the amphion to be 0.18. This value agrees reasonably well with that of 0.16 mol fraction of $N^{\delta 1}$–H tautomer calculated from the observed averaged $^{15}N^{\epsilon 2}$ shift using the values of 131.6 and 211.6 ppm for $N^{\epsilon 2}$ in **3a** and **3b**. The agreement between $N^{\delta 1}$ and $N^{\epsilon 2}$ based calculations is even better for the histidine anion where the observed $^{15}N$ chemical shifts for $N^{\delta 1}$ and $N^{\epsilon 2}$ of 156.7 and 183.9 ppm, respectively, both yield 0.35 for the mole fraction of $N^{\delta 1}$–H tautomer. The slightly less than perfect agreement between the $N^{\delta 1}$ and $N^{\epsilon 2}$ based calculation with the amphion, compared to the perfect agreement with anion, may reflect the presence of the α-amino-$N^{\delta 1}$ H bond which was not taken into account in the calculation. The effect of the H bond can be ignored as the agreement between $N^{\delta 1}$ and $N^{\epsilon 2}$ based calculation for the amphion is still very good and any change resulting from adjustments for the H-bond effect would be insignificant for most purposes.

The 0.35/0.65 $N^{\delta 1}$–H/$N^{\epsilon 2}$–H tautomeric ratio calculated for the histidine anion in aqueous solutions at 25 °C is somewhat larger than that of 0.25/0.75 estimated for histidine at −55 °C in 80% ethanol from the relative intensities of the signals from the slowly exchanging individual tautomers. Apparently the $N^{\epsilon 2}$–H tautomer is even more favored under the conditions used in the low-temperature work. This perhaps explains why the $N^{\delta 1}$–H of the histidine amphion tautomer was not observed as its abundance should be even less than the 0.16 mol fraction observed at 25 °C.

The $^{15}N$ chemical shift derived tautomeric equilibrium constants for the amphion and anion forms of histidine fall in about the middle of the range of values calculated from other parameters. The consistency between the $N^{\delta 1}$ and $N^{\epsilon 2}$ based calculations verifies that the $^{15}N$ shifts observed for the individual tautomers at low temperature in 80% ethanol are valid at 25 °C in $H_2O$. Because of the very large chemical shift differences between protonated and unprotonated nitrogens, we believe that the $^{15}N$ chemical shifts are the best parameters for determining tautomeric

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equilibrium constants for histidine and other imidazoles in general. They are certainly the best parameters for histidyl residues in proteins because the large size of most proteins, and the consequent wide NMR line widths, will make it extremely difficult or impossible to reliably measure the relatively small coupling constants.

It should be noted, however, that the above "canonical" $^{15}N$ chemical shift values for the nitrogen atoms in the individual forms of histidine, used in the above for calculating tautomeric equilibrium constants for histidine, for evaluating the strength of the histidine intramolecular H bond, and for gaining information about H-bond interactions of histidyl residues in proteins, pertain only to histidine or histidyl residues in aqueous or aqueous-like environments. Alcoholic solvents such as methanol or ethanol are apparently sufficiently aqueous-like to have only insignificant effects on these "canonical" $^{15}N$ chemical shifts (Table I). However, solvents of lower dielectric, such as chloroform or benzene, substantially alter the "canonical" values (Table I) as judged by the effects of these solvents on the $^{15}N$ shifts of *N*-methylimidazole (Table I).

The origin of the solvent effect on the $^{15}N$ shifts is not entirely clear, but inspection of the data in Table I suggests that it may reflect the H-bonding properties of the solvent. For example, methanol or ethanol, with dielectric constants of about one-half that of $H_2O$, do not induce much of a change in the chemical shift of either the >N–H type or the >N: type nitrogen, but DMSO with a dielectric constant similar to that of methanol or ethanol induces about a 12 ppm downfield chemical shift change in the >N: type nitrogen and yet does not affect the shift of the >N–H type nitrogen. The explanation may be that alcoholic solvents can act as either H-bond donors (to the >N: type nitrogen) or acceptors (of the >N–H type nitrogen) and can do so with an ability similar to that of $H_2O$, thus no solvent induced chemical changes are observed in alcohols. In contrast, DMSO is a better H-bond acceptor than H-bond donor and therefore can substitute for $H_2O$ only as an H-bond acceptor of the >N–H type nitrogen, but not as an H-bond donor to the >N: type nitrogen. Thus the >N: type nitrogen is significantly affected by this solvent whereas the >N–H type nitrogen is not. Both nitrogen types, however, show substantial solvent shifts on going to chloroform and benzene solvents (Table I), with the N–H type nitrogen having essentially the same shift in chloroform and benzene, about 4 ppm upfield from its position in water, while the >N: type nitrogen has substantially different shifts in the two solvents, 119.3 ppm in chloroform and 111.4 ppm in benzene (Table I). Again, the H-bonding properties provide a possible explanation for the above results. Benzene and chloroform are of course much less able to act as either a H-bond donor or an acceptor than $H_2O$ or alcohol, thereby explaining the upfield movement of the >N–H type nitrogen and the downfield movement of the >N: type nitrogen in these solvents. Furthermore, the chemical structure of these solvents suggests that they would be about equally poor in acting as an H-bond acceptor, but that chloroform might be a better H-bond donor than benzene, thus explaining why the >N: type nitrogen is found about 8 ppm further upfield in chloroform than in benzene while the >N–H type nitrogen has similar chemical shifts in these two solvents. Understanding the effects of solvents on the $^{15}N$ shifts of imidazole ring nitrogens is an area that needs further investigation. The implications, however, for interpreting $^{15}N$ chemical shifts observed for histidyl residues in proteins should be clear. Histidyl residues located in the interior of a protein may be in an environment more like chloroform than water or alcohol and therefore the canonical $^{15}N$ chemical shift values for histidyl residues in aqueous or aqueous-like environment may not apply. With carefully designed experiments, this sensitivity of the $^{15}N$ shifts to hydrophobic environments perhaps can be exploited to investigate the physical properties of protein interiors.

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