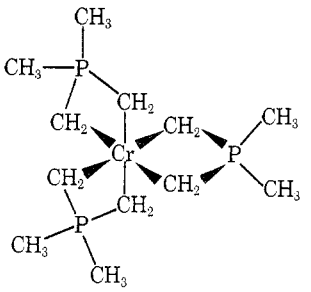

chromium(III) tris(dimethylphosphonium bismethylide) seems to be of special significance. In this octahedral $d^3$ complex, $(CH_3)_2P(CH_2)_2^-$ again appears as the sole ligand comprising all six Cr–C $\sigma$ bonds. The stability of this molecule shows the great potential of metal ylide chemistry, from which many more exciting results are expected.

*The author gratefully acknowledges the cooperation of a series of coworkers, who have contributed to the work from his own laboratory reported in this Account.*

# Observation of Histidine Residues in Proteins by Means of Nuclear Magnetic Resonance Spectroscopy

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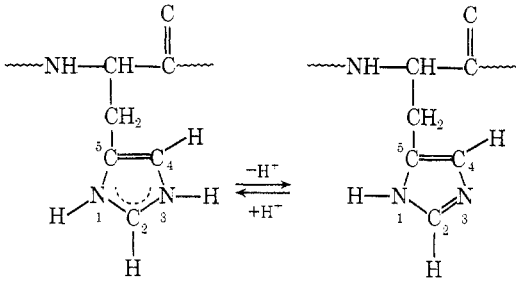
*Received May 28, 1974*

The imidazole ring of histidine plays a critical role in the function of numerous proteins. The unusual importance of this amino acid stems from its acid–base properties. Histidine is the only amino acid whose side chain can serve either as an acid (I) or as a base (II) in the physiological pH range.



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Knowledge of the environments and p$K$ values of key histidine groups in proteins is of interest for learning how proteins work. In many cases one can obtain inferential data from pH–rate profiles of enzymes or from the pH dependence of the chemical modification of histidine residues. However, direct spectroscopic observation of the histidine residues is preferable whenever possible. Only limited success has been achieved with uv spectroscopy of histidine residues, because the histidine spectrum is weak and overlaps the region of stronger aromatic transitions.[1]

John L. Markley was born in Denver, Colorado, in 1941. He received his B.A. degree from Carleton College and the Ph.D. from Harvard University. He worked as a research chemist at Merck Institute, then as NIH Postdoctoral Fellow at Lawrence Berkeley Laboratory with Melvin P. Klein and Melvin Calvin, and since 1972 has been Assistant Professor of Chemistry at Purdue University. His research interests largely concern the structure and function of biological macromolecules, especially proteins. Aside from research, he is a mountaineer and a photographer, and was a member of the first party to kayak the length of the Mahaweli Ganga River in Ceylon.

In 1964, as part of the first proton NMR investigation of proteins at 100 MHz, M. Mandel reported the detection of a composite signal from the imidazole C-2 protons of the histidine residues of ribonuclease A.[2,3] In a prophetic statement, he concluded that, "This provides us with a window to observe the active site [histidines] under various conditions." A year later, J. H. Bradbury and H. A. Scheraga resolved peaks corresponding to three of the four individual histidines of ribonuclease A (using computer averaging at 60 MHz) and published titration curves for these.[4]

Encouraged by these successes, D. H. Meadows, J. S. Cohen, O. Jardetzky, and I embarked on extensive NMR studies at 100 MHz of the histidine residues of several proteins. This led to the elucidation of titration curves for all four histidines of ribonuclease A, the four histidines of staphylococcal nuclease, and the single histidines of chicken and human lysozyme.[5] These and subsequent studies carried out in a number of laboratories concretely exemplify the value of NMR spectroscopy in protein chemistry.

Detailed reviews of much of this work have been published.[6–9] In recent years, investigations have been extended to other proteins, and it is clear that NMR spectroscopy is the method of choice for observ-

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ing the environment and protonation state of histidine residues in small proteins.

This Account summarizes the foundations and scope of NMR studies of histidine residues in proteins. New developments in NMR instrumentation and new experimental techniques have made these experiments much easier to perform and promise to extend application of the method to larger and more complex proteins.

Historically, the first detailed NMR studies of proteins dealt with resonances, like those of the histidine C-2 H, that lie in open regions of the spectrum. Other such resonances include the ring N–H peaks of histidine[10] and tryptophan,[11] and resonances shifted out into the clear by aromatic ring currents or paramagnetic centers.[12,13] The aliphatic regions of both proton and natural abundance carbon-13 spectra of proteins are still difficult to analyze at the current level of instrumentation. However, even these can be simplified by the newer techniques of selective deuteration[14] (in which one removes unwanted overlapping proton peaks by deuterium substitutions) or carbon-13 enrichment[15] (in which one enriches carbon atoms of interest above the 1.1% level of natural abundance). In many respects investigations of the easily resolvable histidine resonances may serve as prototypes for future NMR studies of other amino acid residues in proteins.

At present, three approaches are used to observe histidine residues by NMR spectroscopy.

(1) With low molecular weight proteins (mol wt < 30,000), it is generally possible to resolve C-2 H and in favorable cases C-4 H peaks of the imidazole rings by $^1$H NMR. These spectra are usually taken in $D_2O$ to minimize the influence of the solvent peak and of slowly exchanging N–H peaks. This approach is the most general of the three and has been used most widely.

(2) Imidazole N–H resonances of buried or hydrogen-bonded histidine residues may be visible in the −12 to −18 ppm $^1$H NMR region of proteins dissolved in $H_2O$.[16–19] Only histidine N–H protons that exchange slowly with water may be observed by this approach.

(3) Histidine moieties may also be studied by $^{13}$C NMR spectroscopy.[20–24] The successful studies to date have used $^{13}$C-enriched histidine prepared by chemical synthesis. The labeled amino acid was either fed to microorganisms which incorporated it into proteins of interest[20–23] or synthesized into a peptide used to form a semisynthetic enzyme.[24] This difficult approach is appropriate when the histidine C-2 H resonance cannot be resolved for whatever reason or when $^{13}$C NMR data (which are responsive to different molecular properties than $^1$H NMR data) are required.

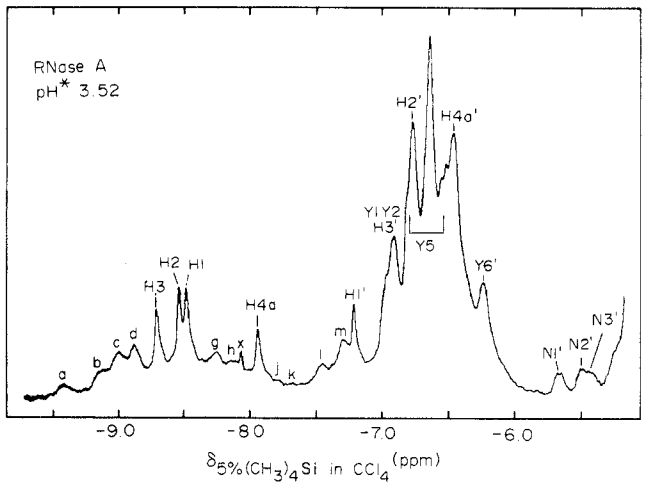


**Figure 1.** Correlation $^1$H NMR spectrum (250 MHz) of the low-field region of bovine pancreatic ribonuclease A. Enzyme concentration, $2 \times 10^{-3}$ *M* in $D_2O$ containing 0.3 *M* NaCl; pH* 3.52; temperature, 32°. Signal averaging: 500 1.5-sec scans. Peak assignments: H1 and H1′, C-2 H and C-4 H of His-105, respectively; H2 and H2′, His-119; H3 and H3′, His-12; H4a and H4a′, His-48; Y5, Tyr-25; Y1, Y2, and Y6, unidentified tyrosines; N1′–N3′, probably abnormal α-CH groups; a-m, partially exchanged, unidentified N–H peaks, (from ref 25).

The present discussion draws heavily on studies of bovine pancreatic ribonuclease A (RNase A) because its histidine residues have been studied in far greater detail by NMR spectroscopy than those of any other protein. RNase incidently is the only protein thus far to be investigated by all three approaches listed above.

## NMR Spectroscopy of Histidine Ring Protons

**Analysis of the Aromatic $^1$H NMR Region.** In general, the low-field or aromatic region of a protein NMR spectrum is the easiest to interpret. The 250-MHz $^1$H NMR spectrum of bovine pancreatic ribonuclease A (RNase A) in $D_2O$ is shown in Figure 1. All peaks that have been identified to date are labeled. Resonances corresponding to the C-2 H (H1, H2, H3, H4a) and C-4 H (H1′, H2′, H3′, H4a′) groups of each of the four histidine residues of RNase A may be recognized by their characteristic chemical shifts, single proton intensities, deuterium exchange properties, and titration behavior. The peaks in Figure 1 labeled with lower case letters correspond to N–H groups that have not exchanged with the $D_2O$ solvent. These probably correspond to the class.IV slowly exchanging protons previously detected by hydrogen-exchange studies.[7] Although these peaks undoubtedly carry interesting information, it is usually advantageous when studying the aromatic region to remove them either by a brief exchange at elevated temperatures in $D_2O$ or by a longer term exposure to $D_2O$ at a lower temperature.[25,26] In the case of 250-MHz spec-

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tra of soybean trypsin inhibitor,[27] chymotrypsin, trypsin,[28] and staphylococcal protease,[29] N–H peaks dominate the lower aromatic region and complicate the resolution of histidine C-2 H peaks. It is only after removal of the N–H peaks by deuterium exchange that the histidine resonances become readily visible.

Using current instrumentation we are able to obtain satisfactory $^1$H NMR spectra of $1 \times 10^{-3}$ *M* protein solutions with 10 min or less of signal averaging.

**Origin of Histidine Chemical Shifts in Protein Spectra.** Three factors contribute to the observed chemical shifts of histidine resonances of diamagnetic proteins: (1) the intrinsic chemical shifts of the histidine side-chain nuclei, which may be determined from spectra of model compounds; (2) the local environment of the histidine side chain as a consequence of the tertiary structure of the protein, which may be different for different nuclei in a given histidine side chain; and (3) the proton dissociation state of the histidine ring, which is a function of its local environment and the hydronium ion concentration. The third factor influences the chemical shifts of all ring nuclei in a proportional manner. (The predominant protonation site of the histidine ring at high pH has recently been determined by $^{13}$C NMR to be the N-1 position).[30] The first factor is constant and determines the region of the spectrum in which the resonance falls. The magnitude of the $^1$H NMR titration shift (3) is generally two or more times larger than the environment shift (2). Thus it is usually easiest to resolve a number of overlapping histidine peaks at pH values in the titration region where the residues may have different fractional charges. As in uv spectroscopy, it is convenient to distinguish between the effects of factors 2 and 3 by naming them the spectroscopic and thermodynamic components, respectively.

## Assignment of Histidine Peaks in NMR Spectra of Proteins

Considerable attention is given to the assignment of nmr peaks to particular residues because this is the key step in interpreting the results of NMR experiments and is often the most difficult step. If the protein contains only a single histidine, the assignment is trivial and automatic as for the lone histidine of lysozyme,[5] neurophysin II,[31] and $\alpha$-lytic protease.[22,23] Two general approaches have been used for making assignments of NMR peaks in the more usual case of a protein having more than one of a given kind of amino acid such as histidine: correlation of NMR spectra with chemical and X-ray data, and the use of mutant proteins. A third approach, deuterium exchange, is unique to histidine and promises to facilitate NMR assignments of this amino acid.

**Correlation of NMR Spectra with X-Ray and Chemical Evidence.** If chemical or X-ray data reveal that a residue is in a peculiar environment or that it should be perturbed when a ligand binds to the protein, this information may be sufficient for assignment purposes. For example, resonances in the spectrum of cyanoferrimyoglobin have been assigned to histidines near the paramagnetic iron based on estimates of expected pseudocontact shifts.[32] A high-field resonance in the spectrum of ferrocytochrome *c* has been tentatively assigned to the C-4 H of His-18 (liganded to the heme) which is shifted 6.84 ppm upfield by the ring current of the porphyrin group.[33] The broad histidine peak H4 of RNase A, which has an abnormal chemical shift at low pH (Figure 1), was assigned[5,34] to His-48 because chemical evidence indicates that the residue is abnormally unreactive or "buried." Supporting evidence for the assignment comes from the observation that H4 becomes more normal when RNase A is converted to RNase S by subtilisin cleavage between residues 20 and 21[34] (His-48 appears more exposed in the X-ray structure of RNase S[35] than in that of RNase A[36]). Unfortunately efforts to predict p$K'$ values of amino acid side chains from X-ray data have not been entirely satisfactory.[37] Thus there can be little confidence in assignments made by comparing the X-ray structure with NMR titration data.

A promising approach for assigning NMR peaks in enzymes having known X-ray structures is the use of a chemically exchanging paramagnetic probe that binds to a single specific location, i.e., a stable nitroxide free radical bound to a competitive enzyme inhibitor[38] or a paramagnetic metal ion used with proteins having a definite metal binding site.[39] The paramagnetic center will cause either broadening or shifting of NMR lines depending on its electronic relaxation time. Relative distances may be calculated from the line-width increases caused by a broadening probe,[40,41] and distances and angular information may be calculated from a shifting probe.[42] Such a study has been carried out using Gd(III) binding to the Ca(II) site of staphylococcal nuclease.[43] The resulting data were consistent with previous assignments of the histidine resonances.[44,45]

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Chemical modification of the protein may also be helpful in making assignments provided that the modification can be shown to affect a local region of the protein structure. Peaks H2 and H3 (Figure 1) were assigned to the active site histidine residues 12 and 119 of RNase A on the basis of chemical modification studies.[34] Since it was found that both peaks H2 and H3 are perturbed in either carboxymethyl-His-12 RNase or carboxymethyl-His-119 RNase, additional information (to be discussed below) is required to distinguish between the two active-site residues. Enzymatic modification may be useful in certain cases. For example, the peak corresponding to the carboxyl terminal His-146$\beta$ of hemoglobin has been assigned by reference to spectra of des-His-146$\beta$-hemoglobin prepared by carboxypeptidase A treatment of the protein.[46,47]

**Assignments Based on Mutant Proteins.** Nature provides chemically modified proteins by way of mutated sequences. One of the histidine peaks of staphylococcal nuclease was assigned by comparing spectra of the protein from the Foggi strain which shows resonances from four histidine residues[5] with spectra from the V8 strain in which peaks corresponding to one residue are missing.[44,48] The sequences of the two enzymes apparently differ only at residue 124, which is histidine in the Foggi and leucine in the V8 protein.[49] Histidine resonances of cytochromes[50,51] and of rat ribonuclease[52] have been assigned by similar means.

**Deuterium Exchange of the Histidine C-2 H as an Assignment Tool.** The C-2 hydrogen of the imidazole ring of histidine has the unique property of being the only carbon-bound proton of an amino acid that exchanges under mild conditions. The rates of C-2 H exchange of imidazole,[53] histidine, and histidine derivatives[54,55] exhibit a sigmoidal increase with increasing pH with the inflection point at the p$K'$ of the exchanging imidazole. In native proteins, other factors including accessibility of the residue to the solvent and catalysis by neighboring groups additionally affect the hydrogen-exchange rates at the histidine C-2 H position.[25,55] Deuterium exchange clearly provides a selective and nonperturbing method of labeling histidine residues of proteins. For example, complete differential labeling of the four histidine residues of RNase A is achieved by exchange at pH 8.0 as shown in Figure 2. The order of exchange is H1 > H2 > H3 > H4.[25]

Provided that differential exchange of all histidines can be achieved (which has been possible with four proteins tried),[55] assignments may be made by cleaving the exchanged protein into peptides containing only single histidine residues, separating the peptides, identifying the location of each peptide in the amino acid sequence, and analyzing the extent of deuterium exchange by NMR spectroscopy. The nonexchanging C-4 H serves as a convenient internal standard. This procedure has the advantage of being completely independent of X-ray and chemical data. The one limitation is that cleavage and separation of peptides must be carried out under conditions where back exchange is minimal. We have recently assigned the two histidines of soybean trypsin inhibitor by this method.[56]

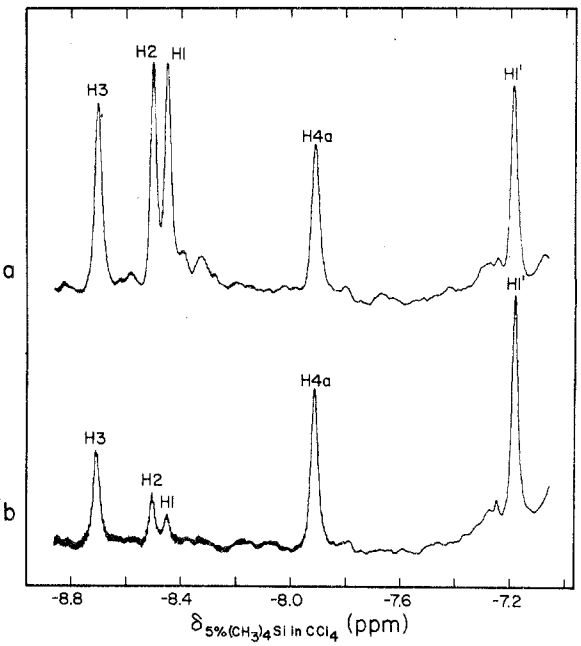


**Figure 2.** Differential deuterium exchange of the four histidine residues of bovine pancreatic ribonuclease A. Spectra taken with 2 × $10^{-3}$ $M$ enzyme samples in $D_2O$ containing 0.3 $M$ NaCl, pH* 3.00, 32°. (a) The histidine C-2 H region at zero time. (b) The histidine C-2 H region after exchange for 5 days in $D_2O$ at pH 8.0 and 40° (from ref 25).

A more elegant assignment procedure makes use of the method of Ohe and coworkers[57] for following tritium exchange into the histidine C-2 H positions of native proteins. After labeling the histidines they cleave the protein enzymatically into peptides containing isolated histidine residues, separate and identify the peptides, and measure the specific tritium incorporation. For RNase A at pH 8.0 they found the order of labeling: His-105 > His-119 > His-12 > His-48.[57] Combining their data with our NMR data gives the assignments:[25] H1 = His-105; H2 = His-119; H3 = His-12; H4 = His-48. These assignments of the NMR peaks of His-48 and His-105 agree with the original assignments of Meadows, et al.,[34] but the assignments of the active site residues His-12 and His-119 are the reverse of those proposed previously.[34,58] The original assignment of the nmr peak of His-12 was based on spectra of reconstituted RNase S′ in which the C-2 H of His-12 in the S peptide had been deu-

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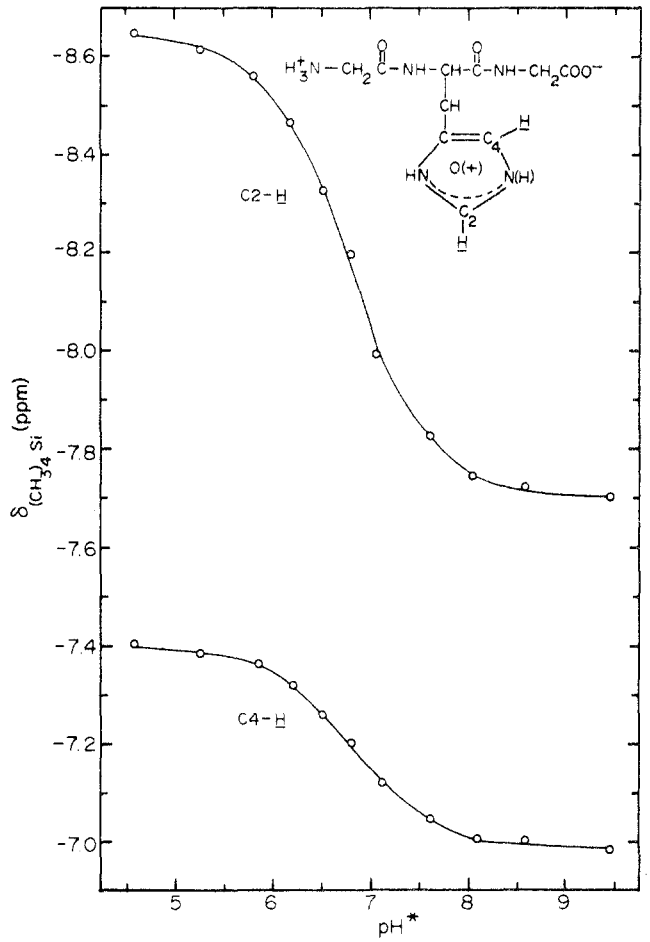



**Figure 3.** $^1$H NMR titration curves for the histidine C-2 and C-4 ring protons of glycyl-L-histidylglycine. Data obtained at 100 MHz using 0.1 *M* tripeptide in $D_2O$ containing 0.3 *M* NaCl.

terated. The missing peak in spectra of the exchanged reconstituted RNase S corresponded to peak H2 of their RNase S control.[34] However, a subsequent investigation of the chemical shifts of the histidine peaks of RNase S[59] demonstrated that the control used by Meadows, et al., must have contained phosphate which shifted peaks H2 and H3 downfield. If the reconstituted RNase S′ contained less phosphate than the control, the original assignment would have been in error. Bradbury and Chapman[58] independently confirmed the assignment of peak H2 to His-12 by carrying out differential exchange of peaks H2 and H3 in the native protein, and by looking for the intensity of the His-12 C-2 H peak in the isolated S peptide. Their assignment, however, rests mainly on a negative result, i.e., their inability to resolve a C-2 H peak in spectra of the isolated S-peptide.[59a]

## Histidine Titration Curves by NMR

**Normal Histidines.** The normal NMR titration behavior of the ring protons of histidine is best illustrated by model compounds.[4,60–62] Figure 3 shows the chemical shift of the C-2 H and C-3 H nuclei of the side chain of glycyl-L-histidylglycine as a function of pH*.[63] This tripeptide appears to be an appropriate model for an internal histidine residue since the p$K'$ values of the amino and carboxyl groups on glycine residues 1 and 3 are sufficiently different from the histidine ring p$K'$ so they do not influence the shape of the histidine titrations. Moreover, the effects of the positive and negative charges of the glycines on the histidine p$K'$ value probably cancel each other out. Since the exchange rates of protons on and off imidazole ring nitrogens are fast (>2000 $sec^{-1}$)[64] compared to the NMR chemical shifts (100–250 Hz), the chemical shift ($\delta_{obsd}$) is a weighted average of the chemical shifts of the protonated ($\delta_{H^+}$) and unprotonated ($\delta_{H^0}$) forms of histidine:

$$\delta_{obsd} = \frac{\delta_{H^+}[His^+] + \delta_{H^0}[His^0]}{[His^+] + [His^0]} \quad (1)$$

The fraction of molecules with neutral histidine is given by $(\delta_{H^+} - \delta_{obsd})/(\delta_{H^+} - \delta_{H^0})$. Titration parameters may be obtained by fitting the NMR data at a number of pH values by nonlinear least-squares analysis to a modified form of the Hill equation[65]

$$\frac{\delta_{H^+} - \delta_{obsd}}{\delta_{H^+} - \delta_{H^0}} = \frac{K_a^n}{K_a^n + [H^+]^n} \quad (2)$$

where $K_a$ is the dissociation constant of the histidine, and $[H^+]$ is the hydronium ion concentration from glass electrode measurements. The Hill coefficient $n$ is a convenient indication of the cooperativity of the dissociation. For a normal titration curve, $n = 1$. Significant deviation of the fitted Hill coefficient from unity suggests that the titration involves more than one dissociating group. In all cases studied thus far, $n \leq 1$. A Hill coefficient less than unity may be explained by the presence of one or more groups in the vicinity of the residue being observed that titrate in the histidine p$K'$ region.

The histidine titration shift ($\delta_{H^+} - \delta_{H^0}$) for the tripeptide Gly-His-Gly is 0.95 ppm for the C-2 H and 0.42 ppm for the C-4 H. As expected, the p$K'$ values derived from chemical shifts of the two ring protons are the same (p$K$ = 6.90) within experimental error, and the Hill coefficients are both unity.

All chemical and physical studies of RNase A indicate that His-105 is exposed to the solvent and has a normal reactivity. NMR titration curves (Figure 4) of both the C-2 H (H1) and C-4 H (H1′) protons of His-105 are perfectly normal and yield p$K'$ values of 6.72 (Table IA) which are close to that of the model tripeptide Gly-His-Gly. The titration shifts ($\delta_{H^+,H^0}$) and

(59) J. S. Cohen, J. H. Griffin, and A. N. Schechter, *J. Biol. Chem.*, **248**, 4305–4310 (1973).

(59a) Note Added in Proof: D. J. Patel, L. L. Canuel, C. Woodward, and F. A. Bovey (personal communication) and C. R. Matthews and J. S. Cohen (personal communication) have independent data which suggest that peak H3 = His-12 of RNase A.

(60) C. C. McDonald and W. D. Phillips, *J. Am. Chem. Soc.*, **85**, 3736–3742 (1963).

(61) D. H. Sachs, A. N. Schechter, and J. S. Cohen, *J. Biol. Chem.*, **246**, 6576–6580 (1971).

(62) J. S. Cohen, R. I. Schrager, M. McNeel, and A. N. Schechter, *Biochem. Biophys. Res. Commun.*, **40**, 144–151 (1970).

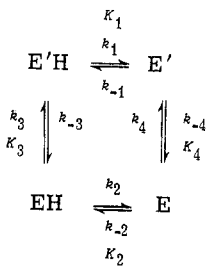
(63) The p$K'$ values of histidine determined by NMR in $H_2O$ agree within experimental error with those determined in $D_2O$ on the basis of uncorrected glass electrode pH meter readings. Thus the isotope effect at the glass electrode (−0.40 unit) [P. D. Glascoe and F. A. Long, *J. Phys. Chem.*, **64**, 188–190 (1960)] appears to be approximately equal and opposite in sign to the isotope effects on the histidine p$K'$ value [N. C. Li, P. Tang, and R. Mathur, *J. Phys. Chem.*, **65**, 1074–1076 (1961)]. It is convenient, therefore, to leave the pH meter readings uncorrected. We use the symbol pH* to indicate an uncorrected glass electrode pH meter reading of a $D_2O$ solution using an electrode standardized with normal $H_2O$ buffers.

(64) M. Eigen, G. G. Hammes, and K. Kustin, *J. Am. Chem. Soc.*, **82**, 3482–3483 (1960).

(65) J. L. Markley, *Biochemistry*, **12**, 2245–2249 (1973).

the Hill coefficient ($n$) are also normal. The only unusual feature of His-105 is the deshielded environment of the C-4 H.

**Involvement of Histidine Residues in Conformational Equilibria.** The general scheme for a conformational equilibrium involving a titrating group is

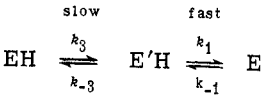


(3)

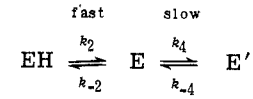
The type of nmr titration curve to be expected depends on the relative magnitudes of the individual rate constants. The cases that have been observed are:

*Slow Exchange between Two Conformations Having Different pK′ Values.* If $k_3$, $k_{-3}$, $k_4$, and $k_{-4}$ are slow on the NMR time scale and if appreciable amounts of both enzyme forms are present, two separate but normal titration curves will be observed. This appears to be the case with His-46 of staphylococcal nuclease.[27,66] If the environments of histidine in the two conformational states are similar, the double peaks may only be visible at pH values near the p$K$'s. Double peaks also have been observed for the single histidine of neurophysin II[31] and for the two interacting histidines of staphylococcal protease.[29] One of the two histidines of modified soybean trypsin inhibitor broadens[65] and appears to split into two peaks near its p$K'$ value; because of the broadening lifetimes of the two forms of approximately 12 msec may be calculated.[67]

*Slow Exchange between Conformational Forms Only One of Which Titrates.* If proton exchange of the histidine ring N–H is slow because of a conformational equilibrium (for example, if the histidine is "buried" and must change its conformation to reach the solvent), eq 3 simplifies either to eq 4 or to eq 5.



(4)



(5)

In the first mechanism (eq 4), the slow conformational change involves the protonated form; in the second (eq 5), the slow process involves the neutral form. Depending on the magnitudes of the equilibrium constants, one will or will not observe resonances from all three enzyme species. Those species linked by rates that are slow compared to their chemical shift differences will be characterized by separate lines that do not shift with pH. In such a case, the

(66) J. L. Markley, M. N. Williams, and O. Jardetzky, *Proc. Nat. Acad. Sci. U.S.A.*, **65**, 645–651 (1970).

(67) J. L. Markley, 5th International Symposium on Magnetic Resonance, Tata Institute of Fundamental Research, Bombay, 1974, No. 7A19.

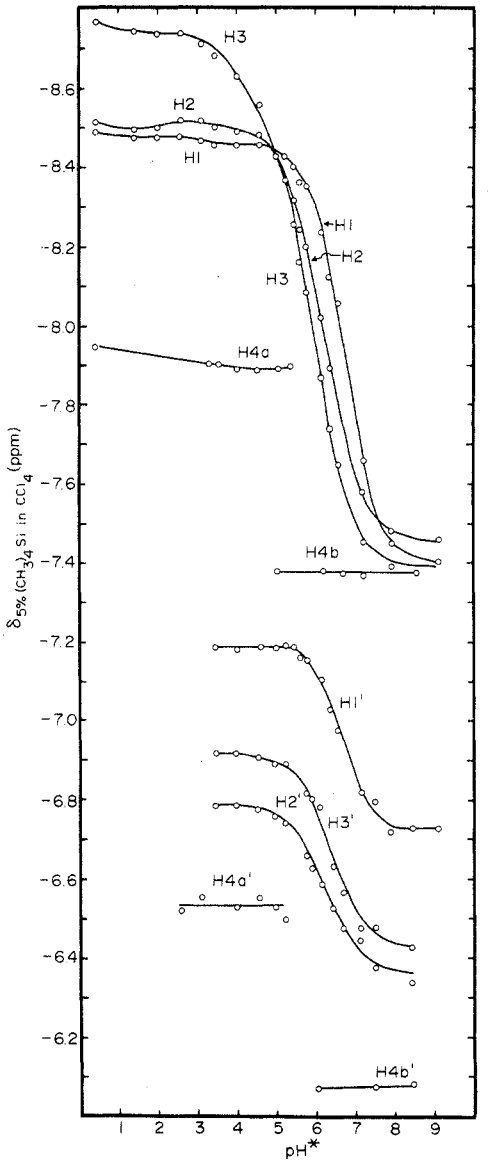



**Figure 4.** $^1$H NMR titration curves at 250 MHz for the histidine C-2 and C-4 ring protons of the four histidines of bovine pancreatic ribonuclease A. Assignments: H1 and H1′, C-2 H and C-4 H of His-105, respectively; H2 and H2′, His-119; H3 and H3′, His-12; and H4a, H4b, H4a′, and H4b′, His-48 (from ref 69 and 70). The crossings of titration curves H3, H2, and H1 at pH* 5 and of H1 and H2 at pH* 7.6 have been verified by studies of ribonuclease A in which the four histidines were differentially deuterated (see Figure 2b).

dissociation constant may be obtained by measuring the areas of the peaks as a function of pH

$$A_{H^0} = 1 - A_{H^+} = \frac{K_a^n}{K_a^n + [H^+]^n} \qquad (6)$$

where $A_{H^+}$ is the sum of the areas of the protonated forms and $A_{H^0}$ is the sum of the areas of the neutral forms of a given histidine (expressed as fractional protons).

The buried histidine in RNase A (His-48) appears to fit a variation of eq 4.[68] Instead of normal rapid-exchange titration curves, separate resonances having pH-independent chemical shifts in the histidine titration region appear for the protonated and dissociated forms (Figure 4[69,70]). The areas of the two pair

(68) J. L. Markley, submitted to *Biochemistry*.

(69) J. L. Markley, submitted to *Biochemistry*.

**Table I**
**Least-Squares Analysis of NMR Titration Data for the Histidine Residues of Ribonuclease A**

| Peak | With Hill coefficient fixed at 1 | With fitted Hill coefficient | Hill coefficient fitted value | Chemical shift, ppm from 5% $(CH_3)_4Si$ in $CCl_4$ $\delta_{H^+}$ | $\delta_{H^0}$ | $\Delta\delta_{H^+,H^0}$ | Variance $\times 10^4$ |
|---|---|---|---|---|---|---|---|
| A. Data Fitted to Single Titration Curves[a] | | | | | | | |
| His-105, H1 | 6.72 ± 0.02 | 6.72 ± 0.02 | 1.01 ± 0.04 | −8.45 ± 0.01 | −7.39 ± 0.01 | 1.06 ± 0.01 | 2.5 |
| H1′ | 6.66 ± 0.04 | | | −7.10 ± 0.01 | −6.72 ± 0.01 | 0.37 ± 0.02 | 1.3 |
| His-119, H2 | 6.19 ± 0.03 | 6.17 ± 0.02 | 0.84 ± 0.03 | −8.53 ± 0.01 | −7.45 ± 0.01 | 1.06 ± 0.02 | 1.3 |
| H2′ | 6.21 ± 0.05 | | | −6.78 ± 0.01 | −6.36 ± 0.01 | 0.42 ± 0.02 | 2.8 |
| His-12, H3 | 5.76 ± 0.04 | 5.79 ± 0.02 | 0.73 ± 0.03 | −8.73 ± 0.01 | −7.36 ± 0.01 | 1.37 ± 0.03 | 4.9 |
| H3′ | 6.38 ± 0.07 | | | −6.92 ± 0.01 | −6.42 ± 0.02 | 0.49 ± 0.03 | 7.1 |

B. The p$K'$ of His-48 Determined from the Area of Peak H4b is 6.9 ± 0.1[b]

| Peak | p$K'$ | | Chemical shift, ppm from 5% $(CH_3)_4Si$ in $CCl_4$ $\delta_{low}$ | $\delta_{high}$ | $\Delta\delta$ | Variance $\times 10^4$ |
|---|---|---|---|---|---|---|
| C. Data for His-12 and His-119 Fitted to Interactive Model (See Text)[c] | | | | | | |
| His-119, H2 | $pK_{10}$ | 6.01 ± 0.02 | −8.51 ± 0.01 | −7.46 ± 0.01 | 1.05 ± 0.02 | 0.6 |
| | $pK_{12}$ | 6.29 ± 0.02 | | | | |
| His-12, H3 | $pK_{20}$ | 5.80 ± 0.03 | −8.59 ± 0.02 | −7.38 ± 0.01 | 1.21 ± 0.03 | 0.6 |
| | $pK_{21}$ | 6.08 ± 0.04 | | | | |
| | $pK_{sp}$ | 3.74 ± 0.16 | −8.74 ± 0.01 | −8.59 ± 0.02 | 0.15 ± 0.03 | 0.6 |

[a] From ref 25 and 69. [b] From ref 68. [c] From ref 70.

of peaks interconvert as the pH is changed. According to this scheme, the p$K'$ of His-48 is 6.9, the pH at which the intensity of H4b = 0.5 proton.

There is NMR evidence for similar titration behavior of the active-site histidine residues of the serine proteases $\alpha$-lytic protease[22,23] and chymotrypsin[28] which appear to dissociate following a slow conformational change.

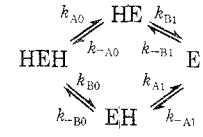
*Effect of a Conformational Transition Occurring Outside the Titration Region.* Conformational transitions that occur outside the titration region of histidine simply affect either $\delta_{H^+}$ or $\delta_{H^0}$. For example, ovomucoid shows a series of three pH-induced conformational transitions at low pH that influence various histidines.[71] These follow the simple scheme

$$\mathrm{HEH} \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} \mathrm{E'H} \qquad (7)$$

**Effect of Adjacent Charged Groups.** The presence of a nearby positively or negatively charged group will respectively lower or raise the p$K'$ of a histidine. It may also produce a spectroscopic perturbation of $\delta_{H^+}$ or $\delta_{H^0}$. If the adjacent group titrates, these effects will be pH dependent. The resulting effects on nmr titration curves have been discussed in detail by Cohen and coworkers.[61,72,73]

The situation is relatively simple if the p$K'$ of the adjacent group is 2 or more pH units removed from that of the histidine. Schechter, et al., have detected at 220 MHz a very small spectroscopic perturbation of His-12 of RNase A centered at pH 8.4, which they have attributed to titration of nearby lysine-41.[73]

On the other hand, if the p$K'$ of the second titrating group falls within the histidine titration region, one must consider the more complicated case of mutual perturbation of p$K'$ values (eq 8). The micro-




(8)

scopic dissociation constants ($K_{A0}$, $K_{B0}$, $K_{A1}$, and $K_{B1}$) may be calculated from the data provided that certain assumptions are made about the magnitude of spectroscopic effects.[72,74] The analysis should normally distinguish between electrostatic and hydrogen-bonded interactions since with a single hydrogen-bonded intermediate species, EH, eq 8 reduces to a three-term form, i.e., $K_{A0} = K_{B0}$ and $K_{B1} = K_{A1}$.

The question of mutual interaction of His-12 and His-119 of RNase A has been controversial. Rüterjans and Witzel[75] found that unlike the p$K'$ value of His-105 the p$K'$ values of the active-site residues are strongly dependent on ionic strength. They proposed for this and mechanistic reasons that the active-site histidine residues are hydrogen bonded in the active enzyme. Their NMR results are also consistent with a simple electrostatic interaction.[7] Schechter, et al., fitted 220-MHz NMR data to a variety of models and chose as a "best fit" one in which there is no direct interaction between the two histidine residues but

(70) J. L. Markley and W. R. Finkenstadt, submitted to *Biochemistry*.
(71) J. L. Markley, *Ann. N.Y. Acad. Sci.*, **222**, 347–373 (1973).
(72) R. Shrager, J. S. Cohen, J. S. Heller, S. R. Sachs, and A. N. Schechter, *Biochemistry*, **11**, 541–547 (1972).
(73) A. N. Schechter, D. H. Sachs, S. R. Heller, R. I. Shrager, and J. S. Cohen, *J. Mol. Biol.*, **71**, 39–48 (1972).
(74) J. T. Edsall, R. B. Martin, and B. R. Hollingsworth, *Proc. Nat. Acad. Sci. U.S.A.*, **44**, 505–518 (1958).
(75) H. Rüterjans and H. Witzel, *Eur. J. Biochem.*, **9**, 118–127 (1969).

rather one in which each histidine is perturbed by one or more external groups having a p$K'$ in the range 4.6–5.0.[73] This is a puzzling result since the proximity of His-12 and His-119 in RNase crystals[36,76] is sufficient in theory[37] to give rise to measurable mutual p$K'$ displacements.

We have recently fitted 250-MHz NMR data to an alternative model in which there is mutual interaction between His-12 (H2) and His-119 (H3) and in which peak H3 is affected by a spectroscopic perturbation outside the titration region at pH 3.7.[70] This model (Table IIB) gives a significantly better fit to our data than those models investigated previously.[73,75] The results appear to rule out a hydrogen-bonded interaction. Provided that the interaction is electrostatic, it is possible to calculate the distance between His-12 and His-119 on the basis of the Tanford–Kirkwood theory.[37] The observed $\Delta pK$ value of 0.28 unit at 0.3 ionic strength corresponds to a calculated distance of 7.7 ± 0.8 Å between the histidine rings.[70] This distance is in excellent agreement with that of 7 Å found in the recent X-ray analysis of uninhibited RNase A.[77]

A similar analysis has been carried out with staphylococcal protease for which the data are consistent with a distance of 7.0 Å between two of the three histidine residues.[29]

## Brief Survey of NMR Studies of Histidine Residues in Proteins

**Classification of NMR Experiments.** The ultimate goal of the NMR studies of histidine residues of proteins has been to investigate the role this amino acid plays in their function. Most of the experiments carried out to date fall into four categories.

*Titration Studies.* The histidine p$K'$ values of those proteins that have been studied by nmr spectroscopy are summarized in Table II (see ref 5, 16, 17, 22, 23, 25, 27–29, 31, 44, 46–48, 51, 52, 65, 68, 71, 78–85). They range from a low of 4.9 for one of the histidines of chinchilla RNase A[52] or less than 4 for the single histidine of $\alpha$-lytic protease[22,23] to a high of 8.1 for two of the histidines of hemoglobin.[46,47] Thermodynamic parameters for the histidine dissociations of two of these proteins, RNase A[86] and lysozyme,[82] have been determined from the temperature dependence of p$K'$ values.

*Protein Modification Studies.* The effects of protein modification, both chemical and enzymatic, on the local environment of histidine residues have been investigated with a number of proteins. The objective usually has been to assign histidine resonances.

*Ligand Binding Studies.* The participation of histidine residues in catalytic and binding mechanisms has been determined by ligand binding experiments (Table III; see ref 5, 19, 31, 39–41, 43, 46, 47, 52, 59, 68, 75, 78–83, 87–95). The region surrounding metal ion binding sites of proteins may be studied in detail by following effects of added diamagnetic and paramagnetic ions.

*Studies of Conformational Transitions.* Conformational transitions may be followed conveniently by NMR spectroscopy. By monitoring a number of groups it is possible to distinguish between limited conformational changes and the more drastic changes that accompany folding and unfolding of proteins. The folding mechanisms of staphylococcal nuclease[45,96,97] and RNase A[98–103] have been investigated in detail by following denaturation shifts of histidine residues. Future studies will certainly make greater use of relaxation measurements in order to determine changes in the mobility of protein side chains during conformational transitions.[26,97]

**Roles that Histidine Residues Play in Protein Structure and Function.** *Catalytic and Binding Sites.* The catalytic role of His-12 and His-119 in RNase A has been confirmed by NMR studies. A detailed mechanism of RNase A has been published that is based on combined chemical, X-ray, and NMR data.[6,104] The interpretation of some of the NMR data for RNase A will have to be modified as a result of the revised assignment of the NMR peaks of the active-site histidines.[25]

The participation of a histidine residue in a "charge relay" system[105] present in serine proteases homologous to chymotrypsin has been proposed. Unfortunately, early attempts to resolve the His C-2 H

(76) H. W. Wyckoff, D. Tsernoglou, A. W. Hanson, J. R. Knox, B. Lee, and F. M. Richards, *J. Biol. Chem.*, **245**, 305–328 (1970).
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(82) J. S. Cohen, *Nature (London)*, **223**, 43–46 (1969).
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(84) J. S. Cohen, H. Hagenmaier, H. Pollard, and A. N. Schechter, *J. Mol. Biol.*, **71**, 513–519 (1972).
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(86) G. C. K. Roberts, D. H. Meadows, and O. Jardetzky, *Biochemistry*, **8**, 2053–2056 (1969).
(87) J. L. Markley and O. Jardetzky, *J. Mol. Biol.*, **50**, 223–233 (1970).
(88) D. H. Meadows, G. C. K. Roberts, and O. Jardetzky, *J. Mol. Biol.*, **45**, 491–511 (1969).
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(91) J. H. Griffin, A. N. Schechter, and J. S. Cohen, *Ann. N.Y. Acad. Sci.*, **222**, 693–708 (1973).
(92) P. H. Haffner and J. H. Wang, *Biochemistry*, **12**, 1608–1617 (1973).
(93) W. Haar, W. Maurer, and H. Rüterjans, *Eur. J. Biochem.*, **44**, 201–211 (1974).
(94) J. S. Cohen, R. I. Shrager, M. McNeel, and A. N. Schechter, *Nature (London)*, **228**, 642–644 (1970).
(95) P. J. Sadler, F. W. Benz, and G. C. K. Roberts, *Biochim. Biophys. Acta*, **359**, 13–21 (1974).
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(99) J. H. Bradbury and N. L. R. King, *Aust. J. Chem.*, **25**, 209–220 (1972).
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(103) C. R. Matthews and D. G. Westmoreland, *Ann. N.Y. Acad. Sci.*, **222**, 240–254 (1973).
(104) G. C. K. Roberts, E. A. Dennis, D. H. Meadows, J. S. Cohen, and O. Jardetzky, *Proc. Nat. Acad. Sci. U.S.A.*, **62**, 1151–1158 (1969).
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**Table II**
**Summary of p$K'$ Values of Histidine Side Chains in Proteins as Determined by NMR Titration**

| Protein | Mol wt | Number of His residues | Number resolved | p$K'$ values (unliganded native proteins) | | | | | | | | | Ref |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | |
| Adenylate kinase (pig) | 21,000 | 2 | 2 | <5.5 | 6.3 | | | | | | | | 78 |
| Carbonic anhydrase (cow, B) | 30,000 | | 3–4 | | | | | | | | | | 79 |
| (human, B) | 29,182 | 11 | 4 | 5.91 | 6.04 | 7.00 | 7.23 | | | | | | 79, 80 |
| (human, C) | 30,000 | | 7 | 5.87 | 5.96 | 6.10 | 6.20 | 6.63 | 7.20 | 7.28 | | | 79 |
| Chymotrypsin $A_6$ (cow) | 25,405 | 2 | 1 | 7.2 | | | | | | | | | 16, 17 |
| Chymotrypsinogen A (cow) | 25,666 | 2 | 1 | 7.2 | | | | | | | | | 16, 17 |
| Cytochrome *c* (horse) | 11,702 | 3 | 2 | 6.41 | | | | | | | | | 51 |
| Hemoglobin (human) | 61,986 | 10$\alpha$ + 9$\beta$ | 9 | 6.8 | 7.0 | 7.0 | 7.1 | 7.2 | 7.13 | 7.7 | 8.1 | 8.1 | 46, 47 |
| Lysozyme (chicken) | 14,314 | 1 | 1 | 5.8 | | | | | | | | | 5, 81 |
| (human) | 14,701 | 1 | 1 | 7.1 | | | | | | | | | 5, 82 |
| Myoglobin (horse) | 16,951 | 11 | 6 | 5.68 | 6.03 | 6.63 | 6.90 | 7.02 | 7.62 | | | | 83, 84 |
| (sperm whale) | 17,199 | 12 | 7 | 5.37 | 5.53 | 6.34 | 6.44 | 6.65 | 6.83 | 8.05 | | | 84 |
| Neurophysin II (cow) | 9,500 | 1 | 1 | 6.87 | | | | | | | | | 31 |
| Nuclease (*Staphylococcus aureus*, Foggi) | 16,800 | 4 | 4 | 5.46 | 5.75 | (5.66) | (5.74) | (6.54)[a] | 6.57 | | | | 27 |
| (*Staphylococcus aureus*, V8) | 16,800 | 3 | 3 | 5.55 | (5.80) | (6.10)[a] | 6.50 | | | | | | 44, 48 |
| Ovomucoid (chicken) | 27,300 | 4 | 4 | 5.94 | 6.71 | 6.75 | 8.07 | | | | | | 71 |
| Protease, $\alpha$-lytic (*Myxobacter* 495) | 19,860 | 1 | 1 | <4 | | | | | | | | | 22, 23 |
| Protease (*Staphylococcus aureus*, V8) | 12,000 | 3 | 3 | 6.99 | 6.87 | 7.20 | | | | | | | 29 |
| Ribonuclease A (coypu) | 14,000 | | 4 | 5.8 | 6.3 | 6.3 | 8.0 | | | | | | 52 |
| (chinchilla) | 14,000 | | 5 | 4.9 | 6.0 | 6.1 | 7.2 | | | | | | 52 |
| (cow) | 13,600 | 4 | 4 | 6.01 | 6.17 | 6.72 | 6.9 | | | | | | 25, 68 |
| (rat) | 14,094 | 5 | 5 | 6.1 | 6.2 | 6.3 | 6.6 | 7.6 | | | | | 52 |
| Ribonuclease $T_1$ (*Aspergillus oryzae*) | 11,089 | 3 | 3 | 7.9 | 8.0 | ? | | | | | | | 85 |
| Trypsin (pig, $\beta$ form) | 25,000 | 4 | 4 | 5.0 | 6.54 | 6.66 | 7.20 | | | | | | 28 |
| Trypsin inhibitor (soybean, Kunitz) | 20,095 | 2 | 2 | 5.27 | 7.00 | | | | | | | | 65 |

[a] The p$K'$ values in parentheses represent the proton dissociation constants of one histidine existing in multiple conformational forms of the enzyme which interconvert slowly.

**Table III**
**Ligand Binding Studies**

| Proteins | Ligand | Ref |
|---|---|---|
| | A. Inhibitors | |
| Adenylate kinase (pig) | ATP | 78 |
| Carbonic anhydrase (human B) | Iodide, *p*-carbobenzoxybenzenesulfonamide | 80 |
| | Acetazolamide | 79 |
| (human C) | Acetazolamide | 79 |
| Hemoglobin (human) | $O_2$ | 46 |
| | CO | 47 |
| Myoglobin (whale) | CO | 83 |
| Lysozyme (chicken) | *N*-Acetylglucosamines | 5, 81 |
| (human) | *N*-Acetylglucosamines | 82 |
| Neurophysin II (cow) | Oxytocin | 31 |
| Nuclease (*S. aureus*, Foggi) | 5′-dTMP, 3′,5′-dTMP | 87 |
| Ribonuclease A (cow) | Sulfate | 75, 88 |
| | Phosphate | 19, 59, 88, 89 |
| | Pyrophosphate | 75 |
| | Nucleotides | 19, 52, 68, 75, 88–93 |
| | Dinucleoside phosphonate | 91 |
| Ribonuclease $T_1$ (*A. oryzae*) | 3′-GMP | 85 |
| | B. Diamagnetic Metal Ions and Complexes | |
| Carbonic anhydrase (human, B) | $Zn^{2+}$ | 80 |
| Nuclease (*S. aureus*, Foggi) | $Ca^{2+}$ | 5, 87, 94 |
| | $La^{3+}$ | 43 |
| Ribonuclease A (cow) | Chlorcplatinite | 95 |
| | C. Paramagnetic Probes | |
| Adenylate kinase (pig) | $Mn^{2+}$ | 78 |
| Lysozyme (chicken) | Tem-pyro-$CH_2$-NAG-NAG (spin-label inhibitor) | 40, 41 |
| Nuclease (*S. aureus*, Foggi) | $Nd^{3+}$ | 39 |
| | $Eu^{3+}$, $Gd^{3+}$, $Dy^{3+}$ | 43 |
| Ribonuclease A (cow) | Tempop (spin-label inhibitor) | 38 |

resonances of serine proteases were unsuccessful.[106] The poor resolution of the early studies appears to have resulted from interference from slowly exchangeable N–H peaks. Alternative methods of observing the histidines have recently been employed. These studies have generated a controversy concerning the actual p$K'$ value of the histidine in the charge relay system. Robillard and Shulman[16,17] have assigned a N–H peak of chymotrypsin that is visible at low temperatures in $H_2O$ solution and that titrates with a p$K'$ of 7.2 to the charge relay histidine. This p$K'$ is in line with the traditional value of 7 assumed for this residue. Richards and coworkers[22,23] introduced $^{13}C$ into the C-2 position of the single histidine of $\alpha$-lytic protease, a bacterial protein whose sequence is homologous to chymotrypsin. In this case, the histidine titration was monitored by changes in the His $^{13}C$–$^1H$ coupling constant which has been shown to be responsive to the protonation state of the ring; the resulting p$K'$ value was less than 4.

Based on this result, Richards and coworkers[22,23] have proposed an inversion of the normal p$K$ values of the aspartic acid and histidine residues in the charge-relay system. They propose that an enzyme with such properties would have a lowered activation energy since there would be less charge separation in the transition complex. We have recently resolved the C-2 H resonances of porcine trypsin and bovine chymotrypsin.[28] Both of these serine proteases contain one abnormal histidine residue having a p$K'$ of 4.5–5.5 which appears to correspond to the active-site histidine.

*Regulatory.* The p$K'$ value of His-146$\beta$ of hemoglobin has been shown by NMR titration to be 7.1 in the carbon monoxy and 8.0 in the deoxy form.[47] This result confirms the role of His-146$\beta$ in the alkaline Bohr effect which is of physiological importance in $CO_2$ transport. In venous blood, His-146$\beta$ of deoxyhemoglobin acts as a base to neutralize the carbonic acid produced by dissolved $CO_2$. In the lungs where hemoglobin is oxygenated, the process is reversed, and $CO_2$ is expelled.

*Influence on Protein Equilibria.* The position of the conformational equilibrium affecting His-46 of staphylococcal nuclease is influenced by binding of calcium ion.[66] Conversely, the binding constant of calcium ion which is required for enzyme activity should depend on the protonation state of His-46.

An interesting role is played by His-71 in the trypsin-catalyzed equilibrium between the virgin (intact) and modified (Arg-63–Ile-64 bond cleaved) forms of soybean trypsin inhibitor. His-71 has a p$K'$ of 5.27 in the virgin form and 5.91 in the modified form.[56,65,71] Since the virgin–modified equilibrium is pH dependent, the change in p$K'$ of His-71 perturbs the equilibrium significantly.[107]

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## Future Prospects

The success of NMR studies of histidine residues in proteins stems largely from the relatively easy resolution and assignment of histidine NMR peaks. The methods of isotopic substitution[14,15] and NMR difference spectroscopy[71,108,109] should permit the extension of this kind of study to other residues. These investigations will certainly be aided by the new techniques of NMR relaxation and double resonance[110-112] and by the increased resolution and sensitivity of newer NMR instrumentation.

*The work described in this Account was initiated in 1966 at the Merck Institute for Therapeutic Research, Rahway, N.J., in an enthusiastic group which included Drs. Jack S. Cohen, Oleg Jardetzky, Donella H. Meadows, Gordon C. K. Roberts, and Myra N. Williams. The recent correlation $^1H$ nmr studies at 250 MHz were carried out at the NMR Facility for Biomedical Research, Carnegie-Mellon University, Pittsburgh, Pa. I am indebted to Drs. Axel A. Bothner-by, Josef Dadok, and Richard F. Sprecher for their hospitality and patience in teaching me how to use this instrument. The recent research has been supported by the National Institutes of Health RR 00292 (to Carnegie-Mellon University) and GM 19901 (to Purdue University), the Research Corporation, and the Pittsburgh Plate Glass Foundation. I thank Dr. Albert Light for advice in preparing this manuscript.*

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