The binding of monosaccharide inhibitors to hen egg-white lysozyme by proton magnetic resonance at 270 MHz and analysis by ring-current calculations

Stephen J. PERKINS,*† Louise N. JOHNSON,* David C. PHILLIPS* and Raymond A. DWEK†

*Laboratory of Molecular Biophysics, University of Oxford, South Parks Road, Oxford OX1 3PS, U.K., and

†Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

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Studies of the binding of the four sugars α - and β -*N*-acetyl-D-glucosamine (GlcNAc) and its α - and β -methyl glycosides to hen egg-white lysozyme (EC 3.2.1.17) by means of high-resolution ^1H n.m.r. at 270 MHz are reported. The details of the binding analyses are described in an Appendix. The results show that the sugars bind independently to more than one site in lysozyme. The apparent fully bound chemical shifts of the inhibitor proton signals show that, although the major binding modes are generally similar for the four sugars, the binding of α GlcNAc is distinct from that of α MeGlcNAc and β MeGlcNAc. The binding of β GlcNAc is intermediate in character between these two modes. The observed shift changes of the inhibitor signals are correlated with the crystal structures of lysozyme–inhibitor complexes by the use of Johnson–Bovey ring-current calculations. Together with consideration of the chemical-shift anisotropy of the GlcNAc amide group, these suggest that the GlcNAc-binding sites in solution are in subsites C and E. The calculations show also that the indole rings of Trp-62 and Trp-63 rotate towards subsite C on the binding of GlcNAc, whereas Trp-108 moves away slightly. These findings indicate a difference between the solution and tetragonal crystal forms of lysozyme–GlcNAc and lysozymes– β MeGlcNAc complexes. In the crystal structure, binding of acetamido monosaccharides is only observed in subsite C, and binding in subsite E is prevented by crystal packing.

The determination of the crystal structure of tetragonal lysozyme (EC 3.2.1.17) (Blake *et al.*, 1965) and studies on the binding of *N*-acetylglucosamine (GlcNAc) and tri-*N*-acetylchitotriose [(GlcNAc)₃] to lysozyme led to the identification of three subsites A, B and C in the active-site cleft of lysozyme. Further model-building studies suggested the existence of three additional subsites D, E and F (Blake *et al.*, 1967) (Fig. 1*a*). Subsite D was then confirmed by crystallographic studies (Beddell *et al.*, 1970; Ford *et al.*, 1974). The 0.2 nm (2 Å)-resolution X-ray-diffraction studies on the binding of

GlcNAc to hen egg-white lysozyme showed that the monosaccharide bound to site C but in two slightly different orientations, one of which corresponded to the binding of the β -anomer (termed subsite C- β and identical with site C in Fig. 1*a*) and the other of which corresponded to the binding of the α -anomer (termed subsite C- α). In subsite C- α (Fig. 1*b*) the sugar ring is rotated (with respect to subsite C- β) about the axis of hydrogen bonds formed between the *N*-acetyl group of the sugar and the main-chain carbonyl group of Ala-107 and the amide group of Asn-59, so that it is now possible to form a hydrogen bond between the α -hydroxy group, O₍₁₎, and the main-chain NH group of residue 109 (Blake *et al.*, 1967; Imoto *et al.*, 1972).

In subsequent n.m.r. studies with the monosaccharides, the methyl resonances of GlcNAc and its methyl glycosides were studied in fast-exchange equilibrium with lysozyme at 60 or 100 MHz. Large upfield shifts were observed for the acetamido methyl groups. These were interpreted by assuming that the monomers bound to subsite C of lysozyme,

Abbreviations used: GlcNAc, *N*-acetyl-D-glucosamine; MeGlcNAc, methyl *N*-acetyl-D-glucosaminoside; r.s.r., real space refinement; Δw_m , apparent fully bound shift; K_d , apparent dissociation constant; RS5D, real-space-refined co-ordinates of native lysozyme (Diamond, 1974).

† Present address: European Molecular Biology Laboratory, Grenoble Outstation, c/o CENG, LMA, 85X, 38041 Grenoble, France.

which brings the *N*-acetyl methyl group close to the aromatic ring of Trp-108 (Dahlquist & Raftery, 1968; Raftery *et al.*, 1969; Sykes & Parravano, 1969; Studebaker *et al.*, 1971).

The present n.m.r. study of inhibitor binding to lysozyme is distinguished from these earlier n.m.r.

studies on the basis of several technical advances. Firstly, it is possible to resolve and interpret completely the ^1H n.m.r. spectra of GlcNAc and its derivatives at 270 MHz, which includes all seven spin-coupled sugar ring protons (Fig. 2) in addition to one or two methyl groups (Perkins *et al.*, 1977).

Fig. 2. 270 MHz ^1H n.m.r. spectrum of $\beta\text{MeGlcNAc}$ at 25°C in $^2\text{H}_2\text{O}$ to show the seven resolved sugar ring resonances in the range 3.4–4.5 p.p.m. from 2,2-dimethyl-2-silapentane-5-sulphonate. The $\text{Me}_{(8)}$ proton signal resonates at 2.026 p.p.m.

Fig. 1. (a) Active site of lysozyme showing the proposed binding of a hexasaccharide substrate in the six subsites A–F in the cleft on the enzyme, and (b) the binding of GlcNAc to subsite C-a in the active-site cleft of lysozyme.

In (a) the sugars in subsites A–C are as observed crystallographically in the binding of tri-*N*-acetylchitotriose, and those in subsites D–F are as suggested from model-building (Blake *et al.*, 1967). Subsites E and F are blocked in the crystal through the intermolecular lattice contacts.

All regions of the sugar molecule within the dimensions of $0.4\text{ nm} \times 0.4\text{ nm} \times 1.0\text{ nm}$ can therefore be studied by eight or nine reference points when the sugar binds to lysozyme. Secondly, real-space refinement methods have been applied to the electron-density maps of lysozyme, lysozyme-GlcNAc and lysozyme- β MeGlcNAc, which have therefore led to sets of co-ordinates to serve as a basis for comparison with the n.m.r. results (Diamond, 1974; Perkins *et al.*, 1978). Finally, the application of ring-current theories to protein crystal co-ordinates for the prediction of chemical-shift changes in n.m.r. spectra have recently been clarified by a detailed model study on lysozyme itself (Perkins & Dwek, 1980).

With these improvements, the present paper describes n.m.r. experiments to characterize in solution the binding of α GlcNAc, β GlcNAc, α MeGlcNAc and β MeGlcNAc to lysozyme at different pH* values and ionic strengths. On the basis of the crystallographic model, most of the chemical-shift changes on the GlcNAc sugars on binding to lysozyme are satisfactorily accounted for by ring-current effects and the chemical-shift anisotropy of the GlcNAc amide group. The ring-current calculations identify the molecular state of the three tryptophan rings that flank subsite C of lysozyme when GlcNAc is bound. The results also demonstrate a difference between the properties of lysozyme in the crystal and solution states, in that the GlcNAc sugars bind to only one subsite in the crystal but to more than one subsite in solution.

Experimental

Materials

GlcNAc and hen egg-white lysozyme were both purchased from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K. α MeGlcNAc and β MeGlcNAc (Nakarai Chemical Co.) were generous gifts from Professor K. Hamaguchi, Osaka University, Osaka, Japan. Before use the lysozyme was dialysed three times at pH 3 to remove acetate anion: 1 g of lysozyme was dissolved in 10 ml of water, acidified to pH 2–3 with HCl and dialysed against 1 litre of water at 4°C , with stirring, for 12 h. A fourth dialysis for 12 h was against water at neutral pH. The lysozyme solution was freeze-dried overnight and stored dry at 4°C until used. Yields were 60–80% by weight. The commercial GlcNAc was found to be in the α -form; no difference between this and GlcNAc crystallized from ethanol/water mixture could be detected in a ^1H n.m.r. spectrum.

For the n.m.r. experiments, 5 mM solutions of the enzyme were prepared by weight (mol.wt. 14 500). For the determination of binding constants, the enzyme concentrations were checked by using the absorbance at 280 nm and absorption coefficient of

$35000\text{ M}^{-1}\cdot\text{cm}^{-1}$ (Chipman *et al.*, 1967). These concentrations were found to vary between 4.6 and 5.1 mM. Dimerization was neglected because this is important only in slightly alkaline conditions above pH 6.1 (Sophianopoulos, 1969). No buffers were used because different results have sometimes been obtained for lysozyme with different buffers (Sykes, 1969; Jones *et al.*, 1974), and to facilitate comparison with the use of lanthanide shift reagents in the comparison study (Perkins *et al.*, 1981) because lanthanides interact with the common buffers.

The pH* titrations were in 99.8% $^2\text{H}_2\text{O}$, with 25 mM- α MeGlcNAc and β MeGlcNAc (mol.wt. 235) or 50 mM- α/β GlcNAc (mol.wt. 221). Solutions of GlcNAc were prepared at least 3 h before they were used, in order to equilibrate the anomers. The pH* was varied by using 1% and 8% solutions of ^2HCl and NaO^2H in 0.2-pH*-unit increments. The reading on the pH-meter, uncorrected, is used and is denoted as pH*.

Binding constants were determined by titrating the 5 mM-lysozyme solution with 250 mM- α/β GlcNAc, with 250 mM- α GlcNAc or with 250 mM- β MeGlcNAc dissolved in a different portion of the same stock solution of 5 mM-lysozyme. The pH* was set at the start of the titration, and was found to remain sufficiently constant for any variation in it during the titration to be neglected. The binding constants of α GlcNAc were determined by weighing out eight batches of 1–7 mg of α GlcNAc into dry n.m.r. tubes, and running the spectrum within 3 min of dissolution in 5 mM-lysozyme set at the appropriate pH*. The α GlcNAc concentrations ranged over 20–80 mM, and the amount of β GlcNAc was always low enough to be neglected in the calculations. The mutarotation equilibrium starting from α GlcNAc at 20°C in water is reached after 3 h (Johnson, 1965). The binding constants for β GlcNAc were calculated from data obtained with α GlcNAc and the equilibrium mixture of α/β GlcNAc (see the Appendix). An α GlcNAc/ β GlcNAc ratio of 1.50:1.00 was used, which was deduced from $[\alpha]_{\text{D}_\alpha} = 82^\circ$, $[\alpha]_{\text{D}_\beta} = -21.5^\circ$, $[\alpha]_{\text{D}_{\alpha/\beta}} = 40.4^\circ$ and 39.5° (Kuhn & Haber, 1953; Johnson, 1965) at 20°C .

To investigate the effect of working with 80 mM-KCl at ionic strengths of 0.1 M, with acetate buffer (Ikeda & Hamaguchi, 1975), a stock buffer solution was prepared from acetic acid (17.4 M) and solid KCl dissolved in $^2\text{H}_2\text{O}$. The pH* was adjusted to 5.1 with 1% and 8% ^2HCl and KO^2H .

N.m.r. studies

^1H n.m.r. spectra were recorded at 25°C at 270 MHz by using the Bruker spectrometer of the Oxford Enzyme Group, including a super-conducting magnet of Oxford Instruments, Oxford, U.K., and incorporating a Nicolet 1085 computer, a

Nicolet 294 disc storage system and a Nicolet 293 pulse controller. Typically 256 transients were accumulated over 8 kilobytes of computer core, with a dwell time (interpoint time) of 166 μ s, a pulse repetition rate of 1.38 s and a pulse angle of 70°. The Fourier transform was calculated over 16 kilobytes of computer core, the zero-filling technique being used to improve the spectral definition (Farrar & Becker, 1971). The spectral width is thus 3012 Hz, corresponding to a digitization of 0.00136 p.p.m. per point. Chemical shifts were measured by using acetone as standard at 2.215 p.p.m. downfield of 2,2-dimethyl-2-silapentane-5-sulphonate.

Fig. 3. Skeletal diagram to show the numbering of the protons and carbon atoms of GlcNAc and its methyl glycosides

The C₍₈₎ and C₍₉₎ carbon atoms are those of the Me₍₈₎ and Me₍₉₎ methyl carbon atoms respectively. In α MeGlcNAc the anomeric configuration corresponds to the proton positions denoted by α H₍₁₎ and α Me₍₉₎.

The chemical shifts of the sugar ring protons were assigned as described previously (Perkins *et al.*, 1977). The individual resonances overlap seriously in the case of α/β GlcNAc. On the basis of the experience gained during the assignment, it proved possible to follow the change in chemical shifts in the presence of lysozyme for most of the signals of the four GlcNAc sugars, taking advantage of the multiplet structure, which remains approximately constant from spectrum to spectrum, and working at 25°C, where the linewidths are sufficiently sharp to avoid serious loss of resolution. For β MeGlcNAc a spectral simplification arises through binding to lysozyme. The H₍₄₎ signal in the free sugar (Fig. 2) is strongly coupled to the H₍₅₎ proton through a near-coincidence of chemical shifts, resulting in the observation of virtual coupling in the H₍₆₁₎ and H₍₆₂₎ signals. In the presence of enzyme, the H₍₄₎ signal is shifted upfield (compare Fig. 2 with Fig. 6) to abolish the virtual coupling. Chemical-shift errors arising from the second-order nature of the n.m.r. spectra of the sugar are less than 0.01 p.p.m. at 270 MHz (Perkins *et al.*, 1977).

The fully bound chemical shifts and dissociation constants were fitted by using a standard least-squares linear regression analysis, with use of an equation of the form $y = mx + c$.

Ring-current calculations

The ring-current calculations that are given in Table 4 were based on the Johnson–Bovey equation and the real-space-refined co-ordinates of lysozyme and lysozyme–inhibitor complexes (Diamond, 1974; Perkins *et al.*, 1978) (see Table 1). Details of the theory and the method of calculation are given elsewhere (Perkins & Dwek, 1980, and references cited therein).

Table 1. GlcNAc and protein co-ordinates used in the ring-current calculations

Lysozyme subsite	Sugar co-ordinates	Lysozyme co-ordinates
Subsite A	(GlcNAc) ₄ lactone*	RS5D§
Subsite B	(GlcNAc) ₄ lactone*	R.s.r. of lysozyme– β MeGlcNAc†
Subsite C- β	(GlcNAc) ₄ lactone*	R.s.r. of lysozyme–GlcNAc†
	R.s.r. of β MeGlcNAc†	
	R.s.r. of GlcNAc†	
Subsite C- α	R.s.r. of GlcNAc†	
Subsite D	(GlcNAc) ₄ lactone*	
Subsite E	Model-building‡	
Subsite F	Model-building‡	

* Ford *et al.* (1974)

† Perkins *et al.* (1978).

‡ Imoto *et al.* (1972).

§ Diamond (1974).

|| Although the lysozyme– β -MeGlcNAc r.s.r. co-ordinates are used in Table 4, the use of those for lysozyme–GlcNAc is mentioned in subsection 2.3.

Results

1. Analysis of the binding of the four GlcNAc monomers to lysozyme

1.1. Evidence for multiple binding sites in solution. The binding of the GlcNAc sugars to lyso-

Fig. 4. Titration plot of the total inhibitor concentration versus the reciprocal chemical-shift change for β MeGlcNAc binding to lysozyme at 270 MHz at 25°C at pH* 5.1 in $^2\text{H}_2\text{O}$

The numbering system for the atoms is given in Fig. 3. The protein concentration is 4.7 mM. The sign of $\Delta w_{\text{obs.}}$ has been omitted; this is negative for $\text{H}_{(2)}$ and $\text{Me}_{(9)}$.

zyme was characterized by plots of the total sugar concentration $[\text{I}_0]$ against the reciprocal chemical-shift difference between the free and bound shifts ($1/\Delta w$). A typical plot for β MeGlcNAc at pH* 5.1 is shown in Fig. 4. Dissociation constants, K_d , were obtained from the negative intercept on the $[\text{I}_0]$ axis. It is clear from Fig. 4 that the experimental K_d values for the different proton signals of the same ligand indicate two apparent dissociation constants, one being 10–20 mM and the other 40–50 mM. That the differences in these apparent dissociation constants are significant is shown by linear-regression analysis. The regression coefficients of the binding plots are better than 0.99 in most cases.

pH* titration curves of the four GlcNAc monomers to lysozyme (Fig. 5; see subsection 1.3 below) established the range of pH* where the binding of sugar to lysozyme significantly affected the ligand shifts. This was found to be the most marked in the range pH* 4–6. To cover this range binding titrations were made at pH* 3, 4, 5.1, 6 and 7 for both anomers of GlcNAc and the two methyl glycosides, and also in buffered solutions of 0.1 M ionic strength at pH* 5.1. The results are summarized in Tables 2 and 3, and show that under all these conditions the phenomenon of unequal apparent dissociation constants was observed.

Reference to the analysis for Case IV in the Appendix shows that these variations in the K_d values are rationalized by the hypothesis that the GlcNAc sugars bind independently to more than one site on lysozyme. The intercepts and slopes are then no longer simply related to the dissociation constants and fully bound chemical shifts [cf. eqn. (38) of the Appendix]. In principle least-squares fitting methods based on the quadratic expression in $[\text{I}_0]$

Table 2. Apparent dissociation constants and fully bound chemical shifts for α MeGlcNAc and β MeGlcNAc obtained from the titrations of α MeGlcNAc and β MeGlcNAc with 3.9–5.1 mM-lysozyme

The dissociation constants in mM are given only where the regression coefficient is greater than 0.95. Asterisked dissociation constants are those with regression coefficients in the range 0.95–0.99. 'KOAc' refers to the titration in acetate buffer, ionic strength 0.1. No binding of α MeGlcNAc to lysozyme was observed at pH* 7.0. The chemical-shift changes are in p.p.m. relative to the free sugar, with positive values symbolizing upfield shift changes.

(a) α MeGlcNAc

pH* ...	Dissociation constant (mM)				
	3.0	4.0	5.1	6.0	5.1 (KOAc)
$\alpha\text{Me}_{(9)}$	109*		49*	57*	39*
$\beta\text{Me}_{(9)}$					
$\text{Me}_{(8)}$	43	36	31	42	33
$\alpha\text{H}_{(1)}$		22*	6		
$\beta\text{H}_{(1)}$					
$\text{H}_{(2)}$	127	70	66	66*	56
$\text{H}_{(3)}$					
$\text{H}_{(4)}$	57	40	32	54	40
$\text{H}_{(5)}$					
$\text{H}_{(61)}$			20		14*
$\text{H}_{(62)}$		27*	23	24*	15

	Chemical shift (p.p.m.)				
$\alpha\text{Me}_{(9)}$	-0.36	-0.17	-0.17	-0.17	-0.14
$\beta\text{Me}_{(9)}$					
$\text{Me}_{(8)}$	0.25	0.45	0.57	0.46	0.51
$\alpha\text{H}_{(1)}^\dagger$		-0.08	-0.06		
$\beta\text{H}_{(1)}$					
$\text{H}_{(2)}$	-0.74	-0.55	-0.62	-0.50	-0.48
$\text{H}_{(3)}$		0.00	0.00	0.00	
$\text{H}_{(4)}$	0.30	0.51	0.65	0.62	0.63
$\text{H}_{(5)}$		0.00	0.00	0.00	0.00
$\text{H}_{(61)}$		0.04	0.13	0.08	0.09
$\text{H}_{(62)}$		0.14	0.22	0.15	0.15

(b) $\beta\text{MeGlcNAc}$

	Dissociation constant (mM)					
pH* ...	3.0	4.0	5.1	6.0	7.0	5.1 (KOAc)
$\alpha\text{Me}_{(9)}$						
$\beta\text{Me}_{(9)}$	37	50	44	75		49
$\text{Me}_{(8)}$	19	21	21	30	70*	20
$\alpha\text{H}_{(1)}$						
$\beta\text{H}_{(1)}$						
$\text{H}_{(2)}$	44	44*	53	97*		57*
$\text{H}_{(3)}$		18*				
$\text{H}_{(4)}$	5	11	13	12*	58*	12
$\text{H}_{(5)}$						
$\text{H}_{(61)}$	5*	13	9*			16*
$\text{H}_{(62)}$			9*			-1*
	Chemical shift (p.p.m.)					
$\alpha\text{Me}_{(9)}$	-0.41	-0.44	-0.44	-0.49		-0.42
$\beta\text{Me}_{(9)}$	0.76	0.63	0.66	0.49	0.30	0.56
$\text{Me}_{(8)}$						
$\alpha\text{H}_{(1)}$						
$\beta\text{H}_{(1)}$	0.00	0.00	0.00	0.00		0.00
$\text{H}_{(2)}$	-0.60	-0.53	-0.61	-0.76		-0.55
$\text{H}_{(3)}$	0.03	0.08	0.05	0.03		0.00
$\text{H}_{(4)}$	0.39	0.45	0.62	0.38	0.29	0.48
$\text{H}_{(5)}$	0.03	0.03	0.05	0.03		0.03
$\text{H}_{(61)}$	0.07	0.11	0.15	0.08		0.16
$\text{H}_{(62)}$	0.10	0.11	0.21	0.15		0.12

† Partly obscured by the HO^2H signal (Perkins *et al.*, 1977).

[eqn. (38) of the Appendix] should lead to the determination of the two dissociation constants K_1 and K_2 and the fully bound shifts Δw_m for each site and proton signal, given the knowledge of $\Delta w_{\text{obs.}}$, $[\text{E}_0]$ and $[\text{I}_0]$. For $\beta\text{MeGlcNAc}$, six of the nine protons experience non-zero fully bound shifts, and thus the analysis would involve a fit to 14 variables based on a total of about 70 experimental points. For the present work, it was found sufficient to take the results of Tables 2 and 3, which were based on linear regressions of the titration data for each proton, to give what are termed apparent K_d and apparent Δw_m values. The apparent linearity of the

binding plots is attributed to the weak binding of the sugars to lysozyme.

The interpretations of these binding data is based on the approximations stated after eqn. (38) in the Appendix. Briefly, a knowledge of the crystal structure with the existence of six subsites for possible binding of the GlcNAc sugars is assumed, together with the assumption that the sources of the Δw_m are short-ranged in space, which means that a large apparent Δw_m would usually arise from one specific interaction on the binding of the GlcNAc sugar to lysozyme. Inspection of Fig. 4 shows in this regard that the $\text{H}_{(4)}$, $\text{H}_{(61)}$, $\text{H}_{(62)}$ and $\text{Me}_{(8)}$

Table 3. *Apparent dissociation constants and fully bound chemical shifts for α GlcNAc and β GlcNAc obtained from the titrations of α GlcNAc and α/β GlcNAc with 3.9–5.1 mM-lysozyme*

For α GlcNAc the dissociation constants in mM are given only where the regression coefficient is greater than 0.95. Asterisked dissociation constants are those with regression coefficients in the range 0.95–0.99. 'KOAc' refers to the titration in acetate buffer, ionic strength 0.1. For β GlcNAc, in place of the determination of apparent dissociation constants, the estimation of F_α , F_β and K_α/K_β is given for the correction of the experimental fully bound chemical shifts for β GlcNAc (Case II in the Appendix). These were obtained indirectly from the titration of lysozyme with α GlcNAc and α/β GlcNAc. For both α GlcNAc and β GlcNAc, the chemical-shift changes are in p.p.m. relative to the free sugar, with positive values symbolizing upfield changes. Blanks correspond to shift changes that are unmeasurable either because of overlap with the HO^2H signal or with other sugar ring proton signals.

(a) α GlcNAc

pH* ...	Dissociation constant (mM)				
	3.0	4.0	5.1	6.0	5.1 (KOAc)
$\alpha\text{Me}_{(9)}$					
$\beta\text{Me}_{(9)}$					
$\text{Me}_{(8)}$	21	10	17*	21	13
$\alpha\text{H}_{(1)}$	31	25	26	29	17
$\beta\text{H}_{(1)}$					
$\text{H}_{(2)}$	31		33*	31	18
$\text{H}_{(3)}$					
$\text{H}_{(4)}$	–4*	–1*	12*	19	13
$\text{H}_{(5)}$					
$\text{H}_{(61)}$					
$\text{H}_{(62)}$					

pH* ...	Chemical shift (p.p.m.)				
	3.0	4.0	5.1	6.0	5.1 (KOAc)
$\alpha\text{Me}_{(9)}$					
$\beta\text{Me}_{(9)}$					
$\text{Me}_{(8)}$	0.80	0.73	0.80	0.78	0.80
$\alpha\text{H}_{(1)}$	–0.49	–0.41	–0.36	–0.36	–0.34
$\beta\text{H}_{(1)}$					
$\text{H}_{(2)}$	–0.57	–0.46	–0.52	–0.50	–0.44
$\text{H}_{(3)}$					
$\text{H}_{(4)}$	0.04	0.15	0.27	0.28	0.24
$\text{H}_{(5)}$					
$\text{H}_{(61)}$					
$\text{H}_{(62)}$					

(b) β GlcNAc

pH* ...	Dissociation constant conversion				
	3.0	4.0	5.1	6.0	5.1 (KOAc)
Mean $F_\alpha^\dagger$	1.07	1.36	1.43	1.30	1.29
Mean $F_\beta^\ddagger$	0.95	0.76	0.71	0.80	0.81
$\frac{K_\alpha}{K_\beta} = \frac{F_\alpha}{F_\beta}$	1.13	1.79	2.01	1.63	1.59

pH* ...	Chemical shift (p.p.m.)				
	3.0	4.0	5.1	6.0	5.1 (KOAc)
$\alpha\text{Me}_{(9)}$					
$\beta\text{Me}_{(9)}$					
$\text{Me}_{(8)}$					
$\alpha\text{H}_{(1)}$	0.87	1.01	0.69	0.51	0.54
$\beta\text{H}_{(1)}^\S$	–0.43	–0.43	–0.62	–0.54	–0.47
$\text{H}_{(2)}$	–0.52	–0.70	–0.75	–0.64	–0.33
$\text{H}_{(3)}$	0.00	0.00			
$\text{H}_{(4)}$	0.53	0.55	0.51	0.31	0.47
$\text{H}_{(5)}$					
$\text{H}_{(61)}$		0.04	0.07	0.18	0.05
$\text{H}_{(62)}$					

protons constitute one type of behaviour whereas the $H_{(2)}$ and $Me_{(9)}$ (and the $\alpha H_{(1)}$ proton of α GlcNAc) form a second group, and that this observation is confirmed more generally in Tables 2 and 3. The rest of this section now makes a comparison of the apparent K_d and Δw_m values for the GlcNAc sugars, and the second section then attempts to rationalize the existence of two groups of titration behaviour in terms of the binding of the GlcNAc sugar to two subsites of lysozyme in the crystallographic model.

1.2. *Apparent fully bound shifts.* The slopes of the binding plots lead to values of the apparent Δw_m for the proton signals. Δw_m reflects the chemical-shift environment of a particular proton on lysozyme. Comparison of the apparent values of Δw_m thus provides a qualitative view at this stage of the similarity or dissimilarity of one mode of sugar binding with the next. The success of this comparison assumes that the relative K_d for the sites are similar, which is justified on inspection of Tables 2 and 3.

The apparent Δw_m values are listed in Tables 2 and 3 also. Greater detail is observed in the α - and β -methyl glycosides, as the problem of overlapping resonances is diminished. The data from 27 binding titrations show that, over the range of four inhibitor monomers, pH^* and change in ionic strength/buffer, the $Me_{(8)}$ and $H_{(4)}$ signals are all consistently shifted upfield, the $\alpha Me_{(9)}$, $\beta Me_{(9)}$ and $H_{(2)}$ signals are all consistently shifted downfield, and the $H_{(3)}$, $H_{(5)}$, $H_{(61)}$ and $H_{(62)}$ signals are almost unperturbed on binding (Fig. 3). This implies that the binding of the four GlcNAc sugars to lysozyme is similar. This result is now qualified in greater detail below.

1.2.1. Δw_m values for the methyl glycosides. Table 2 shows that, for $pH^* 5.1$, the Δw_m data were closely similar for α MeGlcNAc and β MeGlcNAc to within 0.09 p.p.m. At $pH^* 4.0$ and 6.0, and at $pH^* 5.1$ in the presence of acetate buffer, the Δw_m data were similar to within 0.19–0.26 p.p.m. The changes in Δw_m between these pH^* values were again small, being at the most 0.24 p.p.m. for the $H_{(4)}$ proton of β MeGlcNAc. It is thus concluded that α MeGlcNAc and β MeGlcNAc bind similarly to lysozyme in the range $pH^* 4.0$ –6.0.

At $pH^* 3.0$, the Δw_m values for α MeGlcNAc have

changed markedly, in contrast with these values for β MeGlcNAc, which still remained relatively steady. The Δw_m of the $Me_{(8)}$ signal of α MeGlcNAc became very low, a change that was beginning to be detected at $pH^* 4.0$, whereas the Δw_m for $H_{(2)}$ and $Me_{(9)}$ have increased. The apparent K_d at $pH^* 3.0$ for α MeGlcNAc (Table 2a) has also increased. From this it is seen that the binding mode of α MeGlcNAc at $pH^* 3.0$ is markedly different from that of β MeGlcNAc. This parallels previous observations by c.d. measurements (Nakae *et al.*, 1975).

1.2.2. Δw_m values for the anomers. For α GlcNAc, Table 3(a) shows that, over the pH^* range, and irrespective of ionic-strength/buffer effects, the apparent Δw_m values for $Me_{(8)}$, $\alpha H_{(1)}$ and $H_{(2)}$ were similar within a maximum variation of 0.13 p.p.m. This shows that there is a constant protein–sugar environment for these protons over the pH^* range. However, for the $H_{(4)}$ proton it is clear that, although the bound shift for α GlcNAc was similar at $pH^* 5.1$ and 6.0, it began to change at $pH^* 4.0$, and was almost zero at $pH^* 3.0$. This implicates a change in the binding mode of α GlcNAc in the range $pH^* 3$ –4.

The Δw_m values for β GlcNAc in Table 3(b) were less precise, owing to their indirect estimation by the Case II analysis in the Appendix. Table 3(b) shows that the large Δw_m for $Me_{(8)}$ of 0.9–1.0 p.p.m. at low pH^* was decreased by one-half at high pH^* . The other β GlcNAc signals varied within 0.2 p.p.m., except for the $H_{(2)}$ signal at $pH^* 5.1$ (potassium acetate buffer). This suggests that the binding mode of the β -anomer to lysozyme is influenced by an ionization or conformational change different from that of the α -anomer. This implies that the binding of the α - and β -anomers to lysozyme are different, which is consistent with the results from crystallographic analysis of lysozyme–GlcNAc complex (Blake *et al.*, 1967; Imoto *et al.*, 1972).

1.2.3. Δw_m values for the anomers and glycosides. The Δw_m values for the glycosides are first compared with those for α GlcNAc (Tables 2a, 2b and 3a). Larger differences in Δw_m exist than those observed between the α - and β -glycosides. The positive Δw_m values of the $Me_{(8)}$ signal were smaller in the glycosides than in the α -anomer, whereas for the positive Δw_m of the $H_{(4)}$ signal the converse is true. (Also, the negative Δw_m of the $\alpha H_{(1)}$ signal was

Footnotes to Table 3

† With use of the $Me_{(8)}$, $H_{(1)}$, $H_{(2)}$ and $H_{(4)}$ signals of α GlcNAc in the equation:

$$F_\alpha = \frac{\Delta w_{m\alpha(\text{expt.})}}{\Delta w_{m\alpha}} \quad (\text{Appendix})$$

‡ $F_\beta = \frac{x+1-F_\alpha}{x}$ where $x = 1.50$ (Appendix)

§ Partly obscured by the HO^2H signal (Perkins *et al.*, 1977).

much smaller in the α -glycoside than in the α -anomer.) Because the two glycosides are assumed to bind similarly (as deduced above), these changes imply that the binding mode of α GlcNAc is different from those of the glycosides.

With regard to the Δw_m values for β GlcNAc, these tend to be closer to the Δw_m values for the glycosides than the Δw_m values for α GlcNAc (Tables 2a, 2b and 3b). Further, Table 2(b) shows that at pH* 7.0 there is a clear decrease in Δw_m for the $Me_{(8)}$ signal of β MeGlcNAc, which may be compared with the similar decrease of Δw_m for the $Me_{(8)}$ signal of β GlcNAc at pH* 6.0. These similarities between the binding modes of the glycosides and β GlcNAc are, however, limited by the large negative Δw_m of the $\beta H_{(1)}$ proton of β GlcNAc, which is not seen for the $\beta H_{(1)}$ proton of β MeGlcNAc (Tables 2b and 3b). It is thus deduced that the binding

mode of β GlcNAc to lysozyme is intermediate in character between those of α GlcNAc and the methyl glycosides.

1.3. *Effect of pH* on the chemical shifts of the GlcNAc protons.* The pH* titration curves are shown in Fig. 5 for three representative proton resonances of the four GlcNAc monomers in the range pH* 3.0–6.0, where the binding is strong enough to influence the ligand 1H signals. The 1H n.m.r. spectra for β MeGlcNAc are shown in Fig. 6.

At high pH* the four sets of curves for the monomers show a pK of ionization of 6.4 ± 0.1 for all the signals. It is generally accepted that this is the ionization of Glu-35 in the 'dibasic acid' pair of Asp-52 and Glu-35 in the active-site cleft (Kuramitsu *et al.*, 1974), and is in good agreement with the c.d. results obtained by Nakae *et al.* (1975), which show the macroscopic pK of Glu-35 to be perturbed

Fig. 5. pH* titration graphs for the $H_{(4)}$, $Me_{(8)}$ and $Me_{(9)}$ resonances of α GlcNAc (αN , $\circ$), β GlcNAc (βN , Δ), α MeGlcNAc (αMe , $\bullet$) and β MeGlcNAc (βMe , $\blacktriangle$) in the presence of lysozyme

The chemical shifts of the free sugars are indicated on the left by arrows. The positive ordinate axis represents downfield shifts. The titration curves for the six other protons of these sugars are given in Perkins (1977).

Fig. 6. 270 MHz spectra of β MeGlcNAc with lysozyme at 25°C in $^2\text{H}_2\text{O}$ in the pH* range 2.47–8.83 to show the changes in the spectrum with pH* and the upfield shift of $\text{H}_{(4)}$

from 6.0 to 6.6 on binding of the methyl glycosides. At low pH* the curves suggest only one ionization, with a $\text{p}K$ of 3.9 ± 0.3 . Because the titration curve of the ligand resonance reflects changes in both K_d and Δw_m , the sensitivity with regard to one ionization out of two or three with similar $\text{p}K$ is not high. Thus, in view of the c.d. results summarized in Nakae *et al.* (1975), which show several carboxy ionizations with $\text{p}K$ values in the range 3.5–4.4, no firm conclusions on the nature or source of these ionizations as observed in the present work should be inferred. It is noteworthy that the observed $\text{p}K$ of 3.9 ± 0.3 falls between the $\text{p}K$ values of 3.5 and 4.4 reported for Asp-52, Asp-101 and Asp-48, and is very close to the $\text{p}K$ of 3.95 determined for Asp-52 in non-productive complexes of the GlcNAc sugars with lysozyme (Yang & Hamaguchi, 1980). The n.m.r. results are thus in complete conformity with the c.d. results obtained by Hamaguchi and co-workers (see Nakae *et al.*, 1975).

2. Comparison of the n.m.r. shifts with those calculated from the crystal structure of lysozyme

2.1. Chemical-shift anisotropy of the six subsites A–F. The observed Δw_m values are now correlated with the structure of lysozyme at the six possible

GlcNAc-binding sites, i.e. it is assumed that the sugars bind nowhere else on the molecule. The two main sources of shift perturbation occur by through-space ring-current interactions with the aromatic rings of lysozyme and by through-bond sources of chemical-shift anisotropy on the GlcNAc sugar itself. These factors are used to identify the GlcNAc-binding sites to lysozyme in solution, the source of the individual Δw_m , and the positions of Trp-62, Trp-63 and Trp-108 relative to subsite C when bound with GlcNAc sugar.

Ring-current interactions from tryptophan, phenylalanine, tyrosine and histidine aromatic rings account for the aliphatic and aromatic through-space conformation-dependent shifts seen in the ^1H n.m.r. spectrum of lysozyme (Perkins & Dwek, 1980). In that study, the comparison of ring-current shifts calculated from the phase-refined co-ordinates of tetragonal lysozyme with the observed n.m.r. shifts lead to root-mean-square differences of 0.22 p.p.m., with the largest difference between observed and calculated shifts being 0.3 p.p.m. The full ring-current calculation is reported in Table 4. This shows that ring-current effects are important for subsites A, B and C, but not for subsites D, E or F. This results from the proximity of

Trp-62, Trp-63 and Trp-108 to subsites A, B and C. Interestingly, further analysis shows that subsites D and E are characterized by a preponderance of several nearby charged groups or peptide groups, which makes these two subsites relatively polar in content. These calculations have also been made with several other sets of lysozyme–sugar co-ordinates available in the course of this joint n.m.r. and X-ray study, and indicate that the results of Table 4 are representative.

The occurrence of through-bond sources of shift change originates from changes in the disposition of a proton relative to the orientation of chemically anisotropic bonds that can rotate. This can be illustrated for the $H_{(61)}$ and $H_{(62)}$ proton signals in D-glucose and D-galactose. The preferred rotamer population of the $O_{(6)}$ oxygen about the $C_{(5)}-C_{(6)}$ bond (cf. Fig. 3) is influenced by the axial or equatorial substitution of the $O_{(4)}$ hydroxy group on the pyranose ring. This affects the shifts of the $H_{(61)}$ and $H_{(62)}$ protons in the two sugars (De Bruyn & Anteunis, 1976; Perkins *et al.*, 1977). Thus, when the GlcNAc sugar binds to lysozyme, the various rotamer populations about such a rotatable bond in the free sugar become frozen into one orientation, and the result is a contribution to the Δw_m for the proton in question. For the GlcNAc sugar, the importance of this effect is emphasized in the case of the $H_{(2)}$ proton, where its disposition relative to the GlcNAc amide group (Fig. 3) is analogous to that of α -protons of amino acids relative to peptide groups.

It is known that the conformation-dependent shifts of α -protons are to be explained, not by ring-current effects only (Perkins & Dwek, 1980), but also by the chemical-shift anisotropy of the adjoining peptide group (cf. Jackman & Sternhell, 1969).

2.2. Identification of two GlcNAc-binding sites in lysozyme. The GlcNAc-binding sites in lysozyme in solution are identified on the basis of the comparison of the above n.m.r. data with the structure of lysozyme. This is based on the prediction of the chemical-shift changes to be found if the GlcNAc sugar binds in a certain subsite, together with the criterion of self-consistency, which includes the requirement to be able to account for all the observed Δw_m and K_d values, the existence of at least two independent GlcNAc-binding subsites, and earlier crystallographic results on the binding in lysozyme–GlcNAc complexes.

The ring-current calculations are used to identify one GlcNAc-binding site on the basis of the properties of subsites A, B, C and D. The comparison of the apparent Δw_m values at pH* 5.1 in Tables 2 and 3 with the ring-current shifts of Table 4 show that the GlcNAc monomers do not bind in subsite A or B. The large positive shifts predicted for the $\beta Me_{(9)}$ and $\alpha H_{(1)}$ protons in subsite A are different by 1.2 and 1.0 p.p.m. respectively from the large negative Δw_m values for $\beta MeGlcNAc$ and $\alpha GlcNAc$. Subsite A can also be eliminated on the basis of the crystal studies, for no binding is observed in this site, even though it is accessible in

Table 4. Ring-current calculations on the subsites of lysozyme

These calculations are based on the rela-space-refined co-ordinates of lysozyme– $\beta MeGlcNAc$, the sugar co-ordinates of Table 1, the ring-current intensity factors from Perkins & Dwek (1980) and the Johnson–Bovey equation.

GlcNAc proton	Subsite A	Subsite B	Subsite C- α	Subsite C- β †	Sub-site D‡	Sub-site E§
$\alpha Me_{(9)}$ *	0.2	0.5 (0.5, Trp-62)	−0.5 (−0.3, Trp-108)	0.0	—	−0.2
$\beta Me_{(9)}$ *	0.8 (0.8, Trp-62)†	0.2	−0.6 (−0.5, Trp-108)	−0.15	—	0.0
$Me_{(8)}$	−0.04	0.02	0.51 (−0.35, Trp-63; 0.87, Trp-108)¶	2.04 (1.95, Trp-108)	−0.05	0.00
$\alpha H_{(1)}$	0.59 (0.57, Trp-62)	0.36 (0.39, Trp-62)	−0.32	−0.15	−0.16	−0.03
$\beta H_{(1)}$	0.24	0.93 (0.91, Trp-62)	−0.23	−0.03	−0.11	−0.06
$H_{(2)}$	0.18	0.33 (0.35, Trp-62)	−0.15	−0.31 (−0.42, Trp-63)	−0.13	−0.02
$H_{(3)}$	0.06	0.91 (0.86, Trp-62)	−0.05	0.18 (0.32, Trp-108)	−0.13	−0.15
$H_{(4)}$	0.06	0.45 (0.51, Trp-62)	−0.14	0.11¶	−0.14	−0.10
$H_{(5)}$	0.09	2.98 (2.82, Trp-62)	−0.15	0.00	−0.18	−0.12
$H_{(61)}$	0.06	−0.02 (0.63, Trp-62; −0.78, Trp-63)	−0.15	−0.16	−0.37	−0.14
$H_{(62)}$	0.11	0.86 (0.65, Trp-62)	−0.11	−0.05	−0.17	−0.08

* Except for $\beta Me_{(9)}$ in subsite C- β , approximate co-ordinates for the $C_{(9)}$ atom were calculated to within a precision of about 0.1 nm from standard bond angles and distances.

† The individual contributions from aromatic rings are given when these are greater than 0.3 p.p.m.

‡ The mean shifts of the calculation from three sugar co-ordinates in Table 1 are given.

§ Similar shifts are calculated for subsite F.

¶ See the text.

the crystal. For subsite B, the near-zero Δw_m values for $H_{(3)}$ and $H_{(5)}$ are in conflict with calculated ring-current shifts of 0.9–3.0 p.p.m., and hence rule subsite B out. However, the ring-current shifts associated with subsites C- α and C- β are consistent with the experimental findings. Large upfield shifts are predicted for the $Me_{(8)}$ signal as a result of the proximity of the indole ring of Trp-108 to that methyl group. The large upfield shift of the $H_{(4)}$ signal is accounted for by its proximity to the indole ring of Trp-62 (see below). There is also some possibility of a downfield ring-current shift for the $H_{(2)}$ proton in subsite C- β from Trp-63. Then, GlcNAc binding having been identified in subsite C, the requirement for more than one independent site rules out GlcNAc binding in subsites B and D (cf. Case III of the Appendix). Binding in subsite D is also unfavourable on energetic grounds (Imoto *et al.*, 1972).

Consideration of the chemical-shift effects arising from the GlcNAc amide group on the $H_{(2)}$ proton now leads to the identification of a second independent subsite for the binding of GlcNAc to lysozyme. This requires the presence of an interaction between the protein and the acetamide group of the sugar in order to immobilize the amide group relative to the sugar ring itself and to bind the GlcNAc itself in that subsite. Such an interaction has been observed or predicted for subsites A, C, D and E from crystallographic data (Johnson *et al.*, 1968; Ford *et al.*, 1974). On the basis of the arguments above, subsites A and D have already been eliminated, and this leaves subsite E as the best candidate for the second independent sugar-binding site in solution. Subsite E is relatively far from any aromatic residues, and the ring-current calculations predict only small changes in chemical shift (Table 4). That subsite E is the second independent site is supported also by energetic considerations based on the free energies associated with each subsite (Imoto *et al.*, 1972).

The observed Δw_m for the GlcNAc sugars at pH* 5.1 can therefore be accounted for by binding in subsite C for the $Me_{(8)}$ protons, and by binding in subsites C and E for the $H_{(2)}$ proton. Most of the remaining shifts are small, being 0.2 p.p.m. or less, in agreement with the predictions of Table 4. Out of a total of 27 inhibitor shifts for the four sugars in Tables 2 and 3, the large negative apparent Δw_m of the $\alpha H_{(1)}$ and $\beta H_{(1)}$ protons in the anomers, and of the $\alpha Me_{(9)}$ and $\beta Me_{(9)}$ protons in the glycosides, and the large positive apparent Δw_m of $H_{(4)}$, remain to be accounted for.

Since the $H_{(1)}$ shifts are almost zero in the glycosides, the large negative Δw_m for $H_{(1)}$ in the anomers thus implies a specific interaction between the anomeric hydroxy group and lysozyme that cannot take place when the hydroxy group is

replaced by the methoxy group of the glycoside. The source of Δw_m for the $H_{(1)}$ protons of the anomers is hence seen in analogy to the situation with the $H_{(2)}$ proton above. For the large Δw_m of the $\alpha Me_{(9)}$ and $\beta Me_{(9)}$ protons, the shift perturbation is likewise considered to result from restrictions in the steric dispositions of the $OMe_{(9)}$ group relative to the sugar ring when the glycoside is bound to lysozyme. However, it must be added that no specific information on the source of these four large Δw_m is available at present, in distinction to the case of the large Δw_m for the $H_{(2)}$, $H_{(4)}$ and $Me_{(8)}$ protons.

On the basis of these inferences for Δw_m , the observed range of values for K_d listed in Tables 2 and 3 may likewise be rationalized. If the Δw_m for one site is very large relative to Δw_m for the other(s), the apparent K_d corresponds closely to the actual value for K_d for the first-mentioned site [see eqns. (2) and (38) of the Appendix]. Thus K_d for subsite C is given by the apparent K_d for the $H_{(4)}$ (see below) and $Me_{(8)}$ protons; in fact Fig. 4 suggests that the source of most Δw_m values is subsite C. On the other hand, the K_d values for the $Me_{(9)}$ and $H_{(2)}$ protons are always larger, and this is accounted for in the above text by the binding of the GlcNAc sugar in both subsites C and E with significant Δw_m values in both subsites.

2.3. Predicted and calculated shifts for the $H_{(4)}$ and $Me_{(8)}$ protons. To complete the ring-current analysis, it remains to investigate the agreement in the observed and calculated Δw_m values. Up to now, the analysis for the $H_{(4)}$ proton of β GlcNAc, α MeGlcNAc and β MeGlcNAc has not taken into account the fact that the calculated upfield shift for the $H_{(4)}$ proton is so far rather less than the observed shift. Fig. 7 shows that the $H_{(4)}$ proton is in a position to be influenced by upfield shifts from Trp-62 if the ring of Trp-62 is rotated towards the sugar about the indole $C_{(\beta)}-C_{(p)}$ bond. It is seen in Fig. 7 that such a rotation has already occurred in the lysozyme–GlcNAc co-ordinates relative to native lysozyme (Perkins *et al.*, 1978). However, the observed movement is almost certainly underestimated, since the electron-density map of lysozyme–inhibitor complex represents an average of the free (60–80%) and bound (20–40%) forms of the enzyme. There is also a further complication in that, although the electron density for the five-membered ring is well-defined in the map, that for the six-membered ring is diffuse, indicating some mobility of this residue in lysozyme.

In view of these uncertainties in the position of Trp-62 in the fully bound complex, the ring-current program RCCAL was used to rotate the Trp-62 indole ring about the $C_{(\beta)}-C_{(p)}$ bond. It was found that a movement of 20° from the RS5D co-ordinates of Trp-62 towards the $H_{(4)}$ proton of β MeGlcNAc in subsite C- β was sufficient to change

Fig. 7. Movements of the lysozyme tryptophan rings adjacent to subsite C that are observed in the tetragonal crystal structure of lysozyme-GlcNAc complex compared with native lysozyme

The tryptophan residues in bold outline indicate the lysozyme-inhibitor structure. The co-ordinates for this Figure were those from the r.s.r. of lysozyme (Diamond, 1974) and lysozyme/GlcNAc complex (Perkins *et al.*, 1978), in which identical parameters were used in the refinements of the native and lysozyme-inhibitor structure factors.

the calculated ring-current shift of this proton by the required amount to a mean value of 0.60 p.p.m. without seriously affecting any of the other β -MeGlcNAc protons. What other small effects were seen tended to improve the agreement between Table 4 and Table 2(b) for the $H_{(5)}$, $H_{(61)}$ and $H_{(62)}$ protons.

A second feature of the observed and calculated shifts relates to the shifts for the $Me_{(8)}$ protons in subsites C- α and C- β . The observed shifts are larger for subsite C- α than for C- β , whereas the predicted shifts are larger for subsite C- β than for C- α , which is the reverse order. Use of the r.s.r. co-ordinates of lysozyme-GlcNAc complex leads to no improvements. The co-ordinates of both the lysozyme- β MeGlcNAc and lysozyme-GlcNAc complexes show that the $Me_{(8)}$ protons are on average 0.50 nm above the Trp-108 indole ring plane in subsite C- α and 0.26 nm above in subsite C- β , and hence the

$Me_{(8)}$ protons in subsite C- α experience a smaller ring-current shift from Trp-108 (Fig. 1).

Recalculation for subsite C- α by using now the RS5D co-ordinates gives 0.85 p.p.m. in place of 0.51 p.p.m. for the $Me_{(8)}$ protons, in good agreement with Table 3(a). The difference occurs because of the observed movement of the Trp-63 ring plane by 7° towards subsite C on the binding of β MeGlcNAc or β GlcNAc (Fig. 7; Perkins *et al.*, 1978). Trp-63 is not involved in the binding of α GlcNAc in subsite C- α (Fig. 1b). This movement brings the $Me_{(8)}$ protons of subsite C- α from the 'magic angle' region of zero ring-current shift in free lysozyme into the deshielding region of Trp-63 in lysozyme- β MeGlcNAc complex (Table 4). This reasoning for Trp-63 can be studied in more detail by seeing whether or not the large ring-current shift perturbations that are induced by Trp-63 on the Ile-98 1H n.m.r.

signals (Perkins & Dwek, 1980) change in different ways on the addition of α GlcNAc and β MeGlcNAc to lysozyme.

This leaves the comparison of the magnitudes of the $\text{Me}_{(8)}$ shift in subsite C- β , where the observed value is 0.6 p.p.m. and yet a value of 2.04 p.p.m. is calculated. Since the above movement of Trp-63 is almost certainly underestimated because of partial occupancies (see above), the expected full movement of Trp-63 would bring the $\text{Me}_{(8)}$ protons into the deshielding zone of this indole ring and thereby decrease the calculated shift. A second consideration is the precision inherent in the crystallographic co-ordinates, where the shift of 2.04 p.p.m. was obtained as the mean of 1.59, 2.07 and 2.45 p.p.m. calculated from the three sugar co-ordinates of subsite C- β . These reflect differences of up to 0.5 nm in the position of $\text{Me}_{(8)}$ relative to the ring centres of Trp-108. When the ring-current shifts are particularly large, it is difficult to calculate precise shifts from the protein co-ordinates.

Discussion

Multiple binding sites in solution

One of the principal findings of the present work has been the identification of the multiple binding mode of the GlcNAc sugars to lysozyme. The additional resolution of the sugar ring protons at 270 MHz, compared with the 60 MHz and 100 MHz spectrometer frequencies of earlier studies (Studebaker *et al.*, 1971, and references cited therein), was largely responsible for this. Comparison of the present Δw_m values for $\text{Me}_{(8)}$ protons with those of the earlier studies show very good agreement, except for that of β GlcNAc, and this is attributed to the problems of analysis for the two interconverting anomers (Case II in the Appendix). For the $\text{Me}_{(9)}$ signals, agreement in Δw_m is poor also, and this is attributed to the presumption of single-site binding in the earlier study (Raftery *et al.*, 1969; see section 2.2 above).

A conflict in the literature on the binding modes of the GlcNAc sugars to lysozyme at low pH* may now be clarified. Ikeda & Hamaguchi (1975) have argued from the ellipticities in c.d. studies that α GlcNAc and β GlcNAc are associated with different protein conformations in their complexes in acidic media. Studebaker *et al.* (1971) have argued, however, from n.m.r. that α GlcNAc and β GlcNAc bind similarly at low pH, using as evidence the equal Δw_m values for the $\text{Me}_{(8)}$ signals of the two anomers. Comparison of the Δw_m values for α GlcNAc and β GlcNAc at low pH* (Tables 3a and 3b) supports the former hypothesis. The difference of about 0.5 p.p.m. in the Δw_m for $\text{H}_{(4)}$ is considered to be too large to accord these anomers a similar binding

mode at low pH*, even though the Δw_m values for the $\text{Me}_{(8)}$ signals are coincidentally similar. Furthermore, at 270 MHz these methyl resonances are in fact resolved at low pH* (Fig. 5). This example and the one above illustrate the strong advantage of mapping the binding site by using several ligand resonances and not one in order to probe all regions of the site, and in the use of high spectrometer frequencies to assist the resolution of signals.

It is also clear that studies on enzyme–ligand complexes must be accompanied by a good understanding of the enzyme–ligand equilibria involved to avoid misrepresentations. For example, n.m.r. temperature-jump and stopped-flow experiments on the binding of $(\text{GlcNAc})_2$ to lysozyme were interpreted on the assumption of a subsite-C binding mode only (Baldo *et al.*, 1975). It was concluded from the evidence that a two-stage binding mechanism is involved, where one stage is characterized by a small Δw_m for the $\text{Me}_{(8)}$ signal. Accordingly it was postulated that either the inhibitor bound weakly as a first stage, or a conformational change accompanied the process of binding of $(\text{GlcNAc})_2$. Although this may be an explanation, it has first to be shown that $(\text{GlcNAc})_2$ binds in a unique mode and not independently through subsites C and E.

The results from the present n.m.r. study were used to show that the GlcNAc sugars bind to more than one subsite, and were consistent with the binding in subsites C and E. The main implication for the enzymic mechanism is that the binding of the monomeric sugars in subsites C and E reflects a favourable energetic basis for the distortion of the sugar ring in subsite D on the formation of the lysozyme–polysaccharide complex. This hypothesis is supported by the free-energy calculations given by Imoto *et al.* (1972). Subsites C and E are associated with free energies of -19.2 and $-17 \text{ kJ} \cdot \text{mol}^{-1}$, whereas subsites A, B, D and F are associated respectively with free energies of -9.6 , -11.3 , $+12.1/25.0$ and $-7.1 \text{ kJ} \cdot \text{mol}^{-1}$. It should be noted, however, that these are approximate and involved the single-site assumption for the monomers in their estimation; nonetheless it is seen that subsites C and E are energetically comparable.

In general, other solution studies have not revealed the presence of at least two independent GlcNAc-binding sites in lysozyme. Thus u.v. studies have not revealed binding in subsite E because it is far removed from Trp-62, Trp-63 and Trp-108. However, Ikeda & Hamaguchi (1976) used c.d. methods with lysozyme and various GlcNAc inhibitors in the presence of a dye, Beibrich Scarlet, to show that there were two independent sites for the binding of saccharides to lysozyme at pH 7.5, ionic strength 0.3, at 25°C. The free energies calculated for subsites C and E were $-16.7/-17.6$ and $-13.0/-14.6 \text{ kJ} \cdot \text{mol}^{-1}$ respectively, and the disso-

ciation constants for β MeGlcNAc were given as 43 and 330 mM respectively for subsites C and E.

Further evidence for two independent GlcNAc-binding sites on lysozyme in solution comes from the binding of lanthanides to lysozyme (Perkins *et al.*, 1981). These bind strongly ($K_d < 1$ mM) at the carboxy groups of Asp-52 and Glu-35 between subsites D and E, and therefore inhibit the weak binding of the GlcNAc sugar in subsite E. The effects of this were reflected in the n.m.r. study of the pH* and binding titrations.

In the X-ray studies on tetragonal crystals of hen egg-white lysozyme, binding of monosaccharides has only been observed in subsite C. No binding has been observed in subsite E in the tetragonal crystals of hen egg-white lysozyme because this subsite is blocked by a neighbouring molecule in the crystal lattice. The existence of subsite E with its specificity for acetamido sugars was deduced from model-building studies based on the known preference of lysozyme for hexasaccharide substrates and was substantiated by an analysis of acceptor specification in the transglycosylation reaction (evidence reviewed in Johnson *et al.*, 1968). Thus the ^1H n.m.r. studies described in the present paper, which show that there are at least two independent binding sites for the GlcNAc monosaccharides to lysozyme in solution, illustrate a difference between the behaviour of the protein in the crystal and in solution, albeit a difference that may be rationalized on the basis of the crystallographic model. After completion of this study (Perkins, 1977), crystallographic binding studies on turkey lysozyme with the disaccharide *N*-acetylglucosamine-*N*-acetylmuramic acid (Sarma & Bott, 1977) were reported, in which binding in subsite E was also observed.

Ring-current effects have been able to identify various molecular aspects of the conformation of the enzyme in the fully bound state around subsite C. Thus Trp-62 was shown to be in hydrophobic contact with the $\text{H}_{(4)}$ proton of the GlcNAc sugar at the same time as it makes a hydrogen bond between the indole nitrogen and $\text{O}_{(6)}$ of the sugar. Trp-63 is shown to rotate forwards into the cleft in order to form the hydrogen bond between the $\text{O}_{(3)}$ hydroxy group and the indole nitrogen (Fig. 7). Study of the protein n.m.r. signals of Trp-28 (which are perturbed by the ring current of Trp-108) shows also that there is a small movement of Trp-108 away from the cleft and towards Trp-28 (Perkins & Dwek, 1980). Thus details of the enzymic behaviour of all three tryptophan residues flanking subsite C for lysozyme-GlcNAc complexes in solution have been obtained by these calculations. Taken together with other physical studies on the properties of lysozyme in solution and in the crystal state (Blake *et al.*, 1978) and the dynamics of the protein structure (Artymiuk *et al.*, 1979), such studies lead to the

continued accumulation of experimental evidence relating to molecular aspects of the enzymic reaction of lysozyme.

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APPENDIX

Analyses of ligand–macromolecule equilibria studied by nuclear magnetic resonance under fast exchange conditions

This Appendix describes formalisms to deal with several situations that can occur when a ligand binds to a macromolecule and is studied by n.m.r. It shows how the n.m.r. binding titrations can be analysed in terms of fully bound shifts Δw_m and dissociation constants K_d . These derivations have the n.m.r. signals of the ligand in mind, but are equally applicable for n.m.r. signals of the macromolecule itself.

Case I: One ligand binding to one site

Consider the equilibrium between a macromolecule E and a ligand I:

where the dissociation constant K_d is given by $[E][I]/[EI]$, and I binds to one site on E. If the binding of I to E results in a change of chemical shift of a n.m.r. resonance of I, Δw , then under conditions of fast exchange:

$$\Delta w = P_M \Delta w_m$$

where P_M is the fraction bound and Δw_m is the chemical shift of the fully bound form relative to its position in the unbound form. Δw_m is the sum of the chemical-shift changes caused by ring-current interactions, the anisotropy of carbonyl groups, and other possible shift mechanisms. Since $P_M = [EI]/[I_0]$, where $[I_0]$ is the total concentration of I, then assuming $[I_0] \gg [EI]$ we obtain:

$$[I_0] = \frac{\Delta w_m [E_0]}{\Delta w} - K_d \quad (2)$$

where $[E_0]$ is the total macromolecule concentration.

A plot of $[I_0]$ against $1/\Delta w$ gives Δw_m from the slope and K_d from the intercept on the $[I_0]$ axis. If the ligand provides more than one n.m.r. signal, K_d will be the same for each, but in general Δw_m will be different.

Case II: Two interconverting forms of the ligand competing for the same site

Consider two ligands α and β that compete for the same site, but which are in chemical equilibrium between themselves. In the main text, this corresponds to the interconversion between the α and β anomers of GlcNAc, where each anomer binds to lysozyme also at the same site on lysozyme.

The equilibria are described by:

where K_α , K_β and K_1 are the appropriate dissociation constants. If α_0 and β_0 refer to the total concentration of each ligand and I_0 is the total concentration of the ligand, then:

$$[\alpha_0] + [\beta_0] = [I_0] \quad (6)$$

If $[\alpha_0] : [\beta_0] = x : 1$, we can also write that:

$$[\alpha_0] = [I_0] \left(\frac{x}{1+x} \right) \quad (7)$$

Under conditions of fast exchange the observed shifts of each ligand relative to the free position, Δw_α and Δw_β , will be given by:

$$\Delta w_\alpha = \frac{[E\alpha]}{[\alpha_0]} \cdot \Delta w_{m\alpha} \quad \text{and} \quad \Delta w_\beta = \frac{[E\beta]}{[\beta_0]} \cdot \Delta w_{m\beta} \quad (8)$$

where $\Delta w_{m\alpha}$ and $\Delta w_{m\beta}$ are the shifts relative to the free position when the ligand is totally bound to the enzyme.

From eqn. (3) note that:

$$K_\alpha = [E_0 - E\alpha - E\beta][\alpha_0 - E\alpha]/[E\alpha] \quad (9)$$

Making the assumption that the fraction of the bound α is small relative to the total of α , i.e. $[E\alpha] \ll [\alpha_0]$, we obtain

$$K_\alpha = [E_0] \cdot \frac{\Delta w_{m\alpha}}{\Delta w_\alpha} - [\alpha_0] - \frac{[E\beta]}{[E\alpha]} \cdot [\alpha_0] \quad (10)$$

By using eqns. (3) and (4), this may be rewritten:

$$K_\alpha = [E_0] \cdot \frac{\Delta w_{m\alpha}}{\Delta w_\alpha} - [\alpha_0] - \frac{K_\alpha}{K_\beta} \cdot \frac{[\beta]}{[\alpha]} \cdot [\alpha_0] \quad (11)$$

Making the further assumption that:

$$\frac{[\beta]}{[\alpha]} = \frac{[\beta_0]}{[\alpha_0]} = \frac{1}{x}$$

then from eqn. (7) we have:

$$K_\alpha = [E_0] \cdot \frac{\Delta w_{m\alpha}}{\Delta w_\alpha} - [I_0] \left(\frac{x}{1+x} \right) \left(1 + \frac{K_\alpha}{x - K_\beta} \right) \quad (12)$$

The corresponding equation for β is:

$$K_\beta = [E_0] \cdot \frac{\Delta w_{m\beta}}{\Delta w_\beta} - [I_0] \left(\frac{1}{1+x} \right) \left(1 + \frac{xK_\beta}{K_\alpha} \right) \quad (13)$$

Eqn. (12) may be rearranged in a similar format to eqn. (2):

$$[I_0] = \left([E_0] \cdot \frac{\Delta w_{m\alpha}}{\Delta w_\alpha} - K_\alpha \right) \left(\frac{1+x}{x} \right) \left(\frac{1}{1 + \frac{K_\alpha}{xK_\beta}} \right) \quad (14)$$

and correspondingly for β .

For the n.m.r. signals of α and β , plots of $[I_0]$ against $1/\Delta w_i$ ($i = \alpha, \beta$) will then give equal intercepts on the $[I_0]$ axis as:

$$-K_\alpha K_\beta (1+x)/(K_\alpha + xK_\beta) \quad (15)$$

By analogy with eqn. (2), we refer to these as the apparent dissociation constants. The gradients of these plots will be:

$$\Delta w_{mi}[E_0]K_i(1+x)/(K_\alpha + xK_\beta) = \Delta w_{mi}[E_0]F_i \quad (16)$$

where $i = \alpha, \beta$.

If the titration of the macromolecule with α is rapid compared to the interconversion rate between α and β , this allows $\Delta w_{m\alpha}$ and K_α to be determined on the basis of the Case I analysis above (eqn. 2).

The parameters for β are then obtained by combining these results with those of a second titration of E with an equilibrated solution of α and β . The second titration will give values of $\Delta w_{mi}F_i$ ($i = \alpha, \beta$) from eqn. (16). Since $\Delta w_{m\alpha}$ is known, then F_α can be calculated. We can rearrange the expression for F_α and show that:

$$\frac{K_\beta}{K_\alpha} = \frac{1+x-F_\alpha}{xF_\alpha} \quad (17)$$

The ratio F_β/F_α can be obtained from eqn. (16) and is equal to K_β/K_α . Thus, by using eqn. (17), we then have:

$$F_\beta = (1+x-F_\alpha)/x \quad (18)$$

Hence $\Delta w_{m\beta}$ can now be determined.

Errors will arise in this procedure if the ratio $x:1$ of α and β changes as the result of unequal binding of the ligands to the macromolecule. These are minimized by titrating the macromolecule with the equilibrated mixture of α and β over a time that is rapid relative to the interconversion rate of α and β , and by the use of a high concentration of α and β relative to E in the stock solution used for the titration of E.

Case III: One ligand binding to two competing sites

Consider a ligand that binds to two different sites on the macromolecule that are close together. When the ligand is occupying the first site on the macromolecule, the ligand is thus preventing ligation at the second site, i.e. the two sites are competing for the ligand. In the main text, for lysozyme, this corresponds to the hypothesis that there are two positions for Gd(III) in the main lanthanide-binding site between Glu-35 and Asp-52, which was observed in the lysozyme-Gd(III)-complex electron-density maps. It also corresponds to the idea that the GlcNAc sugar binds in two adjacent subsites in the cleft of lysozyme, such as subsites C and D.

Let the ligand be represented by α and its two binding modes on the enzyme be $E\alpha_1$ and $E\alpha_2$. The equilibria are described by

where K_{α_1} , K_{α_2} and K_E are the appropriate dissociation constants. Let $[\alpha_0]$ be the total concentration of α ; then, as before, under conditions of fast exchange the observed shifts Δw_1 and Δw_2 will be given by:

$$\Delta w_{\alpha i} = \frac{[E\alpha_i]}{[\alpha_0]} \cdot \Delta w_{mai} \quad (i = 1, 2) \quad (22)$$

Hence assuming $[E\alpha_1] + [E\alpha_2] \ll [\alpha_0]$ and noting that:

$$[E_0] = [E] + [E\alpha_1] + [E\alpha_2] \quad (23)$$

then:

$$K\alpha_1 = [E_0] \cdot \frac{\Delta w_{m\alpha_1}}{\Delta w_{\alpha_2}} - [\alpha_0] - \frac{[E\alpha_2]}{[E\alpha_1]} \cdot [\alpha_0] \quad (24)$$

which is essentially indistinguishable from eqn. (10). A similar equation can be written for α_2 , and it is then obvious that Case III is formally similar to Case II.

The important conclusion is therefore that the n.m.r. experiment that monitors the ligand (or protein) signals is ambiguous. It cannot distinguish between a single ligand binding to one site or a single ligand binding between two competing sites in conditions of fast exchange. All the dissociation constants will be equal and all the plots of $1/\Delta w$ versus $[I_0]$ will be linear, so experimentally Case I and Case III are indistinguishable.

Case IV: One ligand binding to two independent sites

Consider a ligand I that binds to two independent sites 1 and 2 on the macromolecule E. This is described by the scheme:

This assumes that the sites 1 and 2 are independent of each other, which is to say that there are no co-operative binding effects. The fully bound chemical shifts are then Δw_{m1} and Δw_{m2} respectively. Accordingly the total observed shift, $\Delta w_{obs.}$, under conditions of fast exchange is given by:

$$\Delta w_{obs.} = \frac{[EI_1] + [EI_1 I_2]}{[I_0]} \cdot \Delta w_{m1} + \frac{[EI_2] + [EI_1 I_2]}{[I_0]} \cdot \Delta w_{m2} \quad (25)$$

where $[I_0]$ is the total concentration of ligand and EI_1 , EI_2 and $EI_1 I_2$ denote the three macromolecule-ligand complexes.

The dissociation constants are defined by:

$$K_1 = \frac{[E][I]}{[EI_1]} = \frac{[EI_2][I]}{[EI_1 I_2]} \quad (26)$$

$$K_2 = \frac{[E][I]}{[EI_2]} = \frac{[EI_1][I]}{[EI_1 I_2]} \quad (27)$$

Assuming:

$$[I] \simeq [I_0]$$

and

$$[E] = [E_0 - EI_1 - EI_2 - EI_1 I_2] \quad (28)$$

then substitution of eqn. (28) into eqns. (26) and (27) gives:

$$[EI_1](K_1 + [I_0]) = [E_0] \cdot [I_0] - [EI_2] \cdot [I_0] - [EI_1 I_2] \cdot [I_0] \quad (29)$$

$$[EI_2](K_2 + [I_0]) = [E_0] \cdot [I_0] - [EI_1] \cdot [I_0] - [EI_1 I_2] \cdot [I_0] \quad (30)$$

From eqns. (26) and (27) we obtain also:

$$[EI_1][I_0] = K_2[EI_1 I_2] \quad (31)$$

$$[EI_2][I_0] = K_1[EI_1 I_2] \quad (32)$$

Hence:

$$K_1[EI_1] = K_2[EI_2] \quad (33)$$

and

$$[EI_1 I_2] = \frac{[I_0][EI_1]}{K_2} \quad (34)$$

Starting from eqn. (29), with the use of eqns. (31)–(34):

$$\begin{aligned} [EI_1] &= \frac{[E_0][I_0]}{K_1 \left(1 + \frac{[I_0]}{K_2}\right) + [I_0] \left(1 + \frac{[I_0]}{K_2}\right)} \\ &= \frac{[E_0][I_0]}{(K_1 + [I_0]) \left(1 + \frac{[I_0]}{K_2}\right)} \end{aligned} \quad (35)$$

Eqns. (34) and (35) are now used to obtain an expression that can be directly substituted into eqn. (25), giving after cancellation:

$$[EI_1 + EI_1 I_2] = \frac{[E_0][I_0]}{(K_1 + [I_0])} \quad (36)$$

Similarly for $[EI_2]$ we obtain:

$$[EI_2 + EI_1 I_2] = \frac{[E_0][I_0]}{(K_2 + [I_0])} \quad (37)$$

Hence:

$$\Delta w_{obs.} = [E_0] \cdot \left(\frac{\Delta w_{m1}}{K_1 + [I_0]} + \frac{\Delta w_{m2}}{K_2 + [I_0]} \right) \quad (38)$$

Eqn. (38) is a quadratic in $[I_0]$. If, as before, $1/\Delta w$ is plotted against $[I_0]$ for each n.m.r. signal of the ligand, a different dissociation constant is in principle obtained for each signal. Further, these plots are also in principle curved, although model cal-

Fig. 1. Curve tracing based on eqn. (38) to illustrate the behaviour of the binding titration graphs (cf. Fig. 4 in the main text) in the case of two independent sites on the macromolecule

Vertical axis: total ligand concentration. Horizontal axis: reciprocal chemical shift. The two dissociation constants K_1 and K_2 are 20 mM and 100 mM respectively, and the macromolecule concentration $[E_0]$ is 5 mM. The sum of the fully bound shifts ($\Delta w_{m1} + \Delta w_{m2}$) is 0.2 p.p.m., and the various parabola represent different combinations of values for Δw_{m1} and Δw_{m2} . Note that straight lines e and i are obtained in the cases that Δw_{m1} or Δw_{m2} is set as zero, with intercepts equal to K_2 and K_1 respectively, and slopes equivalent to 0.2 p.p.m. [cf. eqn. (2) of Case I]. The positive quadrant corresponds to the binding titration. The values of (Δw_{m1} , Δw_{m2}) in p.p.m. are as follows: a (0.60; -0.40); b (0.35; -0.15); c (0.30; -0.10); d (0.25; -0.05); e (0.20; 0.00); f (0.15; 0.05); g (0.10; 0.10); h (0.05; 0.15); i (0.00; 0.20); j (-0.05; 0.25); k (-0.10; 0.30); l (-0.40; 0.60).

culations shown in Fig. 1 suggest that this may not be easily observed. Thus Case IV is distinguished from Case I, Case II or Case III on the basis of unequal experimental dissociation constants for different n.m.r. signals.

The chief approximations that can be made in order to analyse Case IV binding of ligands to macromolecules are enumerated below.

(a) If one of Δw_{m1} or Δw_{m2} is zero, eqn. (38) is reduced to eqn. (2) of Case I; when this occurs, the dissociation constant and fully bound chemical shift of the ligand site with the non-zero fully bound shift are determined from the titration. This is not expected to be a rare occurrence because the source of any large shift change is itself short-ranged and is

unlikely to affect more than one n.m.r. signal of the ligand on binding to the macromolecule.

(b) In principle a large negative Δw_m for one site and a large positive Δw_m for another site would lead to cancellations in eqn. (38), and so would suggest an apparent observed Δw_m close to zero. In practice, this is unlikely to occur on the basis of general experience with the observation of large conformation-dependent shifts in protein n.m.r. To have the crystal structure of the protein itself is highly desirable in order to be able to integrate the information available from the apparent Δw_m obtained from binding titrations by using both chemical-shift calculations and the knowledge of the molecular interactions involved.