A Nuclear Magnetic Resonance Study of Association Equilibria and Enzyme-Bound Environments of *N*-Acetyl-D-glucosamine Anomers and Lysozyme*

F. W. Dahlquist† and M. A. Raftery

ABSTRACT: The binding of the inhibitor 2-acetamido-2-deoxy-D-glucose to lysozyme has been studied using nuclear magnetic resonance techniques. A chemical shift of the acetamido methyl proton resonance of the inhibitor resulting from association with the enzyme has been utilized to study the interaction. It has been shown that in the presence of lysozyme the acetamido methyl proton resonances of the α- and β-anomeric forms of *N*-acetyl-D-glucosamine are separated. Line-width measurements of these separated resonances have allowed distinction of the magnetic environments of the acetamido groups of both anomers when enzyme bound, although it has been established that they both bind to the same site.

A study of the mutarotation of α-*N*-acetyl-D-glucosamine and β-*N*-acetyl-D-glucosamine to their equilibrium mixture by nuclear magnetic resonance methods in the presence of lysozyme has allowed calculation of the binding constants of both anomers to a single site on the enzyme and also determination of the chemical shifts of the acetamido methyl protons of the bound form of each anomer relative to the free form. The conclusions reached show that the orientation of α-*N*-acetyl-D-glucosamine is different from that of β-*N*-acetyl-D-glucosamine when bound to to the same site on lysozyme.

The use of nuclear magnetic resonance to study association phenomena of macromolecules has been recognized for some time and the theory dealing with such processes has been outlined by Zimmerman and Brittin (1957) and by Jardetsky (1964). Previous attempts to gain information from such studies by nuclear magnetic resonance methods have employed line-width measurements of resonances attributed to nuclei in the associating small molecule (Jardetsky *et al.*, 1963; Fischer and Jardetsky, 1965; Hollis, 1967). It is also possible for a chemical shift difference to occur for certain nuclei between free and bound species. Such an effect could result from proximity to aromatic systems, electric fields due to polar groups, secondary magnetic field differences arising from induced magnetic moments in neighboring atoms, or indeed from van der Waal effects (Buckingham, 1960; Buckingham *et al.*, 1960). Such a chemical shift difference had not been observed until recently in studies of association equilibria involving enzymes and inhibitors or substrates. A previous communication from this laboratory (Raftery *et al.*, 1968a) has described such an effect in nuclear magnetic resonance studies of the association between lysozyme and NAG.[1-3] It was further demonstrated in the same study that in the presence of lysozyme the acetamido methyl proton resonances of the α- and β-anomeric forms of NAG were resolved and also that the two forms compete for the same binding site(s) on the enzyme.

Our earlier measurements of NAG binding to lysozyme, employing ultraviolet difference spectral techniques (Dahlquist *et al.*, 1966), showed a dissociation constant, K_s, for NAG of 4–6 $\times$ 10^{-2} M, which was confirmed by the results of other workers (Lehrer and Fasman, 1966, 1967; Rupley *et al.*, 1967; Chipman *et al.*, 1967). It was not possible by the techniques used by any of these authors to determine whether both anomeric forms of NAG were bound to the enzyme, and if so, to estimate dissociation constants of both forms. It was demonstrated, however, by use of these spectral techniques that oligosaccharides of chitin showed increases in binding strength to lysozyme up to the trisaccharide (Dahlquist *et al.*, 1966; Rupley *et al.*, 1967). Thus it was evident that there are three strong contiguous binding sites for sugar rings on the surface of lysozyme. Although it was shown in these studies that NAG bound stoichiometrically to the enzyme, it was not possible to relate this 1:1 binding to association with one of the three strong sites rather than to multiple equilibria with all binding sites, with the dissociation constant obtained being a complex entity. This question was recently re-

* Contribution No. 3657 from the Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, California 91109. *Received March 18, 1968.* Supported by U. S. Public Health Service Grant GM-14452-01.

† National Institute of Health Trainee 1967–1968.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: NAG, *N*-acetyl-D-glucosamine; α-methyl-NAG, methyl-2-acetamido-2-deoxy-α-D-glucopyranoside; β-methyl-NAG, methyl-2-acetamido-2-deoxy-β-D-glucopyranoside.

[2] A preliminary account of the work described here was presented at the Gordon Research Conference on Proteins, New Hampton School, N. H., June 27, 1966.

[3] While this work was in progress preliminary accounts of similar observations have recently appeared from other laboratories (Thomas, 1966; Spotswood *et al.*, 1967).

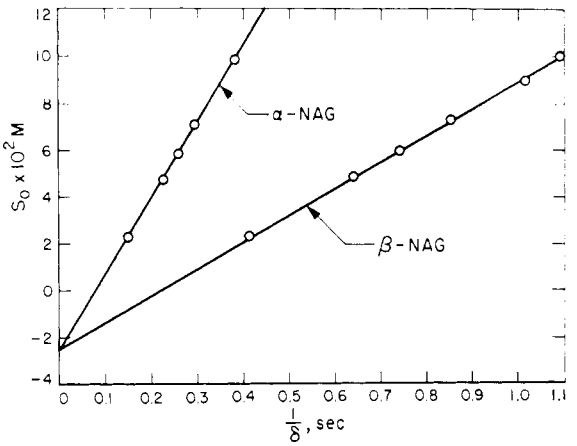

FIGURE 1: Plot of the chemical shift data (from Table I) I) for the acetamido methyl resonances of α-NAG and β-NAG (at mutarotation equilibrium) in association with lysozyme. The concentrations plotted are those of α-NAG plus β-NAG (*i.e.*, total NAG). Measurements were made in 0.1 M citrate buffer (pH 5.5) at 31°, at 100 MHz.

solved (Raftery *et al.*, 1968b) by a nuclear magnetic resonance study of the association of a number of mono-, di-, and trisaccharides and their methyl glycosides with lysozyme. It was demonstrated that NAG binds to only one of the three contiguous binding sites on the enzyme and that the α- and β-anomeric forms of the monosaccharide compete with each other for this site.

The present communication provides a quantitative interpretation of the shifts observed in the NAG methyl group magnetic resonances in the presence of lysozyme in terms of the dissociation constants of α-NAG and β-NAG and of the magnetic environments of their binding sites. It is shown that it is possible to calculate the dissociation constant for each anomer of NAG as well as to probe the environment of each anomer while bound to the enzyme. The results presented show that although α- and β-NAG are competitive for the same over-all site on the enzyme and although they have nearly the same free energy of binding, it is possible to distinguish different magnetic environments for the binding sites of the two anomeric forms.

Quantitation of Chemical Shift Data. Calculation of the dissociation constant and the chemical shift of the bound form of the small molecule is possible for a system in rapid equilibrium E + S $\rightleftarrows$ ES and

$$K_S = \frac{[E][S]}{[ES]} \tag{1}$$

where $[E] = E_0 - [ES]$ and $[S] = S_0 - [ES]$. In this system we regard the chemical shift of the free inhibitor as zero and refer to the observed chemical shift as δ and the chemical shift of the bound inhibitor as Δ. If the exchange lifetime is much less than $1/\Delta$ in seconds, then $\delta = P_b\Delta$, where P_b is the fraction of substrate present in the bound form. Therefore

$$\delta = \frac{[ES]}{S_0}\Delta$$

and

$$[ES] = \frac{\delta S_0}{\Delta} \tag{2}$$

Substitution of eq 1 leads to eq 3.

$$K_S = E_0\left[\frac{\Delta - \delta}{\delta}\right] - \frac{\delta}{\Delta}S_0\left[\frac{\Delta - \delta}{\delta}\right] \tag{3}$$

If the observed shift, δ, is much smaller than the total shift, Δ, and K_S is of the order of S_0, then

$$S_0 = \frac{E_0\Delta}{\delta} - K_S - E_0 \tag{4}$$

A plot of S_0 *vs.* $^1/_\delta$ gives a line whose intercept is $-(K_S + E_0)$ and whose slope is used to calculate Δ.

Table I shows the results obtained from a study of the association of NAG and lysozyme at 100 MHz. The results are plotted in Figure 1 and it is seen that the lines representing the α and β anomers intersect on the S_0 axis.

TABLE I: Chemical Shift Data for the Acetamido Methyl Protons of α-NAG and β-NAG Association with Lysozyme (2.95×10^{-3} M).[a]

NAG × 10^2	δ_α (cps)	δ_β (cps)	$1/\delta_\alpha$ (sec)	$1/\delta_\beta$ (sec)
9.95	2.59	0.92	0.386	1.09
7.17	3.36	1.17	0.298	0.856
5.98	3.85	1.35	0.260	0.741
4.78	4.37	1.55	0.229	0.645
2.39	6.69	2.39	0.150	0.418

[a] Measurements were made at 100 MHz, in citrate buffer, 0.1 M (pH 5.5), 31°.

It has been shown recently (Raftery *et al.*, 1968a) that α-NAG and β-NAG compete with each other for the same site on lysozyme. In view of this competition the the chemical shift data can be quantitated using the simple competitive scheme: E + $\alpha \rightleftarrows$ Eα and $K_\alpha = [E][\alpha]/[E\alpha]$; E + $\beta \rightleftarrows$ Eβ and $K_\beta = [E][\beta]/[E\beta]$. Substitution similar to that of eq 1 (see Appendix A) gives

$$S_0A = \frac{E_0\Delta_\alpha}{\delta_\alpha} - K_\alpha - E_0$$

where

$$A = \frac{[\alpha_0]}{S_0} + \frac{K_\alpha[\beta_0]}{K_\beta S_0} \tag{5}$$

and

$$S_0B = \frac{E_0\Delta_\beta}{\delta_\beta} - K_\beta - E_0$$

where

$$B = \frac{[\beta_0]}{S_0} + \frac{K_\beta[\alpha_0]}{K_\alpha S_0} \tag{6}$$

where S_0 is the total concentration of NAG, E_0 is the total concentration of enzyme, $[\alpha_0]$ is the total concentration of α-NAG, $[\beta_0]$ is the total concentration of β-NAG, and δ_α and δ_β refer to the observed chemical shifts of the α- and β-NAG present in mutarotation equilibrium. Equations 5 and 6 allow interpretation of the data plotted in Figure 1.

The ratio of the slope of the α-NAG data to that of β-NAG (provided $[\alpha_0] = [\beta_0]$) is $K_\beta\Delta_\alpha/K_\alpha\Delta_\beta$ and was found to be 2.7. The intercept for the α form is

$$\frac{-(K_\alpha K_\beta + K_\beta E_0)}{\frac{[\alpha_0]}{S_0}K_\beta + \frac{[\beta_0]}{S_0}K_\alpha} \tag{7a}$$

while for the β form the intercept is

$$\frac{-(K_\alpha K_\beta + K_\alpha E_0)}{\frac{[\beta_0]}{S_0}K_\alpha + \frac{[\alpha_0]}{S_0}K_\beta} \tag{7b}$$

and both were determined to be $(2.5 \pm 0.4) \times 10^{-2}$ M. This figure agrees fairly well with that ($4\text{–}6 \times 10^{-2}$ M) obtained for NAG binding to lysozyme using an ultraviolet difference spectral technique (Dahlquist *et al.*, 1966). Theoretically it is possible to determine the values of K_α and K_β from eq 7a and 7b. However, the error in the intercept for the β-anomeric form (owing to the smaller observed chemical shift associated with this form) is too great to allow this calculation.

Line-Width Measurements. In an attempt to resolve the question of whether K_α and K_β are different or whether Δ_α and Δ_β are different, we have employed line-width measurements. If the observed line width is $1/\pi T_{2\text{obsd}}$, then in the absence of saturation or line broadening due to slow exchange, when the enzyme is present

$$\left(\frac{1}{T_{2\text{obsd}}}\right)_E = P_f\frac{1}{T_2^f} + P_b\frac{1}{T_2^b} + I \tag{8}$$

where P_f and P_b represent fractions of the inhibitor (α-NAG or β-NAG) which are free in solution and bound to the enzyme, respectively, and T_2^f and T_2^b represent the spin–spin relaxation times of the free and bound inhibitor species. The term I represents the inhomogeneity of the field. In the absence of enzyme

$$\left(\frac{1}{T_{2\text{obsd}}}\right)_F = \frac{1}{T_2^f} + I \tag{9}$$

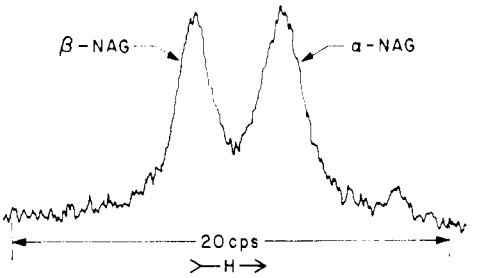

FIGURE 2: Time-averaged spectrum (15 scans) of the acetamido methyl resonances of α-NAG and β-NAG (at mutarotation equilibrium; total NAG concentration = 1.21×10^{-2} M) in the presence of lysozyme (3.0×10^{-3} M). Measurements were made in 0.1 M citrate buffer (pH 5.5) at 31°, at 100 MHz.

Then the contribution to the line width due to association ($1/T_2'$)

$$\frac{1}{T_2'} = \left(\frac{1}{T_{2\text{obsd}}}\right)_E - \left(\frac{1}{T_{2\text{obsd}}}\right)_F \tag{10}$$

$$= (P_f - 1)\frac{1}{T_2^f} + P_b\frac{1}{T_2^b} \tag{11}$$

Since $P_f + P_b = 1$

$$\frac{1}{T_2'} = P_b\left(\frac{1}{T_2^b} - \frac{1}{T_2^f}\right) \tag{12}$$

For the case of small molecules associating with macromolecules $1/T_2^b \gg 1/T_2^f$ and therefore

$$\frac{1}{T_2'} = P_b\frac{1}{T_2^b} \tag{13}$$

This has the same form as eq 2 and from considerations similar to those used to derive eq 5 and 6 it can be shown (see Appendix B) that

$$\frac{(T_2')_\beta}{(T_2')_\alpha} = \frac{K_\beta}{K_\alpha} \times \frac{(T_2^b)_\beta}{(T_2^b)_\alpha} \tag{14}$$

where $(T_2')_\beta/(T_2')_\alpha$ is the ratio of the contributions to the line widths of the α- and β-anomeric forms due to association with the enzyme while $(T_2^b)_\beta$ and $(T_2^t)_\alpha$ represent the relaxation times of the bound species of β-NAG and α-NAG. The ratio $(T_2')_\beta/(T_2')_\alpha$ was determined according to eq 10 to be 1.3 ± 0.1 from the data shown in Figure 2. If the sites occupied by the acetamido side chains of α-NAG and β-NAG are magnetically equivalent, then $\Delta_\alpha = \Delta_\beta$ and $(T_2^b)_\alpha = (T_2^b)_\beta$. We have shown, however, that the ratio of the slopes in Figure 1 is $K_\beta\Delta_\alpha/K_\alpha\Delta_\beta = 2.7$ and above that (K_β/K_α)-$((T_2^b)_\beta/(T_2^b)_\alpha) = 1.3$. Therefore

$$\frac{\Delta_\alpha(T_2^b)_\alpha}{\Delta_\beta(T_2^b)_\beta} = 2.1 \tag{15}$$

Since this ratio is not equal to unity this means that the

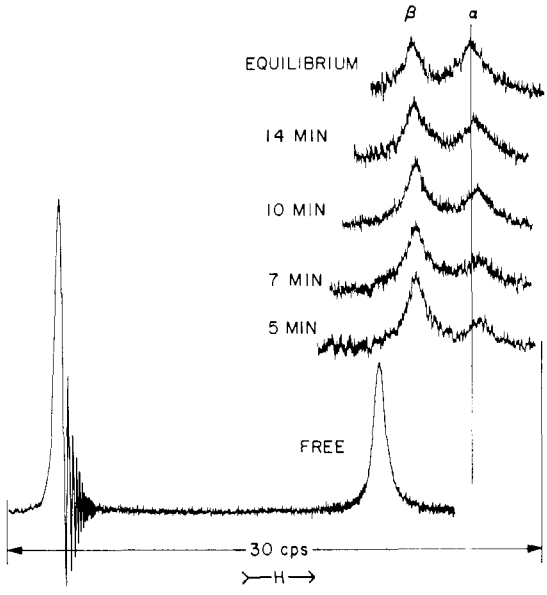

FIGURE 3: Nuclear magnetic resonance spectra of NAG (acetamido methyl protons) free in solution and of β-NAG during mutarotation in the presence of lysozyme (3.0×10^{-3} M). The intense resonance at left is that of the protons of an internal acetone standard. After 5 min in the presence of lysozyme, β-NAG was present in excess over α-NAG. The establishment of mutarotation equilibrium was followed with time until it was attained after approximately 20 min. Spectra were recorded at 100 MHz and 31°. Samples were dissolved in 0.1 M citrate (pH 5.5).

binding sites on the enzyme for the acetamido side chain of α-NAG and β-NAG are not magnetically equivalent. This approach demonstrates that, although α-NAG and β-NAG bind to the same site on lysozyme, they do not occupy that site in an identical manner.

Another possible explanation is that the rates of exchange of the α and β anomers with the enzyme are not identical and "fast" (of the order of 10^5 l. mole^{-1} sec^{-1} for the formation of the enzyme–substrate complex). This could lead to differential broadening of the resonance of one anomer relative to that of the other anomer. The results described above lead to line-width values in the bound state of 10–20 cps which generally agree with the line widths obtained by McDonald and Phillips (1967) for methyl group resonances of lysozyme itself. This suggests that the exchange rates are at or very near the fast exchange limit and probably no extra broadening is caused by exchange phenomena.

The most satisfactory method of proving this argument would be, of course, to determine K_α, K_β, Δ_α, and Δ_β. As mentioned already, this is not possible using the plot of S_0 *vs.* $^1/_\delta$ as has been demonstrated (Raftery *et al.*, 1968a) for α-methyl-NAG and its corresponding β anomer, β-methyl-NAG.

Mutarotation Studies. Our early observations on the use of nuclear magnetic resonance to study the association equilibria of NAG and lysozyme showed that freshly dissolved α-NAG or β-NAG initially displayed in the presence of lysozyme only one acetamido methyl resonance to higher field of the corresponding resonance for free NAG (Raftery *et al.*, 1968a). After about 20 min two separate resonances of almost equal intensity were present, corresponding to the α- and β-anomeric forms of the inhibitor in association with the enzyme after mutarotation equilibrium had been reached. Figure 3 shows the development with time of the resonance due to α-NAG in a solution of lysozyme and β-NAG. This study of the phenomenon at 100 MHz shows that small but measurable changes in the chemical shifts of each anomer occur during the approach to mutarotation equilibrium. Such changes were not reliably obtained during our earlier studies at 60 MHz (Raftery *et al.*, 1968a).

The mutarotation of freshly dissolved α- or β-NAG provides a method of varying the relative concentrations of α-NAG to β-NAG. The changes in chemical shift of the resonances of the two forms as their relative concentration changes can be quantitated as follows from eq 5 and 6.

$$\frac{E_0\Delta_\alpha}{\delta_\alpha} - K_\alpha - E_0 = S_0\left[\frac{[\alpha_0]}{S_0} + \frac{K_\alpha}{K_\beta}\frac{[\beta_0]}{S_0}\right]$$

$$= S_0\left[\frac{[\alpha_0]}{S_0} + \frac{[\beta_0]}{S_0} + \frac{K_\alpha}{K_\beta}\frac{[\beta_0]}{S_0} - \frac{[\beta_0]}{S_0}\right]$$

$$= S_0 + [\beta_0]\left[\frac{K_\alpha}{K_\beta} - 1\right]$$

$$\frac{E_0\Delta_\alpha}{\delta_\alpha} = E_0 + K_\alpha + S_0 + [\beta_0]\left[\frac{K_\alpha}{K_\beta} - 1\right] \quad (16)$$

and

$$\frac{E_0\Delta_\beta}{\delta_\beta} = E_0 + K_\beta + S_0 + [\alpha_0]\left[\frac{K_\beta}{K_\alpha} - 1\right] \quad (17)$$

If the superscript t denotes a measurement before mutarotational equilibrium has been established and e denotes the measurement at mutarotational equilibrium, then

$$\frac{\delta_\alpha{}^t}{\delta_\alpha{}^e} = \frac{E_0 + K_\alpha + S_0 + [\beta_0{}^e]\left[\frac{K_\alpha}{K_\beta} - 1\right]}{E_0 + K_\alpha + S_0 + [\beta_0{}^t]\left[\frac{K_\alpha}{K_\beta} - 1\right]} \quad (18)$$

Similarly for the β-NAG resonances

$$\frac{\delta_\beta{}^t}{\delta_\beta{}^e} = \frac{E_0 + K_\beta + S_0 + [\alpha_0{}^e]\left[\frac{K_\beta}{K_\alpha} - 1\right]}{E_0 + K_\beta + S_0 + [\alpha_0{}^t]\left[\frac{K_\beta}{K_\alpha} - 1\right]} \quad (19)$$

Since $\delta_\alpha{}^t$ and $\delta_\alpha{}^e$ are the observed chemical shifts of the acetamido methyl resonances of the α-anomeric form of a partially and fully mutarotated sample of NAG in the presence of lysozyme, they are measurable. The magnitude of $[\beta_0{}^e]$ and $[\beta_0{}^t]$ can be obtained from integration

TABLE II: Quantitation of Chemical Shift Data for the Acetamido Methyl Protons of α-NAG or β-NAG, in the Presence of Lysozyme, During the Time Necessary to Reach Equilibrium.[a]

$S_0 \times 10^2$	Anomer Form Used	Methyl Resonance Measured	$\delta_\alpha^t/\delta_\alpha^e$	$\delta_\beta^t/\delta_\beta^e$	$\frac{[\alpha_0^e] \times 10^2 \text{ M}}{[\alpha_0^t] \times 10^2 \text{ M}}$	$\frac{[\beta_0^e] \times 10^2 \text{ M}}{[\beta_0^t] \times 10^2 \text{ M}}$
3.65	β-NAG	β		1.055		$\frac{1.84}{1.35}$
3.65	β-NAG	α	0.944		$\frac{1.81}{2.30}$	
3.40	α-NAG	α	0.932		$\frac{1.72}{1.20}$	
6.80	α-NAG	α	0.945		$\frac{3.44}{2.40}$	

[a] Measurements made at equilibrium are given as δ^e, while those made at time t before equilibrium was reached are given as δ^t. The concentration of each anomer at times t and e are shown as $[\alpha_0^t]$, $[\alpha_0^e]$, $[\beta_0^t]$, and $[\beta_0^e]$ and were determined from peak height measurements.

TABLE III: Summary of Chemical Shift Data for the Acetamido Methyl Protons of α-NAG and β-NAG During Mutarotation of Each Anomer in the Presence of Lysozyme (2.8–3.0 $\times 10^{-3}$ M).[a]

$S_0 \times 10^2$	Anomeric Form	K_α/K_β	K_α	K_β	Δ_α (ppm)	Δ_β (ppm)
3.65	β	0.53	1.6×10^{-2}	3.1×10^{-2}	0.71	0.48
3.65	α	0.47	1.5×10^{-2}	3.2×10^{-2}	0.68	0.51
3.40	α	0.40	1.5×10^{-2}	3.6×10^{-2}	0.66	0.55
6.80	α	0.53	1.6×10^{-2}	3.1×10^{-2}	0.71	0.48

[a] Measurements were made in 0.1 M citrate buffer (pH 5.5) at 31°, at 100 MHz.

measurements relative to the α-anomeric resonances at times e and t. Figure 3 shows the progress with time of the resonances of the acetamido methyl groups of α- and β-NAG following mixing of a sample of pure β-NAG with a solution of lysozyme. It is evident from the data that small but measureable chemical shift differences occur for each anomeric methyl resonance between early times where there is a preponderance of one anomeric form and at mutarotational equilibrium where a steady-state concentration of each anomeric form exists. The analogous experiment where pure α-NAG was mixed with a solution of lysozyme and the changes in relative concentrations of the anomeric forms with time recorded by measuring the chemical shifts of the acetamido proton resonances was also performed. The quantitative data obtained from these observations are presented in Table II. Substitution of the values obtained for δ^t/δ^e for either anomer and the concentrations of each anomer present at time t and at equilibrium into eq 18 or 19 yields preliminary estimates of K_α/K_β assuming that K_α or K_β is small relative to $E_0 + S_0$. This ratio was then used to obtain K_α and K_β from eq 7. Such values were resubstituted into eq 18 or 19 to yield a better estimate of K_α/K_β. This was done until the value of K_α/K_β did not change upon further iteration. Table III summarizes the results obtained in this way from four separate determinations. The magnitudes of the dissociation constants obtained ($K_\alpha = (1.6 \pm 0.1) \times 10^{-2}$ M; $K_\beta = (3.3 \pm 0.2) \times 10^{-2}$ M) show that there is very little difference in the relative binding strengths of the two anomeric forms. More interesting, however, is the significant difference in the chemical shifts of the bound forms of each anomer ($\Delta_\alpha = 0.68 \pm 0.03$ ppm; $\Delta_\beta = 0.51 \pm 0.03$ ppm, both to higher field).

Our previous measurements (Raftery *et al.*, 1968a) of the association of α-methyl-NAG and β-methyl-NAG with lysozyme, using nuclear magnetic resonance techniques, showed that the acetamido methyl group of both glycosides displayed a chemical shift of 0.54 ± 0.02 ppm to higher field in the bound state. It was further demonstrated (Raftery *et al.*, 1968b) that both of these glycosides bound to the same site on the enzyme as did α-NAG and that they both were competitive with *N*-

acetyl-(d_3)-α-D-glucosamine. Since it was further shown by similar experiments that α- and β-NAG bind competitively to lysozyme, it is obvious that α-NAG, β-NAG, α-methyl-NAG, and β-methyl-NAG all bind competitively on the enzyme. The chemical shift of the bound form can be used to compare the microenvironment experienced by each inhibitor. Thus, the value of $\Delta = 0.51 \pm 0.03$ ppm obtained for β-NAG (Table II) suggests that its binding orientation is the same as that of α-methyl-NAG and β-methyl-NAG. On the other hand, the chemical shift, Δ, for the bound form of α-NAG in the same binding site was found to be 0.68 ± 0.03 ppm, which means that a different magnetic environment is experienced by its acetamido methyl group. This suggests that the orientation of this inhibitor is different from α-methyl-NAG, β-methyl-NAG, and β-NAG when bound to the same site on lysozyme.

The factor of 2 in relative dissociation constants ($K_{\alpha\text{-NAG}} = 1.6 \times 10^{-2}$ M; $K_{\beta\text{-NAG}} = 3.3 \times 10^{-2}$ M) for the two anomers of NAG binding to the enzyme represents only a small energy difference equal to 0.4 kcal at 31°. This small difference combined with the observation that α- and β-NAG are competitive for the same site on the enzyme (Raftery *et al.*, 1968a) would ordinarily provide good evidence that the binding of the anomers is identical. However, due to the extreme sensitivity of the magnetic resonance method to environmental changes expressed as Δ (the chemical shift of selected protons in the enzyme-bound form), it is clearly shown that the bound orientations of α-NAG and β-NAG are different.

Since the only difference between α-NAG and β-NAG lies in the configuration of hydrogen and hydroxyl group at C_1, it is reasonable to assume that it is this grouping which results in a difference in dissociation constant and in bound orientation of the two anomers. We have been able to show (S. M. Parsons and M. A. Raftery, unpublished data) that the binding energy of β-NAG or β-methyl-NAG can be accounted for in terms of the interactions due to the acetamido side chain and the C-3 hydroxyl group. Since α-NAG has the capability to bind through these two interactions as well as β-NAG and since it binds better than β-NAG, it is logical to conclude that α-NAG forms a bond to the enzyme through its C-1 hydroxyl group, such as a hydrogen bond. It can be further suggested that such a hydrogen bond would involve the hydrogen of this hydroxyl group rather than the oxygen, since α-methyl-NAG does not behave like α-NAG. Formation of such a bond, however, leads to a difference in bound orientation compared with β-NAG. Therefore it is likely that other bonds to the enzyme which are formed by β-NAG are not formed when α-NAG binds, unless conformation changes of the enzyme also occur.

The elegant crystallographic studies of Blake *et al.*, (1967) on association of NAG with lysozyme have shown that in the crystalline enzyme the orientations of α-NAG and β-NAG are different while they bind in an overlapping fashion. The acetamido side chains of both anomers were considered to make the same contacts (probably through hydrogen bonds) with the enzyme but the pyranose rings were orientated in different ways. As a result, the orientation of the acetamido methyl group changed somewhat with respect to a tryptophan residue (no. 108 in the amino acid sequence) on the enzyme. The anisotropy of this aromatic ring could very well be responsible for the change in the magnetic environment of lysozyme-bound α-NAG relative to similarly bound β-NAG.

It is of special interest that our results obtained from use of nuclear magnetic resonance techniques to study enzyme–inhibitor association in solution yield information which is entirely consistent with the aforementioned X-ray analysis studies on crystalline preparations. Further studies of a similar nature on association of oligometric inhibitors with lysozyme (Raftery *et al.*, 1968b; F. W. Dahlquist and M. A. Raftery, unpublished data; R. Wolcott and M. A. Raftery, unpublished data) show further agreement with the X-ray analysis studies of Blake *et al.* (1967). Such consistency between the binding properties of an enzyme in the crystalline state and in solution are taken by us to indicate structural similarity of the enzyme in the two states.

In conclusion, we feel that the results presented in this communication serve to demonstrate the capability of the nuclear magnetic resonance technique to serve as a tool in obtaining information of macromolecular association phenomena such as enzyme–substrate or enzyme–inhibitor interactions. The method yields information, in addition to dissociation constants, such as knowledge of the microenvironment on the macromolecule experienced by the associating small molecule. Such data are a direct result of the sensitivity of nuclei to change in environment which is detectable by the nuclear magnetic resonance method.

Experimental Section

Lysozyme was obtained from Sigma Chemical Co. (lot 96B-8572). Solutions were made up to approximately 3×10^{-3} M lysozyme in 0.1 M citrate buffer (pH 5.5) containing 0.5% acetone as an internal reference. The exact concentration of the enzyme was determined by removing 25 μl, diluting to 5.00 ml with 0.1 M citrate buffer (pH 5.5), and measuring the optical density of the solution at 280 mμ with a Cary Model 14 spectrophotometer. The known extinction coefficient was used to estimate lysozyme concentrations (Sophianpoulos *et al.*, 1962).

N-Acetylglucosamine was obtained from California Corp. for Biochemical Research. Recrystallization of this material from ethanol–water gave α-NAG, mp 202–204°. The β anomer was synthesized by acetylation of β-glucosamine in dimethylformamide according to the procedure of Kuhn and Haber (1953). The melting point of this material was 181–183°.

The measurements of nuclear magnetic resonance spectra were performed on a Varian HA-100 spectrometer in frequency sweep mode. In general, the water resonance was used as the lock signal, although sometimes a capillary of tetramethylsilane was employed for this purpose. Chemical shifts of the acetamido methyl group were measured relative to the internal acetone standard. These shifts were determined by electronic

counting of the difference between the sweep frequency and the manual oscillator frequency using a Hewlett-Packard frequency counter. In each case the spectra were measured three times and the chemical shifts were determined to ±0.03 cps or less from the mean. For those measurements which required time averaging to increase the signal-to-noise ratio, a Varian C-1024 time-averaging computer was used.

All measurements were carried out at a probe temperature of 31 ± 1°. Every sample was preequilibrated at 31° for at least 5 min in a water bath before introduction into the probe.

For experiments in which the acetate impurity in lysozyme interfered with the proton resonances of α-NAG, a sample of acetate-free enzyme was prepared. This was accomplished by multiple ultrafiltrations of a solution of the enzyme in a Diaflo ultrafiltration apparatus (Amicon Corp.) using a UM-1 membrane. The solvent for the protein sample was 0.1 M citrate (pH 5.5). Following the multiple ultrafiltrations exhaustive dialysis against distilled water and lyophilization yielded lysozyme which displayed no nuclear magnetic resonance spectrum for free acetate.

Mutarotation Studies. A weighed amount of crystalline α- or β-NAG was thermally equilibrated in a 1.00-ml volumetric flask in a water bath at 31°. This material was dissolved in a thermally equilibrated solution which contained enzyme, buffer, and acetone. After the sample dissolved, it was transferred to a nuclear magnetic resonance tube, equilibrated in the water bath for 1 min, and placed in the probe. The spectrometer was locked on the water resonance, and the spectrum was recorded. A spectrum could be produced in this manner in 3–4 min from the time the crystalline NAG was dissolved.

Appendix A

Quantitation of chemical shift data for association of an enzyme with an inhibitor which is at equilibrium between two forms:

In the following derivation $[\alpha_0]$ refers to the initial concentration of α-NAG, $[\beta_0]$ to β-NAG, Δ_α and Δ_β to the enzyme-bound chemical shift of a nucleus in α-NAG and β-NAG, respectively, δ_α and δ_β to observed chemical shift differences of nuclei in α-NAG and β-NAG in the presence of the enzyme compared with the chemical shifts of the same nuclei in the absence of the enzyme, $(P_b)_\alpha$ and $(P_b)_\beta$ to the enzyme-bound fractions of the total α-NAG and β-NAG present, $E\alpha$ and $E\beta$ to lysozyme–α-NAG and lysozyme–β-NAG complexes, and K_α and K_β to the dissociation constants of those complexes.

For a system in which α-NAG and β-NAG are binding competitively to lysozyme, we can write

$$\mathrm{E} + \alpha \rightleftharpoons \mathrm{E}\alpha \qquad K_\alpha = \frac{[\mathrm{E}][\alpha]}{[\mathrm{E}\alpha]} \tag{20}$$

$$\mathrm{E} + \beta \rightleftharpoons \mathrm{E}\beta \qquad K_\beta = \frac{[\mathrm{E}][\beta]}{[\mathrm{E}\beta]} \tag{21}$$

and

$$\delta_\alpha = (P_b)_\alpha \Delta_\alpha = \frac{[\mathrm{E}\alpha]}{[\alpha_0]}\Delta_\alpha$$

$$\delta_\beta = (P_b)_\beta \Delta_\beta = \frac{[\mathrm{E}\beta]}{[\beta_0]}\Delta_\beta \tag{22}$$

$$[\mathrm{E}\beta] = [\mathrm{E}\alpha]\frac{[\beta]K_\alpha}{[\alpha]K_\beta}$$

$$K_\alpha = \frac{(E_0 - [\mathrm{E}\alpha] - [\mathrm{E}\beta])([\alpha_0] - [\mathrm{E}\alpha])}{[\mathrm{E}\alpha]} \tag{23}$$

$$K_\alpha = \frac{\left(E_0 - [\mathrm{E}\alpha]\left\{1 + \frac{[\beta]K_\alpha}{[\alpha]K_\beta}\right\}\right)([\alpha_0] - [\mathrm{E}\alpha])}{[\mathrm{E}\alpha]} \tag{24}$$

$$K_\alpha = \frac{\left(E_0 - \frac{\delta_\alpha}{\Delta_\alpha}[\alpha_0]\left\{1 + \frac{[\beta]K_\alpha}{[\alpha]K_\beta}\right\}\right)\left(\alpha_0 - \frac{\delta_\alpha}{\Delta_\alpha}[\alpha_0]\right)}{\frac{\delta_\alpha}{\Delta_\alpha}[\alpha_0]} \tag{25}$$

$$\frac{\delta_\alpha}{\Delta_\alpha - \delta_\alpha}K_\alpha = \left(E_0 - \frac{\delta_\alpha}{\Delta_\alpha}S_0\left\{\frac{[\alpha_0]}{S} + \frac{[\beta][\alpha_0]K_\alpha}{S_0[\alpha]K_\beta}\right\}\right) \tag{26}$$

where $S_0 = [\alpha_0] + [\beta_0]$. Letting

$$A' = \frac{[\alpha_0]}{S_0} + \frac{[\beta]}{S_0}\frac{K_\alpha}{K_\beta}\frac{[\alpha_0]}{[\alpha]} \tag{27}$$

$$K_\alpha \frac{\delta_\alpha}{\Delta_\alpha - \delta_\alpha} = E_0 - \frac{\delta_\alpha}{\Delta_\alpha}S_0A' \tag{28}$$

$$S_0A'\frac{\Delta_\alpha - \delta_\alpha}{\Delta_\alpha} = \frac{\Delta_\alpha - \delta_\alpha}{\delta_\alpha}(E_0) - K_\alpha \tag{29}$$

Using the approximations

$$[\beta]\frac{[\alpha_0]}{[\alpha]} \sim [\beta_0]$$

and

$$\frac{\Delta_\alpha - \delta_\alpha}{\Delta_\alpha} \sim 1$$

$$A' \sim A = \frac{[\alpha_0]}{S_0} + \frac{[\beta_0]}{S_0}\frac{K_\alpha}{K_\beta}$$

and

$$S_0A = \frac{\Delta_\alpha}{\delta_\alpha}E_0 - K_\alpha - E_0 \tag{30a}$$

Similarly

$$S_0B = \frac{\Delta_\beta}{\delta_\beta}E_0 - K_\beta - E_0$$

where

$$B = \frac{[\beta_0]}{S_0} + \frac{[\alpha_0]}{S_0}\frac{K_\beta}{K_\alpha} \tag{30b}$$

Appendix B

Quantitation of line-width measurements for association with an enzyme of an inhibitor which is in mutarotation equilibrium:

In the following derivation $(P_b)_\alpha$ and $(P_b)_\beta$ refer to the fractions of α-NAG and β-NAG which are enzyme bound, $(T_2^b)_\alpha$ and $(T_2^b)_\beta$ to the spin–spin relaxation times of the bound species of α-NAG and β-NAG, $(1/T_2)_\alpha$ and $(1/T_2)_\beta$ to the contributions to the observed line widths due to association with the enzyme of α-NAG and β-NAG (as defined in eq 10), S_0 to the total concentration of NAG, $[\alpha_0]$ and $[\beta_0]$ to the total concentrations of α-NAG and β-NAG, and K_α and K_β to the dissociation constants of $E\alpha$ and $E\beta$ which are defined as the complexes of lysozyme with α-NAG and with β-NAG.

The contributions to the observed line width of nuclei in each anomeric form due to association with the enzyme may be expressed as

$$\left(\frac{1}{T_2'}\right)_\alpha = (P_b)_\alpha\left(\frac{1}{T_2^b}\right)_\alpha \tag{31a}$$

$$\left(\frac{1}{T_2'}\right)_\beta = (P_b)_\beta\left(\frac{1}{T_2^b}\right)_\beta \tag{31b}$$

Since eq 31a and 31b have the same form as eq 21a and 21b in Appendix A, an equation analogous to eq 29 can be written (assuming $A' = A$; see Appendix A).

$$S_0A\frac{\left(\frac{1}{T_2^b}\right)_\alpha - \left(\frac{1}{T_2'}\right)_\alpha}{\left(\frac{1}{T_2^b}\right)_\alpha} = \frac{\left(\frac{1}{T_2^b}\right)_\alpha - \left(\frac{1}{T_2'}\right)_\alpha}{\left(\frac{1}{T_2'}\right)_\alpha}E_0 - K_\alpha \tag{32}$$

which may be rearranged

$$(T_2')_\alpha = \frac{(T_2^b)_\alpha}{E_0}\left[\frac{K_\alpha}{1 - \frac{(T_2^b)_\alpha}{(T_2')_\alpha}} + S_0A\right] \tag{33}$$

Substitution for A gives

$$(T_2')_\alpha = \frac{(T_2^b)_\alpha}{E_0K_\beta}\left[\frac{K_\alpha K_\beta}{1 - \frac{(T_2^b)_\alpha}{(T_2')_\alpha}} + [\alpha_0]K_\beta + [\beta_0]K_\alpha\right] \tag{34a}$$

Similar arguments for the case of the β anomer give

$$(T_2')_\beta = \frac{(T_2^b)_\beta}{E_0K_\alpha}\left[\frac{K_\alpha K_\beta}{1 - \frac{(T_2^b)_\beta}{(T_2')_\beta}} + [\beta_0]K_\alpha + [\alpha_0]K_\beta\right] \tag{34b}$$

Thus

$$\frac{(T_2')_\beta}{(T_2')_\alpha} = \frac{K_\beta(T_2^b)_\beta\left[\frac{K_\alpha K_\beta}{1 - \frac{(T_2^b)_\beta}{(T_2')_\beta}} + [\beta_0]K_\alpha + [\alpha_0]K_\beta\right]}{K_\alpha(T_2^b)_\alpha\left[\frac{K_\alpha K_\beta}{1 - \frac{(T_2^b)_\alpha}{(T_2')_\alpha}} + [\beta_0]K_\alpha + [\alpha_0]K_\beta\right]} \tag{35}$$

Under the conditions of these experiments $1 > (T_2^b)_\alpha (T_2')_\alpha$ and $1 > (T_2^b)_\beta/(T_2')_\beta$ and to within a 5% error

$$\frac{(T_2')_\beta}{(T_2')_\alpha} = \frac{K_\beta(T_2^b)_\beta}{K_\alpha(T_2^b)_\alpha}.$$

References

Blake, C. C. F., Mair, G. A., North, A. C. T., Phillips, D. C., and Sarma, V. F. (1967), *Proc. Roy. Soc. (London) B167*, 365.

Buckingham, A. D. (1960), *Can. J. Chem. 38*, 300.

Buckingham, A. D., Schaefer, T., and Schneider, W. G. (1960), *J. Chem. Phys. 32*, 1227.

Chipman, D. M., Grisaro, V., and Sharon, B. (1967), *J. Biol. Chem. 242*, 4388.

Dahlquist, F. W., Jao, L., and Raftery, M. A. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 26.

Fischer, J. J., and Jardetsky, O. (1965), *J. Am. Chem. Soc. 87*, 3237.

Hollis, D. P. (1967), *Biochemistry 6*, 2080.

Jardetsky, O. (1964), *Advan. Chem. Phys. 7*, 499.

Jardetsky, O., Wade, N. G., and Fischer, J. J. (1963), *Nature 197*, 183.

Kuhn, R., and Haber, F. (1953), *Chem. Ber. 86*, 722.

Lehrer, S. S., and Fasman, G. D. (1966), *Biochem. Biophys. Res. Commun. 23*, 133.

Lehrer, S. S., and Fasman, G. D. (1967), *J. Biol. Chem. 242*, 4644.

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

Raftery, M. A., Dahlquist, F. W., Chan, S. I., and Parsons, S. M. (1968a), *J. Biol. Chem.* (in press).

Raftery, M. A., Dahlquist, F. W., Parsons, S. M., and Wolcott, R. (1968b), *Proc. Natl. Acad. Sci. U. S.* (in press).

Rupley, J. A., Butler, L., Gerring, M., Hartdegen, F. J., and Pecoraro, R. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 1088.

Sophianpoulos, A. J., Rhodes, C. K., Holcomb, D. N., and van Holde, K. E. (1962), *J. Biol. Chem. 237*, 1107.

Spotswood, T. McL., Evans, J. M., and Richards, J. H. (1967), *J. Am. Chem. Soc. 89*, 5052.

Thomas, E. W. (1966), *Biochem. Biophys. Res. Commun.* *24*, 611.

Zimmerman, J. R., and Brittin, W. F. (1957), *J. Phys. Chem.* *61*, 1328.

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

F. W. Dahlquist† and M. A. Raftery

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

* Contribution No. 3652 from the Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, California 91109. *Received March 18, 1968.* This work was supported by a U. S. Public Health Service grant (GM-14452-01).

† National Institutes of Health Trainee.

[1] The authors wish to thank Professor S. I. Chan for use of a Varian HA-100 nuclear magnetic resonance spectrometer.

[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.