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pH and p*K* Determinations by High-Resolution Solid-State ^{13}C NMR: Acid−Base and Tautomeric Equilibria of Lyophilized L-Histidine

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Abstract: Acid−base properties of lyophilized powders of L-histidine have been systematically investigated using parent solutions at pH varying from 1.8 to 10. For the first time, high-resolution solid-state ^{13}C NMR was shown to allow separate observation of all three acid−base pairs in the successive deprotonations of the carboxylic end, the imidazolium cation, and the terminal ammonio group of histidine. ^{1}H CRAMPS NMR spectra directly visualize the absence of the $N_3-H(\pi)$ tautomer in neutral and anionic species. Solid-state titration shifts are enlarged by ∼1−4 ppm with respect to those measured in solution, permitting unambiguous observation of conjugate acid−base pairs. Calculated p*K*'s from solid-state acid-to-base ratios *r* are found equal to those classically measured in solution at 0 °C with a similar ionic strength of 0.1 mol dm^{-3}. This proves that natural-abundance ^{13}C solid-state determinations of *r* can be used to measure p*K*'s in parent solutions without recourse to full titration curves and subsequent curve-fitting procedures. Such an approach also leads to noninvasive characterizations of the acidity of lyophilized powders, i.e., to the prediction of in situ pH of products obtained after rehydration and solubilization of powders. These results show the possibility of measuring the p*K* of nonvolatile acidic substrates dissolved in any sublimable solvent through lyophilization of the investigated solutions; this leads the way to pH and p*K* determinations when electrochemical or spectrophotometric measurements are impossible or ambiguous, e.g., for concentrated solutions, polyacids, or mixtures of acidic solutes, and possibly to the establishment of p*K* scales in nonaqueous solvents and in melts.

Introduction

A cornerstone in physical and structural chemistry is the notion of acidity. This concept relies on the degree of protonation of a given basic site B into its conjugate acid BH, characterized by the molar ratio $r = [BH]/[B]$, from which practical pH and p*K* values are derived on ignoring activity coefficients in a first approximation. Under favorable circumstances where distinct and sharp lines may be observed for species B and BH, molar ratios *r* can be spectroscopically measured from appropriate line intensities. On the other hand, electrochemical measurements may yield the activity ratio. If electrochemical measurements are restricted to fluid solutions, spectroscopic determinations of *r* are quite conceivable in solids. In that perspective, solid-state NMR is especially convenient for this purpose because, in sharp contrast to high-resolution NMR in liquids, separate resonances may be obtained for protonated and unprotonated species due to slow inter- and intramolecular proton exchanges.[1−3] Such line separations were observed in early investigations using, first, ^{15}N NMR spectroscopy on powders of ^{15}N-enriched histidine and imidazole[1] and of α-lytic protease specifically ^{15}N-enriched at His-57[2] and, then, ^{13}C spectroscopy of histidine samples lyophilized at a pH close to the p*K* of the imidazole group.[3] However, the intensity ratio of the protonated and unprotonated ^{15}N lines was not found equal to the population ratio *r* of species B and BH[2]. This can be assigned to the fact that the acidic proton is direcly attached to the observed ^{15}N nucleus, which strongly enhances cross-polarization efficiency in the protonated species BH as compared to that in the unprotonated conjugate base B. ^{13}C nuclei are not directly linked to the acidic protons of histidine, so that differential cross-polarization undesirable effects are expected to bring about errors not exceeding the uncertainty margins which are usually obtained by solid-state ^{13}C NMR spectroscopy (see below).

Lyophilization was used to obtain solid samples from liquid solutions of histidine (see Experimental Section and Results). In fact, a solid matrix was obtained by freezing out the solution, which was the first step in the lyophilization procedure. As suggested by one reviewer, using frozen solutions themselves as samples for solid-state NMR studies might also have been appropriate. Although this is quite conceivable for nonaqueous solutions, or even, more generally speaking, for substrates

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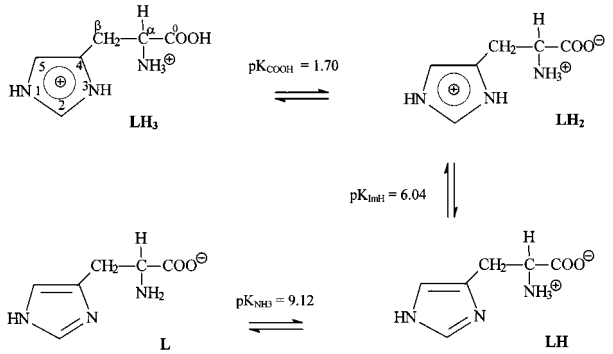
Scheme 1. Ionic Forms LH_3, LH_2, LH, and L of L-Histidine from pH 1.8 to 10



Scheme 2. Tautomers of Imidazole (R = H) and L-Histidine (R = $CH_2CHNH_3^+CO_2^-$)

Table 1. ^{13}C Chemical Shifts of Lyophilized L-Histidine

	carbon					
ionic form	C_0	C_α	C_β	C_2	C_4	C_5
LH_3[a]	170.6[b]	53.3	25.6	135.9	126.6	119.6
LH_2	177.1	55.1	28.2	137.5	129.8	118.4
LH	175.2	57.0	27.2	135.0	137.4	113.6
L	184.4	59.2	33.3	134.7	142.1	111.9

[a] For nomenclature, see Scheme 1. [b] ppm from TMS.

embedded in a solid nonaqueous matrix, for example, in phospholipid layers,[4] this is impossible in the case of aqueous solutions used in this first series of investigations. Abnormal proton mobility phenomena would bring about time averaging of B and BH lines in water by promoting fast intermolecular proton exchanges in the solid as well as in the liquid state. Indeed, there is some residual water in lyophilized powders, but in a too small amount (1−3%) to initiate concerted proton transfers along Grotthus chains of hydrogen-bonded water molecules. A first objective in the present study was therefore to explore the possibility of distinguishing acidic substrates from their conjugate bases in lyophilizates prepared from aqueous solutions over a large pH range, using as NMR probes ^{13}C nuclei in natural abundance. In the present investigations, aqueous solutions of L-α-amino-β-(4-imidazoyl)propionic acid[5] (L-histidine) have been chosen as a starting material. Besides its well-known biological importance as part of the active site in many enzymes, histidine is highly appropriate for model experiments because of the presence of four protonation sites, namely, the carboxylate and amino ends, and the tautomeric two nitrogen atoms N_1 (N-τ) and N_3 (N-π) in the imidazole ring (Schemes 1 and 2). Three successive deprotonations, namely, those of the carboxylic end, of the imidazolium cation, and of the terminal ammonio group, can thus be used to explore aqueous solutions from about pH 1.8 to 10.0 (Table 2).

Table 2. Molar Ratios *r* of Ionic Forms of L-Histidine and p*K*'s Calculated from Each Pair of ^{13}C Resonances over a Series of Lyophilizates Prepared at the Mentioned pH's

		carbon						
pH		C_0	C_α	C_β	C_2	C_4	C_5	average
1.79[a]	*r*	0.85	0.93	0.89	0.86	0.86	0.67	0.84 ± 0.08
	pK_{calc}	1.72	1.76	1.74	1.72	1.72	1.71	1.71 ± 0.04
2.02[a]	*r*	0.36	0.39	0.41	0.53	0.40	0.45	0.42 ± 0.05
	pK_{calc}	1.58	1.61	1.63	1.74	1.63	1.67	1.64 ± 0.05
5.56[b]	*r*	4.54	3.51	3.87	4.43	4.51	3.28	4.02 ± 0.50
	pK_{calc}	6.22	6.11	6.15	6.21	6.21	6.08	6.16 ± 0.05
5.91[b]	*r*	1.86	1.79	1.63	1.67	1.68	1.73	1.73 ± 0.08
	pK_{calc}	6.17	6.16	6.12	6.13	6.14	6.15	6.14 ± 0.02
6.45[b]	*r*	0.53	0.59	0.44	0.49	0.44	0.50	0.50 ± 0.05
	pK_{calc}	6.17	6.22	6.10	6.14	6.10	6.15	6.14 ± 0.04
9.25[c]	*r*	3.21	3.09	3.26	3.53	3.54	3.28	3.32 ± 0.16
	pK_{calc}	9.76	9.74	9.76	9.80	9.80	9.77	9.77 ± 0.02
9.64[c]	*r*	1.23	1.39	1.70	1.36	1.21	1.36	1.37 ± 0.16
	pK_{calc}	9.67	9.78	9.87	9.77	9.72	9.77	9.76 ± 0.06
10.02[c]	*r*	0.60	0.73	0.61	0.66	0.64	0.57	0.64 ± 0.05
	pK_{calc}	9.80	9.88	9.80	9.84	9.83	9.78	9.82 ± 0.03

[a] Ionization step: $LH_2 \rightarrow LH_3$. [b] Ionization step: $LH \rightarrow LH_2$. [c] Ionization step: $L \rightarrow LH$.

The second objective in these studies was to bring definite information on a basic problem, namely, whether there is conservation of p*K* values from parent solutions to their lyophilizates. Indeed, it may be wondered whether hydroxonium ions contained in the initial aqueous solution remain attached to residual water molecules or to the basic solute(s) B, possibly altering the acid-to-base ratio on solvent removal. One may also suspect the acid−base properties of solutes to be deeply changed after desolvation of species in the solid phase, as observed when shifting from an aqueous solution to a gas phase.[6−8] It was therefore necessary to perform accurate comparisons of acid-to-base ratios *r* in lyophilized histidine with those encountered in the parent solutions. Comparisons were preferably performed between p*K* values calculated from the molar ratios *r* measured in the lyophilizates and the corresponding values obtained in solution as primary information from electrochemical measurements (see Discussion). This comparison is indeed of fundamental importance, since a positive answer may lead to pH and p*K* determinations when electrochemical or spectrophotometric measurements are impossible or ambiguous. This is particularly the case for concentrated or viscous solutions, polyacids, or mixtures of monoacids or polyacids. Under favorable circumstances, these studies could even be extended to lyophilizates prepared from parent hydroorganic or nonaqueous solutions, thus bringing straightforward new information in the vast field of p*K* scales in aqueous and nonaqueous solvents or even, possibly, in melts.[9]

Experimental Section

Lyophilization. An 80-mg sample of free base L-histidine (Sigma Ultra >99%) or 30 mg of imidazole (Fluka >99%) was dissolved in bidistilled water, adjusting the volume to 10 mL (0.051 or 0.044 molar

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solutions). The pH was adjusted with NaOH and $HClO_4$ at 25 °C using an Orion 901 pH meter equipped with an Orion 91-03 electrode which was previously standardized with appropriate buffer solutions. Samples were frozen out in a 50-mL rotating flask immersed in liquid nitrogen, to obtain a thin solid layer presenting a great surface area. The flask was then detached from the rotating system (Büchi Rotavapor) and fitted to a lyophilizator Chris Alpha 1-4 for 10 h under heat and pressure regulation at −40 °C and 300 Pa. The water content in the lyophilizates was measured using the classical Karl Fischer method (Methrom E 547).

NMR Spectroscopy. $\{^1H\}$ ^{13}C High-resolution NMR was performed on a Bruker DRX 400 spectrometer at 100.6 MHz using aqueous solutions at 25 °C added with 5% D_2O (lock). Chemical shifts were referred to internal dioxane (δ = 67.4 ppm from TMS) and then converted to the TMS scale (estimated precision, 0.01 ppm). ^{13}C spectra of lyophilizates were recorded on a Bruker DSX 300 spectrometer at 75.46 MHz equipped with a 4-mm CP/MAS[10] probe using spinning frequencies between 5 and 8 kHz and cross-polarization contact time of 3 ms. Chemical shifts were expressed with respect to TMS using the CH_2 signal of adamantane (δ = 38.56 ppm) as an internal reference (estimated precision, ∼0.1 ppm). All samples were examined at a constant temperature of 25 °C to eliminate the possibility of temperature-dependent changes of the ^{13}C chemical shift tensor.[11] ^1H CRAMPS NMR spectra were recorded on a Bruker MSL 300 spectrometer at 300 MHz. Homonuclear multiple-pulse decoupling was achieved using the MREV-8 pulse sequence[12] with a 90° pulse width of 2.5 μs, a cycle time of 42 μs, and a spinning frequency of 3.5 kHz. A set of step-by-step tuneup procedures for accurate spectrometer adjustment was systematically applied.[13−17]

Results

Lyophilization. Any quantitative acid−base titration should satisfy a number of basic requirements, which, at first sight, seem incompatible with solid-state operations, namely: (i) a perfect equilibration between the titrated and titrating acid−base pairs; in practice, relative acidities are appreciated by opposing in a common mobile medium the relevant acid−base pair BH/B to a reference pair, which in many cases is the solvent itself, acting as a proton reservoir; (ii) the availability of a series of media with gradually increasing acidity level, to check the validity of mass action law and obtain the p*K* value(s) of the substrate.

As far as solid samples are concerned, an obvious procedure consists of putting the relevant solid in contact with a fluid medium containing a mobile titrant in variable concentration. Yet, it is clear that superficial acidities only can be measured in this way, unless the solid can be reduced to a finely grained powder, ideally ground up to the molecular level. However, surface acidities are highly relevant to catalytic operations, and NMR measurements involving gaseous un-ionized Lewis acids in contact with the solid substrate have been carried out to characterize the acidity of active sites on the surface of various catalysts.[18−22] The determination of acidities in the bulk solid seems to require the following two-step procedure: the solid sample is dissolved in an appropriate solvent which is maintained at a given pH (i). The solvent is removed (ii) in order to allow NMR measurements on the recovered solid. This procedure obviously requires acid−base solutes that are less volatile than the solvent. A major problem is the necessity of maintaining the acid-to-base molar ratio(s) *r* constant during solvent removal. A slow evaporation, as commonly used in fractional crystallization, involves the successive precipitation of solids in the sequence of their solubilities. In this case, the overall composition of the solid phases is continuously varying in the course of solvent removal; it becomes compulsory to pursue drying to obtain a composition of the solids that is as close as possible to that of the initial solution. However, the elimination of the last amounts of solvent is not an easy task: the lowering of solvent vapor pressure in the concentrated remaining liquid forces operation at a higher temperature, especially if one of the investigated compounds remains soluble, giving rise to oily end fractions, as is often the case for polymeric compounds and biologic substrates such as albumin. This may then result in unwanted chemical or biochemical degradation of the solid products and also in their partial vaporization from the solution. Moreover, the solids obtained in this way often contain residual solvent molecules. Finally, another drawback of the crystalline powders, as far as subsequent NMR analyses are concerned, remains the possibility of obtaining nonequivalent positions of individual molecular or ionic species in the crystal lattice[23−24] or even several allotropic crystals. This results in the appearance of several resonances for each individual chemical site, and line assignment to each component, B or BH, of the investigated acid−base pairs becomes highly ambiguous.

Most of these drawbacks can be circumvented by operations at low temperature, in which the solution is suddenly frozen out by immersion of the sample in liquid nitrogen. The solvent, typically water, is then removed by sublimation at low temperature and under reduced pressure. This is the well-known lyophilization procedure, widely used in organic chemistry, biochemistry, the food industry, pharmaceuticals, and medicine.[25−26] Besides the obvious advantages for the preservation of substrates, lyophilized products consist of amorphous powders that are highly suitable for solid-state NMR spectroscopy. Despite the low vapor pressures, which are necessary for sublimation, the solvent is easily removed, presumably because the sudden freezing out of the solution results in the deposition of metastable nearly pure solid phases without the possibility for extended solid solutions, thus avoiding the lowering of solvent vapor pressure due to concentration effects according to Raoult's laws, as mentioned above in the case of slow evaporation.

Operational pH Ranges. Lyophilizates were prepared from a series of L-histidine solutions at pH's ranging from about 2

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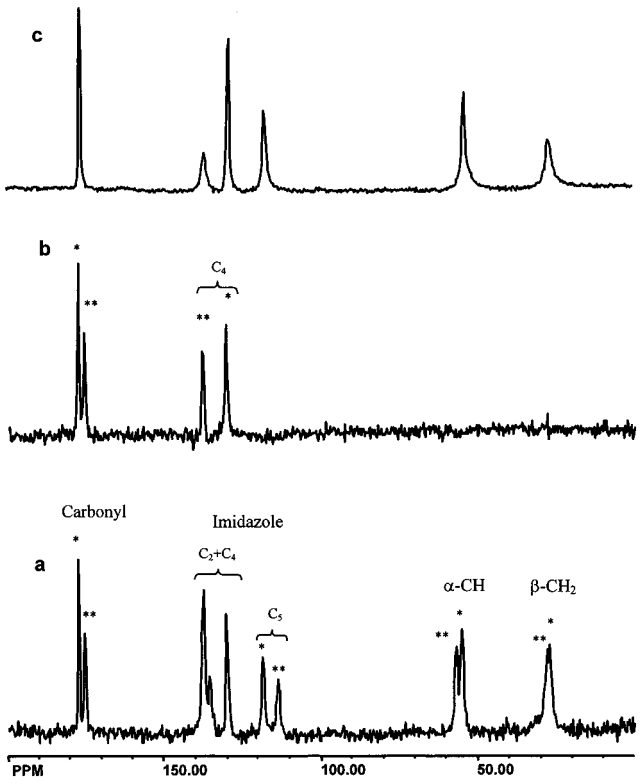

Figure 1. ^{13}C CP/MAS NMR spectra of L-histidine lyophilized from solutions at pH 5.91, without (a) or with (b) suppression of protonated carbons, and of pure L-histidine hydrochloride orthorhombic crystals (c). Starred lines refer to different states of protonation of histidine: LH_2 (*) and LH (**).

to 10. To be sufficiently accurate, NMR measurements require the acid-to-base molar ratio *r* to be between about 0.1 and 10. This means that spectroscopic measurements are restricted to a pH range of about two units about the p*K* of the investigated acid−base pair. In the case of L-histidine, three p*K*'s are to be considered, ignoring the problem of tautomerism in this section. As shown in Scheme 1, they are relative to the carboxylic end ($LH_3 \rightarrow LH_2$) $pK_{COOH} = 1.70$, to the imidazolium ion ($LH_2 \rightarrow$ LH) $pK_{ImH} = 6.04$, and to the ammonium end (LH → L) $pK_{NH_3} = 9.12$ in solution (see below). NMR measurements in solution would thus be restricted to three pH regions only, pH 2 ± 1, 6 ± 1, and 9 ± 1, respectively; these restrictions proved to fit for the solid case as well. An important point is that these pH ranges do not overlap: this will permit line assignments for each of the three relevant acid−base pairs separately. pH values mentioned in the following should be understood as those measured on the parent initial solutions.

Line Assignments. The main groups of lines in the lyophilizates can be identified by comparison with the corresponding resonances in solution due to the relatively small chemical shift variations from the liquid to the solid state; they refer to C_α, C_β, imidazolic C_2, C_4, C_5, and carboxylic C_0 carbons, respectively, when going from high to low fields (Figure 1a and Table 1). In the appropriate pH ranges, each carbon-13 atom in lyophilized L-histidine leads to the appearance of two signals, as shown at pH 5.91 in Figure 1a. When the resulting doublets are clearly resolved, the intensities of their components are in a common ratio for all carbons, reflecting a constant acid-to-base molar ratio *r*, $[LH_2]/[LH]$ in this example (see below). Despite large splittings, imidazolic C_2 and C_4 are so closely spaced that individual components cannot be clearly distinguished. This difficulty was overcome by using the dipolar dephasing method,[27] which suppresses the resonances from protonated carbons, thus leaving alone the C_4 doublet (Figure 1b). In the case of a monoacid, the two components in each pair of lines can be simply identified by a "continuity" method, on comparing lyophilizates from solutions at gradually higher pH and assigning acid and basic sites to lines whose intensities are decreasing or increasing, respectively. In the case of a polyfunctional compound such as histidine, some doubt remains as regards the nature of the observed acid−base pair, that is, whether acidic properties are changed or not from the liquid to the solid state. This is all the more necessary as an opposite assignment seems to have been previously suggested for the carboxylic doublet at pH ∼5−7.[3] Most ambiguities can be removed in the present example by recording the ^{13}C spectrum of pure L-histidine hydrochloride orthorhombic crystals, themselves prepared from the same solution at pH 5.91 by slow evaporation. Studies of these crystals by X-ray and neutron diffraction[28] have established their composition (species LH_2) and showed the existence of one crystallographic site per unit cell. The single resonance signals obtained from these crystalline structures (Figure 1c) are then compared to the pairs of associated resonance signals from the lyophilizate (Figure 1a) to unambiguously identify lines assigned to species LH_2 (starred lines). The other component in each doublet was then assigned to LH species (doubly starred lines). On increasing the pH from 5.91 (Figure 1a) to 6.45 (Figure 2b), LH lines grow at the expense of LH_2 lines, which themselves fully disappear above pH ∼7.5. Increasing the pH of parent solutions above 9 results in the appearance of a new set of lines (triply starred) at the expense of species LH (Figure 2c and d), assigned to the fully deprotonated anionic species L by continuity. Conversely, decreasing the pH of parent solutions below 5.91 first results in the disappearance of species LH and then the appearance around pH 2 of a new set of resonances (lines marked +) at the expense of species LH_2, which were then assigned to the fully protonated cation LH_3 (Figure 2a).

pH Dependence of NMR Spectra. The ^{13}C chemical shifts of the four chemical species LH_i ($i = 0$ to 3) involved in the three acid−base equilibria of L-histidine were measured over their respective operational pH ranges (see above). A series of nearly identical chemical shifts was thus obtained for each chemically nonequivalent carbon within each species (Figure 3). Standard deviations for each set of chemical shifts are less than 0.01 ppm, and chemical shifts reported in Table 1 were obtained as their arithmetic means. The successive steps of deprotonation are accompanied by stepwise variations of chemical shifts which are large for carboxyl C_0 carbon (by 13.8 ppm from LH_3 to **L**) and quaternary C_4 imidazolic carbon (15.5 ppm), medium-sized for C_α (5.9 ppm), C_β (7.7 ppm), and imidazolic C_5 (7.1 ppm) carbons, and small for imidazolic C_2 carbon (2.0 ppm). Integrated line intensities of corresponding resonance signals in the lyophilizate have been measured at eight pH's of the parent solution. The ratio of the integrated intensities for each carbon in the relevant acid−base pair LH_i/LH_{i-1} ($i = 1, 2, 3$) at the mentioned pH directly yields the corresponding molar ratio *r* (Table 2). At each pH of the parent solution, six

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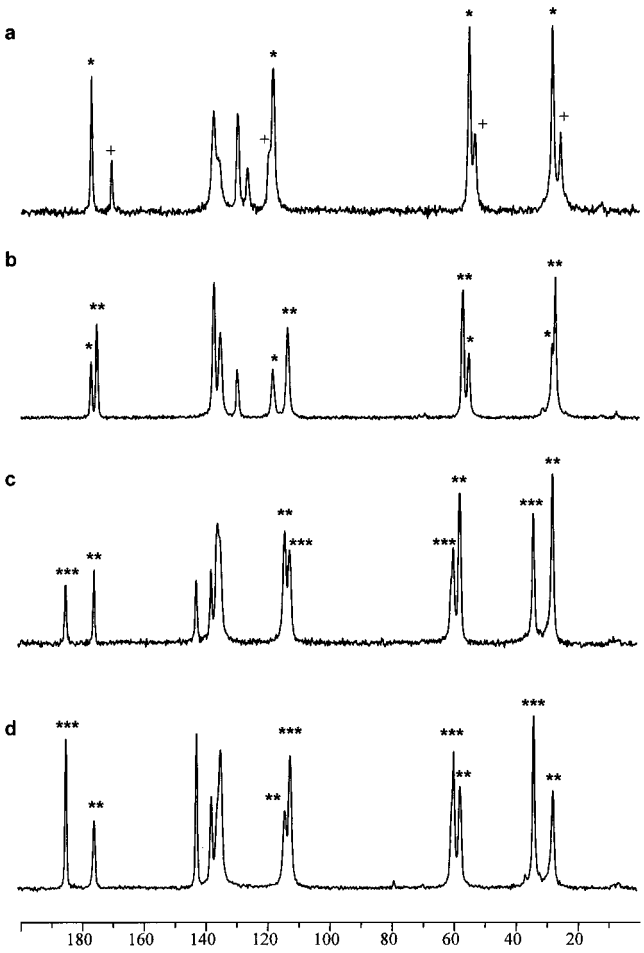

Figure 2. ^{13}C CP/MAS NMR spectra of L-histidine lyophilized from solutions at pH 2.02 (a), 6.45 (b), 9.60 (c), and 10.02 (d). Marked lines refer to species LH_3 (+), LH_2 (*), LH (**), and L (***).

independent determinations of *r* were thus carried out using each of the six available ^{13}C resonances. Averaged values and standard deviations to the mean are displayed in the last column of Table 2 for each of the eight pH's. It can be seen that the precision of measurement ranges from 4.6 to 12.4%, which is a typical order of accuracy for ^{13}C solid-state NMR measurements.

An important point to notice is the random character of the distribution of *r* values over the six carbons of histidine given in Table 2: deviations of individual data to the mean on each row of Table 2 are not appearing in a same order according to their sign (above or below the mean) or magnitude. Such random distributions of errors strongly suggest the absence of systematic and significant deviations due to cross-polarization effects, which could invalidate population ratios *r*. This point deserves some more comments. First, the set of protons covalently attached to carbon atoms is left unchanged in protonation/deprotonation processes. Magnetization transfer occurring in cross-polarization processes under Hartmann−Hahn conditions[29] is based on dipolar interactions between ^{13}C and ^{1}H nuclei.[13] Such interactions are very strong for carbons bearing protons, which is the case for the secondary C_β and the tertiary C_α, C_2, and C_5 carbons in histidine. For all of them, a coherent magnetization transfer is achieved, essentially in the same manner, in a few tens of microseconds. This short initial period is followed by an equilibration of magnetization within the proton system via a spin-diffusion mechanism. The whole process requires a magnetization transfer period, the so-called contact time, typically 1 ms. In rigid organic solids, due to a huge difference in natural abundance of protons and carbon-13 atoms and to a very efficient intra- and intermolecular spin exchange between protons, small changes in the number of protons of a given species in protonation/deprotonation processes are not able to change by any means the final cross-polarization enhancement. As mentioned above, our studies only require the ratio of line intensities in each doublet, which is representative of one given ^{13}C nucleus in both conjugated species **BH** and **B**. Possible differences in cross-polarization dynamics for chemically inequivalent carbons are therefore immaterial for our purposes. As far as quaternary carbons are concerned, an appropriate contact time was determined by checking that the ratio *r* remained constant for contact times greater than 1 ms. Although they do not bear directly attached protons, quaternary carbons C_0 and C_4 yield cross-polarization enhancements and *r* values in line with those from secondary and tertiary carbons. In our case, even at relatively high spinning speeds, the contact time of 3 ms is long enough to achieve complete and uniform magnetization transfers from the proton reservoir to all ^{13}C nuclei of histidine, being simultaneously not too long to suffer from subsequent relaxation effects.

Discussion

Protonation Shifts. Titration shifts can be obtained by subtracting chemical shifts of species LH_{i-1} from those of species LH_i, line by line in Table 1. Three solid-state protonation shifts Δ_i^s ($i = 1-3$) corresponding to carboxyl ($LH_2 \rightarrow LH_3$), imidazole ($LH \rightarrow LH_2$), or amino ($L \rightarrow LH$) titrations, respectively, are thus obtained for each carbon. Upfield shifts due to protonation are defined as positive, and each of them is compared to the value measured in the parent solutions (Table 3). ^{13}C NMR studies of amino acids, peptides, and proteins in solution indicate that protonation of either a carboxyl or an amino group generally results in upfield shifts of ^{13}C resonances, whose magnitude decreases as the number of bonds between the observed nucleus and the site of protonation increases.[31−34] However, upon amino titration of amino acids, larger ^{13}C upfield shifts are observed for C_β than for C_α carbons. Other noticeable points are the very large protonation shifts observed for carboxyl and bridgehead aromatic carbons in both carboxyl and amino titrations. Various attempts were made to account for these trends, using either electric field effect theory[32,35] or semiempirical[31] and ab initio[36] calculations. However, despite great progress in this field, rms deviations of ∼6 ppm are still found between experimental and predicted chemical shifts,[36] which represents the same order of magnitude as titration shifts

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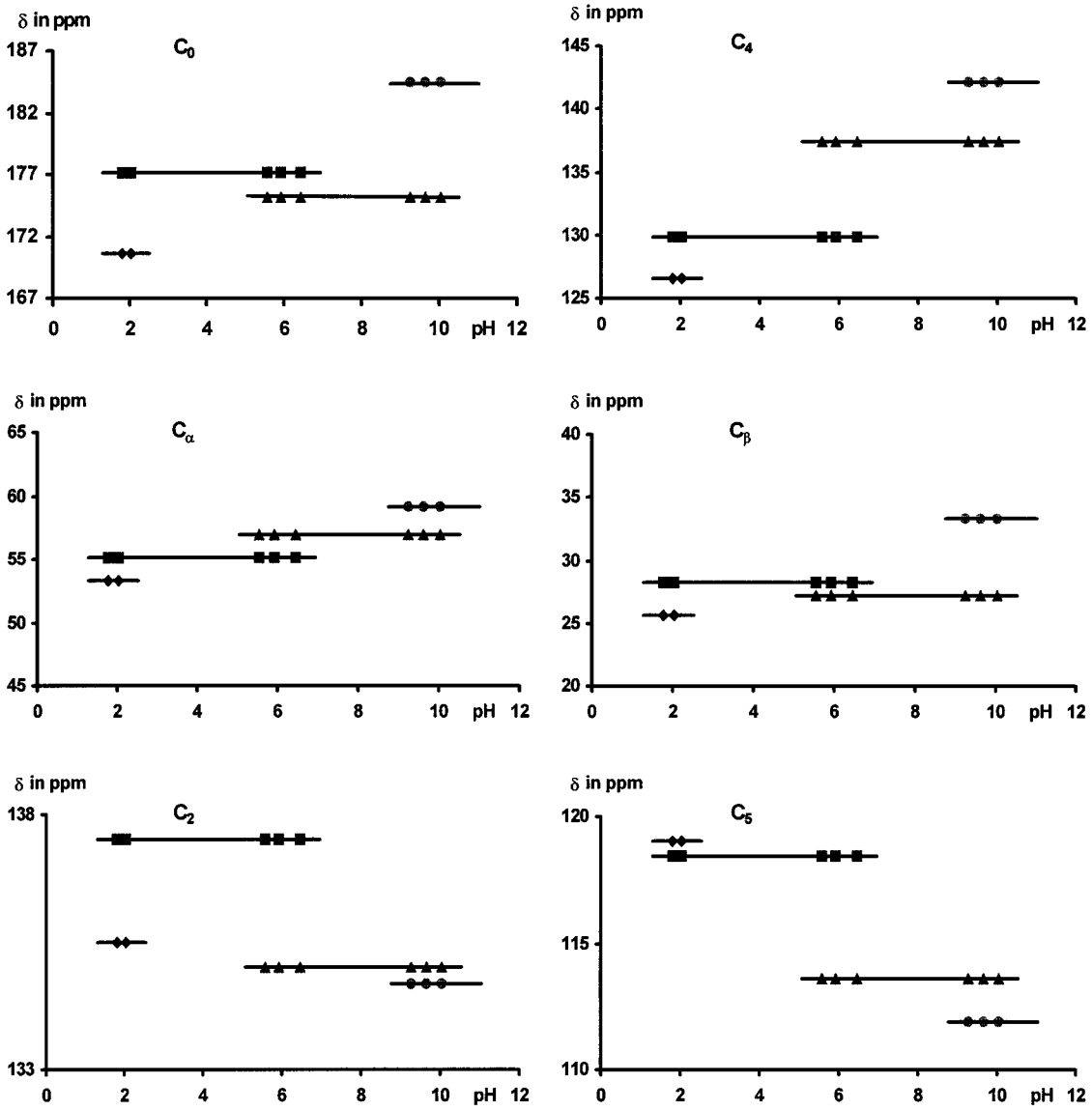

Figure 3. ^{13}C chemical shifts of lyophilized L-histidine in species LH_3 (◆), LH_2 (■), LH (▲), and L (●), as a function of the pH of parent solutions.

Table 3. Titration Shifts of L-Histidine in Three Ionization Steps, Measured Either in the Solid State (Δ_1^s, Δ_2^s, Δ_3^s) or in Solution (Δ_1^l, Δ_2^l, Δ_3^l)

		carbon					
ionization step		C_0	C_α	C_β	C_2	C_4	C_5
$LH_2 \rightarrow LH_3$	Δ_3^s	+ 6.5[a]	+ 1.8	+ 2.6	+ 2.4	+3.2	− 1.2
	Δ_3^l	+2.33	+0.79	+1.64	−0.32	+1.11	−0.56
$LH \rightarrow LH_2$	Δ_2^s	−1.9	+1.9	−1.0	−2.50	+7.6	−4.8
	Δ_2^l	+1.32	+1.10	+2.20	+2.40	+4.80	−0.70
$L \rightarrow LH$	Δ_1^s	+9.2	+2.2	+6.1	−0.3	+4.7	−1.70
	Δ_1^l	+8.10	+0.90	+3.90	−0.60	+1.60	+0.80

[a] ppm; upfield shifts for protonation are taken positive.

themselves. Then, whatever the exact origin of these shifts, L-histidine indeed shows large amino and carboxyl titration shifts in solution on carbonyl, C_β, and C_4 (bridgehead aromatic-like) carbons (entries Δ_1^l and Δ_3^l in Table 3).

These trends are still reinforced in the solid state, since upfield shifts of the relevant carbons are enlarged by 1−4 ppm with respect to those measured in solution (entries Δ_1^s and Δ_3^s in Table 3). In the frame of electric field theory, variations of titration shifts on a given carbon nucleus are generally assigned to changes in molecular conformations and, consequently, in electric field orientation.[32] However, in the present case, three relevant carbon resonances are simultaneously shifted to higher fields when going from the solution to the lyophilizate. This suggests the existence of an additional common event accounting for these shifts, in connection with the removal of solvent molecules. In this view, dehydration of L-histidine on lyophilization is likely to produce a decrease in the local dielectric constant (toward vacuum permittivity), which increases the electric field on the observed nuclei and, consequently, the resulting titration shifts. In the frame of chemical bond theories, this amounts to assuming that hydration of L-histidine results in a partial delocalization of point charges onto neighboring hydrogen-bonded water molecules and, consequently, in decreased electronic effects on nuclear shieldings. Ab initio quantum mechanical calculations seem promising as regards the quantitative prediction of chemical shift anisotropy (CSA) tensors, and, consequently, of isotropic chemical shifts.[37] In that perspective, it would be especially interesting to examine not

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only the effect of hydrogen bonding but also the influence of protonation on CSA, to account for titration shifts in the solid state. However, CSA calculations have so far been carried out for a variety of crystalline substrates, essentially amides and peptides,[37−38] and have not yet been applied to amorphous powders in the absence of the necessary structural information. From a practical point of view, increased titration shifts in lyophilizates should permit better line separation of conjugate acid−base pairs, which could be a potential advantage for performing solid-state determinations of protonation sites and of titration shifts.

Tautomeric States of the Imidazole Ring. Protonation of histidine around physiological pH is a well-documented topic, since histidine residues are frequently assumed to monitor protein function due to the ligating properties of the imidazole ring, themselves controlled by the protonation of nitrogens N_1-(τ) and N_3(π). In aqueous solution, on the basis of pH-dependent ^{13}C chemical shifts,[39] it was concluded that the N_1-H tautomeric form of the imidazole ring was preferred over the N_3-H tautomer (Scheme 2) by ∼4:1 in the zwitterionic form of histidine **LH**. In these experiments, only one time-averaged resonance signal was observed for each carbon in both tautomers due to fast proton transfer. Conclusions on the existence of each tautomer were then based on a comparison with N_1-CH_3 and N_3-CH_3 derivatives, which were assumed to have the same chemical shifts as the N_1-H and N_3-H tautomers of the parent histidine molecule. These conclusions were later on confirmed by experiments using ^{15}N chemical shifts,[1−2,40−42] ^{14}N chemical shifts,[43] and $^{15}N-^{13}C$ coupling constants.[40,44] In the case of imidazole itself, both tautomers are chemically equivalent; the two nonequivalent C_4 and C_5 carbons in each tautomer give rise to a time-averaged singlet whose intensity is twice as high as that of the C_2 singlet. In sharp contrast, distinct singlets of equal intensities were obtained for carbons C_4 and C_5 of pure solid imidazole due to slow intra- or intermolecular proton exchange on the NMR time scale.[45]

On the basis of these experiments, two resonances with intensities in a 4:1 ratio were tentatively expected for each carbon of species LH and L in lyophilized L-histidine. In fact, only singlets were actually obtained for each carbon in both species LH and L (see Figure 1), in agreement with earlier experiments using ^{15}N NMR.[1−2] This unexpected result may be explained by two different possibilities: (i) weak chemical shift differences between the two tautomers, associated with fast proton exchange or (ii) the absence of one tautomer. Although the aforementioned experiment using pure imidazole is not in favor of the former assumption, it could be suspected that the small amount of residual water or the simultaneous presence of species in various states of protonation (LH_2, LH, L) in the lyophilizate might promote fast intermolecular proton transfers. Two pieces of evidence are presented in the following paragraphs in order to reject this assumption and to support the presence of only one tautomer.

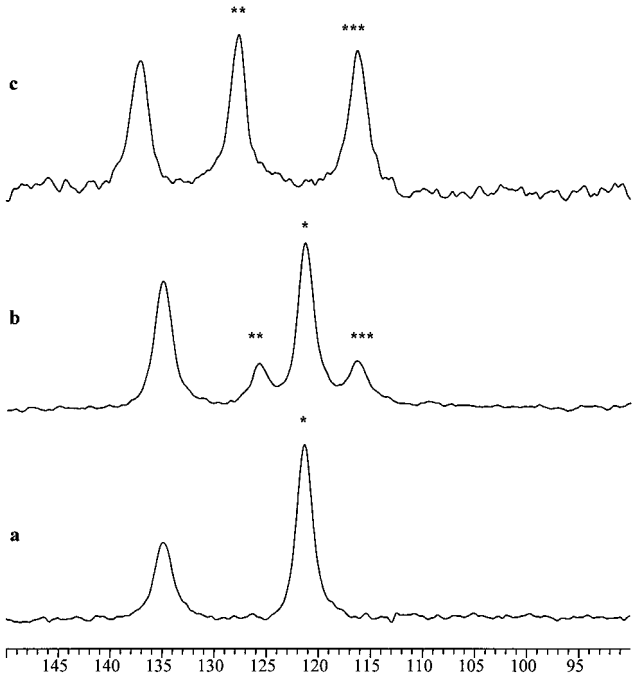

Figure 4. ^{13}C CP/MAS NMR spectra of imidazole lyophilized from solutions at pH 5.0 (a), 6.5 (b), and 9.0 (c). Starred lines refer to carbons ($C_4 + C_5$) of the imidazolium cation (*) and to carbons C_4 (**) and C_5 (***) of imidazole.

Table 4. ^{13}C Chemical Shifts and Titration Shifts of Lyophilized Imidazole

	carbon		
	C_2	C_4	C_5
δ_{ImH}[a]	134.9	121.3	121.3
δ_{Im}[b]	137.0	127.6	116.2
Δ_{Im}[c]	+2.1	+6.3	−5.1
Δ_{Im}[d]	−2.4	−2.7	−2.7

[a] Chemical shifts of lyophilized imidazolium cation; ppm from TMS. [b] Chemical shifts of lyophilized imidazole. [c] Titration shifts of lyophilized imidazole; upfield shifts for protonation taken as positive. [d] Titration shifts of imidazole in solution from ref 39.

First, proton transfers were shown to be presumably slow in parallel experiments using lyophilized imidazole. In imidazole, the inequivalence of C_4 and C_5, if actually observed, can reveal slow proton exchange as mentioned above. Using parent solutions at variable pH, the spectrum at pH 5, where the imidazolium cation ImH is predominant by ∼99%, consists of two singlets in a 2:1 ratio (Figure 4a). The high-field intense singlet is assigned to both C_4 and C_5, which have the same isotropic chemical shifts in species ImH, and the low-field singlet to C_2. At the other end of the pH range explored (pH ∼9), a three-line spectrum is obtained (Figure 4c), closely analogous to the one previously described for pure imidazole Im:[45] three singlets are observed for C_5, C_4, and C_2, in this order toward lower fields. A comparison between Figure 4a and c shows that C_4 and C_5 are shifted by about the same extent (∼5 ppm), but in an opposite sense (Table 4), on protonation of imidazole, presumably as the result of concerted electron transfer between both nitrogen atoms in the imidazole ring. At intermediate pH's of the parent solutions, the lyophilizate contains

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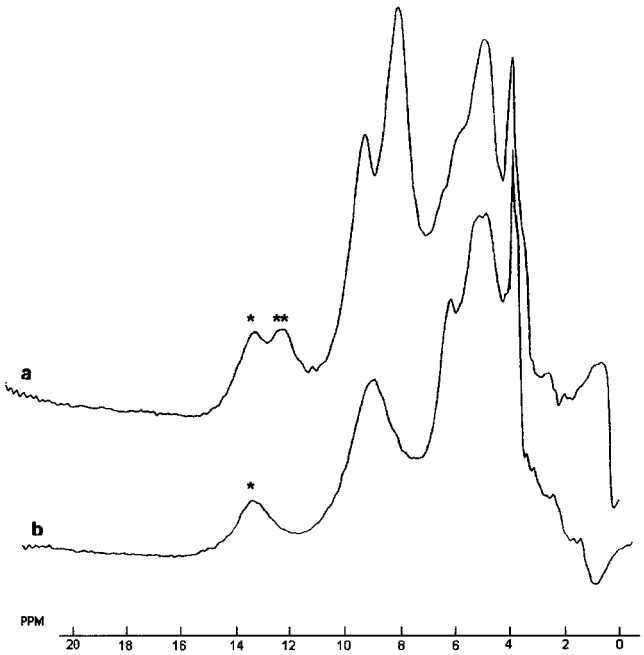

Figure 5. ^{1}H CRAMPS NMR spectra of L-histidine lyophilized from solutions at pH 5.91 (a) and 9.10 (b). Starred lines refer to protons N_1-H (*) and N_3-H (**).

mixtures of both species ImH and Im whose spectra appear superimposed in a variable ratio. The (C_4 + C_5) singlet from species ImH thus appears approximately midway between the two equally intense C_4 and C_5 singlets from species Im. Side components of this apparent triplet simultaneously grow at the expense of the central component as the pH increases from the acidic to the basic end of the explored pH range (encompassing the p*K* value of imidazole). These experiments show that proton transfer in lyophilized imidazole is slow on the NMR time scale; i.e., its frequency should be clearly smaller than the frequency shift of ~200−250 Hz between the exchanging sites. Assuming that time scales for proton exchanges are of the same order of magnitude in both lyophilized imidazole and L-histidine, time-averaging of tautomeric lines in L-histidine is a highly unlikely event. Carbon C_2, which, along the same lines, is subject to mutually canceling effects from adjacent nitrogens N_1 and N_3, gives rise to an undecoalesced singlet at any pH. (Figure 4b). This singlet, however, is continuously shifted downfield up to 2.1 ppm in the course of deprotonation of ImH (as it is also the case**,** albeit at a lesser extent, for C_4). Consequently, the absence of line splitting should result from parallel shifts of both ImH and Im C_2 resonances, presumably on account of additional effects of intermolecular hydrogen bonding on the adjacent N_3-(π) nitrogen (see below).

We have brought further evidence for the presence of a single tautomer in lyophilized L-histidine by ^{1}H CRAMPS NMR spectroscopy. Using first an acidic parent solution, two broad resonances were observed at low field and assigned to protons N_1-H and N_3-H of the imidazolium cation ImH (Figure 5a). Besides clear visualization of the deprotonation of the terminal ammonio group as the pH of the parent solution is increased, CRAMPS spectra show that one of these two low-field signals, namely, that at higher field, progressively vanishes, leaving the other resonance at low field alone at pH ~9 (Figure 5b). This proves that deprotonation of the imidazolium cation exclusively takes place on one nitrogen, either N_1 or N_3 , and, once again, clearly points out the existence of only one of the two possible tautomers. Our following objective was the assignment of the tautomeric form that was actually present in the solid. Titration shifts $\Delta_2{}^s$ (LH → LH_2**)** of carbons C_4 and C_5 are large and, respectively, occur upfield and downfield in lyophilized L-histidine (Table 3) as in lyophilized imidazole, which strongly favors deprotonation of nitrogen N_3 and, consequently, the existence of the $N_1-H(\tau)$ tautomer. Additional straightforward evidence for this assignment results from earlier investigations using histidine enriched in ^{15}N at the π position only and at both π and τ positions.[1−2,40] This conclusion in its turn allows us to assign the unique low-field ^{1}H resonance at pH 9 (Figure 5b) to the N_1-H tautomer. By continuity, the two N−H resonances at low and high field in species LH_2/LH_3 containing fully protonated imidazole rings should be assigned to $N_1-H(\tau)$ and $N_3-H(\pi)$ protons, respectively, i.e., in an order which is opposite to that previously proposed on other grounds for pure histidine hydrochloride crystals.[17]

The ^{1}H CRAMPS spectra from Figure 5 bring definite evidence for the presence of the $N_1-H(\tau)$ tautomer only in lyophilized histidine. One may wonder why the $N_3-H(\pi)$ tautomer, present at ~20% in solution, is absent in the lyophilizate. Such changes are commonly observed in 4-R-substituted imidazoles where tautomer preferences seem to be governed by a variety of factors, themselves highly dependent on the physical state of the sample. The case of histamine, a bifunctional analogue of histidine (R = $CH_2CH_2NH_2$), is particularly well-documented in this view. In gaseous phase, as observed by rotational jet spectroscopy, neutral histamine contains equal proportions of N_1-H and N_3-H tautomers under various conformations.[46] On the basis of theoretical computations and of gas-phase basicity measurements, the protonation of the side-chain amino group seems to bring about the predominance of the N_3-H tautomer[8] (in fact, protonation on the imidazole ring is an equiprobable event in this description). This behavior contrasts with that observed in solution, where, as in L-histidine, there is a predominance of the N_1-H tautomer over the N_3-H tautomer by ~4:1, both in neutral histamine and in its ammonio monocation.[47] Still at variance with these results, solid histamine was shown to exist as the N_3-H tautomer in the free base[48] and as the N_1-H tautomer in the hydrobromide salt.[49]

Internal chelating hydrogen-bonding $N_3\cdots HNH_2^+$ between the protonated amino group and the imidazolic nitrogen N_3 has been invoked to account for the increasing proportion of the N_1-H tautomer upon protonation of the terminal amino group of histamine (and of histidine[40] as well) in solution or in the gas phase. However, this is not a suitable explanation in cases where intermolecular hydrogen bonding is weak or absent[47] and where, however, the preference for the N_1-H tautomer is maintained. Examples of such situations are basic solutions of (neutral) histamine and of L-histidine (species L) or histidyl residues in polypeptides. Yet, and more conclusively in this view, recent computations[50] show the predominance of the N_1-H tautomer even in the absence of functional side chain,

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as demonstred for 4-methylimidazole (R = CH_3), in agreement with experimental observations. Thus, it appears that, besides additional effects from intramolecular hydrogen bonding, the major factor determining the tautomer preference is the electronic effect of the side-chain R on the 4-R-substituted imidazole ring. Predictions as to the exact tautomer ratio in solution can thus only result from complete theoretical treatments including solvent interactions. The situation is still different in the solid state, where intermolecular interactions may be locally strong in the crystal lattice. Thus, the crystalline structure of neutral histamine consists of chains of molecules linked together by intermolecular hydrogen bonds between the N_3-H hydrogen of one molecule to the nitrogen atom of the terminal amino group of its nearest neighbor.[48] By contrast, in the monocation crystal, histamine molecules form dimers in which the N_3 nitrogen of one molecule is hydrogen-bonded to the ammonio group of its nearest neighbor.[49] This contrasting behavior accounts for the predominance of either the N_3-H or the N_1-H tautomer in crystalline neutral histamine or its monocation, respectively. Such rationalizations of experimentally observed tautomer preferences are, however, impossible in the case of lyophilized powders where local environments are not known. We can only notice that the N_1-H tautomer exclusively present in lyophilized L-histidine is the same as in crystalline histidine[28] and that deprotonation of the terminal ammonio group does not change the tautomer preference.

States of Protonation of Lyophilized L-Histidine. A key point in these investigations is to determine whether there is conservation of acid-to-base ratios *r* from the aqueous solutions to the lyophilizates. Such comparisons require a parallel knowledge of acid-to-base ratios r_l in solution from electrochemical measurements and the classical equation

$$pK = pH + \log r_l + \log \gamma_{BH}/\gamma_B \quad (1)$$

In this formula, p*K* stands for either of three values pK_1, pK_2, pK_3 relative to the successive ionizations of histidine, depending on the pH (Scheme 1 and Table 2). Along the same lines, γ_{BH} and γ_B refer to the corresponding acid−base pair BH/B, namely, LH/L, LH_2/LH, or LH_3/LH_2, respectively. In fact, activity coefficients are generally unknown and even often impossible to estimate since, besides deviations from the laws of ideal mixtures, they also include a variety of ill-known factors such as ionic effects, ion pairing, and solvent and aggregation effects. Comparisons of pH and p*K* values should therefore be performed under similar experimental conditions that are assumed to ensure constant values to activity coefficients. This especially requires a common temperature *T* and ionic strength *I*. This amounts to using practical apparent p*K*:

$$pK^{app} = pK - \log \gamma_{BH}/\gamma_B \quad (2)$$

and concentrations instead of activities in the modified formula 1:

$$pK^{app} = pH + \log r_l \quad (3)$$

Even under these restrictive conditions, the p*K* values reported in the literature usually show large deviations from one reference to another (see below). For these reasons, we preferred to check first the internal consistency of our measurements before any comparison. At each measured pH (Table 2), we computed a trial value pK^{app} from eq 3 on assuming r_l equal to *r*. This value itself results from an average over the six carbon nuclei of histidine. Individual and averaged values with their standard deviations are reported on the bottom row of each entry in Table 2. The first two entries yield the averaged values $pK_3^{app} = 1.71$ and 1.64 at pH 1.79 and 2.02; the next two entries, $pK_2^{app} =$ 6.16, 6.14, and 6.14 at pH 5.56, 5.91, and 6.45; and the last three entries, $pK_1^{app} = 9.77$, 9.76, and 9.82 at pH 9.25, 9.64, and 10.02. In each of these three sets of experiments, computed pK^{app} values are fairly close to each other; this strongly supports both the validity of the procedure used to compute them and the coherence of experimental data. The best values for apparent p*K*'s are obtained as averages within each set of experiments, together with the corresponding standard deviations:

$$pK_1^{app} = 9.78 \pm 0.03$$

$$pK_2^{app} = 6.15 \pm 0.01$$

$$pK_3^{app} = 1.68 \pm 0.04$$

The apparent p*K*'s can now be compared with a good degree of confidence to the apparent p*K*'s of histidine measured in aqueous solutions by standard electrochemical titrations and collected over the years 1970−1996 in the IUPAC Stability Constants Database.[51] Among the 75 titrations of L-histidine quoted in this document, we extracted a set of 19 experiments[52] all performed in aqueous solution at 25 °C and a common ionic strength $I = 0.1$ mol dm^{-3}, i.e., under conditions closely similar to those in the parent solutions of lyophilized L-histidine (see Appendix A, Supporting Information). pK_1^{app}, pK_2^{app}, and pK_3^{app} values selected in this way range from 9.06 to 9.24, 5.91 to 6.14, and 1.58 to 1.92, respectively. Uncertainty margins were reduced to reasonable limits by computing averaged values and their standard deviations:

$$pK_1^{app} = 9.12 \pm 0.04$$

$$pK_2^{app} = 6.04 \pm 0.05$$

$$pK_3^{app} = 1.70 \pm 0.10$$

These values, although of the same order of magnitude as those from lyophilization experiments, are smaller than those reported above with the exception of pK_3^{app}, the differences ΔpK_1, ΔpK_2, and ΔpK_3 between both sets of numbers being 0.66, 0.11, and −0.02, respectively. The discrepancy increases from the carboxyl to the amino group titration, where experimental pK_1^{app} is clearly outside the range of values in the database. These differences may be tentatively assigned to temperature effects. Although parent solutions of lyophilizates were all standardized at a common temperature of 25 °C, the freezing out of aqueous solutions necessarily starts at temperatures close to 0 °C. Even if reliable p*K* data are not available from the literature at this temperature, p*K* variations δpK on decreasing the temperature from 25 to 0 °C can be estimated

(51) Powell, J. F. *The IUPAC Stability Constants Database*; Academic Software and IUPAC: 1992−2000.
(52) References in Appendix A, Supporting Information.

by using the corresponding equilibrium enthalpies quoted[53] in the IUPAC database, ΔH_1, ΔH_2, $\Delta H_3 = -44.0$, -29.0, -2.9 kJ mol^{-1}; hence δpK_1, δpK_2, $\delta pK_3 = 0.71$, 0.46, and 0.05, respectively. Temperature corrections δpK have indeed the same sign as the ΔpK differences mentioned above with the exception of ΔpK_3 and δpK_3, both of which are in fact very close to zero. They also have appropriate orders of magnitude, with the exception of δpK_2 which is somewhat larger than expected from these premises. We may thus conclude from this section that p*K*'s computed from acid-to-base ratios measured in lyophilized L-histidine are very close to those measured in solution by standard titrations, eventually after appropriate temperature corrections, and, consequently, that the pH of the parent solutions is connected to the molar ratio *r* in the lyophilizate through equation

$$\mathrm{pH} = \mathrm{p}K^{\mathrm{app}} - \log r \qquad (4)$$

This means that a conventional pH can be assigned to the lyophilized powder, namely, the pH measured in the parent liquid, or otherwise, the one that is obtained by redissolving the powder in the same volume of water. The latter statement deserved experimental confirmation; we effectively checked an exact recovery of the pH after back-rehydration of lyophilized L-histidine. This reversibility is certainly due to the conservation of matter during lyophilization, which means that all acid−base pairs in the sample should clearly be less volatile than water, including ingredients used to adjust the pH (e.g., $HClO_4$ or H_2SO_4 rather than HCl). Along the same lines, lyophilization is best achieved at a pH close to the p*K*('s) of the lyophilized compound, i.e., in a buffered system abstracting almost all protons from the solution. When the pH is decreased or increased far from the p*K* of the lyophilized substrate, there is a noticeable excess of either the added acid, e.g., $HClO_4$, or the added base, e.g., NaOH, strongly associated with water molecules. This is likely to slow or even prevent sublimation of the last amounts of water and increase the water contents found in the lyophilizates.

Conclusions

In this work, the suitability of high-resolution solid-state ^{13}C NMR for accurate determination of acid−base properties of L-histidine has clearly been demonstrated. ^{13}C NMR is less subject to significant changes of cross-polarization efficiency in the protonation process than is ^{15}N NMR, which improves the reliability of measured population ratios *r*. Another advantage is the possibility for observing several sets of nonequivalent nuclei in ^{13}C spectra, which allows several independent determinations of population ratios *r*, permitting in turn checking the validity of the experimental procedure and improving the precision of measurements. Acid-to-base ratios *r*, and consequently calculated p*K*'s, are unaltered from parent solutions to lyophilizates within reasonable uncertainty margins (a few tenths of p*K* units at most). However, attention must be paid to possible shifts in tautomer equilibria. This allows us to conventionally assign pH and p*K* values as defined in aqueous solution to the derived lyophilizates and to apply the classical formula 3, where the acid-to-base ratio is measured not in the liquid but in the solid powder. The advantage in combining liquid (pH and p*K*) and solid-state (*r*) measurements arises from the unambiguous determination of *r* in the lyophilizate due to the existence of well-separated signals in pairs of conjugate species. This independent knowledge of acid-to-base ratios *r* allows in turn p*K* determinations from the single measurement of the corresponding pH's. In contrast to ordinary acid−base titrations, the exact quantity and even the nature of the acid or base added to adjust the pH is not required, as well as the conventional pH/titrant plot and the subsequent curve-fitting procedures to extract p*K* values. Solid-state pH and p*K* determinations allow measurements even in the case of dilute aqueous solutions by taking advantage of the high concentration obtained in the solid residue. They also should allow operations when electrochemical or spectrophotometric measurements are impossible or ambiguous, e.g., in the case of viscous, concentrated, or turbid solutions. These advantages should be even greater for mixtures of acidic compounds or of polyfunctional compounds, especially natural products and biochemical substrates, where the nature and the sequence of protonation steps can be clearly isolated and identified by observing successive ^{13}C line-splittings in the lyophilizate.

Finally, another feature from these investigations is the possibility to characterize the acidity of lyophilizates, i.e., to predict the pH of the product obtained after rehydration and solubilization of the powder, without any alteration of the sample in the analysis procedure. This noninvasive in situ "pH" determination of lyophilized powders could have potential use in various fields of application, for example, in biochemistry, the food industry, and pharmaceuticals. From a more fundamental point of view, this amounts to saying that a model compound such as histidine can be used in turn as a pH indicator in lyophilized powders to anchor p*K* scales in the solid state. In other words, after addition of histidine to a newly investigated parent solution, solid-state ^{13}C spectra of histidine can be used to calculate the pH of the initial solution from known values of pK_1^{app} pK_2^{app}, and pK_3^{app} and then the p*K*'s of all other substrates from their own respective ^{13}C spectra and the above pH value. In that perspective, well-chosen sets of acidic compounds could be alternatively used as test samples or pH indicators to obtain complete p*K* scales in the solid state and possibly without recourse to any measurement in parent solutions. It should be remembered that the present experiments on histidine show that the solid-state p*K* scale in the lyophilizate reflects the p*K* scale in water. By extension, solid-state p*K* scales should depend on the nature of any other solvent possibly used to prepare parent solutions; in fact, they should faithfully reflect the p*K* scale in each particular solvent, thus offering entirely new perspectives to the long-lasting controversial problem of p*K* scales in nonaqueous solvents.[9]

Supporting Information Available: The material contained in Appendix A (see the text) is available free of charge via the Internet at http://pubs.acs.org. See any current masthead page for ordering information and Web access instructions.

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