The simultaneous binding of lanthanide and *N*-acetylglucosamine inhibitors to hen egg-white lysozyme in solution by ^1H and ^{13}C nuclear magnetic resonance

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(Received 19 June 1980/Accepted 7 October 1980)

Lanthanide ions and the *N*-acetylglucosamine (GlcNAc) sugars are able to bind simultaneously to hen egg-white lysozyme (EC 3.2.1.17). The present study characterizes the properties of the ternary complexes with lysozyme, which involve up to seven paramagnetic lanthanides and two diamagnetic lanthanides, together with α GlcNAc, β GlcNAc, α MeGlcNAc and β MeGlcNAc. pH titrations and binding titrations of the GlcNAc sugars with lysozyme–La(III) complexes show that the GlcNAc sugars bind in at least two independent sites and that one of them competes with La(III) for binding to lysozyme. Given the known binding site of lanthanides at Asp-52 and Glu-35, the competitive binding site of GlcNAc is identified as subsite E. A simple analysis of the paramagnetic-lanthanide-induced shifts shows that the GlcNAc sugar binds in subsite C, in accordance with crystallographic results [Perkins, Johnson, Machin & Phillips (1979) *Biochem. J.* **181**, 21–36]. This finding was refined by several computer analyses of the lanthanide-induced shifts of 17 proton and carbon resonances of β MeGlcNAc. Good fits were obtained for all the signals, except for two that were affected by exchange broadening phenomena. No distinction could be made between a fit for a two-position model of Ln(III) binding with axial symmetry to lysozyme, according to the crystallographic result, or a one-position model with axial symmetry where the Ln(III) is positioned mid-way between Asp-52 and Glu-35. Although this work establishes the feasibility of lanthanide shift reagents for study of protein–ligand complexes, further work is required to establish the manner in which lanthanides bind to lysozyme in solution.

The use of paramagnetic lanthanide cations in complexes with biological macromolecules has attracted attention in view of their utility in n.m.r. studies of macromolecules. They have been used as structural probes, aids to assignments, spectral simplifiers and probes of dynamic information (Dwek, 1973; Nieboer, 1975; Reuben, 1975; Dobson & Levine, 1976). Applications have previously been described for lysozyme and lysozyme–inhibitor complexes (Butchard *et al.*, 1972; Dwek *et al.*,

1971; Jones *et al.*, 1974; Campbell *et al.*, 1975a; Dobson & Williams, 1977).

Evidence from protein crystallographic studies, ultraviolet data and n.m.r. data show that lysozyme has a strong natural binding site at the catalytic carboxylic acids Asp-52 and Glu-35, which lie between subsites D and E in the cleft of lysozyme (Fig. 1) (Kurachi *et al.*, 1975; Secemski & Lienhard, 1974; Campbell *et al.*, 1975a; Dobson & Williams, 1977; Perkins *et al.*, 1979). The solution studies, with that of Ostroy *et al.* (1978), show that a 1:1 enzyme–metal ion complex is formed, and that the dissociation constants lie in the range 0.2–0.8 mM. Proton relaxation enhancement studies, however, give a larger dissociation constant, about 13 mM (Dwek *et al.*, 1971; Jones *et al.*, 1974). The activity of lysozyme is inhibited by lanthanide binding (Ostroy *et al.*, 1978). The high-resolution crystallographic studies at pH 4.5–4.7 show that there are

Abbreviations used: GlcNAc, *N*-acetyl-D-glucosamine; MeGlcNAc, methyl *N*-acetyl-D-glucosaminide; Pipes, 1,4-piperazinebis(ethanesulphonic acid); Δw_m , apparent fully bound shift; K_d , apparent dissociation constant; Ln(III), lanthanide; RS5D, real-space-refined coordinates of native lysozyme (Diamond, 1974).

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Fig. 1. Active-site cleft in native lysozyme drawn by using the RS5D co-ordinates (Diamond, 1974) to show the position of the two Gd(III)-binding subsites determined by high-resolution crystallographic studies (Kurachi *et al.*, 1975; Perkins *et al.*, 1979) and the sugar-binding subsites C and E

The binding of Gd(III) will competitively inhibit the binding of the sugar monomers in subsite E as postulated from ^1H n.m.r. studies. The Gd(1) site is to the lower right and the Gd(2) site is to the upper left.

two Gd(III) positions in one main binding site between Asp-52 and Glu-35, which are separated by 0.36 nm in both triclinic and tetragonal lysozyme (Kurachi *et al.*, 1975; Perkins *et al.*, 1979). This

observation has led to three different proposals to describe the way in which lanthanides bind to lysozyme (Dobson & Williams, 1977; Agresti *et al.*, 1977; Perkins *et al.*, 1979).

The present n.m.r. investigation of the GlcNAc sugar bound to lysozyme and probed by lanthanides differs from its predecessor (Butchard *et al.*, 1972) in that many more than one or two GlcNAc resonances are studied, and that lanthanide shift reagents are used in place of the broadening reagent Gd(III). The use of ^1H and ^{13}C n.m.r. in the case of $\beta\text{MeGlcNAc}$ leads to the study of a total of 17 resonances, which have been assigned in a previous study (Perkins *et al.*, 1977). Furthermore, the solution study of lysozyme–lanthanide–GlcNAc ternary systems can be compared with the results obtained for tetragonal crystals of lysozyme–Gd(III) complex and lysozyme–Gd(III)– $\alpha/\beta\text{GlcNAc}$ complex (Perkins *et al.*, 1979). There, preliminary co-ordinates of the protein and GlcNAc sugar were obtained by real-space-refinement methods (Diamond, 1974). The present n.m.r. study confirms the crystallographic results for Gd(III) and GlcNAc binding, and therefore the feasibility of the use of lanthanide shift reagents to map the position of GlcNAc relative to lanthanide in solution when both are bound to lysozyme. It also confirms the finding in the companion study (Perkins *et al.*, 1981) that the GlcNAc sugars bind in both subsites C and E. However, the results of computer analyses, though giving some insight into the problem, were unable to distinguish conclusively between the various models for the binding of lanthanides to lysozyme, and this is discussed.

Theory

The theory of lanthanide-induced shifts has already been described in detail (Dwek, 1973; Nieboer, 1975; Reuben, 1975; Dobson & Levine, 1976), and only a brief account is given here. Paramagnetic-induced shifts arise either through a through-bond (contact) or a through-space (pseudo-contact) mechanism. Both occur in the present work, but only the latter is important for the paramagnetic mapping of GlcNAc relative to lanthanide. The pseudo-contact shift at a nucleus Δp is given by:

$$\Delta p = D' \left(\frac{3 \cos^2 \theta - 1}{r^3} \right) - D'' \left(\frac{\sin^2 \theta \cdot \cos 2\phi}{r^3} \right) \quad (1)$$

where D' and D'' are ligand field parameters, and r , θ and ϕ are the spherical co-ordinates of the nucleus with the lanthanide at the origin. θ defines the angle between the principal symmetry of the metal axis and the vector joining the metal ion and nucleus, and ϕ is the angular position in the plane around the metal-ion symmetry axis.

This equation has been found empirically in many studies (Dobson & Levine, 1976) to reduce to the form for axial symmetry. This may arise because D'' is zero as a result of the axial symmetry, or because

the $\cos 2\phi$ term is effectively zero as the result of dynamic averaging in solution. Alternatively D'' may be small relative to D' . In such circumstances, ratios of the pseudo-contact shift for different nuclei, i and j , in the same molecule and with the same lanthanide:

$$\frac{\Delta p_i}{\Delta p_j} = \frac{(3 \cos^2 \theta_i - 1)r_j^3}{(3 \cos^2 \theta_j - 1)r_i^3} \quad (2)$$

may be used to eliminate D' . These geometrical ratios are the base of the computer searches in the programs MTS and MTS2 described below.

Experimental

Materials

GlcNAc and hen egg-white lysozyme were both purchased from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K. $\alpha\text{MeGlcNAc}$ and $\beta\text{MeGlcNAc}$ (Nakarai Chemical Co.) were generous gifts from Professor K. Hamaguchi, Osaka University, Osaka, Japan. All the lysozyme used was first dialysed at pH 3 to remove the bound acetate anion (Perkins *et al.*, 1981). Stock solutions (500 mM) of lanthanides at pH < 2 [except for approx. 400 mM-Pr(III) and 50 mM-Gd(III)] were prepared by dissolving the oxides (Koch–Light Laboratories, Colnbrook, Bucks, U.K.), with heating, with 35% ^2HCl and $^2\text{H}_2\text{O}$ until the solutions were clear. For Pr(III), which is available only as the mixed oxide, chlorine is liberated together with a white semi-soluble precipitate. The reaction mixture was warmed overnight at 70–80°C and micro-filtered through Millipore filters to remove the precipitate. All concentrations were checked by titrations. The concentrations of the stock solutions used for the ^1H n.m.r. experiments were obtained from titration with standard EDTA, with Eriochrome Black T as the indicator. Those used for the ^{13}C n.m.r. experiments were determined by adding an excess of a standard EDTA solution, and acidifying and back-titrating this against a standard solution of CuSO_4 in the presence of acetate, with 1-(2-pyridylazo)-2-naphthol indicator (Fisons, Loughborough, Leics., U.K.). For all the lanthanides except Pr(III), concentrations were found to be within less than 1% of that expected from weighing.

The pH* of the solutions in $^2\text{H}_2\text{O}$ was taken as the observed pH-meter reading, and was 5.1 for all but a few experiments for which another value is given in the text. In the presence of lysozyme, the reading was found to remain constant to within ± 0.1 unit throughout a titration if both the stock and probe solutions had been set initially at pH* 5.1. No buffers were used, to avoid complications through the formation of complexes between lysozyme and the buffer (Sykes, 1969), or between the lanthanide

and the buffer. It was found that pH glass electrodes deteriorated with Ln(III), in particular when they were being used concurrently for other purposes. This problem was avoided by reserving one electrode in the laboratory for lanthanides only, or by soaking the electrode in 0.4 M-EDTA or 0.1 M-HCl.

N.m.r. spectra

^1H n.m.r. spectra at 270 MHz were recorded as described elsewhere (Perkins *et al.*, 1981).

^{13}C n.m.r. spectra were recorded at 25°C at 25.2 MHz, with a Varian XL-100 spectrometer. Spectra were accumulated under conditions of complete proton decoupling, the spectra being stored over 8 kilobytes of core with an acquisition time of 0.8 s and pulse delay of 1.0 s, generally for 1000 transients in the absence of enzyme and 2000 transients in the presence of enzyme. The free induction decay was multiplied by an exponential with a time constant of 0.4 s before Fourier transformation. Shifts were measured relative to an internal reference of dioxan as chemical standard. The digitization is 0.048 p.p.m. per point.

GlcNAc and lanthanide titrations

For a standard n.m.r. experiment, 5 mM solutions of lysozyme were prepared by weight (mol.wt. = 14 500) in $^2\text{H}_2\text{O}$. Enzyme concentrations were checked by using the absorbance at 280 nm and an absorption coefficient of $35\,000\text{M}^{-1}\cdot\text{cm}^{-1}$ (Chipman *et al.*, 1967). These were found to vary in the range 4.7–5.3 mM. Sugar concentrations were 25 mM- $\alpha\text{MeGlcNAc}$ and $\beta\text{MeGlcNAc}$ or 50 mM- $\alpha/\beta\text{GlcNAc}$ in the ^1H n.m.r. experiments, and were 50 mM and 100 mM respectively in the ^{13}C n.m.r. experiments.

Apparent fully bound chemical shifts, Δw_m , and dissociation constants, K_d , for αGlcNAc , βGlcNAc , $\alpha\text{MeGlcNAc}$ and $\beta\text{MeGlcNAc}$ were determined by using ^1H n.m.r. in order to characterize GlcNAc binding to lysozyme in solutions containing 5 mM-lysozyme and 80 mM-La(III) at 25°C (Cases II and IV in the Appendix to Perkins *et al.*, 1981).

In the ^1H n.m.r. lanthanide shift titrations, two solutions were used, both of which contained 5 mM-lysozyme, 25 mM- $\beta\text{MeGlcNAc}$ and 80 mM-total lanthanide. In one solution La(III) was used as the diamagnetic blank. The titration of the ternary complex was thus performed at constant ionic strength by mixing these two solutions in 17 increments of La(III)/shift-reagent ratios. Each titration took up to 2 h. Likewise, for the ^{13}C n.m.r. shift titrations with $\beta\text{MeGlcNAc}$, 12 increments of La(III)/shift-reagent ratios were used to cover the range 0–80 mM-lanthanide, where in this case the sugar concentration was 50 mM. Each titration took about 18 h. Blank titrations were made in all cases in which the lysozyme was omitted. The observed

lanthanide-induced shifts for the lysozyme–Ln(III)–sugar titration could therefore be corrected for the Ln(III)–sugar complex by determining the percentages of free sugar and lanthanide in the solutions with lysozyme. For this, it was assumed that the lysozyme was fully bound with lanthanide, and the dissociation constant for sugar binding was arbitrarily taken to be 20 mM; the correction factor obtained from this is insensitive to the values taken for the sugar dissociation constant.

A 80 mM-lanthanide concentration was used with lysozyme in these titrations in order to ensure that lysozyme was fully bound with metal ion, given that lanthanides and GlcNAc sugars compete for the same binding site on lysozyme. Lanthanides were found to stabilize lysozyme in solution, which is in agreement with the observations made by Norton & Allerhand (1977). A 0.48 M concentration of NaCl, which is the same ionic strength as 80 mM-lanthanide, is appropriate to crystallization conditions for lysozyme.

Computer analyses

Three FORTRAN IV programs were used.

RCCAL. This is the ring-current program described before (Perkins & Dwek, 1980), but with the addition of a sub-routine for the analysis of lanthanide-induced paramagnetic effects. Starting from the crystallographic co-ordinates, the proton co-ordinates were generated by using standard sub-routines (Ford, 1974). From these, r^{-3} and r^{-6} terms for each proton could be calculated, where r is the distance between the proton and one or two specified metal-ion positions. Methyl groups were represented as 12 equally populated proton rotamers spaced out in 30° steps starting from the eclipsed conformer with the adjacent bond, and the mean r^{-3} or r^{-6} values of the 12 were calculated.

MTS. This program, which has also been described previously (Barry *et al.*, 1973), was used for the calculation of shift $[(3\cos^2\theta - 1)r^{-3}]$ and relaxation (r^{-6}) data for any trial conformation of the molecule in question. The metal-ion position is scanned together with its principal symmetry axis until the experimental shift and broadening ratios agree to within a set tolerance (i.e. acting as a filter) of the theoretically predicted ratios for that geometry. A goodness-of-agreement index, R :

$$R = \frac{\sum(\text{observed} - \text{calculated})^2 / (\text{tolerance})^2}{(\text{no. of observations})^{\frac{1}{2}}} \quad (3)$$

is printed for each solution. Minor modifications improved the averaging of a methyl group in terms of 12 proton rotamers. The direction of the metal symmetry axis is defined by two angles α and β , the rotation angles about the crystallographic x and z axes respectively, where $\alpha = 0^\circ$ is the z direction,

and denoted as α/β . It should be noted that an α or β scan from 20° to 180° is equivalent to that of 200° to 360° for reason of symmetry. Although a typographical error reversed the definition of Campbell *et al.* (1975b), the α and β values of that study are directly comparable with this one.

MTS 2. This is a double-up version of MTS for analysis of the two-position model (below) for lysozyme. The principal symmetry axes of the two metal positions are scanned, and the metal ions are given a relative weighting.

The lanthanide, sugar and protein co-ordinates in these analyses are defined in Table 1.

Results

1. Characterization of the lysozyme-lanthanide-sugar ternary system

The determination of the relative orientation of lanthanide and GlcNAc sugar when both are bound to lysozyme requires the prior analysis of three systems. These are the lysozyme-sugar and lanthanide-sugar binary complexes, as well as the ternary complex itself. The lysozyme-sugar equilibria have been described in detail in the preceding paper (Perkins *et al.*, 1981).

1.1. Metal ion-sugar binary complex. To deter-

Table 1. Co-ordinates used in the calculations

Lanthanide co-ordinates		Co-ordinates (nm) in the right-handed system of axes (Imoto <i>et al.</i> , 1972)			Distance (nm) from the nearest oxygen atom of	
Name	Source	<i>x</i>	<i>y</i>	<i>z</i>	Asp-52	Glu-35
Gd(1)	Lysozyme-Gd(III)-GlcNAc at	0.839	2.470	1.840	0.410	0.246
Gd(2)	0.25 nm resolution (Perkins <i>et al.</i> , 1979)	1.035	2.379	2.147	0.233	0.570
0.6 nm Gd	0.6 nm resolution Gd(III) co-ordinate used by Campbell <i>et al.</i> (1975a)	0.989	2.383	2.179	0.193	0.554
N.m.r.	N.m.r. fit (Agresti <i>et al.</i> , 1977)	0.84 ± 0.20	2.27 ± 0.01	1.69 ± 0.04	0.513	0.328
Sugar co-ordinates						
As defined previously (Table 1 of Perkins <i>et al.</i> , 1981), but including also the GlcNAc co-ordinates from the real-space refinement of lysozyme-Gd(III)-GlcNAc (Perkins <i>et al.</i> , 1979)						
Protein co-ordinates						
Lysozyme native co-ordinate set RS5D (Diamond, 1974)						

Table 2. Change in chemical shift of the ligand resonances on titration (a) from 25 mM- β MeGlcNAc/80 mM-La(III) to 25 mM- β MeGlcNAc/80 mM-Ln(III) at 25°C , pH* 5, by using ^1H n.m.r., and (b) from 50 mM- β MeGlcNAc/80 mM-La(III) to 50 mM- β MeGlcNAc/80 mM-Ln(III) at 25°C , pH* 5, by using ^{13}C n.m.r.

Positive shifts are upfield shifts. The two sets of ^1H n.m.r., and ^{13}C n.m.r. shifts for a given lanthanide are not on the same scale. The $\text{C}_{(7)}$ signal (not reported) shifts by an order of magnitude greater than the $\text{C}_{(8)}$ signal.

		$10^3 \times \text{p.p.m.}$						
Nucleus		Pr	Nd	Eu	Dy	Ho	Er	Yb
(a)	$\beta\text{Me}_{(9)}$	6	1	1	6	-43	42	21
	$\text{Me}_{(8)}$	-157	-49	32	-300	21	-60	-74
	$\beta\text{H}_{(1)}$	-27	-13	11	-11	-20	20	6
	$\text{H}_{(2)}$	-63	-14	-4	414	560	-398	-227
	$\text{H}_{(3)}$	-11	-14	-5	-17	-42	52	17
	$\text{H}_{(4)}$	0	-1	-7	95	73	-73	-28
	$\text{H}_{(5)}$							-7
	$\text{H}_{(61)}$	-1	-4	-1	20	-1	3	6
	$\text{H}_{(62)}$	1	-1	-3	31	36	-17	-4
(b)	$\beta\text{C}_{(9)}^*$				100	20	40	20
	$\text{C}_{(8)}$				-1700	-530	-320	-100
	$\text{C}_{(1)}$				-20	-40	-120	-70
	$\text{C}_{(2)}^*$				220	220	-120	-100
	$\text{C}_{(3)}$				-70	-60	-80	-40
	$\text{C}_{(4)}$				120	50	-40	-20
	$\text{C}_{(5)}$				110	60	-30	-40
	$\text{C}_{(6)}$				90	40	-40	0

* The previously reported assignments of the $\text{C}_{(2)}$ and $\beta\text{C}_{(9)}$ signals (Perkins *et al.*, 1977) were reversed in the course of publication.

mine if lanthanides bind to the GlcNAc sugars, titrations of β MeGlcNAc were made at constant ionic strength with several lanthanides, with the use of both ^1H and ^{13}C n.m.r. No differences in chemical shifts were observed between β MeGlcNAc alone and in the presence of 80 mM-La(III), the diamagnetic blank. Table 2, however, shows that large shifts are induced in the β MeGlcNAc signals when paramagnetic lanthanides are used. These increase to magnitudes of up to 0.6 p.p.m. in the ^1H n.m.r. spectra and up to 1.7 p.p.m. in the ^{13}C n.m.r. spectra. Because the shift perturbations are the greatest at the $\text{C}_{(8)}$ and $\text{C}_{(7)}$ signals, it is inferred that the lanthanides bind at the carbonyl group of the GlcNAc amide. The relative shifts of the $\text{H}_{(2)}$ and

$\text{Me}_{(8)}$ signals (or the $\text{C}_{(2)}$ and $\text{C}_{(8)}$ signals) are not constant from lanthanide to lanthanide, and, if it is assumed that the metal ion binds in identical fashion from lanthanide to lanthanide, this shows that the shifts are caused by a contact mechanism (Dwek, 1973). A pH titration of 25 mM- β MeGlcNAc and 80 mM-Yb(III) in 25 mM-Pipes buffer shows that the shift perturbations are not dependent on pH^* between $\text{pH}^* 4.3$ and 5.8. Hence variations in the lanthanide-induced shifts in the paramagnetic ternary complexes with pH^* will not influence the results of those pH^* titrations (see below).

1.2. *Lysozyme-metal ion-sugar ternary complex.* The ternary system was characterized by titrating lysozyme-La(III) solutions with the four GlcNAc

Fig. 2. Binding plot for β MeGlcNAc in 5 mM-lysozyme and 80 mM-La(III) at 270 MHz at 25°C, $\text{pH}^* 5.1$, in $^2\text{H}_2\text{O}$. The total inhibitor concentration is plotted versus the reciprocal chemical-shift change, where the sign of Δw_{obs} has been omitted. Note that some curvature is now apparent in the plots for $\text{H}_{(4)}$ and $\text{Me}_{(8)}$ (cf. Case IV in the Appendix of Perkins *et al.*, 1981); nonetheless these plots were fitted by a linear regression line.

sugars, and by performing pH* titrations on the ternary system with both the diamagnetic and paramagnetic lanthanides. Previous work has identified two independent sugar-binding sites of lysozyme in solution to be subsites C and E (Perkins *et al.*, 1981). Further, it is well-established that lanthanides bind between subsites D and E. The GlcNAc sugars should therefore compete with lanthanides for subsite E, and the results below are fully consistent with this expectation.

1.2.1. Titrations of lysozyme–La(III) complex with GlcNAc sugars. In the titrations of 5 mM-lysozyme and 80 mM-La(III) with the GlcNAc sugars at pH* 5.1 and 6.0, the plot of total GlcNAc concentration against the reciprocal GlcNAc shift change shown in Fig. 2 shows that the intercepts on

the $[I_0]$ axis are not equal. These give values of the apparent dissociation constant K_d . Linear regression analyses give values of K_d in the ranges 20–60 mM for α GlcNAc, 20–120 mM for α MeGlcNAc and 10–60 mM for β MeGlcNAc (Table 3). This reinforces the conclusion that there are at least two independent GlcNAc-binding sites on lysozyme–La(III) complex, as before (Perkins *et al.*, 1981). Comparison of the values of K_d in the presence and in the absence of La(III) shows that certain K_d values have increased whereas others have decreased. For β MeGlcNAc at pH* 5.1, K_d for Me₍₈₎ decreases from 20 mM to 8 mM on the addition of La(III), whereas K_d for H₍₂₎ increases from 53 mM to 88 mM. The preceding paper (Perkins *et al.*, 1981) has shown that K_d for Me₍₈₎ largely reflects GlcNAc sugar

Table 3. Values of the apparent dissociation constants and fully bound chemical shifts from titrations of 5 mM-lysozyme and 80 mM-La(III) solutions in 2H_2O at 25°C with the α - and β -anomers and methyl glycosides of GlcNAc

The apparent dissociation constants are given only when the regression coefficient of the linear regression is better than 0.95, and are asterisked when it is in the range 0.95–0.99. In the case of α GlcNAc, the values not in parentheses refer to dissociation constants obtained directly from titrations with α GlcNAc. For α GlcNAc and β GlcNAc, the values in parentheses are those dissociation constants obtained from titration of equilibrated α/β GlcNAc with lysozyme–La(III) complex (Case II in the Appendix of Perkins *et al.*, 1981). The chemical-shift changes are in p.p.m. relative to the free sugar, with positive values symbolizing upfield shift changes. Blanks when appropriate to the sugar refer to shift changes that are unmeasurable as the result of overlap with the HO²H signal or other sugar ring proton signals. The values for α GlcNAc are directly obtained from the titration of lysozyme–La(III) complex with α GlcNAc, whereas those for β GlcNAc are indirectly calculated from the titrations of lysozyme–La(III) complex with α GlcNAc and α/β GlcNAc.

	Dissociation constant (mM)							
	α GlcNAc pH* 5.1	α GlcNAc pH* 6.0	β GlcNAc pH* 5.1	β GlcNAc pH* 6.0	α MeGlcNAc pH* 5.1	α MeGlcNAc pH* 6.0	β MeGlcNAc pH* 5.1	β MeGlcNAc pH* 6.0
α Me ₍₉₎					56*	60*		
β Me ₍₉₎							62	54
Me ₍₈₎	16 (13)	26 (24*)	(25)	(23)	24	17	8	10
α H ₍₁₎	48* (74)	38* (50)						
β H ₍₁₎			(127)					
H ₍₂₎	65* (72)	64 (65)	(54*)	(70*)	101*	115	88	85
H ₍₃₎								
H ₍₄₎	18* (10)	40* (30)	(29)	(28)	49	56	7	5
H ₍₅₎								
H ₍₆₁₎							19*	8*
H ₍₆₂₎								
	Chemical shift (p.p.m.)							
	α GlcNAc pH* 5.1	α GlcNAc pH* 6.0	β GlcNAc pH* 5.1	β GlcNAc pH* 6.0	α MeGlcNAc pH* 5.1	α MeGlcNAc pH* 6.0	β MeGlcNAc pH* 5.1	β MeGlcNAc pH* 6.0
α Me ₍₉₎					–0.18	–0.20		
β Me ₍₉₎							–0.59	–0.54
Me ₍₈₎	0.45	0.41	0.71	0.62	0.24	0.20	0.41	0.46
α H ₍₁₎ †	–0.35	–0.24						
β H ₍₁₎ †			–0.78				0.00	0.00
H ₍₂₎	–0.71	–0.62	–0.75	–0.89	–0.80	–0.95	–1.06	–1.08
H ₍₃₎	0.00	0.00			–0.02	–0.02	0.03	0.04
H ₍₄₎	0.26	0.40	0.75	0.59	0.53	0.59	0.43	0.41
H ₍₅₎					0.00	0.00	0.03	0.03
H ₍₆₁₎	0.00	0.00			0.05	–0.03	0.15	0.11
H ₍₆₂₎	–0.07	–0.08			0.10	0.14	0.11	0.13

† The α H₍₁₎ and β H₍₁₎ proton signals of α MeGlcNAc and β GlcNAc are partly obscured by the HO²H signal.

binding in subsite C, whereas that for $H_{(2)}$ largely reflects binding in subsites C and E. The inhibition of GlcNAc binding in subsite E by La(III) accounts for this variability of K_d . Furthermore, that there is evidence of at least two independent GlcNAc sites on lysozyme-La(III) complex implies that the affinity of La(III) and the GlcNAc sugars for subsite E are comparable.

The slopes of the plots in Fig. 2 lead to values of the apparent fully bound shift, Δw_m . These reflect the chemical environment of the GlcNAc-binding sites of lysozyme. The Δw_m values of Table 3 show that the four sugars are broadly similar in this respect. The $Me_{(8)}$ and $H_{(4)}$ signals are shifted strongly upfield; the $Me_{(9)}$ and $H_{(2)}$ signals are shifted downfield, and the $H_{(3)}$, $H_{(5)}$, $H_{(61)}$ and $H_{(62)}$ signals are weakly perturbed. As before, the main differences are found in the shift perturbations of the anomeric protons $\alpha H_{(1)}$ and $\beta H_{(1)}$, which are alike in the glycosides and for the anomers but differ between them. These differences arise from the different substitutions at the anomeric centre of the four sugars.

A more detailed comparison of the Δw_m values in Table 3 shows that the Δw_m values for $\alpha MeGlcNAc$ and for $\beta MeGlcNAc$ at $pH^* 5.1$ and 6.0 are very similar at the two pH^* values, although the Δw_m values between the two glycosides are more different. Comparison of Δw_m values for $\alpha GlcNAc$ and $\beta GlcNAc$ at $pH^* 5.1$ and 6.0 shows that, unlike the

glycosides, there are differences at the two pH^* values for each anomer, as well as between the anomers. This is now examined in more detail from the results of pH^* titrations with $\alpha/\beta GlcNAc$ and with $\beta MeGlcNAc$.

1.2.2. pH^* titration of lysozyme-Ln(III)-sugar complex. The pH^* titration curves for the $Me_{(8)}$ and $H_{(4)}$ signals of lysozyme-GlcNAc complex and lysozyme-La(III)-GlcNAc complex are compared in Fig. 3. This shows that there are two end points, which are $pK 3.9$ and 6.5 for both anomers of GlcNAc in the absence of La(III), and $pK 3.9$ and 4.9 ± 0.2 in the presence of La(III). The difference in Δw_m seen for the anomers at $pH^* 5.1$ and 6.0 is then the consequence of the second pK of 4.9 ± 0.2 .

The pH^* titration curves for all nine proton resonances of $\beta MeGlcNAc$ are compared in Fig. 4 for four ternary systems differing in the lanthanide. La(III) and Lu(III) are the diamagnetic blanks and are the first and last lanthanides of the rare earths. Pr(III) and Yb(III) are the third and second-to-last lanthanides of the rare earths, and have conventional pseudo-contact shifts that are downfield and upfield respectively (Dwek, 1973). The behaviour of the $H_{(3)}$, $H_{(61)}$, $H_{(62)}$ and $Me_{(9)}$ signals lead to a pK of 4.3 ± 0.2 , and from $pH^* 5$ to precipitation of the lanthanide at $pH^* 6-7$ there are no further end points. The constancy of the lanthanide-induced shifts of the $\beta MeGlcNAc$ signals above $pH^* 5$ from both the Δw_m data above and the pH^* titration is

Fig. 3. pH^* titration curves for the $H_{(4)}$ and $Me_{(8)}$ n.m.r. signals of 50 mM- $\alpha/\beta GlcNAc$ in 5 mM-lysozyme and in 5 mM-lysozyme/80 mM-La(III)

○, $\alpha GlcNAc$ /lysozyme; △, $\alpha GlcNAc$ /lysozyme/La(III); ●, $\beta GlcNAc$ /lysozyme; ▲, $\beta GlcNAc$ /lysozyme/La(III).

Fig. 4. pH^* titration curves for (a) the $H_{(11)}$, $H_{(12)}$, $H_{(3)}$, $H_{(4)}$, $H_{(5)}$ and $H_{(61)}$ n.m.r. signals, and (b) the $H_{(62)}$, $Me_{(8)}$ and $Me_{(9)}$ n.m.r. signals of β MeGlcNAc in solutions of 5 mM-lysozyme, 25 mM- β MeGlcNAc and 80 mM-Ln(III) at 25°C in D_2O . The resonance numbering is given in Fig. 3 of Perkins *et al.* (1981). The chemical-shift positions in the free sugar are arrowed on the left. O, 80 mM-Lu(III), Δ , 80 mM-Lu(III); $\bullet$, 80 mM-Pr(III); $\blacktriangle$, 40 mM-Yb(III) and 40 mM-Lu(III).

Table 4. Change in chemical shift of the ligand resonances on titration (a) from 25 mM- β MeGlcNAc/80 mM-La(III)/5 mM-lysozyme to 25 mM- β MeGlcNAc/80 mM-Ln(III)/5 mM-lysozyme at 25°C, pH* 5.1, on using ^1H n.m.r., and (b) from 50 mM- β MeGlcNAc/80 mM-La(III)/5 mM-lysozyme to 50 mM- β MeGlcNAc/80 mM-Ln(III)/5 mM-lysozyme at 25°C, pH* 5.1, on using ^{13}C n.m.r.

Positive shifts are upfield shifts. The two sets of ^1H n.m.r. and ^{13}C n.m.r. shifts for a given lanthanide are not on the same scale. Lysozyme concentrations varied in the range 4.7–5.3 mM, and the correction factor for the metal–sugar equilibrium varied between 0.78 and 0.81. The digitizations of the Fourier-transformed n.m.r. spectra are 1.3 per point for the ^1H n.m.r. spectra and 48 per point for the ^{13}C n.m.r. spectra on the same units that are used in the Table. The GlcNAc C₍₇₎ signal is not readily observed in ^{13}C n.m.r. spectra for reason of its low intensity and overlapping with over 150 carboxylic signals of lysozyme in the same region of the ^{13}C n.m.r. spectrum.

		$10^3 \times \text{p.p.m.}$						
Nucleus		Pr	Nd	Eu	Dy	Ho	Er	Yb
(a)	$\beta\text{Me}_{(9)}$	140/224	129	–249				–929
	$\text{Me}_{(8)}$	–41	–20	31	–262	210	83	74/115
	$\beta\text{H}_{(1)}$	120	55	–115				–307
	$\text{H}_{(2)}$	–35	–3	1	–57		213	–30
	$\text{H}_{(3)}$	8	11	–13	372		–102	–41
	$\text{H}_{(4)}$	0	11	–8	137		–100	–43
	$\text{H}_{(5)}$	56	–7	–17			–112	–134
	$\text{H}_{(61)}$	28	20	–21	421		–127	–60
	$\text{H}_{(62)}$	21	25	–17				–59
(b)	$\beta\text{C}_{(9)}$				1980	910	–690	–280
	$\text{C}_{(8)}$				–300	–90	–10	20
	$\text{C}_{(1)}$				640	270	–260	–70
	$\text{C}_{(2)}$				220	130	–30	–20
	$\text{C}_{(3)}$				240	80	–30	–20
	$\text{C}_{(4)}$				200	120	–50	0
	$\text{C}_{(5)}$				450	110	–60	–10
	$\text{C}_{(6)}$				350	190	–40	–8

important for the paramagnetic mapping studies below. It shows that the ternary complex is fully formed with a single binding mode above pH* 5; in other words, the possible binding of lanthanides to other carboxy groups near to the GlcNAc site (such as Asp-101) can be neglected. Furthermore, the direction of the paramagnetic shifts with Pr(III) and Yb(III) above pH 4.3 are in accordance with the opposite signs of their pseudo-contact shifts and with the expected magnitudes (Dwek, 1973). In contrast, the shift changes with pH* in the cases of the diamagnetic blanks La(III) and Lu(III) are generally small, with the minor exception of the $\text{Me}_{(8)}$ signal.

Apparent pK values for Glu-35 have been obtained by monitoring the signals of Trp-108 by ^1H n.m.r. (Campbell *et al.*, 1975b). In free lysozyme, this has a value greater than 6. The binding of lanthanides as a function of various lanthanide concentrations has been monitored by using the Thr-51 methyl resonance (Dobson & Williams, 1977). From these studies, a pK of approx. 4 at 25°C in the presence of 80 mM-lanthanide may be assumed. This pK may now be compared with the value of 4.3 ± 0.2 in the presence of 25 mM- β MeGlcNAc and 4.9 ± 0.2 in the presence of 50 mM- α/β GlcNAc. The change of pK in lyso-

zyme–La(III) complex on the addition of the GlcNAc sugars is evidence again for the competitive binding of the sugar and lanthanide to subsite E of lysozyme with comparable dissociation constants. Although complicated by the use of twice the α/β GlcNAc concentration, α/β GlcNAc appears to inhibit La(III) binding more strongly than does β MeGlcNAc. This is consistent with a competition between La(III) binding at Asp-52 and α GlcNAc binding in subsite C- α (Perkins *et al.*, 1979).

2. Paramagnetic mapping of the sugar relative to the metal ion in the lysozyme–Ln(III)– β MeGlcNAc ternary system

Of the four GlcNAc sugars, β MeGlcNAc is best suited for mapping in the lysozyme–Ln(III)–sugar complex. The apparent K_d given in Table 3 suggests it binds more strongly than α MeGlcNAc in the ternary complex. Its use avoids the complications of the α/β GlcNAc anomeric equilibrium, and its ^1H n.m.r. spectrum is simpler.

2.1. Calculation of the shift ratios with the use of ^1H n.m.r. and ^{13}C n.m.r. The lanthanide-induced shift data for 17 ^1H n.m.r. and ^{13}C n.m.r. signals of β MeGlcNAc are listed in Tables 2 and 4, and typical

titration plots for Yb(III) and Dy(III) are shown in Fig. 5(a) and 5(b) for the ^1H n.m.r. and ^{13}C n.m.r. titrations respectively. It is seen that the lanthanide-induced shifts resulting from the metal-ion-sugar interaction are of magnitudes comparable with those observed in the lysozyme-Ln(III)-sugar ternary complex. A factor in the range 0.78–0.81 was estimated on the basis of assumptions for the binding of metal ion and sugar to lysozyme (see the Experimental section), and this was used to correct the data of Table 2 before subtraction from the data of Table 4 in order to give the paramagnetic-lanthanide-induced shifts of the ternary complex. The use of different lanthanides [particularly for

Pr(III), Nd(III) and Eu(III), where the metal ion-sugar shifts are smaller] diminishes the uncertainties of this correction; furthermore, Fig. 6 shows that the correction is linear with concentration of shift reagent and is therefore easily applied.

To place the lanthanide-induced shifts on a common scale, shift ratios were now calculated, and these are given in Table 5. These were obtained by using as denominator the sum of the moduli of the shift perturbations of Table 4 for the ^1H n.m.r. sets and ^{13}C n.m.r. sets separately. In principle the ^1H n.m.r. and ^{13}C n.m.r. data sets could have been matched by running the one titration on both

Fig. 5. Titration plots for (a) the ^1H n.m.r. signals of 25 mM- $\beta\text{MeGlcNAc}$ in the presence of 80 mM-Yb(III) and of 80 mM-Yb(III)/5 mM-lysozyme at 25°C, pH* 5.1, and (b) the ^{13}C n.m.r. signals of 50 mM- $\beta\text{MeGlcNAc}$ in the presence of 80 mM-Dy(III) and of 80 mM-Dy(III)/5 mM-lysozyme at 25°C, pH* 5.1

○, Binary system of sugar/Ln(III); ●, ternary system of sugar/Ln(III)/lysozyme.

Fig. 6. Influence of temperature on the 270 MHz n.m.r. spectra of 25 mM- β MeGlcNAc/80 mM-Pr(III)/5 mM-lysozyme at pH* 5.1

spectrometers, but this proved to be impracticable. The shifts induced by Pr(III), Nd(III), Eu(III) and Yb(III) were too small compared with the window of ^{13}C n.m.r. shifts. The shifts induced by Dy(III), Ho(III) and Er(III) in the ^1H n.m.r. spectra were accompanied by pronounced broadening of the sugar resonances, thus obscuring them. Table 5 then shows that the shift ratios are similar from one lanthanide to the next. This indicates that the usual assumptions of fast exchange between the metal ion and lysozyme- β MeGlcNAc complex, effective axial symmetry of the metal ion and the structural homology of the ternary complexes are satisfactory.

Two further points relating to the results of Table

5 are noted. Firstly, the apparent Δw_m of the $\text{H}_{(2)}$ and $\text{Me}_{(8)}$ signals when β MeGlcNAc binds to lysozyme are large. The use of La(III) in all titrations was therefore mandatory in order to allow for this by keeping the total lanthanide concentration as well as the ionic strength constant. This point becomes unimportant for the ^{13}C n.m.r. data because the Dy(III)- and Ho(III)-induced shifts are large in comparison. Secondly, Fig. 6 shows that, in the ^1H n.m.r. spectra at 25°C, considerable line-broadening is observed for the $\text{Me}_{(9)}$ signal at 3.4 p.p.m. The $\text{H}_{(1)}$ signal is likewise affected (not shown). The effects of the onset of intermediate exchange conditions were overcome by extrapolation of the titration plots for the $\text{Me}_{(9)}$ and $\text{H}_{(1)}$ signals (Fig. 5a) to 80 mM-Ln(III). At higher temperatures, Fig. 6 shows that the fast-exchange condition is restored for $\text{Me}_{(9)}$.

2.2. Initial analysis of the shift ratios of the ternary complex. The high-resolution crystal structures of lysozyme-Gd(III) complex show that there are two positions for Gd(III) between Asp-52 and Glu-35 (Kurachi *et al.*, 1975; Perkins *et al.*, 1979). However, previous analyses of the binding of Ln(III) to lysozyme have assumed that there was one main site (Campbell *et al.*, 1975a; Agresti *et al.*, 1977), which was justified in terms of a ligation at least to Glu-35 (Dobson & Williams, 1977). They are termed the two-position and one-position models in the present paper, and the purpose of the following analysis is to compare the two models.

The first step of this analysis was to identify the site of β MeGlcNAc in the ternary complex as subsite C- β . It is also necessary to scale together the ^1H n.m.r. and ^{13}C n.m.r. shift ratios. Both of these were achieved by assuming initially that the angular term of eqn. (2) (Theory section) can be neglected and therefore that the lanthanide-induced shifts were proportional to r^{-3} . These r^{-3} terms were calculated for the subsites of lysozyme by using the Gd(1) and Gd(2) co-ordinates and sugar co-ordinates specified in Table 1. They were expressed as r^{-3} ratios for comparison with the experimental shift ratios given in Table 6. The most effective comparisons were those of the $\text{H}_{(1)}$ -versus- $\text{H}_{(4)}$ ratios and the $\text{C}_{(1)}$ -versus- $\text{C}_{(4)}$ ratios, because these correspond to the extremities of each subsite with respect to the metal ion. Subsites C- α , E and F were thus eliminated at once. Subsites C- α , D and E were eliminated also on the grounds that the van der Waals radii of the sugar and lanthanide were violated. This leaves subsites A, B and C- β , of which subsites A and B were eliminated on the basis that the r^{-3} ratios for $\text{H}_{(1)}$ and $\text{H}_{(4)}$ were not sufficiently different as required by the experimental shift ratios. The paramagnetic shift data thus confirm subsite C- β to be the β MeGlcNAc-binding site in solution. This conclusion is supported also by the evidence from Δw_m in

Table 5. Calculation of the shift ratios from the n.m.r. solution studies of the β MeGlcNAc–Ln(III)–lysozyme ternary complex for (a) the ^1H n.m.r. signals and (b) the ^{13}C n.m.r. signals

The two sets of ^1H n.m.r. and ^{13}C n.m.r. shift ratios are not on the same scale. For any given nucleus, they should be similar if the usual assumptions of fast exchange, axial symmetry and structural homology are valid. The ^{13}C n.m.r. ratios are expected to be significantly more accurate for Dy(III) and Ho(III) than for Er(III) or Yb(III) because the shift perturbations are large relative to the spectral digitization. The ^1H n.m.r. ratios are expected to be more accurate for Pr(III), Nd(III) and Eu(III) because the ionic radii of these lanthanides are the closest to that of La(III), the diamagnetic blank (see the Experimental section.).

		Shift ratio						
Nucleus		Pr	Nd	Eu	Dy	Ho	Er	Yb
(a)	$\beta\text{Me}_{(9)}$	1.25/2.00	1.98	3.36				4.44
	$\text{Me}_{(8)}$	−0.37	−0.31	−0.42	−0.21		−0.13	−0.35/−0.55
	$\beta\text{H}_{(11)}$	1.07	0.85	1.55				1.47
	$\text{H}_{(2)}$	−0.31	−0.05	−0.01	−0.05		−0.34	0.14
	$\text{H}_{(3)}$	0.07	0.17	0.18	0.30		0.16	0.20
	$\text{H}_{(4)}$	0.00	0.17	0.11	0.11		0.16	0.21
	$\text{H}_{(5)}$	0.50	0.26	0.23			0.18	0.64
	$\text{H}_{(61)}$	0.25	0.31	0.28	0.34		0.20	0.29
	$\text{H}_{(62)}$	0.19	0.38	0.23				0.28
(b)	$\beta\text{C}_{(9)}$				0.83	0.92	1.44	1.89
	$\text{C}_{(8)}$				−0.13	−0.09	0.02	−0.14
	$\text{C}_{(1)}$				0.27	0.27	0.54	0.47
	$\text{C}_{(2)}$				0.09	0.13	0.06	0.14
	$\text{C}_{(3)}$				0.10	0.08	0.06	0.14
	$\text{C}_{(4)}$				0.08	0.12	0.10	0.00
	$\text{C}_{(5)}$				0.19	0.11	0.13	0.07
	$\text{C}_{(6)}$				0.15	0.19	0.08	0.05

the comparison study of lysozyme–sugar binding (Perkins *et al.*, 1981).

The ^1H n.m.r. and ^{13}C n.m.r. data of Table 5 were then scaled together on the basis of the r^{-3} ratios for subsite C- β given in Table 6. In justification, Table 6 shows that the r^{-3} ratios are very similar to the moduli of the experimental shift ratios. For the computer searches below, the minimum filters or tolerances for a given computer fit were taken from (i) the range of shift ratios seen experimentally with the different lanthanides and (ii) a variation of 6–12% in the scaling of the r^{-3} ratios, which is estimated from the r^{-3} terms calculated in the cases that Ln(III) is bound only to Glu-35 or is bound only to Asp-52.

2.3. Mapping of β MeGlcNAc relative to Ln(III) on the one-position model. Four two-parameter searches with the program MTS were made with large filters (tolerances) (Table 7) in order to compare the four Gd(III) co-ordinates of Table 1. The parameters varied were α and β , which define the direction of the principal symmetry axis of the metal. The restriction is imposed that the sugar ring nuclei are upfield-shifted with Pr(III) and Dy(III), namely that the nuclei are outside the $(3\cos^2\theta - 1)$ cone where $54^\circ < \theta < 90^\circ$. In each case about 60–160 solutions were found out of 1296 tested, with similar values for α and β , which range from $\alpha/\beta = 95^\circ/5^\circ$ and passing by 5° increments to finish

at $125^\circ/65^\circ$. Finally, a five-parameter search with large filters was made, in which the three spherical co-ordinates of the metal ion as well as α and β were varied. The metal ion was allowed to move within a 0.5 nm sphere centred at the mean of the Gd(1) and Gd(2) co-ordinates of Table 1. Many similar fits were obtained, in which α and β varied only in the range from $70^\circ/30^\circ$ to $140^\circ/70^\circ$. These values for α and β compare well with typical values of $100^\circ/70^\circ$ and $110^\circ/100^\circ$ for α/β , which were obtained from computer searches based on the lanthanide-induced protein resonances and the position of Gd(III) derived from a 0.6 nm resolution study of lysozyme–Gd(III) complex (C. C. F. Blake, personal communication) (Campbell *et al.*, 1975a; Dobson & Williams, 1977).

This final range of α/β values was examined in more detail with small filters and small stepping intervals, based on three spheres of 0.12 nm radius centred at the mean of the Gd(1) and Gd(2) co-ordinates, the Gd(1) co-ordinate, and the Gd(2) co-ordinate. These searches resulted in 879, 575 and 103 solutions out of 34 560 tested geometries. At each of the Gd(1) and Gd(2) co-ordinates, a two-parameter variation of the 1296 possible values of α and β with the small filters gave few solutions. More solutions were thus found between the Gd(1) and Gd(2) positions rather than at either of them. The *R* index for these solutions did not suggest any

Table 6. *Calculation of the shift ratios on the basis of an r^{-3} distance-dependence of the lanthanide-induced-shift ratios*
 This is based on the co-ordinates defined in Table 1 for the sugar residues in the subsites of lysozyme and the Gd(1) and Gd(2) co-ordinates, which were given a 0.5:0.5 weighting. For direct comparison with Table 5, the r^{-3} ratios were calculated in the same manner as for those of Table 5. The mean experimental shift ratios are the average of the shift ratios for Pr(III), Nd(III) and Eu(III) for the ^1H n.m.r. ratios, and the average of the shift ratios for Dy(III) and Ho(III) for the ^{13}C n.m.r. ratios.

Nucleus	Mean experimental shift ratios	Subsite A	Subsite B	Subsite C- α	Subsite C- $\beta^\dagger$	Subsite D †	Subsite E	Subsite F
$\beta\text{Me}_{(9)}$	2.32				1.81*			
$\text{Me}_{(8)}$	-0.37	0.19	0.24	0.01	0.23	0.14	0.01	0.05
$\beta\text{H}_{(1)}$	1.16	0.28	0.35	0.07	0.47	2.09	0.10	0.08
$\text{H}_{(2)}$	-0.12	0.22	0.24	0.07	0.26	0.30	0.07	0.14
$\text{H}_{(3)}$	0.14	0.17	0.19	0.05	0.18	0.37	0.39	0.22
$\text{H}_{(4)}$	0.09	0.18	0.16	0.22	0.15	0.10	0.44	0.44
$\text{H}_{(5)}$	0.33	0.20	0.21	0.13	0.20	0.13	0.57	0.15
$\text{H}_{(61)}$	0.28	0.24	0.16	0.64	0.17	0.09	0.09	0.15
$\text{H}_{(62)}$	0.27	0.19	0.22	0.36	0.15	0.05	0.06	0.32
$\beta\text{C}_{(9)}$	0.88				0.90*			
$\text{C}_{(8)}$	-0.11	0.13	0.16	0.01	0.14	0.06	0.00	0.04
$\text{C}_{(1)}$	0.27	0.19	0.21	0.08	0.26	0.37	0.03	0.08
$\text{C}_{(2)}$	0.11	0.15	0.16	0.05	0.17	0.32	0.03	0.10
$\text{C}_{(3)}$	0.09	0.12	0.12	0.05	0.11	0.11	0.15	0.20
$\text{C}_{(4)}$	0.10	0.12	0.11	0.13	0.09	0.05	0.57	0.29
$\text{C}_{(5)}$	0.15	0.14	0.13	0.18	0.13	0.06	0.18	0.14
$\text{C}_{(6)}$	0.17	0.14	0.12	0.50	0.10	0.03	0.02	0.15

† Average of the three sugar co-ordinates of Table 1, except as asterisked (*).

Table 7. *Results of the paramagnetic mapping with the $\beta\text{MeGlcNAc}$ co-ordinates*

The shift ratios are expressed relative to $\text{C}_{(1)}$ at 100, and are scaled by using the ratios of the r^{-3} terms for $\beta\text{MeGlcNAc}$. The computer filters (tolerances) are the 'small' tolerances, and the large filters in the text are double the 'small' ones. All R indices (goodness of agreement) given in the text are based on the small tolerances.

Nucleus	Shift ratios	Computer filters (tolerances)	Good fit on the one-position model*	Good fit on the two-position model †
$\beta\text{Me}_{(9)}$	529	300	427	371
$\text{Me}_{(8)}$	-84	50	-56	-62
$\beta\text{H}_{(1)}$	264	125	175	172
$\text{H}_{(2)}$	-27	50	-18	-17
$\text{H}_{(3)}$	32	35	54	45
$\text{H}_{(4)}$	21	35	22	10
$\text{H}_{(5)}$	75	70	81	87
$\text{H}_{(61)}$	64	50	72	81
$\text{H}_{(62)}$	62	50	45	47
$\beta\text{C}_{(9)}$	318	100	340	340
$\text{C}_{(8)}$	-43	35	-60	-57
$\text{C}_{(1)}$	100	—	—	—
$\text{C}_{(2)}$	38	40	22	23
$\text{C}_{(3)}$	35	35	35	29
$\text{C}_{(4)}$	35	35	36	29
$\text{C}_{(5)}$	62	50	68	66
$\text{C}_{(6)}$	59	50	57	55

* $R = 0.36$, metal co-ordinates 0.847, 2.425, 1.994 $\alpha/\beta = 110^\circ/60^\circ$. Obtained from the search made by varying three spherical co-ordinates plus α/β . The co-ordinates are consistent with the van der Waals radii of the proton co-ordinates generated from the RS5D native lysozyme co-ordinates.

$^\dagger R = 0.35$; Gd(1) α/β $75^\circ/205^\circ$; Gd(2) α/β $130^\circ/80^\circ$.

well-defined 'best fit', for they were all uniformly fair. A typical good solution with $R = 0.36$ is given in Table 7, where the metal ion is positioned 0.09 nm from the mean of the Gd(1) and Gd(2) co-ordinates. Only the $\text{Me}_{(9)}$ and $\beta\text{H}_{(1)}$ shift ratios deviate from this solution.

2.4. Two-position model. The MTS2 calculations were four-parameter searches, where the α and β values for each of Gd(1) and Gd(2) were varied in order to search for fits on the basis of variations of the principal symmetry axes of the two metal ions. The two metal-ion positions were given relative weightings of 1:1, 2:1, 1:2 and 0.23:0.38 in a standard set of four runs. The latter weighting corresponded to the observed occupancies of Gd(1) and Gd(2) in the tetragonal lysozyme-Gd(III) complexes (Perkins *et al.*, 1979). A total of 6561 trial geometries was tested by stepping in 20° increments from 20° to 180° , and these gave 370–520 solutions with the large filters. The α and β solutions of the four runs correspond to a single family of solutions related by single steps of α and β . For Gd(1) α and β ranged from $80^\circ/160^\circ$ to $120^\circ/200^\circ$, and for Gd(2) from $80^\circ/40^\circ$ to $140^\circ/120^\circ$.

Those initial ranges of α and β values were then searched again with small filters and 5° increments of α and β in order to examine 16929 geometries. The best solutions were found with the 1:1 weighting, where 153 solutions had an R index that was better than 0.41, in distinction to the 2:1 and 1:2 weightings for Gd(1) and Gd(2), which gave 19 and 0 solutions respectively, which were better than an R index of 0.41. Because the minimum was close to the limit of the scanning range, this was increased, and 92055 geometries were scanned in a final run that used the 1:1 weighting in order to confirm the minimum. This 'best fit' was determined to have α/β values of $75^\circ/175^\circ$ to $80^\circ/210^\circ$ for Gd(1) and α/β values of $120^\circ/70^\circ$ to $130^\circ/80^\circ$ for Gd(2), with an R index of 0.35 to 0.36. It turns out that these best values for α/β are similar to the α/β values of the Gd–O and O–C bonds calculated from the crystal structure (Table 1), where Gd(III) ligates to lysozyme. These are respectively $100^\circ/182^\circ$ and $95^\circ/182^\circ$ for Gd(1) and $102^\circ/48^\circ$ and $113^\circ/89^\circ$ for Gd(2). Hence the determined best fit for α/β has a physical meaning in terms of axial symmetry in the two-position model to within about 20° . A typical good fit is given in Table 7, where again the $\text{Me}_{(9)}$ and $\beta\text{H}_{(1)}$ signals are exceptions to what is otherwise a satisfactory agreement.

Discussion

For lysozyme, the main outcome of this present study has been evidence to show that the GlcNAc

sugars bind to both subsites C and E of lysozyme in solution, together with the provision of insight into the way in which lanthanides bind to lysozyme. Since the GlcNAc binding in subsites C and E has already been discussed (Perkins *et al.*, 1981), we do not return to it here. Several aspects of the binding of lanthanides to lysozyme have also been discussed before (Perkins *et al.*, 1979).

Satisfactory mapping results were obtained for 15 of the 17 signals of $\beta\text{MeGlcNAc}$, where the two signals that could not be well-fitted were associated with the onset of intermediate exchange conditions and a difficult extrapolation (Fig. 5a). The computer analyses could not distinguish clearly between the one-position and two-position models. One reason for this is the limit to which the lanthanide-induced shifts of the ternary complexes could be measured when they were small. Study of the observed and calculated fits listed in Table 7, together with the estimated tolerances, give an indication of this. It is, however, important to note that three of the 17 shifts were of opposite sign to the remaining shifts. This shows that the cone of zero shift [where $3\cos^2\theta = 1$ in eqn. (2)] passes through the sugar ring, and this was an important guide for the computer analyses. Comparison should be made with reference to Fig. 1, where the separation between the lanthanide sites in the two-position model is 0.36 nm, and the distance between the mean of the lanthanide sites and the centre of the sugar ring is 0.83 nm. Although arithmetically equally satisfactory, the two-position model seems to offer some conceptual advantages over the one-position model. It is a four-parameter fit, and not a five-parameter fit as with the one-position model, and may give slightly improved fits in terms of the R index. A physical explanation is available for the directions of the best-fitted symmetry axes in terms of the direction of the Gd–O–C monodentate bonds. It is consistent with the crystal structures of tetragonal and triclinic lysozyme-Gd(III) complexes. At this stage, however, we prefer to leave a clear choice between the one-position and two-position models in solution for further experimental study.

It is instructive to compare this study of 17 n.m.r. signals of $\beta\text{MeGlcNAc}$ in the ternary complex with lysozyme and lanthanides with earlier studies that analysed 16 assigned and three unassigned protein ^1H n.m.r. signals in lysozyme-lanthanide complexes (Campbell *et al.*, 1975a). The two studies are complementary in the sense that different regions of the lysozyme molecule are perturbed by the lanthanide-induced shifts. Because the lanthanides bind almost at the protein surface and not at the centre, the solid space of lysozyme that is perturbed by lanthanides corresponds to almost over half-a-sphere. A large channel is missing from this

half-sphere, and this corresponds to the lysozyme cleft above and below Asp-52 and Glu-35 (Fig. 1). The 19 protein signals originate from the half-sphere minus the channel [where almost all the assigned signals are closer to site Gd(1) at Glu-35 than they are to Gd(2) at Asp-52]. The 17 β MeGlcNAc signals originate from the channel above the lanthanide site, where the dimensions of the sugar are $1.0\text{ nm} \times 0.4\text{ nm} \times 0.4\text{ nm}$. It is highly satisfying that, despite these differences in location, very similar computer fits in terms of the lanthanide symmetry axis were obtained on the one-position model for the protein and ligand signals. This demonstrates by two independent approaches and groups of workers that the application of paramagnetic mapping with lanthanides to lysozyme is so far self-consistent.

S. J. P. thanks the Medical Research Council for a Scholarship (1973–1976). We thank Dr. J. Feeney for generous opportunities to use a Varian XL-100 spectrometer for the ^{13}C n.m.r. measurements, and Professor K. Hamaguchi for generous gifts of α MeGlcNAc and β MeGlcNAc. This paper is a contribution from the Oxford Enzyme Group.

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