THE USE OF NUCLEAR MAGNETIC RESONANCE TO DESCRIBE RELATIVE MODES OF BINDING TO LYSOZYME OF HOMOLOGOUS INHIBITORS AND RELATED SUBSTRATES*

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Abstract and Summary.—Proton magnetic resonance has been used to study the association of inhibitors and substrates with hen egg-white lysozyme. Changes in chemical shift, due to association, of acetamido methyl group resonances of the small molecules have been quantitated. This has allowed definition of magnetic parameters for three contiguous binding subsites on the enzyme surface. The relative modes of occupancy of these sites by *N*-acetyl-D-glucosamine (NAG), chitobiose, chitotriose, their methyl glycosides, and chitotetraose have been delineated. In addition, the binding to these sites of *N*-acetyl-D-muramic acid (NAM) and a cell-wall disaccharide, NAG-NAM, have been studied. There is good, although not complete, agreement between the results obtained and X-ray analysis studies of the binding of some of these inhibitors to crystalline lysozyme. Binding of synthetic substrates, such as *p*-nitrophenyl-2-acetamido-4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-glucopyranoside (NAG-Gluc-ϕNO_2), has also been studied by the magnetic resonance technique described.

We have recently demonstrated the use of nuclear magnetic resonance for studies of enzyme-inhibitor association.[1-4] The changes in chemical shift observed for selected nuclei in the inhibitors as a result of binding to a macromolecule, such as lysozyme[1-5] and chymotrypsin,[6] allow not only a comparison of binding strength of various small molecules but also of their magnetic environments in the enzyme-bound state.

Many enzymes act on polymeric substrates and may be expected to have several contiguous binding subsites to accommodate the monomeric residues of the polymers. Previous studies of lysozyme in this laboratory[7] and elsewhere[8-10] have suggested that there are on the enzyme surface three contiguous *strong* binding subsites for *N*-acetyl-D-glucosamine residues.

In this communication we report the results of studies, by nuclear magnetic resonance methods, of the association with lysozyme of a series of oligosaccharide inhibitors and related substrates.

Experimental.—Materials: Synthesis and resolution of *d*- and *l*-*trans*-2-acetamido-cyclohexanol (ACHol) was accomplished as previously described.[11] Methyl-β-chitobioside and methyl-β-chitotrioside were prepared by Koenigs-Knorr synthesis from the peracetyl sugars.[4] Other materials were obtained as described previously.[1-4]

Methods: Spectra were obtained with a Varian HA-100 nuclear magnetic resonance (NMR) spectrometer, operating in frequency-sweep mode.[1-4]

Results.—Quantitation of chemical shifts: We have previously shown[2] that for the equilibrium

$$E + S \rightleftharpoons ES,$$

describing enzyme-inhibitor or enzyme-substrate association,

$$K_s + ([E_0] - [ES]) = [E_0]\frac{\Delta}{\delta} - [S_0]. \quad (1)$$

The bracketed terms describe the concentrations of the various species and K_s is the dissociation constant for the complex. The quantity δ is the observed change in chemical shift for a nucleus of the small molecule, whereas Δ is the chemical shift for the same nucleus in its enzyme-bound form. Equation (1) may be simplified and rearranged to give

$$S_0 = E_0\frac{\Delta}{\delta} - K_s. \quad (2)$$

Values of K_s and Δ may be obtained from a plot of S_0 versus $(1/\delta)$. Table 1 illustrates how this simplification of equation (1) has no significant effect on the determined K_s and Δ over a wide range of values for K_s and S_0.

The association of monosaccharide inhibitors with lysozyme: A typical result is shown in Figure 1 for the association of methyl-β-NAG with lysozyme. The acetamido methyl group of the inhibitor underwent a chemical shift to higher field, as well as some line-broadening, due to association with the enzyme. A plot of the chemical shift data is shown in Figure 2, and the values of K_s and Δ are listed in Table 2. Data obtained from studies of α-NAG, β-NAG, methyl-α-NAG, acetamido-cyclohexane, and the *d*- and *l*-diastereomers of *trans*-2-acetamido-cyclohexanol are also presented. These serve to illustrate that the amide functionality is obviously important for binding to the enzyme. The average magnetic environments experienced in the complexed state by the acetamido groups of β-NAG, methyl-β-NAG, and methyl-α-NAG are the same, whereas that encountered by α-NAG is different. This suggests that the C_1 hydroxyl proton of this inhibitor interacts with the enzyme and causes a change in its bound orientation. The results obtained from studies of the *d*- and *l*-diastereomers of *trans*-2-acetamido-cyclohexanol suggest that in addition to the

TABLE 1. *The use of equation (2) to calculate K_s and Δ.**

K_s (*M*)†	(−) Intercept‡	$1/\Delta$§
1×10^{-4}	1.1×10^{-4}	0.9999
5×10^{-4}	5.6×10^{-4}	0.9996
1×10^{-3}	1.1×10^{-3}	0.999
5×10^{-3}	5.6×10^{-3}	0.996
1×10^{-2}	1.1×10^{-2}	0.993
5×10^{-2}	5.3×10^{-2}	0.982

* All enzyme concentrations were taken as 3×10^{-3} *M*.

† For values of K_s between 1×10^{-2} *M*, values of S_0 used were 3–15 $\times 10^{-2}$ *M*, which gave a range for bound inhibitor or substrate of 1–10%. For $K_s = 5 \times 10^{-2}$ *M*, values of S_0 used were 1–10 $\times 10^{-2}$ *M*.

‡ Determined from plots of S_0 vs. $(1/\delta)$.

§ Arbitrary value of Δ = 1.0000.

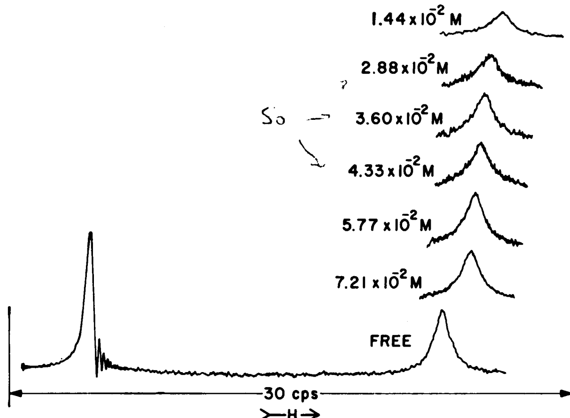

FIG. 1.—PMR spectra of the acetamido methyl resonance of methyl-β-NAG free and in the presence of lysozyme (3×10^{-3} M). The sharp resonance to lowest field is for an internal standard of acetone.

amide functionality, the C_3 hydroxyl of β-NAG is also indispensable, whereas the C_1 hydroxyl is not.

The association of chitin oligosaccharides with lysozyme: The acetamido methyl group resonances at 100 MHz of NAG, chitobiose, chitotriose, and chitotetraose are shown in Figure 3. In each case the resonance to highest field corresponds to the methyl group at the reducing end of the saccharide. The resonance to lowest field in each case corresponds to the methyl group at the nonreducing end of the saccharide. For the tri- and tetrasaccharides, the broader resonances that occur about 0.45 Hz to higher field of the low-field resonances have been assigned to the acetamido methyl protons of the middle pyranose ring in chitotriose and of the central rings in chitotetraose. The chemical shifts of these resonances relative to an internal acetone standard are listed in Table 3.

In the presence of lysozyme at pH 5.5 and 20°C, the effects on the acetamido methyl group resonances of chitobiose were (1) an extensive broadening of the resonance to higher field and (2) a chemical shift to higher field of this same resonance. These effects are shown in Figure 4. The extrapolated line-width of the reducing-end acetamido methyl resonance for the enzyme-bound disaccharide was 200–250 cps. This was unexplicable in view of the normal line-widths of 15–20 cps observed for methyl groups in proteins.[12] Under the conditions

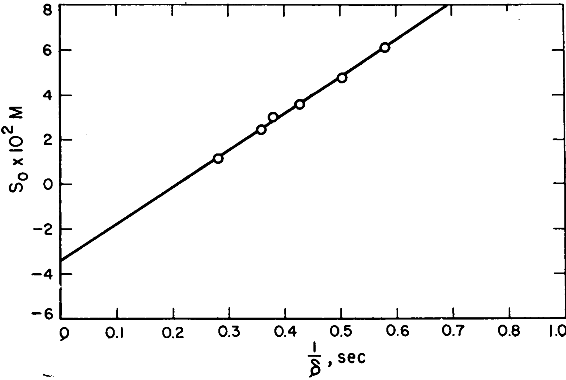

FIG. 2.—Plot of the reciprocal of the observed chemical shift (δ) for the acetamido methyl group resonance of methyl-β-NAG vs. varying concentrations (S_0) of the inhibitor in the presence of a constant concentration of lysozyme (3×10^{-3} M).

TABLE 2. *Data for binding of NAG various analogues to lysozyme.*

Inhibitor		$K_s(M)$	Δ (ppm) (a) $-NH-C(=O)-CH_3$	Δ (ppm) (b) OCH_3
β-NAG		$3.3 (\pm 0.2) \times 10^{-2}$	0.51 ± 0.03	—
α-NAG		$1.6 (\pm 0.1) \times 10^{-2}$	0.68 ± 0.03	—
β-Me-NAG		$3.3 (\pm 0.5) \times 10^{-2}$	0.54 ± 0.04	0.17 ± 0.03
α-Me-NAG		$5.2 (\pm 0.4) \times 10^{-2}$	0.55 ± 0.02	0
ACH		$12.6 (\pm 2.7) \times 10^{-2}$	0.34 ± 0.07	—
ACHol (*l*)		$15.7 (\pm 2.4) \times 10^{-2}$	0.22 ± 0.06	—
ACHol (*d*)		$0.95 (\pm 0.16) \times 10^{-2}$	0.91 ± 0.03	—

used, pH 5.5 and 20°C, the enzyme-chitobiose complex was too long-lived to yield a spectrum representative of a weighted average of enzyme-bound and free forms of the disaccharide. At 40°C and pH 5.5, this condition was fulfilled and (as shown in Fig. 4) it resulted in sharper resonances and an increase in the observed chemical shift.

The resonance due to the acetamido methyl group on the nonreducing end of chitobiose did not undergo any change in chemical shift upon association with lysozyme. At pH 9.7 and 45°C, effects similar to those at pH 5.5 were seen. In contrast to NAG, no separate resonances for the α- and β-anomeric forms of chitobiose were seen in the presence of lysozyme at any pH value or temperature, even when pure α-chitobiose was allowed to mutarotate.[2]

Binding of chitotriose to lysozyme (at pH 9.7) at low temperature (10–30°C) gave little change in the methyl group resonances, although it was known that all the enzyme (3.1×10^{-3} *M*) was complexed with the trisaccharide ($S_0 = 9.62 \times 10^{-2}$ *M*), since $K_s = 6 \times 10^{-6}$ *M*.[7] As the temperature was increased from 31 to 55°C, broadening of the reducing-end acetamido methyl resonance occurred

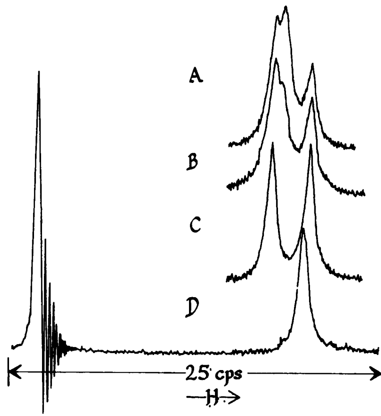

FIG. 3.—PMR spectra of the acetamido methyl groups of (*A*) chitotetraose, (*B*) chitotriose, (*C*) chitobiose, and (*D*) NAG. The sharp and intense resonance to lowest field is for an acetone internal standard.

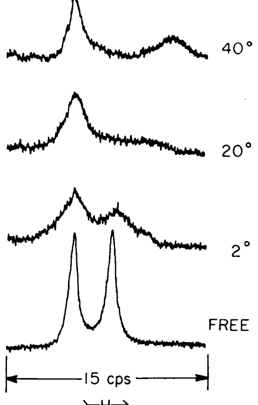

FIG. 4.—PMR spectra of the acetamido methyl group of chitobiose (5×10^{-2} *M*) free and in the presence of lysozyme (3×10^{-3} *M*) at various temperatures.

and this same resonance underwent a chemical shift. Upon a further raise in temperature (to 65°C), the increase in exchange rate gave rise to a sharper resonance and to an increase in the observed chemical shift. It is possible that the observed spectrum still was not representative of a weighted average of free and bound species,[3] but this condition was closely approximated. The value of Δ obtained was 0.61 ± 0.12 ppm to higher field. The resonance corresponding to the central acetamido methyl group of the trisaccharide did not undergo any change in chemical shift, whereas the resonance corresponding to the acetamido methyl group at the nonreducing end displayed a small *downfield* chemical shift ($\Delta = -0.08 \pm 0.01$ ppm).

On addition of lysozyme to a solution of chitotetraose at pH 9.7 and 65°C, broadening of the acetamido methyl resonances of the tetrasaccharide was observed. In addition, the resonance due to the reducing-end acetamido methyl

TABLE 3. *Chemical shift data (apparent maxima) for methyl groups in chitin oligosaccharides and their methyl-glycosides.*

Inhibitor	Resonance*				
	CH_3-N_1†	CH_3-N_2†	CH_3-N_3†	CH_3-N_4†	OCH_3‡
NAG	17.71				
Methyl-β-NAG	18.79				14.72
Chitobiose	18.35	15.63			
Methyl-β-chitobioside	19.51	15.33			13.94
Chitotriose	18.32	16.28	15.83		
Methyl-β-chitotrioside	19.53	16.28	15.90		13.33
Chitotetraose	18.35	16.54	16.54	15.98	

All chemical shifts are in Hz at 100 MHz.
* The acetamido methyl groups are numbered 1, 2, 3, 4, beginning at the reducing or glycosidic termini of the molecules.
† Values relative to acetone; all chemical shifts to higher field.
‡ Values relative to methanol; all chemical shifts to lower field.

TABLE 4. *Chemical shift data for inhibitors and substrates complexed with lysozyme at various pH and temperature values.*

Compound	Temp. (°C)	pH	Δ (ppm)* CH_3-N_1†	CH_3-N_2†	CH_3N_3†	—OCH_3‡
Methyl-β-NAG	31	4.9–5.4	0.54 ± 0.04	—	—	0.17 ± 0.03
	55	"	0.51 ± 0.03	—	—	0.16 ± 0.05
Chitobiose	45	"	0.57 ± 0.04	0	—	—
Methyl-β-chitobiose	35	"	0.60 ± 0.05	0	—	0.20 ± 0.05
Methyl-β-NAG	31	9.7	0.36	—		0.16 ± 0.02
Chitobiose	55	"	0.77 ± 0.04			
Methyl-β-chitobioside	55	"	0.80 ± 0.04	0		0.16 ± 0.02
Chitotriose	65	"	0.61§ ± 0.12	0	0.08	
Methyl-β-chitotrioside	65	"	0.63§	0	0.08	0.19

* The acetamido methyl groups are numbered 1, 2, 3 beginning at the reducing or glycosidic termini of the inhibitor molecules.
† Values relative to acetone; all chemical shifts to higher field.
‡ Values relative to methanol; all chemical shifts to lower field.
§ Not at fast exchange limit.

group was shifted to higher field. The spectrum was reminiscent of those for other saccharides when the fast-exchange limit was not achieved.

The association of chitin oligosaccharide glycosides with lysozyme: The NMR spectra of the acetamido methyl groups of methyl-β-chitobioside and methyl-β-chitotrioside in the presence of lysozyme showed that in each case the resonance due to the acetamido methyl group proximal to the glycosidic group was shifted to higher field. The results obtained are summarized in Table 4. The resonances due to the acetamido methyl group distal from the glycosidic group in methyl-β-chitobioside and the central acetamido methyl group in methyl-β-chitotrioside were not shifted, whereas the resonance of the acetamido methyl group distal from the glycosidic end of methyl-β-chitotrioside displayed a small downfield shift. Formation of a complex also resulted in chemical shifts of the resonances due to the glycosidic methyl groups. In the case of *p*-nitrophenyl-β-chitobioside, the resonance due to the acetamido methyl group proximal to the *p*-nitrophenyl residue was shifted to higher field, whereas that due to the acetamido methyl group distal from the glycosidic residue was not shifted.

The association of other inhibitors and substrates with lysozyme: Complex formation between lysozyme and 2-acetamido-3-O(D-1-carboxyethyl)-2-deoxy-β-D-glucopyranoside (NAM) showed no effect on the acetamido methyl group resonance of the saccharide. It was demonstrated, however, by competition[1] with NAG association that NAM did bind to the enzyme. The disaccharide NAG-NAM showed a small downfield shift of the resonance due to the acetamido methyl group of the NAG moiety, whereas that due to the NAM moiety was not shifted. Substrates[13] such as the disaccharides *p*-nitrophenyl-β-D-glucopyranosyl-β(1-4)-D-glucosaminide (NAG-Gluc-φ-NO_2) and methyl-β-D-glucopyranosyl-β(1-4)-D-glucosaminide (NAG-Gluc-OCH_3) displayed upfield chemical shifts of their acetamido methyl groups in the presence of lysozyme.

Discussion.—It has been suggested[14, 15] that the basis for differential broadening in the NMR spectra of small molecules in the presence of macromolecules is restricted movement when bound with resultant large dipolar fields that cause relaxation. It is of considerable interest that the especially broadened reso-

	A	B	C	D	E
1	NAG–	NAG–	NAG–	NAG–α, β	
2		NAG–	NAG–	NAG<β α	
3			NAG–	NAG<β α	
4/5				NAG–β; NAG–α	
6				ACH	
7				l-ACHol	
8				d-ACHol	
9		NAG–	NAG–	NAG–β R	
10			NAG–	NAG–β R	
11			NAG–	NAG–β PhN	
12				NAG–β R	
13				NAG–α R	
14				NAG–	GLU–β PhN
15				NAG–	GLU–β R
16			NAM<β α	/	NAM<β α
17		NAG–	NAM<β α		

FIG. 5.—Scheme for relative modes of association with lysozyme of various saccharide inhibitors and substrates. Where α- and β-anomeric forms are indicated on a single line, no information on relative binding modes was obtained. Where α- and β-forms are depicted separately (as with α-NAG and β-NAG), different binding modes were elucidated. Where α- and β-forms are shown on the same molecule on two levels, both anomeric forms bind identically. Methyl groups are depicted by *R*, nitrophenyl groups by *PhN*.

nances observed in the present investigation are also those that show *measurable chemical shifts* due to association with a macromolecule. The effects of temperature on the observed spectra strongly suggest that the "selective" broadening is due to slow exchange of the saccharides between the free and enzyme-bound states.

The data presented in Table 4 suggest that the various subsites (labeled *A*, *B*, and *C* in Fig. 5) which are associated with the binding of chitotriose may be assigned specific magnetic parameters. Based on this, the relative manner in which chitobiose and NAG occupy these sites may be delineated. In addition, the methyl glycosides of all three saccharides fit nicely into the scheme and further reinforce it in that the glycosidic methyl resonances all undergo the same chemical shift. The results are summarized in Figure 5. The α- and β-anomeric forms of the di- and trisaccharide bind identically, whereas insufficient information was available to judge whether both anomeric forms of the tetrasaccharide bind.

The results obtained in these studies are in good agreement with those for the binding of several inhibitors to crystalline lysozyme.[16] There are minor differ-

ences, however, such as the identical mode of binding of chitobiose anomers suggested by the NMR results, whereas the X-ray analysis studies suggested anomalous binding for α-chitobiose. Furthermore, it would appear from the present work that the preferential mode of binding of the cell-wall disaccharide, NAG-NAM, is in subsites A and B, rather than an anomalous mode, which again is suggested from the X-ray analysis results. The general agreement, however, on the relative modes of association of homologous chitin oligosaccharides with lysozyme as studied by difference Fourier analysis for crystalline preparations and by NMR methods for solutions is encouraging.

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