Short Communications

pH-Dependence of ^{13}C Chemical Shifts and $^{13}\text{C},\text{H}$ Coupling Constants in Imidazole and L-Histidine

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The pH-dependence of selected ^{13}C chemical shifts reflects the state of ionization of the imidazole ring in both imidazole and L-histidine. Titration of the amino and carboxyl groups of histidine also perturbs the shifts. The coupling constants $^1J(^{13}\text{C}_{(2)},\text{H})$ and $^1J(^{13}\text{C}_{(5)},\text{H})$ for both compounds also vary with pH, but in L-histidine these constants are relatively insensitive to the titration of groups outside the imidazole ring.

The application of carbon nuclear magnetic resonance (^{13}C n.m.r.) to studies of amino acids, peptides and proteins has been demonstrated in several instances. Histidine in particular possesses certain characteristic resonances that, coupled with its normally low abundance in polypeptides and proteins, render possible studies on the environment of the residue in these molecules. From the ^{13}C chemical shift–pH profiles of imidazole, histidine, 1-methylhistidine and 3-methylhistidine, Reynolds *et al.* (1973a) have shown that the predominant tautomeric form of the imidazole ring in histidine in basic solution is the 1-H tautomer* (II, below), and they estimated the degree of predominance as about 4:1:

Similar studies (Deslauriers *et al.*, 1974) have shown the 1-H tautomer to be the predominant form in the tripeptide thyrotrophin-releasing factor. These and other studies (Horsley & Sternlicht, 1968; Christl & Roberts, 1972; Freedman *et al.*, 1973; Quirt *et al.*, 1974; Tran-Dinh *et al.*, 1974) have demonstrated that the pH-dependence of chemical shifts reflects the titration of functional groups within the molecule. Typically, apparent pK_a values of $\sim 6.3 \pm 1.0$ are observed for the imidazole ring, along with downfield

shifts of approx. 2.5 p.p.m. on going from tautomer (I) to tautomer (II), when the $\text{C}_{(2)}$ chemical shift is monitored. Changes in the $\text{C}_{(2)}$ chemical shift are also observed on deprotonation of the amino and carboxyl groups of histidine (0.29 and 0.32 p.p.m. respectively, both upfield), but that nevertheless reflect the expected pK_a values of these groups. In contrast, the $\text{C}_{(5)}$ chemical shift of histidine is much more sensitive to the titration of the amino and carboxyl groups (1.79 p.p.m. downfield and 0.56 p.p.m. upfield respectively, compared with 0.95 p.p.m. upfield on deprotonation of the imidazole ring). On the basis of these observations and of CNDO/2 calculations, Reynolds *et al.* (1973b) suggested that these changes in ^{13}C chemical shifts can be accounted for by a π -polarization mechanism where the observed chemical shifts in the aromatic system are dependent on the distance of the charged groups from the conjugated π -electron system.

The low natural abundance of ^{13}C generally precludes detailed investigation of individual amino acid residues of proteins. Recently, however, the $\text{C}_{(2)}$ carbon of histidine residues in tryptophan synthetase (Browne *et al.*, 1973) and in α -lytic protease (Hunkapiller *et al.*, 1973) have been selectively isotopically enriched with ^{13}C . In the protease both the $\text{C}_{(2)}$ chemical shift and the carbon–hydrogen coupling constant, $^1J(^{13}\text{C}_{(2)},\text{H}_{(2)})$, were monitored as a function of pH. Although the behaviour of the $\text{C}_{(2)}$ chemical shift corresponded closely to the titration curve of the imidazole ring (apparent pK_a 6.75), the coupling constant $^1J(^{13}\text{C}_{(2)},\text{H}_{(2)})$ remained essentially constant in the pH range 5.2–8.2. This latter observation led the authors to conclude that the histidine residue in α -lytic protease remained unprotonated throughout this pH range, and that the observed chemical-shift behaviour was due to the titration of a neighbouring aspartate residue in the catalytic site. This implies that the coupling constant might provide a

* Alternatively, the τ -H tautomer [Biochem. J. (1972) 126, 775].

better measure of the state of ionization of the imidazole ring than the chemical shift. However, there have been few studies reported on the pH-dependence of coupling constants and the effects of neighbouring groups on this parameter. We have measured the pH-dependence of the $^{13}\text{C}_{(2)}$ and $^{13}\text{C}_{(5)}$ chemical shifts and also the coupling constants $^1J(^{13}\text{C}_{(2)},\text{H})$ and $^1J(^{13}\text{C}_{(5)},\text{H})$ in both imidazole and histidine. We were particularly interested in examining the influence of carboxyl- and amino-group titrations on the $^1J(\text{C},\text{H})$ values in histidine.

Materials and methods

Imidazole and L-histidine were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Solutions were made up in 0.2M-KCl solutions. Imidazole concentration was 3.5M in all cases. L-Histidine concentrations were in the range 0.2–0.5M, depending on the pH. The pH of each solution was adjusted to the desired value with HCl or NaOH solutions, and measured at 35°C before and after the n.m.r. run.

N.m.r. measurements were carried out at approx. 35°C on a Varian CFT-20 spectrometer, with data acquisition times of 2.925s or longer (1 data point every 0.34Hz). Sensitivity enhancements were always 0.3s or longer, thus ensuring that the linewidths were not broadened by more than approx. 1Hz. A coaxial tube containing $^2\text{H}_2\text{O}$ provided the necessary deuterium lock for the CFT-20 spectrometer. All chemical shifts are accurate to at least ± 0.1 p.p.m., and the coupling constants are accurate to ± 0.7 Hz or better.

Results and discussion

The chemical shift of the $\text{C}_{(2)}$ and $\text{C}_{(5)}$ carbon atoms of histidine as a function of pH are given in Figs. 1(a) and 1(b) respectively. All chemical shifts are given in p.p.m. relative to the chemical shift observed for the $\text{C}_{(2)}$ carbon at pH 11.0. Chemical shifts to high field of this arbitrary zero are given negative values. The results obtained here are in good agreement with those obtained by Quirt *et al.* (1974).

Fig. 1. pH-dependence of ^{13}C n.m.r. parameters of L-histidine
(a) $\text{C}_{(2)}$ chemical shift; (b) $\text{C}_{(5)}$ chemical shift; (c) $^1J(\text{C}_{(2)},\text{H})$; (d) $^1J(\text{C}_{(5)},\text{H})$.

Table 1. $^1J(C,H)$ values for imidazole

^{13}C -proton nuclear spin-spin coupling constants for imidazole and the imidazolium cation are shown.

	$^1J(C_{(2)},H)$ (Hz)	$^1J(C_{(5)},H)$ (Hz)
Imidazole (neutral)*	208.8 ± 0.5	190.8 ± 0.5
Imidazole (cation)†	222.0 ± 1.5	202.5 ± 0.5

* Values at pH 10.0.

† Values at pH 4.5.

Plots of the one-bond $^{13}\text{C},\text{H}$ coupling constants $^1J(C_{(2)},H)$ and $^1J(C_{(5)},H)$ in histidine against pH are given in Figs. 1(c) and 1(d). From Figs. 1(a)–(d) it is evident that both the ^{13}C chemical shifts and $^1J(C,H)$ values reflect the ionization of the imidazole ring of histidine in the pH range 4–8. Also, within experimental error the same $\text{p}K_a$ value (6.25 ± 0.2) is obtained from each of the four curves. Observed $^1J(C,H)$ values for imidazole are summarized in Table 1. Plots of $^1J(C_{(2)},H)$ and $^1J(C_{(5)},H)$ against pH for imidazole gave a sigmoid titration curve, with an apparent $\text{p}K_a$ 6.9, similar to the curves shown in Figs. 1(c) and 1(d). For histidine it is important to note that the coupling constants $^1J(C_{(2)},H)$ and $^1J(C_{(5)},H)$ are relatively insensitive to the titration of the amino and carboxyl groups. Although $^1J(C_{(5)},H)$ shows some change on titration of the amino and carboxyl groups, the relative magnitude of this change is small; for $^1J(C_{(2)},H)$ changes in these regions are not observed, within experimental error.

The insensitivity of $^1J(C_{(2)},H)$ and $^1J(C_{(5)},H)$ to the ionization of the amino and carboxyl groups in histidine may be qualitatively rationalized by the following simple argument. It is well known that directly bonded ^{13}C -proton coupling constants are completely dominated by the Fermi contact mechanism (Maciel *et al.*, 1970). Further, excellent correlations between observed $^1J(^{13}\text{C},\text{H})$ values and values of $P_{\text{S}(\text{C})\text{S}(\text{H})}^2$ calculated by using semi-empirical MO calculations at the INDO level of approximation have been observed (Maciel *et al.*, 1970). Here $P_{\text{S}(\text{C})\text{S}(\text{H})}$ represents the bond order between the carbon $2s$ orbital and hydrogen $1s$ orbital. Since the $C_{(2)}$ and $C_{(5)}$ carbon atoms in histidine are at least four bonds away from the site where ionization of the amino and carboxyl group occurs, one would not expect these ionizations to cause a significant perturbation to the $\text{C}(2s)\text{--H}(1s)$ sigma bonds (relative

to the perturbation that occurs on ionization of the imidazole ring).

On the other hand the chemical shift of a nucleus depends in a complex manner on the orbital motion of the surrounding electrons. [See Raynes (1974) for a recent review.] A theoretical interpretation of the ^{13}C chemical shifts in histidine as a function of the state of ionization state has been given elsewhere [Quirt *et al.* (1974), and references cited therein]. It may be noted that proton chemical shifts of the $C_{(2)}$ proton of the histidine residue have also been observed to be very sensitive to the titration of remote groups [e.g. in proteins as well as in the amino acid itself (Cohen *et al.*, 1973)].

In conclusion, either chemical shifts or $^1J(C,H)$ values may be used to monitor the ionization of the imidazole ring. Our results indicate that the titration of a remote group appears to have little influence on the $^1J(C,H)$ values.

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