NUCLEAR MAGNETIC RESONANCE STUDIES OF THE STRUCTURE AND BINDING SITES OF ENZYMES, II. SPECTRAL ASSIGNMENTS AND INHIBITOR BINDING IN HEN EGG-WHITE LYSOZYME

By Jack S. Cohen and Oleg Jardetzky

DEPARTMENT OF BIOPHYSICS AND PHARMACOLOGY, MERCK, SHARP AND DOHME RESEARCH LABORATORIES, RAHWAY, NEW JERSEY

Communicated by Max Tishler, March 8, 1968

Inhibitor binding to HEW lysozyme has been studied by X-ray crystallography,[1, 2] ultraviolet[3, 4] and fluorescence[5, 6] spectroscopy. Much consistent information regarding the nature of the binding subsites for oligosaccharides has been elicited. Recent progress in the application of NMR to problems of protein structure and function[7–17] has made it possible to obtain more detailed information on the behavior of the enzyme in solution. In this paper we wish to report partial assignments in the low-field (aromatic) region of the HEW lysozyme spectrum, a comparison of several denatured conformations and spectral changes resulting from inhibitor binding.

Materials and Methods.—HEW lysozyme (twice crystallized, salt-free) was obtained from Worthington and was lyophilized from D_2O. Unless otherwise indicated, 10% solutions (0.0068 *M*) in D_2O containing 0.1 *M* NaCl were used. pH was measured with a Radiometer pH-meter 26 and a Beckman microcombination electrode (no. 39030) and was adjusted with 0.1 *M* DCl or NaOD. pH values given are actual meter readings uncorrected for deuterium isotope effects and were checked after the sample had been in the spectrometer.

NAG was obtained from Mann Laboratories, Inc. Di- and tri-NAG were prepared from chitin.[18] A Bio-Gel P-2 (200–400 mesh) (Bio-Rad Laboratories) column (75 × 2 cm) was used to purify aliquots (*ca.* 70 mg) of the initially separated oligomers. The materials were characterized by specific rotation, paper chromatography, and NMR. Inhibitors and urea were lyophilized from D_2O. Peptides were obtained from Miles-Yeda Laboratories. Insoluble material was spun down in a Clay-Adams Microchemistry centrifuge. Precision-bore NMR cells (0.5 cm OD), requiring 0.35 ml of solution, and coaxial inserts were obtained from the Wilmad Glass Co.

Spectra were recorded with frequency sweep on a Varian Associates HA 100 NMR spectrometer equipped with internal lock system and autoshim device. Sweep rate was 1 cps/sec at 250 cps sweep width, unless otherwise stated. The offset and sweep width were calibrated directly using a Varian Associates Counter (V 4315) and a C-1024 CAT. Chemical shift values are given relative to external HMS to the nearest cps. (DSS is 33 cps downfield from HMS.) For observation of the high-field region, benzene was used as the lock signal.

Ambient probe temperature was 32°, and spectra were recorded at this temperature unless otherwise stated. For higher temperatures, the Varian Variable Temperature Controller (V 4343) was used.

Results and Discussion.—Denaturation and spectral assignments: The simplest NMR spectrum of HEW lysozyme is obtained by totally denaturing the enzyme and breaking the four disulfide bonds[19, 20] with excess 2-mercaptoethanol[21] in 8 *M* urea at 65°. From the fit of the spectra of the aromatic amino acids and their relative chemical shifts (Fig. 2), it is possible to assign peaks in the enzyme spectrum as shown in Figure 1*C*. The major peak at 767 cps which is absent in

the other enzyme spectra (Fig. 1*A*, *B*) arises from the equivalence of 15 Phe protons. The major peak at 761 cps is assigned to the sharp singlet of the Try H-2 protons. The doublet at 720 cps corresponds to the high-field Tyr doublet. Hence, the region 730–755 cps consists of the superposition of the low-field Tyr doublet and the Try H-5,6 peaks (Fig. 2). In view of the overlap and the possible change in multiplicity of the Try H-5,6 peaks as a result of small relative differences in chemical shifts (since $\Delta\delta \simeq J_{H-5,6}$),[22] this region is difficult to analyze. Similarly, the H-4,7 region 770–800 cps, although largely separated, clearly shows a change in intensity ratios compared to the simple Try spectrum

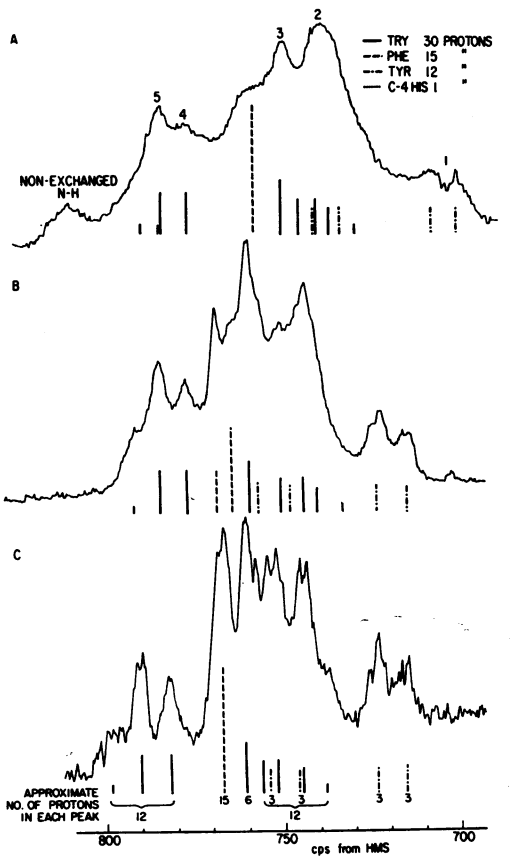

FIG. 1.—100 Mc/sec NMR spectra of the aromatic region of HEW lysozyme. (*A*) 8 M urea, pH 4.5, 32°, 45 CAT scans; (*B*) 0.1 M NaCl, pH 4.6, 75°, 25 scans; (*C*) 8 M urea + excess 2-mercaptoethanol, 65°, 25 scans.

(Fig. 2*G*). There is a Try-Try sequence (residues 62, 63) in the enzyme,[20] and the peptide Try-Try shows unequal intensity ratios in this region, even in 8 *M* urea.[23]

Temperature denaturation[10, 12] occurs over a range of a few degrees. At 75° the spectrum (Fig. 1*B*), although containing some sharp peaks as a result of thermal motion, is less sharp than the spectrum with the disulfide bonds broken (Fig. 1*C*), reflecting the rigidity due to these bonds. The Try H-4,7 region is shifted upfield, and there is a change in the Try H-5,6–Tyr region (peak 2),

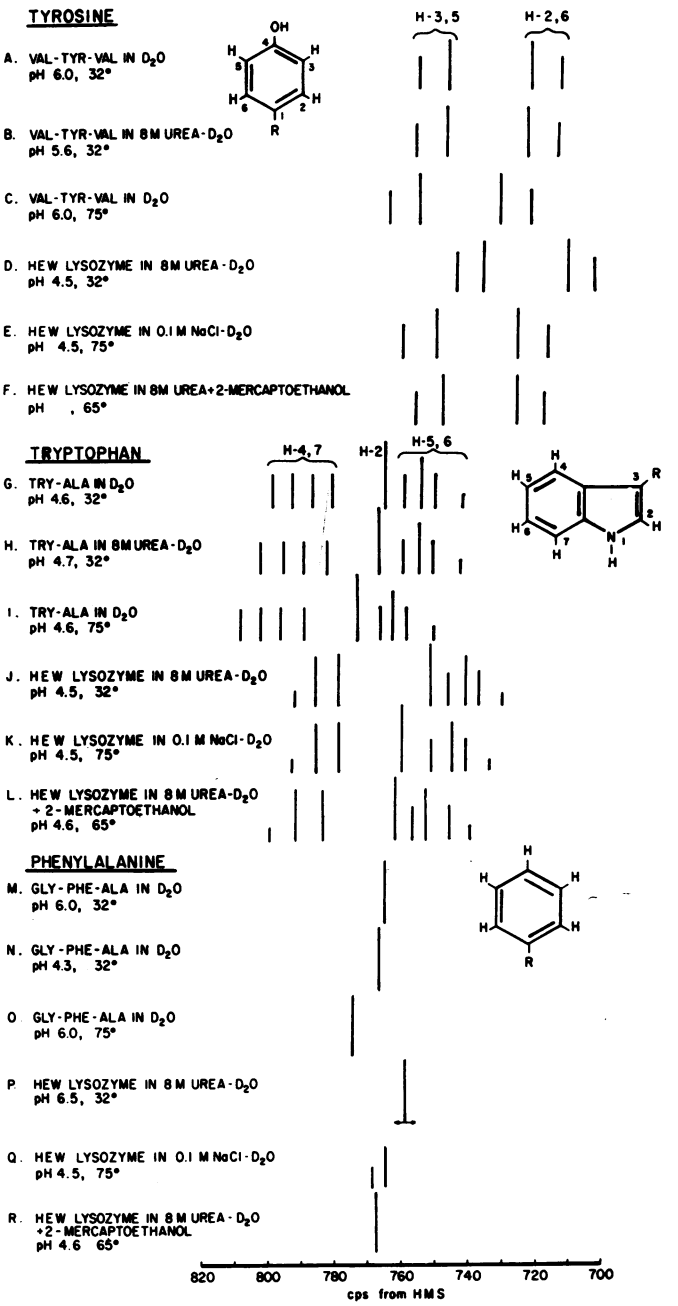

FIG. 2.—Spectra of the aromatic region of Tyr, Try, and Phe in peptides and HEW lysozyme under various solvent and temperature conditions (R = $CH_2CH(NH_2)COOH$).

partly due to broadening of these peaks. The sharp peak at 768 cps (Fig. 1*B*) must be due to a single Phe residue with free rotation (as in Fig. 1*C*) and is assigned to Phe at position 3 near the amino terminal end in the protein sequence.[20] The peak due to the other two Phe residues appears to be upfield by 4 cps and to be much broader than the Phe-3 peak. (The area of this shoulder is *ca.* 20% of the total with the use of the DuPont 310 curve resolver.) The residues Phe-34 and -38 are therefore less mobile and more shielded.

Denaturation with increasing molarity of urea (Fig. 3) results in the gradual shift of a peak from *ca.* 740 to 750 cps in 8 *M* urea. This peak is assigned (Fig. 1*A*) to the Try H-2 protons. Since there are few other changes from the spectrum of the native enzyme, no major conformational change could have occurred.

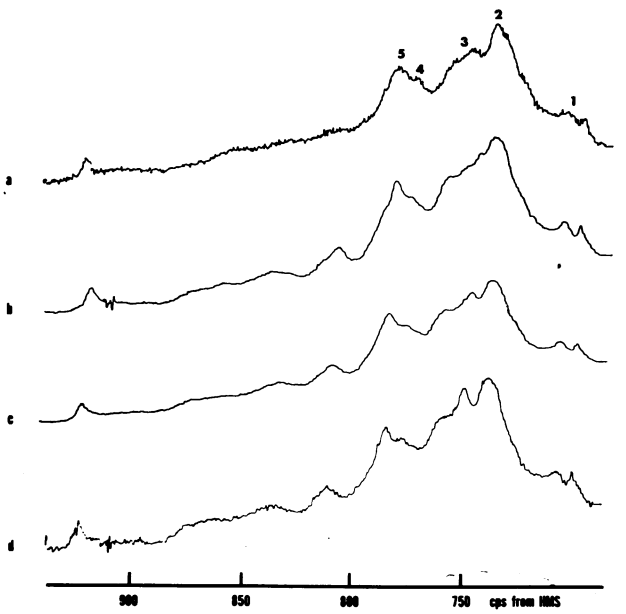

FIG. 3.—100 Mc/sec NMR spectra of HEW lysozyme at pH 4.55 ± 0.05 with increasing molarity of urea. (*a*) alone, 35 CAT scans; (*b*) 4 M urea, 220 scans; (*c*) 6 M, 109 scans; (*d*) 8 M, 45 scans.

This is consistent with the known stability of the enzyme in urea from other measurements.[24] Comparison of the spectrum to those of the heated samples (Fig. 1*B*, *C*) indicates a number of major spectral differences, apart from the changes in line widths. The Try H-2, Phe, and Tyr peaks have all shifted upfield as expected from the temperature shifts of the peptides (Fig. 2). However, the Tyr doublet at 705 cps has shifted 14–15 cps upfield, in contrast to only 8 cps for Val-Tyr-Val (Fig. 2). This partly accounts for the changes in relative intensities of peaks *2* and *3*.

It is quite clear from the above that different conformations of the molecule exist under the different denaturing conditions described. Previously, there have only been a few indications of such states.[25–28] The changes observed, notably increased shielding of Phe, Tyr, and Try residues in going from Figure 1*C* to Figure 1*A*, are indicative of the formation of a compact highly aromatic region, as described for the structure determined by X-ray crystallography.[2]

Furthermore, the downfield shift of the Try H-2 peak in 8 *M* urea (Fig. 3) indicates the presence of magnetically nonequivalent highly shielded Try residues in the native enzyme. For Try-Try, the H-2 singlet is at 750 cps, 13 cps upfield from the position in Try-Ala (Fig. 2*G*). This shielding indicates base stacking in Try-Try, and may also be expected for the Try-Try sequence in the native enzyme.[2]

The mode of action of such denaturants as urea was clarified in parallel studies which were carried out on the high-field region of the lysozyme spectrum (Fig. 6*A*) recently described by McDonald and Phillips.[10, 29] Peaks in this region are thought to arise from the shielding of aliphatic protons by aromatic ring currents.[10, 12] *No changes* occurred in these peaks in 8 *M* urea, even though they vanish at 75°.[10] Studies of solvent structure perturbations in denaturing solutions with ultrasonic attenuation have indicated gross breakdown of water structure.[30, 31] The results are consistent with the increased solubilization observed for hydrocarbons[32] and aromatic amino acids[33] in aqueous urea solutions. Hence, it has been suggested[31, 33] that breakdown of hydrophobic bonds in the native enzyme are a prime cause of denaturation by urea. This does not appear to be the case for lysozyme where certain hydrophobic bonds, as indicated above, remain intact in 8 *M* urea solution. The major effect of urea would appear to result in a deshielding of Try H-2 protons (Fig. 3). Those residues (Try 62, 63, and 108) which occupy the cleft containing the active site[2] would be expected to be the most susceptible to solvent effects.

Binding studies with di- and tri-NAG: Above pH 4.5[34] or 5,[35] HEW lysozyme is reported to aggregate in solution. This is reflected in a general loss of resolution of the NMR spectrum with increase in pH due to broadening of the peaks.[9] Thus, all binding studies were carried out at pH 4.5–4.6 to avoid superposition of phenomena. Slight broadening of the aromatic region of the spectrum of HEW lysozyme resulted at pH 4.5 with high molar ratios of NAG. However, with di- and tri-NAG selective effects were observed at inhibitor to enzyme ratios of 0.5–15:1 (Figs. 4 and 5). These changes were essentially complete with 7.5 *M* ratio of di-NAG and 2 *M* ratio of tri-NAG, reflecting differences in their association constants.[6] It should be noted that the C-2 His peak (915–917 cps) and the high-field tyrosine doublet (peak *1*, 705 cps) do not shift on addition of inhibitor. Also the shoulder on peak *3* (Fig. 4, 756 cps) assigned to Phe (Fig. 1*A*) appears to remain stationary.

The systematic shifts which occur can be followed most readily for di-NAG (Fig. 4). Thus, with 1 *M* ratio a peak appears at 746 cps and shifts a further 9 cps downfield. This behavior is strikingly similar to that observed on urea denaturation (Fig. 3), and in that case the peak was assigned to the H-2 protons of Try (Fig. 1*A*). Concurrently, peak *4* in the enzyme spectrum (772 cps) shifts downfield with di-NAG a total of 12 cps. This peak is assigned to the H-4,7 region of the Try spectrum (Fig. 1). Thus, it appears that some (probably two or three) of the Try residues with H-2 peaks at unusually high field in the native enzyme experience a deshielding on binding inhibitor. This could result from H-bonding of Try 62 and 63 with the inhibitor molecule[2] or from increased van der Waals contact with adjacent amino acid residues. Peak *2* in the enzyme

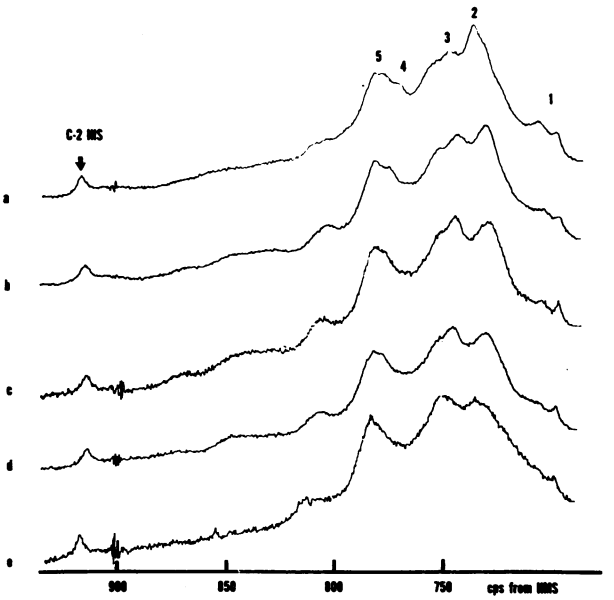

FIG. 4.—100 Mc/sec NMR spectra of the aromatic region of HEW lysozyme (10% in 0.1 M NaCl/D_2O) at pH 4.55 ± 0.05 with increasing molar ratios of tri-NAG. (*a*) alone, 35 CAT scans; (*b*) plus 0.5 M ratio, 38 scans; (*c*) 1 M, 25 scans; (*d*) 5 M, 20 scans.

spectrum is also affected by addition of inhibitor. However, this peak arises from the overlap of a number of components (Fig. 1*A*) and no clear deductions can be made.

The spectra obtained on saturation of HEW lysozyme with di- or tri-NAG (Figs. 4*E*, 5*D*) are almost superimposable, which would tend to indicate equivalent involvement of aromatic, particularly Try residues, in the binding site(s) for each. This is consistent with the view[2, 6] that subsites B and C are implicated in the strong binding of both di- and tri-NAG, and that subsite C involves Try residues 62 and 63. Perturbation of the spectra of these and Try 108 would also be expected on binding as a result of changes of their intramolecular bonds (hydrogen or hydrophobic).[5, 6]

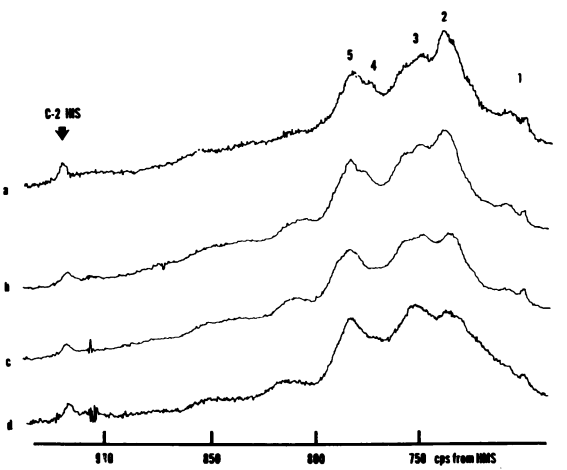

FIG. 5.—100 Mc/sec NMR spectra of the aromatic region of HEW lysozyme (10% in 0.1 M NaCl/D_2O) at pH 4.55 ± 0.05 with increasing molar ratios of di-NAG. (*a*) alone, 62 CAT scans; (*b*) plus 1 M ratio, 48 scans; (*c*) 2.5 M, 19 scans; (*d*) 5 M, 30 scans; (*e*) 10 M, 20 scans.

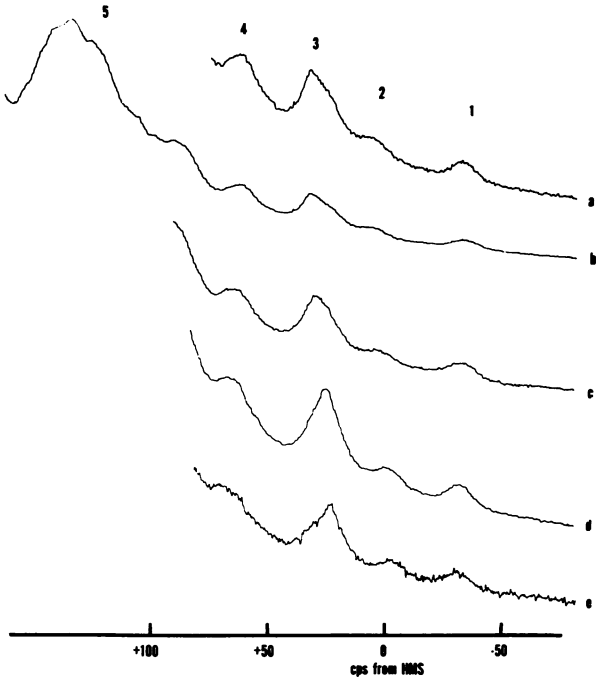

FIG. 6.—100 Mc/sec NMR spectra of the high field region of HEW lysozyme (10% in 0.1 M $NaCl/D_2O$). (*a* alone, pH 4.55 ± 0.05, 35 CAT scans; (*b*) same, lower gain; (*c*) plus 0.5 M ratio of tri-NAG, 19 scans; (*d*) 1 M ratio, 63 scans; (*e*) 5 M ratio, 6 scans.

The high-field region of the lysozyme spectrum on addition of tri-NAG is shown in Figure 6. Peak *1* remains almost stationary, while peaks *2* and *3* shift concurrently upfield by 9 cps, and peak *4* shifts downfield by 9 cps. Peak *5* represents the majority of the methyl group protons in the enzyme. It is quite clear from its distorted shape that peak *3* results from the superposition of two or three components. Furthermore, peaks *1–3* cannot be fitted with Lorentzian shaped curves with the use of the DuPont curve resolver.

An attempt to derive an association constant[36] from the shifts of peaks *2* and *3* was unsuccessful. The upfield shifts on binding indicate greater shielding for the protons comprising peaks *2* and *3*. Since these are thought[10] to arise from the close juxtaposition of aliphatic protons with aromatic moieties (hydrophobic bonds), this result would indicate even closer proximity of these groups during the binding process. This is consistent for solution with the observation from X-ray difference analysis[2] that the cleft containing the active site closes somewhat on binding inhibitor, bringing i-Leu 98 and Try 63 (from opposite sides of the cleft) closer together. Thus, peaks *2* and/or *3* (or components thereof) can be tentatively assigned to the side-chain protons of i-Leu 98.

We wish to thank M. Feil for technical assistance, and G. Roberts, J. Markley, and D. Meadows for constructive criticism.

Abbreviations used: NMR, nuclear magnetic resonance; HEW lysozyme, hen egg-white lysozyme; NAG, *N*-acetyl glucosamine; HMS, hexamethyldisiloxane; CAT, computer of average transients; DSS, 2,2-dimethylsilapentane-5-sulphonate.

[1] Johnson, L. N., and D. C. Phillips, *Nature*, **206**, 761 (1965).

[2] Phillips, D. C., these PROCEEDINGS, **57**, 484 (1967); Blake, C. C. F., L. N. Johnson, G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sarma, *Proc. Roy. Soc. (London), Ser. B*, **167**, 378 (1967).

[3] Rupley, J. A., L. Butler, M. Geering, F. J. Hartdegen, and R. Pecoraro, these PROCEEDINGS, **57**, 1088 (1967).

[4] Dahlquist, F. W., L. Jao, and M. Raftery, these PROCEEDINGS, **56**, 26 (1966).

[5] Lehrer, S. S., and G. D. Fasman, *J. Biol. Chem.*, **242**, 4644 (1967); Lehrer, S. S., *Biochem. Biophys. Res. Commun.*, **29**, 767 (1967).

[6] Chipman, D. M., V. Grisaro, and N. Sharon, *J. Biol. Chem.*, **242**, 4388 (1967).

[7] Markley, J. L., T. C. Hollocher, A. S. Brill, and O. Jardetzky, in *Physical Methods in Biochemistry*, ed. D. Moore (New York: Academic Press, in press).

[8] Cohen, J. S., in *Experimental Methods in Molecular Biology*, ed. C. Nicolau (New York: John Wiley and Sons, in preparation).

[9] Meadows, D. H., J. L. Markley, J. S. Cohen, and O. Jardetzky, these PROCEEDINGS, **58**, 1307 (1967).

It has recently been suggested that proteins can be divided into *two* categories on the basis of the width of their C-2 His peaks, taken only as an indication of the rigidity of His within the molecule.[11] This would appear to be invalid in view of the routine observation of the relationship of line width to molecular weight. The width of the C-2 His peak of HEW lysozyme (at 50 cps sweep width) increased by 25% from pH 4.55 (3.7 cps) to 5.64 (4.8).

[10] McDonald, C. C., and W. D. Phillips, *J. Am. Chem. Soc.*, **89**, 6332 (1967).

[11] Bradbury, J. H., and P. Wilairat, *Biochem. Biophys. Res. Commun.*, **29**, 84 (1967).

[12] Sternlicht, H., and D. Wilson, *Biochemistry*, **6**, 2881 (1967).

[13] Bak, B., E. J. Pedersen, and F. Sundby, *J. Biol. Chem.*, **242**, 2637 (1967).

[14] Markley, J. L., D. H. Meadows, and O. Jardetzky, *J. Mol. Biol.*, **27**, 25 (1967).

[15] Morlino, V. J., and R. B. Martin, *J. Am. Chem. Soc.*, **89**, 3107 (1967).

[16] Ferreti, J. A., *Chem. Commun.*, 1030 (1967).

[17] Nakamura, A., and O. Jardetzky, these PROCEEDINGS, **58**, 2212 (1967).

[18] Rupley, J. A., *Biochem. Biophys. Acta*, **83**, 245 (1964).

[19] Jollès, J., J. Jauregui-Adell, and P. Jollès, *Compt. Rend.*, **258**, 3926 (1964).

[20] Canfield, R. E., and A. K. Liu, *J. Biol. Chem.*, **240**, 1997 (1965).

[21] Anfinsen, C. B., and E. Haber, *J. Biol. Chem.*, **236**, 1361 (1961).

[22] Emsley, J. W., J. Feeney, and L. H. Sutcliffe, *High Resolution Nuclear Magnetic Resonance Spectroscopy* (New York: Pergamon Press, 1966).

[23] Cohen, J. S., and O. Jardetzky, unpublished results.

[24] Hamaguchi, K., and K. Rokkaku, *J. Biochem. (Tokyo)*, **48**, 358 (1960).

[25] Jirgensons, B., *Arch. Biochem. Biophys.*, **41**, 333 (1952).

[26] Hamaguchi, K., and A. Kurono, *J. Biochem. (Tokyo)*, **54**, 111 (1963).

[27] Biltonen, R. L., and R. Lumry, *J. Am. Chem. Soc.*, **87**, 4208 (1965).

[28] Martin, C. J., and G. M. Bhatnagar, *Biochemistry*, **6**, 1638 (1967).

[29] McDonald, C. C., and W. D. Phillips, in *Proceedings of the Second International Conference on Magnetic Resonance* (New York: Pergamon Press, 1967).

[30] Hammes, G. G., and P. R. Schimmel, *J. Am. Chem. Soc.*, **89**, 442 (1967).

[31] Hammes, G. G., and J. C. Swann, *Biochemistry*, **6**, 1591 (1967).

[32] Wetlaufer, D. B., S. K. Malik, L. Stoller, and R. L. Coffin, *J. Am. Chem. Soc.*, **86**, 508 (1964).

[33] Nozaki, Y., and C. Tanford, *J. Biol. Chem.*, **238**, 1596 (1963).

[34] Bruzzesi, M. R., E. Chiancone, and E. Antonini, *Biochemistry*, **4**, 1796 (1965).

[35] Sophianopoulos, A. J., and K. E. Van Holde, *J. Biol. Chem.*, **239**, 2516 (1964).

[36] Nakano, M. N., I. Nakano, and T. Higuchi, *J. Phys. Chem.*, **71**, 3954 (1967).