*NUCLEAR MAGNETIC RESONANCE STUDIES OF THE STRUCTURE AND BINDING SITES OF ENZYMES, I. HISTIDINE RESIDUES**

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To date, the most successful studies on the structure of proteins have been carried out using X-ray crystallography.[1-4] This technique is, however, subject to certain disadvantages, and the extent to which its findings are relevant to the conformation of proteins in solution is still subject to appreciable uncertainty. NMR spectra of a number of proteins and enzymes have been published,[5-8] but little detailed analysis and little structural information have resulted thus far. Nevertheless, high-resolution NMR is in principle one of the best methods for the study of the structure and interaction of proteins in solution. The difficulties of deciphering the information obtained can be overcome by systematic studies of suitable model peptides,[9-12] the preparation of selectively deuterated proteins,[13] and the development of accurate curve-fitting methods.

In this series of papers we wish to report a detailed comparative analysis of the NMR spectra of several enzymes, and of the effects produced on them by the binding of inhibitors and metal ions. Information on the structure of several protein small-molecule complexes has been obtained by Jardetzky and co-workers,[14-17] utilizing effects on the NMR spectrum of the small molecule, which is present in excess in the solution. The present investigation has established that additional information can be obtained by direct observation of the macromolecule in the binding process.

Using a high-resolution spectrometer operating at 100 Mc and time-averaging with an internal lock system, it is possible to obtain meaningful spectra of small (mol wt 10,000–25,000) proteins at concentrations as low as 0.005 *M*. Under these conditions the aromatic amino acids give a clearly separated low-field absorption. In particular, the C-2 proton of the imidazole group of histidine shows a single resonance downfield from the rest of the aromatic envelope.[8] We have been able to resolve each of the C-2 peaks of the four His residues of bovine pancreatic RNase and staph. nuclease, and the C-2 and C-4 peaks of the single His residues of human and HEW lysozymes. The effects on each of these peaks due to pH changes and the presence of metal ions and inhibitors of enzymatic activity can be followed individually, yielding data not obtainable by any other technique.

Materials and Methods.—RNase A (lyophilized and phosphate-free), staph. nuclease, and HEW lysozyme were obtained from Worthington Biochemical Corp.

The solvent for all RNase NMR samples was a 0.2 *M* acetate buffer, pH 5.25, made from CD_3COOD, NaOD, and 99.85% D_2O. The 3′-CMP–RNase solutions were made by introducing the appropriate amount of a freshly made 0.01 *M* cytidine cyclic monophosphate (Sigma) solution in deuteroacetate buffer into a 0.01 *M* (12%) RNase solution. The mixture was stirred for 1 hr to allow complete digestion of the cyclic phosphate to 3′-CMP, and then the pH was adjusted to the desired value. Cytidine and 5′-CMP were products of the Sigma Chemical Co.

The commercial staph. nuclease was prepared by the method of Heins, Taniuchi, and An-

finsen.[18] The nuclease was dialyzed against 6 liters of 1 mM EDTA for 24 hr with 3 changes, and subsequently against 6 liters deionized H_2O for 36 hr with 4 changes to remove divalent cations.[19] The pore size of the cellophane dialysis tubing (Visking) was reduced by boiling in 1% $NaHCO_3$ for 10 min.[20] The enzyme solution was lyophilized to remove H_2O. Subsequently the enzyme was lyophilized twice from D_2O solution in order to deuterate exchangeable groups. Thymidine-5'-monophosphate was obtained from Calbiochem. The solvent for all staph. nuclease NMR spectra was 99.85% D_2O containing 0.30 *M* NaCl, 0.15 *M* CD_3COOD, and 0.10 *M* sodium tetraborate. The initial enzyme concentration was 9% (0.0053 *M* on the basis of mol wt 17,000 for the enzyme).[21] The above solvent system was used to make up 5.00 *M* $CaCl_2$ and 0.700 *M* TMP solutions. These were added to the enzyme solutions with a micrometer syringe.

Ten per cent solutions (0.0068 *M*) of HEW lysozyme (twice crystallized) were made up in D_2O. For inhibition binding studies with D_2O-lyophilized NAG, salt-free enzyme was used. Appropriate amounts of solutions containing from 2.0 to 30 molar ratios of inhibitor were added. A sample of human lysozyme was kindly supplied by Dr. E. Osserman of Columbia University College of Physicians and Surgeons, and was obtained from the urine of patients suffering from leukemia.[22]

All spectra were recorded on a Varian Associates HA 100 NMR spectrometer equipped with internal lock system and autoshim device. A Varian C1024 CAT was used, and spectra shown are averages of 20–180 sweeps. Probe temperature was 28°C. Precision-bore NMR cells (0.5 mm OD) with coaxial inserts were supplied by the Wilmad Glass Co. HMS was the external standard. The pH of all solutions was adjusted with 1.0 *M* DCl or NaOD solutions using a Radiometer titrator (TTT11) and pH meter (model 26) with microelectrodes. All pH values given are actual meter readings uncorrected for deuterium isotope effects.

Results and Discussion.—Ribonuclease: Representative NMR spectra of native RNase at different pH's are shown in Figure 1. The spectra are similar to those reported by Bradbury and Scheraga[23] at 60 Mc, except that in our case all four C-2 protons of the histidine residues at positions 12, 48, 105, and 119 in the peptide chain[24] are clearly resolved. It was therefore possible for us to follow the chemical shift of each peak as a function of pH. The resulting titration curves are shown in Figure 2. The pK's of the four histidines (not corrected for the presence of D_2O) are roughly 5.6, 6.0, 6.2, and 6.4. Peak 5 is one of the C-4 protons of the His

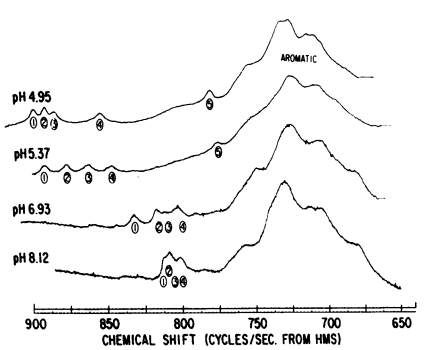

FIG. 1.—100 Mc NMR spectra of the aromatic region of 0.012 *M* RNase A in deuteroacetate buffer (pH 4.95, 100 CAT scans; pH 5.37, 14 CAT scans; pH 6.93, 51 CAT scans; pH 8.12, 57 CAT scans). Peaks *1–4* are C-2 imidazole peaks of the four histidine residues. Peak *5* is a C-4 imidazole resonance. The envelope labeled "aromatic" includes three other C-4 imidazole peaks as well as peaks from six tyrosine and three phenylalanine residues.

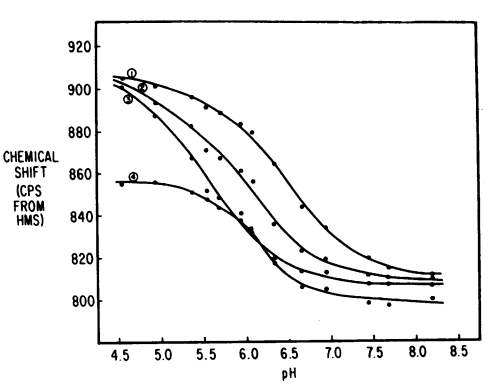

FIG. 2.—Titration curves of C-2 peaks of histidine residues of RNase A. Curves 1–4 correspond to peaks 1–4 of Fig. 1. Approximate pK's are: *curve 1*, 6.3; *curve 2*, 5.9; *curve 3*, 5.6; *curve 4*, 6.1.

residues, the other three of which are buried under the large aromatic envelope of Phe and Tyr absorptions.

The proton of peak 4 appears to be in a quite different magnetic environment from those of the other three histidine residues, especially in the protonated state. Its chemical shift, about 50 cps upfield from the other C-2 peaks, suggests that this histidine is in the vicinity of a negatively charged residue or one with a strong magnetic anisotropy. The more normal chemical shift of His peak 4 after deprotonation of the imidazole group could be due to a conformation change in its immediate environment, or to the disappearance of a coulombic attraction due to the removal of the positive charge, or to both factors. In any case this histidine is apparently much more influenced by its neighbors than His residues 1–3 and might tentatively be identified as His 48, which is known to be chemically inactive,[25] and which seems to be partially buried in the X-ray crystallographic model.[26]

The association of peak 4 with His 48 is also suggested by studies of the RNase spectrum in the presence of various enzyme inhibitors, as illustrated in Figure 3. On the addition of the competitive inhibitor 3′-CMP at a molar ratio as low as one 3′-CMP/ten RNase, there is a marked downfield shift (5 cps) and broadening of His peak 3, while the other three peaks are unaffected. When 5′-CMP is added in a ratio of one 5′-CMP/five RNase, peaks 2 and 3 are both shifted downfield. It is significant that only one or two of the four His residues seem to be affected at this pH (5.38), near which the inhibitor-binding constants are maximal.[27, 28] The presence of the nucleoside cytidine, which is believed not to inhibit RNase,[29] has no effect on the positions or line-widths of the His peaks at these concentration ratios. As the inhibitor/enzyme ratio approaches 1, however, in the presence of 3′-CMP, 5′-CMP, or cytidine, all four peaks shift to differing degrees, suggesting a conformation change in the enzyme. Peaks 1 and 4 show the smallest total shift and remain unchanged at low inhibitor concentrations.

Since it is well established that His 12 and His 119 are involved in the enzymatic activity[30] and are located near each other at the active site,[3] it is tempting to believe

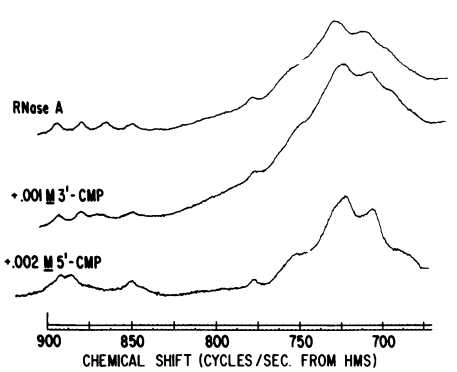

FIG. 3.—Spectra of 0.1 *M* RNase A at pH 5.37 in the presence of inhibitors (RNase A alone, 14 CAT scans; RNase A + 0.001 *M* 3′-CMP, 50 CAT scans; RNase A + 0.002 *M* 5′-CMP, 25 CAT scans).

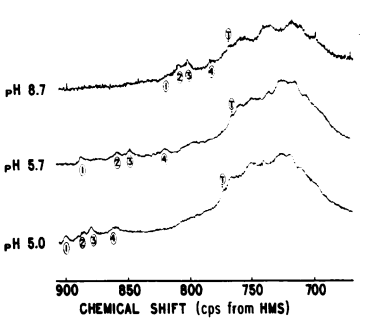

FIG. 4.—Spectra of the aromatic region (4 His, 1 Trp, 3 Phe, 7 Tyr) of calcium-free staphylococcal nuclease (Foggi strain) at three pH values. Solutions contained: 0.005 *M* staph. nuclease, 0.3 *M* NaCl, 0.15 *M* CD_3COOD, 0.1 *M* sodium tetraborate. Peaks *1–4* are C-2 imidazole peaks of the four histidine residues. Peak *T* is the main resonance of the single tryptophan residue. Number of CAT scans: 48 (pH 5.4), 58 (pH 6.4), 56 (pH 8.3).

that peaks 2 and 3, which are most affected by the binding of inhibitors, correspond to His 12 and His 119. However, a positive identification of the four NMR peaks must await the completion of the study of RNase histidine derivatives currently in progress in collaboration with Dr. H. A. Scheraga.

Staph. nuclease: NMR spectra of calcium-free staph. nuclease at three pH values are shown in Figure 4. Four His peaks can be resolved in the pH range from 5 to 9 in agreement with the published amino acid composition of the enzyme.[21] The titration curves of these His residues are seen in Figure 5. The main peak of the single tryptophan residue may also be distinguished. The slight (6 cps) shift in this peak (Fig. 5) probably is a reflection of the titration of one or more histidine residues which are located near the tryptophan side chain. Precipitation of the enzyme occurs below pH 5 at the concentrations used in this study; therefore, titration curves 2, 3, and 4 are incomplete. It is possible, however, to make a rough estimate of the pK values of the four His residues. These are 5.6, 5.9, 6.1, and 6.6.

Spectra of staph. nuclease in the presence of calcium ion are shown in Figure 6. The addition of Ca^{2+}, which is required for activity of the enzyme, causes a selective downfield shift in His peak 1 above pH 6.8. There is apparently an interaction between calcium ion and the uncharged imidazole ring. At a Ca^{2+}/staph. nuclease molar ratio of 30, the downfield shift in peak 1 is 16 cps in the pH range 7.5–9.5. The other three His peaks are shifted downfield only slightly (less than 6 cps) in this pH range. Their shift may be the result of nonspecific interactions. The main tryptophan peak remains unshifted in the presence of Ca^{2+}.

The effect of the inhibitor 5′-TMP on the aromatic region of the NMR spectrum

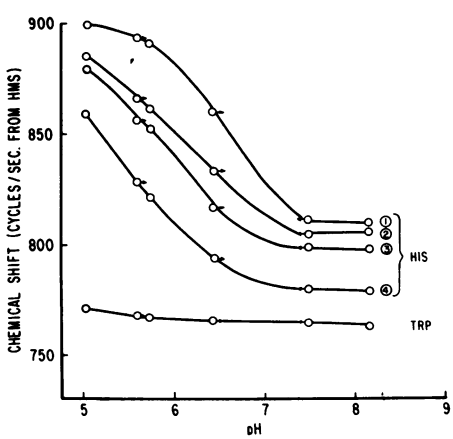

FIG. 5.—Titration curves of the C-2 peaks of staph. nuclease. Curves *1–4* correspond to peaks *1–4* of Fig. 4. Approximate pK values for curves *1–4*: 5.6, 5.9, 6.1, 6.6. The pH of the solution was measured before and after the spectra were taken. The points of the arrows indicate the final pH reading when it differed from the initial.

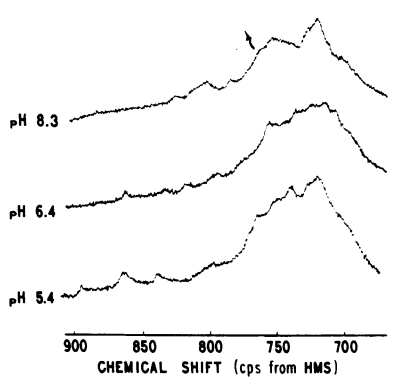

FIG. 6.—Spectra of the aromatic region of staph. nuclease in the presence of calcium ion at three pH values. Solutions are identical to those of Fig. 4 except for the addition of Ca^{2+} at a Ca^{2+}/enzyme molar ratio of 30. In comparison with the Ca^{2+}-free titration curve (Fig. 5), it may be seen that the lowest field C-2 His proton is shifted downfield significantly at pH 8.3. Number of CAT scans: 52 (pH 5.4), 48 (pH 6.4), 46 (pH 8.3).

of staph. nuclease was investigated in the presence of Ca^{2+} which reportedly is necessary for nucleotide binding.[31] Inhibitor-to-enzyme molar ratios of $1/20$ to $1/4$ were studied at pH 6.4 and 9.3 with a Ca^{2+}/enzyme molar ratio of 30. No large selective shifts in the histidine peaks of the kind seen in ribonuclease A were observed under these conditions. Thus the two nucleases clearly have different mechanisms of inhibitor binding.

Systematic changes in the main aromatic envelope of the staph. nuclease spectrum (comprising 7 Tyr and 3 Phe) are seen on nucleotide binding and are under further study.

HEW and human lysozymes: Spectra of HEW and human lysozymes at various pH's are shown in Figures 7 and 8. The peaks corresponding to the C-2 and C-4 protons of the single histidine residue in each enzyme[22, 32, 33] are clearly identified. The titration curves for these peaks are shown in Figure 9. The much higher microscopic pK (7.6) observed for the His of the human as compared to the HEW lysozyme (5.8) and the other enzymes is surprising. This retarded titration may result from the effects of hydrogen bonding or the proximity of a positively charged group. It seems unlikely that this His residue in this case is buried in the interior of the molecule since the resonance peak is at least as sharp as those of the His protons in the other enzymes studied.

Addition of the NAG inhibitor to solutions of HEW lysozyme in molar ratios of 2–30/1, respectively, gave no systematic shifting of the C-2 His peak (Fig. 9). Similarly no systematic broadening was observed. This result should be compared to the very marked selective broadening and shifting observed for two of the C-2 His peaks of RNase at much lower molar ratios of inhibitor to enzyme. It is therefore clear that the His residue of HEW lysozyme is not implicated in the binding site for NAG in solution. This is consistent with the known structure of the crystalline inhibitor-enzyme complex obtained from X-ray crystallography.[34]

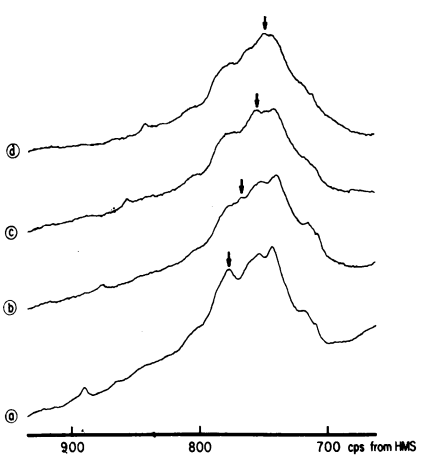

FIG. 7.—Spectra of the aromatic (6 Trp, 3 Phe, 3 Tyr, 1 His) region of hen egg-white lysozyme (10% in D_2O). (*a*) pH 5.0, 140 CAT scans; (*b*) pH 5.3, 40 CAT scans; (*c*) pH 5.7, 40 CAT scans; (*d*) pH 6.0, 188 CAT scans. The arrow indicates the position of the C-4 His peak.

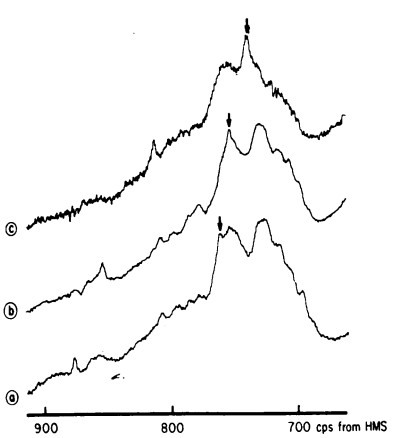

FIG. 8.—Spectra of the aromatic region of human lysozyme (10% in D_2O). (*a*) pH 4.9, 45 CAT scans; (*b*) pH 6.9, 114 CAT scans; (*c*) pH 7.8, 26 CAT scans. The arrow indicates the position of the C-4 His peak.

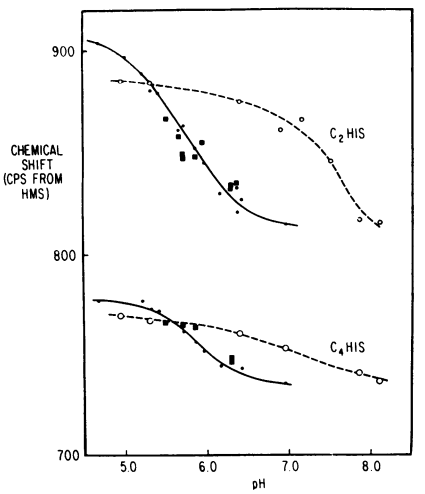

FIG. 9.—Titration curve for C-2 and C-4 histidine protons of human (-○-) and HEW (-●-) lysozymes (■, latter in presence of 2–30 molar ratio of NAG). Approximate pK's are 7.6 and 5.8, respectively.

Certain other changes of the aromatic envelope of the HEW lysozyme spectrum are, however, observed on the addition of NAG, particularly the aromatic region of the spectrum which becomes progressively sharpened at pH's 5.9 and 6.3. It is relevant to note that in solutions of the enzyme alone, the spectrum becomes gradually broadened as the pH is increased (Fig. 7), until above pH 7 very little fine structure can be resolved. This must result from reduced mobilities of the aromatic residues due to molecular association in solution.[35] Such a phenomenon is not observed with human lysozyme (Fig. 8). Addition of NAG sharpens the aromatic region of the spectrum of HEW lysozyme at a given pH, which would seem to indicate that the formation of the NAG-lysozyme complex effects a dissociation of the enzyme aggregates.

Changes in relative peak heights of the aromatic envelopes observed on inhibitor binding are being analyzed using the DuPont 310 curve resolver, and results will be presented in forthcoming papers.

Summary.—100 Mc NMR spectra of the aromatic absorption regions of the enzymes bovine pancreatic ribonuclease A, staphylococcal (Foggi) nuclease, and hen egg-white and human lysozymes have been obtained. The imidazole C-2 proton resonances of the four histidine residues of ribonuclease and staphylococcal nuclease and the single histidine residues of the two lysozymes have been clearly resolved. Plots of the shifts of these peaks as a function of pH give an individual titration curve and pK for each histidine residue. In ribonuclease, the pK values are 5.6, 6.0, 6.2, 6.4; in staphylococcal nuclease, 5.6, 5.9, 6.1 and 6.6; in HEW lysozyme, 5.8; and in human lysozyme, 7.6, reflecting differences in protein structure. Addition of nucleotide inhibitors to ribonuclease causes selective shifting and broadening of at most two of the four histidine C-2 peaks. The presence of inhibitors did not affect the histidine regions of the spectra of staphylococcal nuclease or hen egg-white lysozyme. The formation of the inhibitor–hen egg-white lysozyme complex did, however, result in sharpening of the aromatic region of the spectrum, indicating dissociation of enzyme aggregates. One histidine C-2 peak of staphylococcal nuclease has been found to shift downfield by 16 cps upon addition of Ca^{2+}. The positions of the other three peaks were only slightly affected by Ca^{2+}.

Abbreviations used: RNase, ribonuclease; NMR, nuclear magnetic resonance; 3′-CMP, cytidine-3′-phosphate; 5′-CMP, cytidine-5′phosphate; 5′-TMP, thymidine-5′-phosphate; HEW lysozyme, hen egg-white lysozyme; staph. nuclease, staphylococcal (Foggi strain) nuclease; NAG, *N*-acetyl glucosamine; HMS, hexamethyldisiloxane; CAT, computer of average transients; EDTA, ethylenediaminetetraacetate.

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