Nuclear Magnetic Resonance Titration Curves of Histidine Ring Protons

I. INFLUENCE OF NEIGHBORING CHARGED GROUPS

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SUMMARY

Several imidazole derivatives bearing titratable charged groups adjacent to the imidazole ring have been studied by nuclear magnetic resonance. Curves relating the chemical shift values of the C-2 and C-4 ring proton resonance signals of these compounds to the solvent pH have been examined. These curves clearly demonstrate effects corresponding to the titration of the neighboring charged groups in addition to the effect of protonation of the imidazole ring in each case. The conclusions drawn from these data may be useful in interpreting similar titration curves of the histidine ring proton signals of proteins.

Of the many hydrogen atoms present in proteins, those at the C-2 and C-4 positions on the imidazole ring of the histidine residues are most easily resolved by nuclear magnetic resonance because of the unique downfield positions of their resonance signals. Changes in chemical shift values of these protons during physical or chemical perturbations of protein solutions can therefore be used as a sensitive monitor of local structural and electronic effects within protein molecules (1). To this end, the chemical shift values of the histidine C-2 and C-4 ring protons in several globular proteins have been followed during sequential changes of pH (2–8). In most cases plots of such chemical shift measurements as a function of pH have yielded simple titration curves, as would be predicted by the Henderson-Hasselbalch equation for protonation of the imidazole ring (6, 9). However, for two of the four histidine residues of ribonuclease A the titration curves do not follow this predicted relationship but show an inflection in the acid region of the curve (10–12).

The physical bases for the asymmetric titration curves of these two histidine residues have been the subject of considerable interest because of their possible implications for the stereochemistry of the active site (10–12). Such asymmetry suggests the interaction of these histidine rings with one or more neighboring titratable groups. Limited experimental information is available, however, on the influence that such neighboring charged groups might be expected to have on the titration curves of the C-2 and C-4 imidazole protons (13). We have therefore examined the titration curves of the C-2 and C-4 imidazole protons in the presence and absence of neighboring carboxyl and amino functions with histidine and several histidine analogues as model compounds. Our results indicate that the titration of these adjacent functional groups is indeed reflected in the titration curves of the imidazole protons.

MATERIALS AND METHODS

Chemicals—L-Histidine and l-histidine methyl ester were purchased from Fox Chemical Corporation, Los Angeles, California. Imidazole was purchased from Cyclo Chemical Company. N-Acetyl histidine was prepared as follows. Two grams of L-histidine (Fox) were dissolved by heating in 5 ml of glacial acetic acid. The solution was brought to 95°, whereupon 3 ml of acetic anhydride were added at once and the mixture was stirred for 2 min without further heating. The reaction flask was cooled under the tap, 20 ml of water were added, and the mixture was evaporated to an oil under vacuum. The oil was dissolved in 20 ml of 95% ethanol by heating, and 10 ml of acetone were added with swirling. The solution was allowed to cool, yielding 1.6 g of crystalline product which was directly pure: m.p. 144–146° (with gassing).

C_{10}H_{12}N_{2}O_{2}·H_{2}O
Calculated: C 44.60; H 6.05; N 19.50
Found: C 44.40; H 6.09; N 18.52

NMR Spectra—All spectra in this study were taken on a Varian A-60 spectrometer at a probe temperature of 33–34°. Samples were dissolved in one-half of the appropriate volume of 99.7% D_{2}O (Aldrich) to produce the specified molarity, and an equal volume of 0.2 M NaCl in 99.7% D_{2}O was added. Except where otherwise stated, results reported are for 0.1 M solutions of analogues in 0.1 M NaCl. For studies in salt concentrations other than 0.1 M, the appropriate amount of NaCl was added directly to the sample. The pH of solutions was measured with a Radiometer pH meter No. 26 with an Instrumentation Laboratories No. 14200 electrode which could be conveniently inserted.
into the NMR tube after being rinsed with D$_2$O. The pH meter was calibrated every few readings with standard buffers (Fisher, Harelco, B & A) in the range being measured. No correction was made for the difference between activities of hydrogen and deuterium ions at the glass electrode since this difference has been shown to be approximately equal and opposite to the difference in activities of these ions with respect to the titratable group (3).$^7$ Additions of acid or base (DCI, 1 M or 0.1 M in D$_2$O or NaOD 1 M or 0.1 M in D$_2$O) were performed by means of a 0.2-ml micrometer syringe (Gilmore) adapted to a narrow Teflon catheter which could be threaded into the NMR tube with the electrode in place. The pH of the solution was measured before and after each reading, the values generally agreeing within 0.03 pH unit. The final pH value was used for calculations.

Spectra were run at a sweep width of 250 Hz with an external tetramethylsilane standard before and after each spectrum. For some of the small but significant changes of chemical shifts (e.g. the acid deflection for the C-2 proton of L-histidine) with which we were concerned, the small measurement errors inevitably introduced by changing the sweep offset were unacceptable. Therefore this control was never manipulated once a series of spectra had been started. Instead, the position of the tetramethylsilane standard could be conveniently recorded in the same region as the C-2 and C-4 titrations by dialing in a -500 Hz field offset at the same sweep width of 250 Hz. This manipulation was found not to introduce any significant measurement errors and corrections for the small drift of the tetramethylsilane peak could conveniently be added to C-2 and C-4 readings when the final measurements were made. Chemical shift values are reported as ppm downfield from the tetramethylsilane standard.

RESULTS

L-Histidine—The complete NMR spectrum of L-histidine in D$_2$O at pH 8.7 is shown in Fig. 1. The peaks centered at 4.00 ppm were attributed to the proton on the α-carbon and the peaks centered at 3.16 ppm to the protons on the β-carbon. The complex splitting pattern of these signals indicates that the two protons on the β-carbon are not equivalent, undoubtedly as a result of the adjacent asymmetric center (14). The C-2 and C-4 proton resonances were clearly distinguished as the two peaks furthest downfield (δ = 7.76 ppm and δ = 7.08 ppm, respectively at this pH). A plot of chemical shift of each of these protons as a function of pH (Fig. 2) showed two distinct transitions in addition to the expected major transition$^8$ with inflection at pH 6.1. The additional inflections occurred at pH 2.0 and 9.2 in each case, corresponding to the titration of the adjacent carboxyl and amino functions, respectively. They accounted for a much larger fraction of the total change in chemical shift in the titration of the C-4 proton than in that of the C-2 proton.

N-Acetyl Histidine—The complete NMR spectrum of this analogue confirmed its structure, showing in addition to the resonances seen in histidine, a singlet at 1.99 ppm which integrated to 3 protons as would be expected for the methyl hydrogen atoms of the acetyl derivative. In the pH range from 1 to 10 the titration curves of the C-2 and C-4 imidazole protons of this derivative (Fig. 3) showed only one inflection in addition to that corresponding to the imidazole ring protonation (pH 7.2). This was in the acid region of the titration curve of both protons, at a pH of about 3.75. As was the case for free histidine, this acid transition was more pronounced in the titration curve of the C-4 proton than in that of the C-2 proton.

L-Histidine Methyl Ester—The complete NMR spectrum of this compound was similar to that of histidine except for the presence of a methyl singlet at 3.95 ppm attributable to the methyl protons of the methyl ester. The titration curves of the
NMR Titration Curves of Histidine Ring Protons

Fig. 3. N-acetyl histidine. Chemical shifts of the C-2 and C-4 imidazole ring protons are plotted against pH. Note presence of secondary transitions in the acid regions of the curves only.

Fig. 4. L-histidine methyl ester. Chemical shifts of the C-2 and C-4 imidazole ring protons are plotted against pH. Note the asymmetry of the two titration curves.

Fig. 5. Imidazole. Chemical shifts of the C-2 and C-4 (and C-5) protons are plotted against pH. Note the absence of asymmetries and secondary transitions in either curve.

C-2 and C-4 protons of histidine methyl ester (Fig. 4) also showed an inflection in addition to that corresponding to the imidazole ring protonation. This inflection was in the alkaline region of the titration curves as one would expect for the titration of an amino group. However, in this case exact pK values were difficult to assign by inspection because of the close overlap between the two transitions. Again the C-4 proton titration curve demonstrated the additional titration more markedly than did the C-2 proton titration curve.

Imidazole—The complete NMR spectrum of this compound at pH 7.00 showed two resonance peaks at 8.53 ppm and 7.52 ppm, with areas in the ratio 1:2. This was as expected for free imidazole in which the C-4 and C-5 protons are equivalent. The titration curves of the C-2 and C-4, 5 protons (Fig. 5) each showed a single transition with inflection at pH 7.15, conforming to the Henderson-Hasselbalch relationship.

Effects of Varying Solute and Salt Concentration—Small but inevitable changes in solute and salt concentrations were produced by the addition of acid and base solutions during the titrations. It was therefore of practical as well as theoretical interest to determine the effect which such variables might have on the observed titration curves. No significant changes in the C-2 and C-4 proton titration curves of histidine or of histidine methyl ester were found on varying the solute concentration in the range from 0.05 to 0.5 M. The titration curves for histidine were unaffected by changes in salt concentration in the range of 0.05 to 1.0 M. In addition, identical curves were obtained when titrations were performed in either direction with respect to the pH range tested.

DISCUSSION

The NMR titration curve of the C-2 imidazole proton of histidine has previously been thought to conform to the pre-
dieted Henderson-Hasselbalch relationship for the protonation of the imidazole ring, and not to reflect the titration of the adjacent carboxyl and amino functions (2, 9). Our data indicate, however, that when the extremes of pH are carefully examined, the titration curves of both the C-2 and C-4 protons of histidine clearly show inflections corresponding to the neighboring titratable groups (Fig. 2). The lack of influence of solute concentration on these inflections suggests that the effects due to the carboxyl and amino titrations are mediated intramolecularly.

Blocking of either the carboxyl or amino group results in the loss of the corresponding inflection from the titration curves (Figs. 3 and 4). In addition, such modification changes the electrostatic environment of the remaining group, bringing its pKₐ closer to neutrality. This is also reflected in the C-2 and C-4 proton titration curves, the acid inflection of N-acetyl histidine occurring at a higher pH and the alkaline inflection of histidine methyl ester occurring at a lower pH than the corresponding inflections of free histidine.

It is apparent from these titration curves that the pKₐ of a neighboring titratable group can be readily determined by inspection if it is more than about two pH units away from that of the imidazole ring. Thus for N-acetyl histidine, for example, a pKₐ of 3.75 for the carboxyl titration can be assigned directly. However, when the pKₐ of the neighboring titratable group approaches that of the imidazole ring, as it does in the case of histidine methyl ester, the corresponding transition merges with that due to the imidazole titration, and one cannot assign a precise value for this pKₐ by inspection. In such cases an accurate pKₐ value can be extracted from the data by an analysis in terms of the microscopic equilibrium constants for the two simultaneous titrations (15). This analysis can be performed most easily by computer curve fitting, and the program which has been developed for this purpose as well as the mathematics involved will be presented in a subsequent paper. In the case of histidine methyl ester such analysis leads to a value of 7.54 for the pKₐ of the amino group titration, which is in agreement with the accepted value for the pKₐ of this group (16).

Also of note is the effect of adjacent ionic charges on the observed pKₐ value of the imidazole ring titration itself. That this is accurately reflected by the C-2 and C-4 proton titration curves is shown by the close correlation between the values we have obtained and literature values obtained by potentiometric titrations in water² (Table I). The presence of an adjacent positively charged amino group has a greater effect in lowering the imidazole pKₐ than does the presence of an adjacent negatively charged carboxyl group in raising it. The reason for this difference is not entirely clear, but may involve the delocalization of electronic charge in the case of the carboxyl group due to the two possible resonance forms. The difference in magnitude of the effect of neighboring titratable groups on the C-2 and on the C-4 proton titration curves presumably arises as a result of the greater proximity of the C-4 proton to the charged group. The relative spatial proximities are apparent in the space-filling model of histidine (Fig. 6). However, attempts to quantitate this difference in terms of intramolecular distance and angle functions have so far been unsuccessful.

It is apparent that a plot of chemical shift values of the NMR titration curves of the histidine ring protons as a function of pH

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2 Reference 17.

3 Reference 16.

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![Fig. 6. Space-filling (CPK®) model of L-histidine. The C-4 imidazole ring proton is closer to both charged neighboring groups than is the C-2 proton.](http://www.jbc.org/)

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### Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>Neighboring charge</th>
<th>Imidazole pKₐ (potentiometric)</th>
<th>Imidazole pKₐ (C-2 NMR)</th>
<th>Imidazole pKₐ (C-4 NMR)</th>
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<td>5.62</td>
<td>5.52</td>
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of such parameters on the observed NMR titration curves of the histidine ring protons.

REFERENCES