The Stepwise Binding of Small Molecules to Proteins. Nuclear Magnetic Resonance and Temperature Jump Studies of the Binding of 4-(*N*-Acetylaminoglucosyl)-*N*-acetylglucosamine to Lysozyme†

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ABSTRACT: The binding of 4-(*N*-acetylaminoglucosyl)-*N*-acetylglucosamine to lysozyme was studied by both nuclear magnetic resonance (NMR) and temperature-jump methods under comparable conditions. The NMR measurements on the inhibitor spectrum were carried out over a range of inhibitor concentrations including levels at which most of the inhibitor was bound to the enzyme. Data in this region were obtained by a novel difference method in conjunction with correlation spectroscopy. The results from the combination of both experimental techniques demonstrated the existence of a two-step binding mechanism and produced both values for all of the individual rate constants and also the NMR spectral data for the inhibitor in the two enzyme-inhibitor complexes. The latter data characterize the environment experienced by the inhibitor at each stage in the binding process and thus provides both a three-dimensional and a dynamic picture of the interaction.

The binding of small molecules to proteins is a subject which has received considerable attention in the study of enzyme mechanisms and substrate recognition processes. The observation of such binding by nuclear magnetic resonance (NMR) methods is dependent upon the existence of a measurable change in at least one NMR parameter of the system resulting from the binding. A change in either the relaxation times (T_1 or T_2) or the chemical shift of any observable nucleus in the system may be employed, where the system includes both the small molecule, the protein, and *any other* species present in the solution such as the solvent. In almost all of the cases in which NMR methods have been employed in such studies (Sykes and Scott, 1972, and references therein), the results have been interpreted, at least initially, in terms of a simple bimolecular binding process (eq 1). Studies on the comparable systems using other

$$E + I \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} EI \qquad (1)$$

methods, particularly temperature jump (T-J) and stopped-flow, have in many cases suggested that the binding process is more complex and involves a bimolecular step followed by a unimolecular step (Holler et al., 1969; Hammes and Schimmel, 1970; Gutfreund, 1971)

$$E + I \underset{k_1'}{\overset{k_{-1}'}{\rightleftharpoons}} EI \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} EI' \qquad (2)$$

For this scheme the interpretation of the NMR results becomes more difficult because the number of parameters is increased. It is still possible in principle, however, to use NMR methods to determine if mechanism 1 or 2 applies provided there is an observable difference in the chemical shift of some nucleus in the system between the two bound species. Under these conditions the two step mechanism, unlike the one step mechanism, offers the possibility of observing chemical exchange line broadening *when all the inhibitor is bound to the enzyme.* However, the data which can be obtained from the NMR experiments are generally insufficient to uniquely determine all of the unknown parameters, so that a complete analysis is not possible from NMR alone and data from other methods such as T-J or stopped flow methods must also be used.

To accurately obtain these parameters from the NMR results on a system described by mechanism 2 requires NMR spectra of samples in which substantially all of the inhibitor is bound to the enzyme. For such samples, the enzyme and inhibitor will be at nearly equimolar concentrations. ^{1}H NMR is well suited for studies at low concentrations from the standpoint of sensitivity, and such spectra are both well understood and widely obtainable. However, the selection of ^{1}H NMR will generally mean the spectrum of the enzyme and the inhibitor will interfere with one another. In addition, since it is desirable to do the study in H_2O rather than D_2O to facilitate comparison with other methods and to more closely represent the in vivo situation, the large H_2O resonance will dominate the ^{1}H NMR spectrum.

The problem of buried and overlapping resonances can be solved in principle by using difference NMR spectroscopy. The simple subtraction of the enzyme spectrum from that of the enzyme and inhibitor proves, in general, to be unsatisfactory since changes in the enzyme spectrum caused by inhibitor binding are likely to be numerous and of the same order of magnitude as the changes in the inhibitor spectrum. Accordingly we have developed a technique of selective deuteration of the inhibitor at the resonance(s) of interest, followed by the preparation of two samples, identical

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with the exception of the deuterated inhibitor in place of the protonated inhibitor in one of the samples. The difference between spectra taken of such matched samples is then an easily interpreted difference spectrum containing only the resonance(s) representing the deuterated position(s) on the inhibitor. Acquiring ^1H NMR spectra in H_2O places additional constraints on the NMR techniques used. Several techniques are capable of producing spectra of species at low concentration in H_2O, but we have found correlation spectroscopy (Dadok and Sprecher, 1974; Gupta et al., 1974) to be well suited to our studies.

We have applied the technique of difference correlation spectroscopy to the binding of GlcNAc-β(1 → 4)-GlcNAc (NAG_2)[1] to lysozyme. By combining these results with those obtained from T-J experiments under nearly identical conditions, we have obtained detailed information possible only from NMR about the stepwise approach of NAG_2 to the active site of lysozyme. Earlier NMR studies on NAG_2 binding to lysozyme had either been interpreted on the simple bimolecular mechanism (Sykes and Parravano, 1969) or concerned solely with the extraction of chemical shift data (Raftery et al., 1969). Previous T-J experiments on this system have been carried out (Chipman and Schimmel, 1968; Holler et al., 1969) but only at pH values above 6, where it has been shown that the dimerization of the protein interferes with the interpretation of NMR experiments done at high protein concentrations (Studebaker et al., 1971).

Theory

NMR. For either mechanism 1 or mechanism 2 there is an exchange of the inhibitor between free solution and the binding site on the enzyme. This exchanges nuclei on the inhibitor or the enzyme between sites of different local magnetic environments characterized by different relaxation times T_1(i) and T_2(i) and different chemical shifts ω_i (in radians sec^{-1}). The resultant line shape can be calculated from the Bloch equation formalism as modified by McConnell to include the effects of chemical exchange (McConnell, 1958).

The general three site exchange formalism has been developed by Patterson and Ettinger (1960). For mechanism 2 (E + I ⇄ EI ⇄ EI′) we can write the problem as

$$\alpha \underset{k_1'[E]}{\overset{k_{-1}'}{\rightleftharpoons}} \beta \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} \gamma \qquad (3)$$

with $1/\tau_\alpha = k_1'[E]$, $1/\tau_{\beta\alpha} = k_{-1}'$, $1/\tau_{\beta\gamma} = k_2$, $1/\tau_\gamma = k_{-2}$ where $1/\tau_{\beta\alpha}$ is the lifetime of the inhibitor in the site (EI) before exchange to the site I, and $1/\tau_{\beta\gamma}$ is the lifetime of the inhibitor on the site EI before exchange to the site EI′. The line shape is obtained by solving the following simultaneous equations to obtain an expression for steady-state magnetization G, where G is the sum of the magnetization of the individual sites G_α, G_β, and G_γ, and D_j is defined as $1/T_{2j} + i(\omega - \omega_j)$.

$$\begin{aligned}(D_\alpha + 1/\tau_\alpha)G_\alpha &= -i\omega_1 M_\alpha^0 + G_\beta/\tau_{\beta\alpha}\\ (D_\beta + 1/\tau_{\beta\alpha} + 1/\tau_{\beta\gamma})G_\beta &= -i\omega_1 M_\beta^0 + G_\alpha/\tau_\alpha + G_\gamma/\tau_\gamma\\ (D_\gamma + 1/\tau_\gamma)G_\gamma &= -i\omega_1 M_\gamma^0 + G_\beta/\tau_{\beta\gamma}\end{aligned} \qquad (4)$$

The spectral line shape is given by the real part of G.

In certain limits these equations can be simplified to yield insight into the behavior of the line width and chemical shift as a function of concentration. Consider mechanism 1 (E + I ⇄ EI) as a two site exchange problem for a nucleus on the inhibitor molecule

$$A \underset{k_1[E]}{\overset{k_{-1}}{\rightleftharpoons}} B \qquad (5)$$

with $1/\tau_A = k_1[E]$, $1/\tau_B = k_{-1}$. In the limit of fast exchange [$(\tau\Delta)^2 \ll 1$, where $\tau = \tau_A\tau_B/(\tau_A + \tau_B)$ and $\Delta = (\omega_A - \omega_B)$], which has been shown to be a close approximation to the case for the reactions considered (Sykes, 1969; Sykes and Parravano, 1969), the observed resonances from the two sites have coalesced into a single resonance with chemical shift and line width given by

$$\omega_{obsd} = f_A\omega_A + f_B\omega_B \qquad (6A)$$

$$\pi\Delta\nu_{obsd} = \frac{1}{T_2(obsd)} = \frac{f_A}{T_2[A]} + \frac{f_B}{T_2[B]} + f_A^2 f_B^2(\tau_A + \tau_B)\Delta^2 \qquad (6B)$$

where $f_A = [A]/([A] + [B])$ and $f_B = [B]/([A] + [B])$.

Normally rather complex expressions derived from eq 4 are written for the resultant line shape in the presence of exchange for mechanism 2 even in the fast exchange limit. However, approximate expressions for the observed line shape for mechanism 2 can be written by inspection using the expression for mechanism 1 under certain conditions. If mechanism 2 is applicable, then the first step will generally be fast on the NMR time scale (given the range of rate constants k_1' and k_{-1}' and possible chemical shifts normally encountered). The assumption that the first step is rapid is often used in the analysis of T-J data (see below) to decouple the first and second steps (Halford, 1972). If the first step is assumed to be in fast exchange, then we can substitute for sites α and β an effective site ϵ whose chemical shift and relaxation times are a weighted average of those of sites α and β. Mechanism 2 can then be written as a two-site problem (Patterson and Ettinger, 1960) involving exchange between site ϵ and site γ,

$$\epsilon \rightleftharpoons \gamma \qquad (7)$$

with $1/\tau_\epsilon = \{[EI]/([I] + [EI])\}k_2$, $1/\tau_\gamma = k_{-2}$. If the second step is also in the fast exchange limit $\{(\tau'(\omega_\epsilon - \omega_\gamma))^2 \ll 1$, $\tau' = \tau_\epsilon\tau_\gamma/(\tau_\epsilon + \tau_\gamma)$, $\omega_\epsilon = ([I]\omega_I + [EI]\omega_{[EI]})/([I] + [EI])\}$, the observed coalesced resonance from all three sites has chemical shift and line width given (in complete analogy with mechanism 1) by

$$\omega_{obsd} = f_\epsilon\omega_\epsilon + f_\gamma\omega_\gamma \qquad (8A)$$

$$\pi\Delta\nu_{obsd} = \frac{1}{T_2(obsd)} = \frac{f_\epsilon}{T_2[\epsilon]} + \frac{f_\gamma}{T_2[\gamma]} + f_\epsilon^2 f_\gamma^2(\tau_\epsilon + \tau_\gamma)(\omega_\epsilon - \omega_\gamma)^2 \qquad (8B)$$

where $f_\epsilon = [\epsilon]/([\epsilon] + [\gamma])$ and $f_\gamma = [\gamma]/([\epsilon] + [\gamma])$.

The important thing to note is that the last term in the expression for the line widths (eq 6B and 8B) has a very different concentration dependence for mechanism 1 vs. mechanism 2 and therefore the two mechanisms may in principle be distinguished by an examination of the line width as a function of fraction inhibitor bound (fraction bound is defined as the ratio of enzyme–inhibitor complexes over total inhibitor present in solution). For mechanism 1, as the fraction of inhibitor bound goes from zero to one, $f_A^2 f_B^2$ goes

[1] Abbreviation used is: GlcNAc-β(1 → 4)-GlcNAc = (NAG_2), 2-acetamido-2-deoxy-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-glucopyranose (4-(*N*-acetylaminoglucosyl)-*N*-acetylglucosamine).

from zero through a maximum and back to zero; that is to say that there is no line broadening if the inhibitor is all in one site (I) or the other site (EI). However, for mechanism 2, as the inhibitor goes from completely free (I) to completely bound (EI + EI'), $f_\epsilon^2 f_\gamma^2$ starts at zero and increases but does not necessarily go through a maximum and then decrease to zero. For example, if [EI]/[EI'] = 1, $f_\epsilon^2 f_\gamma^2$ increases to a maximum and then levels off. Thus there may well be exchange still present between sites ϵ and γ when the inhibitor is completely bound to the enzyme. Of course, the behavior of the line shape will now also depend on the dependence of $(\omega_\epsilon - \omega_\gamma)^2$ on concentration. This means that even though there may be exchange still present in mechanism 2 when the inhibitor is completely bound, the magnitude of the line broadening will depend upon whether ω_β and ω_γ are similar or different (remembering that for completely bound inhibitor $\omega_\epsilon \rightarrow \omega_\beta$).

In principle, therefore, the dependence of NMR line broadening on concentration can be used to determine whether mechanism 1 or 2 applies. However, this requires data over the complete range of inhibitor concentration including the region where the inhibitor is predominately bound to the enzyme. Not only are these data the most difficult to obtain experimentally, but they are the most subject to errors. In addition, as the fraction bound becomes higher, the term $f_\gamma/T_2(\gamma)$ can become significant. Without data in this region or data from other methods, the rate constants cannot be uniquely determined from the NMR data. While the above fast exchange approximations are very useful in conceptualizing the line width behavior as a function of concentration, deviations from the predicted fast exchange limit behavior were seen in the present example in the region of high inhibitor concentration. This indicated that the fast exchange approximation was not entirely valid, and that full line shape analysis expression would have to be used.

Temperature Jump. Eigen and de Maeyer (1963) have derived equations relating relaxation times in T-J experiments with individual rate constants and reactant concentrations for a number of reaction schemes.[2] The only limitation to these derivations is that the perturbation from equilibrium must be small so that the rate equations may be linearized. For mechanism 1

$$1/\tau = k_{-1} + k_1(\bar{E} + \bar{I}) \tag{9}$$

where τ represents the relaxation time and $\bar{E}$ and $\bar{I}$ are the respective equilibrium concentrations of free reactants.

For mechanism 2, the relaxations that arise from the perturbation of the two-step equilibrium are coupled

$$\frac{1}{\tau_1} + \frac{1}{\tau_2} = k_{-1}' + k_1'(\bar{E} + \bar{I}) + k_{-2} + k_2 \tag{10}$$

and

$$\frac{1}{\tau_1}\frac{1}{\tau_2} = k_{-1}'k_{-2} + k_1'(k_{-2} + k_2)(\bar{E} + \bar{I})$$

If mechanism 2 consists of a fast binding step followed by a slower rearrangement ($\tau_1 \ll \tau_2$), eq 10 can be decoupled (Eigen and de Maeyer, 1963)

$$\frac{1}{\tau_1} = k_{-1}' + k_1'(\bar{E} + \bar{I}) \tag{11}$$

$$\frac{1}{\tau_2} = k_{-2} + \frac{k_2}{\left(1 + \frac{K_{D1}}{(\bar{E} + \bar{I})}\right)} \tag{12}$$

where $K_{D1} = k_{-1}'/k_1'$.

In most experimental situations and for a variety of reasons, only the relaxation corresponding to τ_2 is observed (Gutfreund, 1971; Halford, 1972). If $(\bar{E} + \bar{I}) \ll K_{D1}$, then eq 12 reduces to

$$\frac{1}{\tau_2} = k_{-2} + \frac{k_2}{K_{D1}}(\bar{E} + \bar{I}) \tag{13}$$

which has the same functional form as eq 9. Only when $(\bar{E} + \bar{I}) \geq K_{D1}$ will the concentration dependence of τ_2 be indicative of a two-step mechanism.

Experimental Section

Sample Preparation. The NAG_2 was prepared as described by Rupley (1964) with additional purification steps. The *N*-acetylglucosamine dimer was obtained from chitin (Sigma, Practical) by acid hydrolysis in concentrated HCl at 40° for 2 hr. After neutralization of the acid, the sugar was subjected to chromatography on a carbon–Celite column and was eluted with a gradient running from H_2O to 60% ethanol in H_2O. The sugar peaks in the effluent were detected by using the Park–Johnson test (Park and Johnson, 1949) and dried under partial vacuum. The *N*-acetylglucosamine dimer prepared in this manner had an impurity which strongly bound to lysozyme: this was removed by N-acylation and chromatography on Sephadex G-15. The purified sugar ran as a single spot on thin-layer chromatography.

The NAG_2-d_6 was prepared by N-acylation from the analogous glucosamine. The glucosamine dimer was prepared from chitosan (Chemical Procurement Laboratories) by acid hydrolysis and then separated by column chromatography on Dowex 50-X8 according to Distiler and Roseman (1962). The sugar was then N-acylated by converting it to the free base form in methanol with the addition of sodium methoxide and N-acylated with the addition of acetic-d_6 anhydride according to Inouye et al. (1956).

Hen egg white lysozyme (from PL Biochemicals, three times crystallized) was dialyzed for 18 hr (three changes) against water adjusted to pH 3.9 with HCl, in order to remove acetate present in the commercial sample. The protein was then lyophilized. For NMR experiments, solutions of lysozyme and saccharide were made up in 0.01 *M* citrate buffer containing 0.1 *M* KCl, 2.5% D_2O, and acetone as an internal NMR standard, at a final pH of 4.9. The lysozyme concentration was determined from the absorption at 280 nm ($E^{280} = 3.5 \times 10^4\ M^{-1}$)(Chipman et al., 1967). Significant errors in the concentrations of the two samples of lysozyme with either protonated or deuterated NAG_2, which would have affected the NMR difference spectra, were avoided by taking great care in the preparation of the samples.

NMR. All the spectra were taken on a Varian XL100 spectrometer slightly modified for correlation spectroscopy (Baldo, 1974; Patt, 1975). The desired frequency range was swept with the voltage controlled oscillator driven by a Varian 620i computer generated voltage ramp. The unfiltered output of the spectrometer was taken from across the DC observe switch on the power control and scope selector panel and was passed through a low pass filter (Ithaco 4215) and then a signal amplifier (Baldo, 1974) to boost the

[2] The authors apologize for any confusion caused by the use of "relaxation times" to describe both NMR and temperature jump experiments.

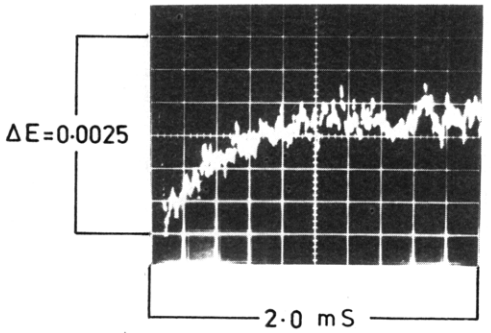

FIGURE 1: Spectrophotometric record at 365 nm of a temperature-jump perturbation of the binding equilibrium of lysozyme (0.24 m*M*) and NAG_2 (0.25 m*M*) in 0.10 *M* KCl with 2,4-dinitrophenol (0.20 m*M*) at pH 4.4 and a final temperature of 31°.

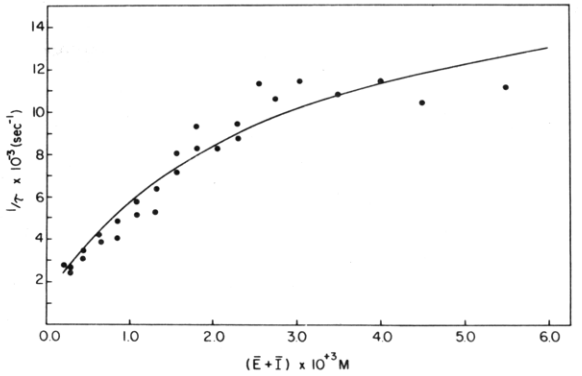

FIGURE 2: Plot of experimental values of $1/\tau$ obtained in T-J studies on the lysozyme–NAG_2 system as a function of $(\bar{E} + \bar{I})$, the concentration of free reactants. The curve represents the optimal computer fit to the data using eq 12. Each data point is the average of three relaxation times measured on a single solution of the enzyme–inhibitor mixture. The solutions, containing between 0.1 and 0.3 m*M* lysozyme and between 0.1 and 5.5 m*M* NAG_2, were made up in 0.1 *M* KCl with 0.20 m*M* 2,4-dinitrophenol. For each sample, the pH was adjusted to 4.4 and the final temperature after the jump was 31°.

signal level to take full advantage of the dynamic range of the computer's analog to digital converter. The spectrometer was modified so that the rf power could be switched off during the retrace portion of the ramp to avoid ringing and other fast passage effects caused by passage through the spectrum on the retrace. The accumulated spectra were stored on magnetic tape (Computer Operations tape drive) for later analysis.

The fast passage spectra were manipulated as described by Dadok and others (1974; Gupta et al., 1974) to obtain the slow passage spectra. The Fourier transform of the accumulated spectrum was obtained using a fast Fourier transform routine. The Fourier transform of a theoretical line with no decay (zero width) was generated and the two were multiplied together to accomplish the correlation. The resultant complex signal could be then further conditioned by the application of an appropriate weighting function or corrected by a desired phase angle correction by rotation of the data in the complex plane. The slow passage spectrum was obtained by inverse Fourier transformation. Spectra obtained from two matched samples were then subtracted to obtain the desired difference spectra.

The deuterium lock used for field frequency stabilization was obtained by making the buffer 2.5% in D_2O. The internal HDO lock was chosen over other external lock schemes due to the large signal degradation caused by the presence of a capillary tube. The NMR probe was thermostated to prevent long term drifts of the spectrum due to the sensitivity of the HDO chemical shift to temperature changes. Significant improvement was obtained in the difference spectra by allowing for the shifting of the two spectra relative to one another prior to subtraction to further compensate for long term drift.

A typical set of experimental conditions for the NMR experiments were: rf level, 69dB; sweep rate 40 sec/250 Hz; low pass filter cut off 160 Hz, and between 16 and 2048 accumulations depending on the inhibitor concentration.

T-Jump. Solutions of lysozyme and NAG_2 for T-J experiments were made up in 0.1 *M* KCl containing 0.20 m*M* 2,4-dinitrophenol, and the pH was titrated to 4.4. The binding of NAG_2 to lysozyme at pH 4.4 results in the release of protons into solution (Rupley, 1967) (due to the lowering of the pK_a of aspartate-101 in the enzyme–inhibitor complex) which can be monitored by a pH indicator. The pK_a of 2,4-dinitrophenol was 3.8. No evidence of an interaction between 2,4-dinitrophenol and lysozyme could be detected by uv difference spectroscopy (see also Chipman and Schimmel, 1968).

The T-J experiments were done on an apparatus designed by Dr. L. C. de Maeyer and built by Messanlagen G.m.b.H. (Gottingen, Germany). Starting from 27°, a temperature rise of 4° was achieved with a heating time of 10 μsec by discharging 30 kV through the sample of total volume 5.5 ml. The relaxation of the chemical system to the new equilibrium position was followed spectrophotometrically at 365 nm, the absorption maximum of 2,4-dinitrophenylate ion. A 100-W mercury lamp was used as the light source. The transients were recorded on a Tektronix storage oscilloscope and the relaxation times evaluated as described previously (Halford, 1972).

Results

Temperature-Jump. Following the application of a temperature-jump to an equilibrium mixture of lysozyme and NAG_2, only a single relaxation was observed with the 2,4-dinitrophenol indicator on the T-J time scale (10 μsec–1 sec). An oscilloscope record of a typical relaxation is shown in Figure 1. No relaxations were observed with this system in the absence of NAG_2. The relaxation times for a series of samples of varying concentration of lysozyme and NAG_2 were determined by the T-J method. Using the overall dissociation constant (K_D) measured previously (Chipman and Sharon, 1969), the concentrations of the free lysozyme and NAG_2 were calculated and a plot of their sum $(\bar{E} + \bar{I})$ vs. $1/\tau$ is given in Figure 2. A non-linear least-squares fitting program was used to obtain the best fit to the data using eq 12. Given that K_D was known independently for these conditions and was used to calculate $(\bar{E} + \bar{I})$, the following constraint was imposed upon the analysis of the T-J data

$$K_{D1} = K_D[1 + (k_2/k_{-2})] \quad (14)$$

From the computer fit, values for k_2 and k_{-2} were obtained and hence also a value for K_{D1}, the dissociation constant for the first step (values given in Table I). The resultant calculated line is plotted in Figure 2 over the experimental data.

NMR. A series of samples consisting of lysozyme and NAG_2 was made up over a range of fraction NAG_2 bound from 0.0 to 0.9. In all cases where the fraction NAG_2 bound was greater than 0.1, pairs of samples were made and spectra obtained by the difference methods described above. Representative correlation spectra of the *N*-acetyl region of these samples are shown in Figure 3. The spectra at frac-

Table I: Parameters Derived from the Temperature-Jump and NMR Studies on the NAG_2–Lysozyme System.

Kinetic Data		
Parameter	Value	Source
K_D	2.0×10^{-4} M	Chipman and Sharon, 1969
k_1	$4 \pm 1 \times 10^7$ M^{-1} sec^{-1}	NMR[a]
K_{D1}	$2.9 \pm 0.2 \times 10^{-3}$ M	T-J
k_2	$1.7 \pm 0.2 \times 10^4$ sec^{-1}	T-J
k_{-2}	$1.3 \pm 0.1 \times 10^3$ sec^{-1}	T-J

	Line-Shape Data[a] (N-Acetyl Resonances)		
	I (Hz)	EI (Hz)	EI′ (Hz)
Reducing NAG			
Shift	0	0[b,c]	118[b]
Line width	1.5	7[d]	7[c]
Nonreducing NAG			
Shift	−3	+4	+4
Line width	1.5	7[d]	7

[a] Obtained from the NMR analysis for three site exchange (Patterson and Ettinger, 1960). [b] Uncertainties in the values of the reducing shift for the EI and EI′ sites were ±50 and ±3 Hz, respectively. [c] The value of the chemical shift of site EI and to a lesser extent the line width of site EI′ are sensitive to the value of (k_{-2}/k_2). [d] The fit to the data is insensitive to the line width of the EI site.

tions bound of 0.2 and 0.5 are examples of typical difference spectra obtained. The chemical shift and line-width data were obtained from the spectra either by direct measurement or by matching the spectrum with the best fit of a sum of two calculated Lorentzian lines. The latter method allowed a more accurate determination of the shifts and line widths especially in the low fraction bound region of the series where the two N-acetyl peaks overlapped somewhat.

The difference between the chemical shifts of the two acetyl peaks in each spectrum was taken. This effectively removed any nonspecific contributions to the chemical shift that were common to both acetyl peaks. In any case the total chemical shift of the nonreducing N-acetyl peak (≃7 Hz) was very much less than that of the reducing N-acetyl peak (≃110 Hz). For simplicity the difference between the chemical shifts of the reducing and nonreducing acetyl resonances (2.9 Hz) in the free spectrum was also subtracted from each of the previously mentioned differences. These values for the chemical shift are plotted in Figure 4. The line-width data plotted in Figure 5 were obtained by subtracting the line width of the nonreducing N-acetyl resonance from that of the reducing N-acetyl resonance.

The values for the bound chemical shifts for sites EI and EI′ (i.e., sites β and γ in mechanism 2) and k_1 were determined so that the chemical shifts and line widths calculated from the general three site expression, using the values of K_{D1}, k_2, and k_{-2} determined from the T-J results and the value of K_D (Chipman and Sharon, 1969), best fit the values obtained experimentally. Using the generated parameters, plots of the calculated chemical shifts and line widths are plotted over their respective data in Figures 4 and 5. The best values for the parameters are listed in Table I.

Discussion

The value of difference spectroscopy is clearly demonstrated by this work. Only for samples of NAG_2 where the fraction bound is below 0.1 is it sufficient to use single spectra. In this range of relative concentrations, the enzyme peaks have not yet become significant. However, when the fraction bound is higher than 0.1, the amplitude of the reducing end N-acetyl methyl peak has been markedly re-

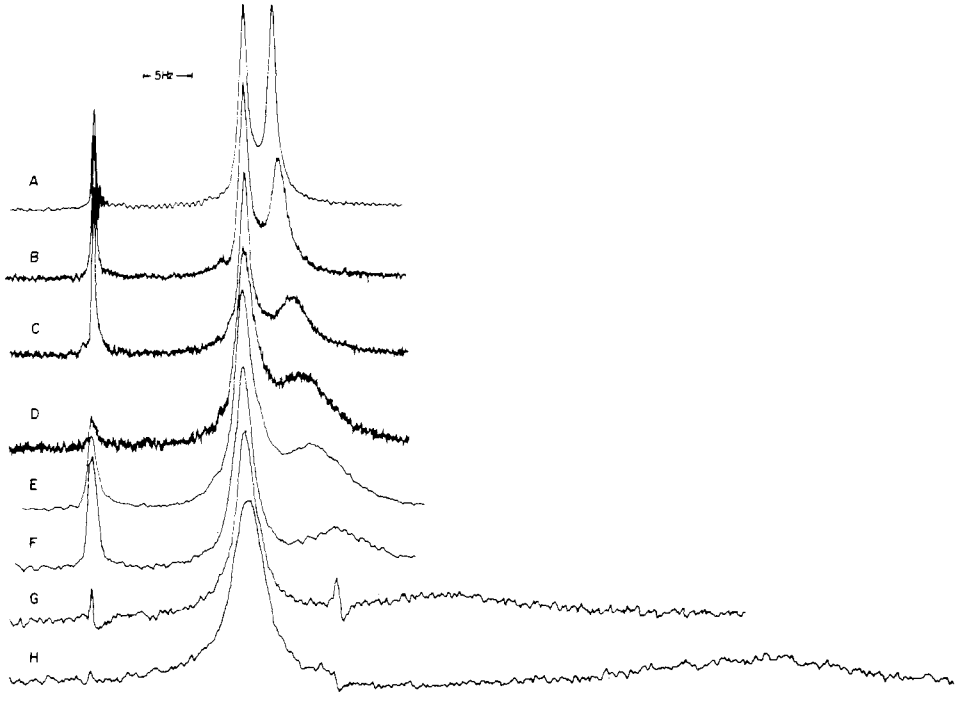

FIGURE 3: Representative NMR spectra. Spectra G and H are typical difference spectra. The solutions were made up in 0.1 M KCl, 0.01 M sodium citrate buffer (pH 4.9); 2.5% D_2O was added to provide a field frequency lock. The temperature was 29°. The spectra correspond to the following values of fraction inhibitor bound: A, free; B, 0.0089; C, 0.027; D, 0.045; E, 0.063; F, 0.087; G, 0.20; H, 0.49. The lowest field resonance is acetone added as an internal standard; the next two resonances in spectrum A are the respectively nonreducing and reducing end N-acetyl methyl groups of the inhibitor. The small resonance upfield of the nonreducing end N-acetyl methyl group in spectra G and H is residual acetate from the enzyme.

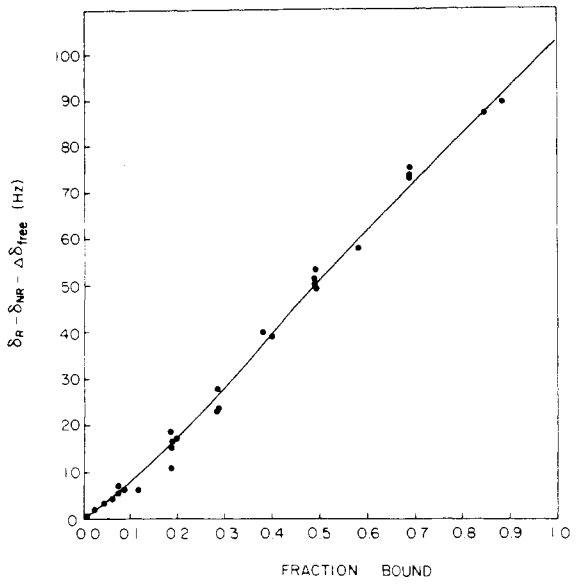

FIGURE 4: Plot of the observed chemical shift of the reducing end *N*-acetyl methyl group of NAG_2 (δ_R) as a function of the fraction of NAG_2 bound to lysozyme. The small chemical shift of the nonreducing end *N*-acetyl methyl group (δ_{NR}) and the difference between δ_R and δ_{NR} for the free inhibitor ($\Delta\delta_{FREE}$) have been subtracted from δ_R. The theoretical chemical shift calculated from the experimental parameters listed in Table I is drawn through the data.

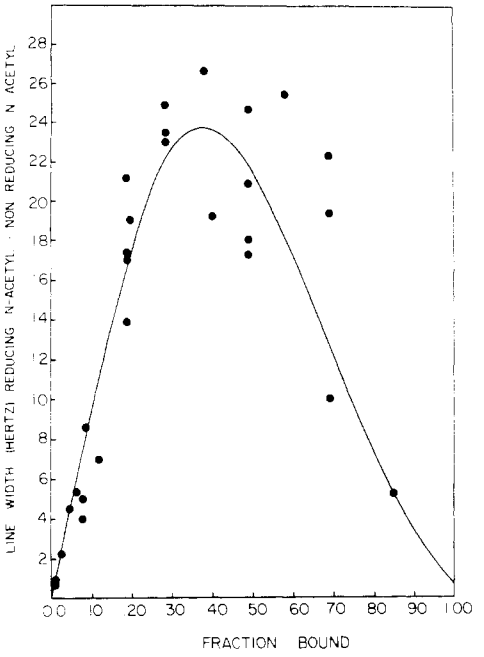

FIGURE 5: Plot of the difference in line width between the methyl resonances of the reducing and the nonreducing end *N*-acetyl groups as a function of the fraction NAG_2 bound to lysozyme. The theoretical line width calculated from the parameters listed in Table I is drawn through the data.

duced by exchange broadening and shifted into an enzyme methyl region in the NMR spectrum. Determination of the line width and shift of the reducing end *N*-acetyl methyl peak in a normal manner becomes impossible under these conditions and only by subtracting off the unwanted peaks can one obtain the necessary data. This is demonstrated in Figure 6 where it is clear that the data for the spectrum corresponding to a fraction bound of 0.5 could not be obtained without subtraction. Without the data obtained in the region where a large fraction of the inhibitor is bound to the enzyme, one is unable to observe the behavior of the chemical exchange broadening over the full concentration range. Moreover the observed chemical shifts can have the appearance of being in the complete fast exchange limit but extrapolate to an erroneous low bound chemical shift (see, as an example, Figure 4).

In cases as these where the line widths of the *N*-acetyl methyl peaks at high fraction bound are difficult to measure even using difference methods because of the extremely low inhibitor concentrations necessary, it is difficult to determine the value of the line widths of the bound species. If the bound line width is small, it then also becomes difficult to determine whether mechanism 1 or 2 should apply. The parameters for mechanism 2 have already been given, but from the NMR results alone one could just as well obtain parameters $k_1 = 4 \times 10^6\ M^{-1}\ \text{sec}^{-1}$ and $\delta_{EI} = 110$ Hz for mechanism 1 and fit the line-width behavior equally well. In fact if the T-J study was not carried out to higher values of $(\bar{E} + \bar{I})$, one could easily adopt mechanism 1 and obtain a value of $4 \times 10^6\ M^{-1}\ \text{sec}^{-1}$ for k_1 from the linear region of the plot of $1/\tau$ vs. $(\bar{E} + \bar{I})$. However, the complete T-J data clearly require that the mechanism be a two-step mechanism, and it is this key piece of information that allows one to use mechanism 2 to obtain the parameters in Table I. A close examination of the T-J data reveals a deviation from the theoretical curve but this is probably due to partial invalidity of the decoupling assumption of $\tau_1 \ll \tau_2$. These results are in close agreement with those previously obtained by Holler et al. (1969) for NAG_2 binding at pH 7.0.

Attempts were made to keep the conditions for the T-J and NMR studies as close as possible so that the results could be pooled. The ionic strength of the solutions used was the same in both cases (0.1 *M* KCl). The pH values in the studies differed by 0.5 pH unit, but a control series was done on the NMR at pH 4.4 and no significant differences were observed. The NMR runs were done at 29° while the final temperature for the T-J experiments was 31°. The NMR samples contained a 0.01 *M* citrate buffer for long term stability while the T-J samples contained an indicator dye but no supplementary buffer. Thus while the conditions were not identical, they were similar enough to permit the results to be pooled. Also values for the dissociation constant between 1.7 and 2.5×10^{-4} *M* have been reported for the lysozyme-NAG_2 system. A value of 2.0×10^{-4} *M* (Chipman and Sharon, 1969) was used here.

Combining of NMR and T-J data, as we have seen, gives a full set of kinetic parameters for the two-step mechanism. It also gives us information which is unattainable if each method was used alone. A spatial picture of the lysozyme-NAG_2 interaction is obtained, in addition to the rate constants, from the chemical shifts for the species EI and EI′. The large upfield shift of the *N*-acetyl methyl group on the reducing saccharide results from its position over the indole ring of tryptophan-108 (Sykes, 1969) and this is observed only when the inhibitor is in site EI′. Tryptophan-108 is located under subsite C in the lysozyme cleft (Blake et al., 1967). Thus, while it is more difficult to determine exactly the chemical shift of the reducing end *N*-acetyl methyl group in site EI, these protons are nevertheless experiencing a significantly different magnetic environment from that of the EI′ site and most likely a similar environment to the solvent. From these facts one can envision the interaction between the inhibitor and lysozyme as the following two-stage sequence: the first where the inhibitor is

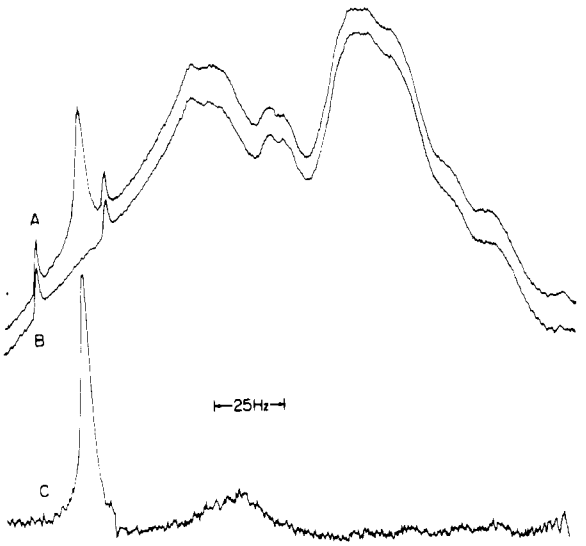

FIGURE 6: Representative primary enzyme plus inhibitor spectra and the resultant difference spectrum. (A) 5.9 m*M* NAG_2 and 2.9 m*M* lysozyme; (B) 5.9 m*M* NAG_2-d_6 and 2.9 m*M* lysozyme; (C), the difference spectrum (A − B). Spectrum C also appears in Figure 3H. The lowest field resonance in spectra A and B is acetone added as an internal standard. The next resonance in spectrum A is the nonreducing end *N*-acetyl methyl group of NAG_2. The resonance slightly upfield of this resonance in spectrum A is residual acetate from the enzyme.

initially in contact with lysozyme in the cleft and experiencing an (averaged) environment with $\delta_I - \delta_{EI} = 0$, and the second where the inhibitor is locked in place and experiencing the shielding effect of the tryptophan-108 indole ring. Alternatively the NAG_2 could be similarly positioned in the active site in the complexes EI and EI′ but between the two complexes, the enzyme has undergone a large conformational change involving tryptophan-108. Fluorescence studies on a similar reaction, the binding of NAG_3 to lysozyme, are consistent with this picture in that the perturbation of tryptophan-108 occurs solely upon the unimolecular step of the binding process (S. E. Halford, unpublished observation).

Unfortunately the observed spectra are very insensitive to the value of $\Delta\nu_{EI}$ and no information was obtained about the motional restriction of the inhibitor on the site EI. Note, in addition, that it is in fact not possible to tell from the data obtained here by either NMR or T-J methods whether or not the inhibitor loses contact with lysozyme while going from site EI to EI′

$$\begin{array}{ccc} E + I & \rightleftharpoons & EI \\ + & & \\ I & & \\ \uparrow\downarrow & & \\ EI' & & \end{array}$$

This mechanism has been further discussed by Viale (1971). However, the possibility of an isomerization in either the enzyme or the inhibitor prior to complex formation is eliminated by the T-J results (Halford, 1972).

This line of reasoning can easily be extended to the interaction of lysozyme with cell wall oligomers. One could envision the first binding step to be an initial association between the lysozyme and a strand of oligosaccharide. Then, depending upon whether or not the contact was such that its site D was near a muramic acid, it would either slide into the tighter site EI′ or dissociate and try again. However, the rearrangement of the enzyme–substrate complex in subsites A–F (Blake et al., 1967) would involve some interactions in addition to those observed here in nonproductive complex formation at subsites B and C.

Conclusion

This combined T-J and difference NMR study of the NAG_2–lysozyme system has yielded more than a complete set of kinetic constants. It has yielded a feeling for the spatial relationships between the inhibitor and the enzyme that supplement the rate constants to give a fuller understanding of the overall mechanism. It has thus pointed to the value of pooling the results of two methods to obtain a more comprehensive understanding of the system in question.

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