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A Nuclear Magnetic Resonance Study of Enzyme–Inhibitor Association. The Use of pH and Temperature Effects to Probe the Binding Environments*

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ABSTRACT: The effects of temperature and pH on the dissociation constant of methyl 2-deoxy-2-acetamido-β-D-glucopyranoside and lysozyme have been measured. In addition the effect of temperature and pH on the chemical shifts of the acetamido and glycosidic methyl groups when bound to the enzyme have been determined. It has been shown that no conformation changes occur in the binding site of lysozyme with increases in temperature up to 53°, or as a result of pH changes in the range 2.5–10. In the absence of such changes it has been concluded that the ionizable groups which affect the binding constant and the acetamido methyl group chemical shifts are very close to the inhibitor binding site. Since it has also been demonstrated that the binding properties of lysozyme in solution, as determined by nuclear magnetic resonance spectroscopy, parallel those of the crystalline enzyme, the pK_a values determined in the present study can be assigned to carboxyl residues at the binding site of the enzyme. Thus it is concluded that the pK_a of glutamic acid residue 35 is 6.1 and those of aspartic acid residues 101 and 103 are 4.2 and 4.7, respectively.

We have previously shown that lysozyme causes a shift in the nuclear magnetic resonance spectrum[1] of the acetamido methyl group of various *N*-acetyl pyranosides (Raftery *et al.*, 1968a) and analogs of sugars (S. M. Parsons and M. A. Raftery, unpublished data). It has been further demonstrated (Dahlquist and Raftery, 1968) that the observed chemical shift, δ, can be quantitated for the equilibrium $E + S \rightleftharpoons ES$ and $K_s = E \cdot S/ES$. If Δ represents the chemical shift of the acetamido methyl group in its binding site on the enzyme and δ represents the observed chemical shift, $S_0 = (E_0\Delta/\delta) - K_s - E_0$, where E_0 and S_0 represent the total concentrations of enzyme and sugar molecules, respectively. The quantity Δ is a measure of the magnetic environment of the binding site of the enzyme and can be determined for any or all of the nonequivalent protons of the sugar molecule.

The present paper describes a study of the binding of methyl 2-deoxy-2-acetamido-β-D-glucopyranoside to lysozyme, using nuclear magnetic resonance methods. In this study both the glycosidic and acetamido methyl resonances were observed in the presence of the enzyme and the effects were quantitated as a function of temperature and pH. The results obtained show that a substrate or inhibitor molecule may be utilized as an effective probe of its binding site on the enzyme.

The Temperature Dependence of β-Methyl-NAG[2] Binding. Figure 1 shows the temperature dependence of the dissociation constant, K_s, for β-methyl-NAG and lysozyme. To determine the enthalpy of the binding equilibrium at pH 5.5 in 0.1 M citrate buffer, pK_s ($-\log K_s$) was plotted *vs.* $^1/_T$. A value of −5.5 kcal was obtained for ΔH under these conditions. Unfortunately, an interpretation of this value in terms of specific types of interaction between the sugar and enzyme is difficult. In this regard it has been demonstrated (Rupley *et al.*, 1967) that the binding of saccharides to lysozyme involves the displacement of protons from the enzyme. Thus, the over-all reaction whose enthalpy has been measured involves contributions from the loss of protons by the enzyme and interactions of these protons with the solvent, as well as from the binding interactions of interest. Even if the contributions of interest could be isolated, the binding is most probably a complex combination of apolar and hydrogen-bonding interactions, whose separate contributions to the total enthalpy would be difficult to evaluate.

The determined values of Δ for the acetamido methyl protons remained constant within experimental error

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[2] Abbreviations used that are not listed in *Biochemistry* *5*, 1445 (1966), are: β-methyl-NAG, methyl 2-deoxy-2-acetamido-β-D-glucopyranoside; di-NAG, chitobiose; tri-NAG, chitotriose.

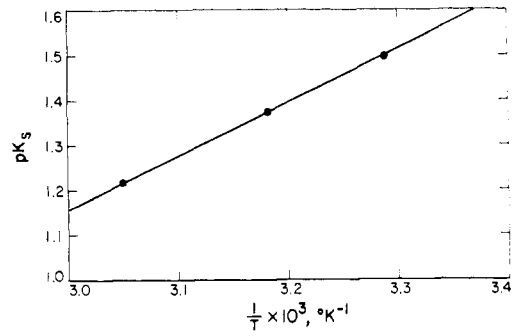

FIGURE 1: Plot of the temperature dependence of the dissociation constant, K_s, for β-methyl-NAG and lysozyme.

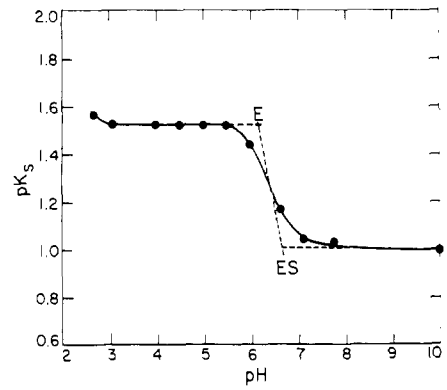

FIGURE 2: The effect of pH on the dissociation constant, K_s, for β-methyl-NAG and lysozyme. To determine the pK_a values of ionizable groups on the enzyme and the enzyme–inhibitor complex, $-\log K_s$(pK_s) was plotted with pH.

over the temperature range studied and were found to be 0.51–0.54 ppm. This would suggest that there were no significant conformational changes taking place in the binding site over the temperature range studied. This observation is in agreement with the nmr studies of lysozyme carried out by McDonald and Phillips (1967) at 220 MHz and Sternlicht and Wilson (1967) at 100 MHz. These investigators found that at temperatures below 75–80°, the transition temperature for reversible thermal denaturation, the lysozyme nuclear magnetic resonance spectrum did not appear to undergo any gross changes.

The pH Dependence of the Binding Constant. The pH dependence of the dissociation constant for β-methyl-NAG and lysozyme at 31° is shown in Figure 2. The data are presented as pK_a *vs.* pH as recommended by Dixon and Webb (1958). Interpretation of this data, according to Dixon's theory concerning the effects of pH on substrate binding, allows us to say that one ionizable group on the enzyme is perturbed by the association with β-methyl-NAG. This group has a pK_a of 6.1 in the free enzyme, which is perturbed to a value of 6.6 in the enzyme–inhibitor complex. Our previous measurements on the binding of tri-*N*-acetylchitotriose to lysozyme by optical techniques (Dahlquist *et al.*, 1966) showed that association with the trisaccharide affected the pK_a's of two ionizable groups on the enzyme. A group of pK_a = 4.2 in the free enzyme was changed to a value of 3.6 in the enzyme–substrate complex, and a second group of pK_a = 5.8 in the free enzyme was perturbed to 6.3 in the complex. The results of Rupley *et al.* (1967) and Lehrer and Fasman (1966) obtained from similar studies of the binding of tri-*N*-acetylchitotriose and lysozyme by related optical methods support these earlier findings.

Thus, the binding of β-methyl-NAG as studied by nuclear magnetic resonance methods affects the ionization of a group whose determined pK_a is only slightly different from the pK_a of one of the ionizable groups perturbed by tri-NAG binding. It is probable that these are indeed the same ionizable group on the enzyme. The pK_a associated with this ionizable group suggests it is either a histidine residue of a carboxyl of unusually high pK_a. The titration data of Donovan *et al.* (1960) indicate a carboxyl group of pK_a = 6.3 to be present in lysozyme, while Meadows *et al.* (1967) have demonstrated that the pK_a of the single histidine residue in lysozyme is 5.8 and is not affected by substrate binding. Therefore, it appears reasonable to say that the ionization of a carboxyl group of pK_a = 6.1 in lysozyme is affected by the binding of β-methyl-NAG and also of tri-NAG. The group of lower pK_a (4.2) in lysozyme which is affected by tri-NAG binding shows no effect on the binding of β-methyl-NAG and it is reasonable to infer that this also is a carboxyl group. This group must interact with a sugar ring(s) of the trisaccharide in binding regions other than the β-methyl-NAG binding site.

Work from this laboratory (Raftery *et al.*, 1968b) concerning the relative binding orientations of NAG, di-NAG, and tri-NAG has shown that the reducing end of each saccharide occupies the same binding site. This observation, as well as our demonstration that α-NAG and β-NAG bind with different orientations in a single binding site (Dahlquist and Raftery, 1968), is in complete agreement with the X-ray analysis studies of Blake *et al.* (1967) on lysozyme–saccharide complexes in the crystalline state. It would appear therefore that for lysozyme the binding process is quite similar in the crystal and in solution. We regard the demonstrations of similar binding properties in the crystalline state and in solution as indirect but nevertheless convincing proof of identical structure in the two states. This is of course restricted, at least in detail, to the region of the molecule to which inhibitors bind. Examinations of a space-filling model of lysozyme built in this laboratory from Corey–Pauling–Koltum (CPK) models using the coordinates obtained from the X-ray analysis data of Blake *et al.* (1967) allows us to speculate as to the specific amino acid residues whose ionizations are being observed. There are at least two carboxyl residues whose ionizations could obviously affect β-methyl-NAG binding, aspartic acid 52 and glutamic acid 35, and it would appear that the group of pK_a = 6.1 is associated with one of these residues. Since the environment of glutamic acid 35 is nonpolar, Blake *et al.* (1967) have suggested that it would be expected to have an abnormally high pK_a and we therefore assign the pK_a value of 6.1 to this group.

The Effect of pH on Chemical Shifts of β-Methyl-NAG Methyl Groups. The pH dependency of Δ, for two res-

onances of β-methyl-NAG have been measured. Figure 3 shows the effect of pH on the acetamido methyl resonance of NAG, and also similar data for the glycosidic methyl group resonance. The glycosidic methyl group shows a relatively small downfield shift which is pH dependent, within experimental error. Such a result enables us to state that no significant conformation changes at the binding site of the enzyme take place as a result of pH effects in the range studied, since chemical shifts are extremely sensitive to changes in distance from the source of the effect causing them (Buckingham *et al.*, 1960).

This result does not exclude the possibility of a conformation change in the enzyme resulting in poor binding of the glycoside. It does, however, suggest that such a conformation change, if it occurs, is independent of pH. The acetamido methyl resonance, however, shows two breaks due to ionizations on the enzyme–substrate complex which affect the magnetic environment of the acetamido group while bound. The pK_a of a group whose ionization affects the magnetic environment of a particular nucleus may be obtained in the following manner. If for the scheme ESH $\rightleftharpoons$ ES + H and K_a = [ES][H]/[ESH] Δ_1 is a chemical shift associated with ESH and Δ_2 a chemical shift similarly associated with with the unprotonated form ES, then (neglecting charges the observed chemical shift, Δ, will be

$$\Delta = \frac{[ESH]}{[ES] + [ESH]}\Delta_1 + \frac{[ES]}{[ES] + (ESH]}\Delta_2$$

$$\Delta = \frac{\left(\frac{[ESH]}{[ES]}\right)\Delta_1 + \Delta_2}{([ESH]/[ES]) + 1}$$

$$\Delta = \frac{\left(\frac{[H]}{K_a}\right)\Delta_1 + \Delta_2}{\left(\frac{[H]}{K_a}\right) + 1}$$

when K_a = [H] or pK_a = pH, $\Delta = (\Delta_1 + \Delta_2)/2$. Taking the plateau values of Δ for particular states of ionization of the enzyme–substrate complex, as shown in Figure 3, pK_a's of 4.7 ± 0.1 and 7.0 ± 0.5 are obtained for the ionizable groups in the enzyme–substrate complex. The higher pK_a is consistent with the idea that the same ionizable group which affects the binding of β-methyl-NAG also has an effect on the magnetic environment of the β-methyl-NAG acetamido methyl protons. The lower value of 4.7 is interesting in that the ionization of the group involved has no noticeable affect on binding. This implies that the pK_a of this group remains unperturbed in the presence of β-methyl-NAG.

The most probable causes for pH-dependent changes in the magnetic environment of a binding site are changes in the ionization state of a group on the enzyme which affects: (1) the electric field in the binding site, (2) the magnetic anisotropy of the group in question, (3) the conformation of the macromolecule, or (4) the state of aggregation of the enzyme, which can be regarded as a conformational change. A gross conformational

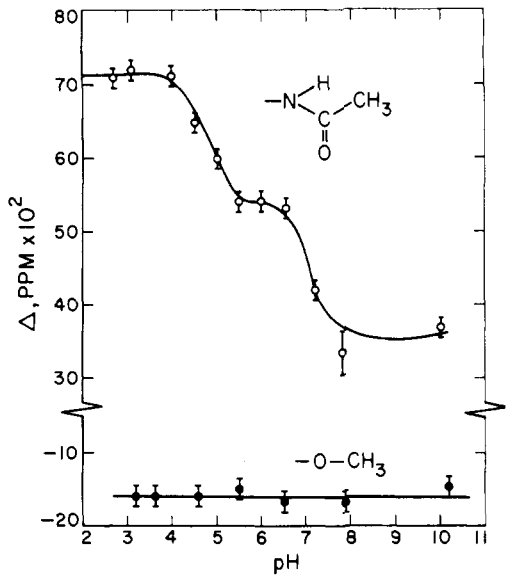

FIGURE 3: The pH dependencey of the chemical shifts, Δ, of the glycosidic methyl proton (—●—) and the acetamido methyl protons (—○—) of β-methyl-NAG when bound to lysozyme.

change should have some affect on the glycosidic methyl resonance as well as the acetamido methyl group resonance. However, the chemical shift of the glycosidic methyl group is pH independent. Furthermore, in the pH region 3.0–5.5, the dissociation constant of β-methyl-NAG does not change, which would not be expected if the conformation of the binding site were changing. It is, therefore, unlikely that the group of pK_a = 4.7 which affects the chemical shift of the acetamido methyl group when bound is associated with conformational changes in the enzyme. The effect of this group is most likely manifested as an electric field or magnetic anisotropy change which affects the magnetic environment of the binding site. Such electric field effects have been shown (Buckingham *et al.*, 1960) to fall off as the square of the distance to the nuclei being observed, while anisotropy effects fall off as the cube of the distance. Therefore, this group must be near the binding site of the acetamido methyl group of β-methyl-NAG. The determined pK_a value of 4.7 furthermore suggests that it is a carboxyl group.

Our present results therefore indicate two groups (pK_a = 4.7 and 6.1) in the enzyme which are close to the binding site of β-methyl-NAG and our previous results (Dahlquist *et al.*, 1966) show two groups are affected by the binding of tri-NAG. Examination of the space-filling model, shows a total of four carboxyl residues near the β-methyl-NAG binding site. These are the carboxyl side chains of glutamic acid 35 and aspartic acids 103, 101, and 52. We have already assigned to glutamic acid residue 35 a pK_a value of 6.1. We can assign, on the basis of the X-ray analysis results of Blake *et al.*, (1967) when compared with our previous studies (Dahlquist *et al.*, 1966; Raftery *et al.*, 1968b), a pK_a value of 4.2 to aspartic acid residue 101, since the trisaccharide interacts with it, while monosaccharides do not. Aspartic acid 52 is involved

in structural stabilization of the enzyme through hydrogen bonding and should have a low pK_a. It has been shown (Sophianpoulos and Weiss, 1964) that the thermal stability of lysozyme is affected by the ionization of groups on the enzyme of low pK_a, approximately pK_a = 2. Therefore aspartic acid 52 is possibly associated with such a low pK_a. The only remaining acidic group near the binding cleft is aspartic acid 103. This is solvent accessible and should have a normal pK_a. This residue is, we feel, most probably associated with the pK_a value of 4.7.

At present, two derivatives of lysozyme have been prepared in this laboratory (S. M. Parsons and M. A. Raftery, unpublished data) both of which are monoethyl esters of lysozyme. These derivatives have binding properties differing from those of the native enzyme, and further investigation of these binding properties should hopefully provide support for our present assignment of the various pK_a's of the acidic residues which occupy positions at the binding site of lysozyme.

Experimental Section

All solutions contained approximately 3×10^{-3} M lysozyme (Sigma Chemical Co., lot no. 96B8572) and 0.5% each of methanol and acetone as internal nuclear magnetic resonance standards. The buffers were made by mixing 0.1 M citric acid with either 0.1 M sodium citrate, 0.1 M disodiumphosphate, or 0.1 M trisodiumphosphate. At pH 5.5, identical results were obtained using either a citrate buffer or a citrate–phosphate buffer.

Methyl 2-acetamido-2-deoxy-β-D-glycopyranoside was prepared according to the procedure described by Conchie and Levvy (1963). The melting point of this material was 202–204, and was free of *N*-acetylglucosamine as determined by the Park–Johnson (1949) method.

For measurements at pH 4.5, lysozyme was dialyzed against an excess of 10^{-3} M acetic acid-d_3 (Tracerlab Inc.) to exchange the acetate impurity in the enzyme preparation, which interfered at this pH with the acetamido methyl resonance of the substrate, and finally lyophilized.

All spectra were obtained with a Varian HA-100 nuclear magnetic resonance spectrometer, operating in frequency sweep mode. The water resonance was used as a lock signal, and each sample was allowed to come to thermal equilibrium before measurements were taken. For measurements at temperatures other than 31°, the operating temperature of the probe, a Varian V-4341 variable-temperature accessory was employed. The difference in chemical shift of methanol as a function of temperature was used to determine the probe temperature.

Chemical shifts were determined by electronic counting of the difference between the sweep frequency and manual oscillator frequency using a Hewlett-Packard counter.

The chemical shift of the glycosidic methyl group in the bound state was determined by measurement of the ratio of the glycosidic methyl group shift to the acetamido methyl group shift. This ratio was then multiplied by the value of the chemical shift, Δ, of the bound acetamido group which was determined separately from a concentration study of the observed chemical shift of the acetamido group resonance. This method was used because the glycosidic methyl group displayed only a small observed shift, and the error in a concentration study of its resonance to determine K_s and Δ would have been very large.

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