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Binding mode of N,N',N'',N'''-tetraacetylchitotetraitol to hen egg white lysozyme

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Abstract

The binding of N,N',N'',N'''-tetraacetylchitotetraitol [$(GlcNAc)_4$-ol] to hen egg white lysozyme was investigated by fluorescence and ^1H NMR spectroscopy. From observation of changes in the fluorescence intensity, the association constants of $(GlcNAc)_4$-ol and $(GlcNAc)_3$ were found to be 0.70×10^5 and 1.07×10^5 M^{-1}, respectively, at pH 5.0 and 30°C. The lack of a substantial difference between the association constants suggests that the binding mode of the $(GlcNAc)_3$ moiety of $(GlcNAc)_4$-ol is basically similar to that of $(GlcNAc)_3$, but that the *N*-acetylglucosaminitol residue of $(GlcNAc)_4$-ol does not interact significantly with lysozyme. On the other hand, ^1H NMR spectroscopy revealed a minor difference in the binding modes of the two saccharides. For most of the ^1H signals responding to saccharide binding, such as those of Trp 63 H^2, Trp 28 H^5, and Ile 98 $H^{\gamma 1}$, the chemical shift changes induced by $(GlcNAc)_4$-ol were almost identical to those induced by $(GlcNAc)_3$. However, the effect of binding on the signals of Asn 59 H^α and Trp 108 indole N^1H, which are located near subsite C, was different for $(GlcNAc)_4$-ol and $(GlcNAc)_3$. Thus it is inferred that the binding mode of the first sugar residue of $(GlcNAc)_4$-ol to subsite C is somewhat different from that of $(GlcNAc)_3$.

Keywords: Binding mode; Chitotetraitol, N,N',N'',N'''-tetracetyl; Hen egg white lysozyme

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1. Introduction

The modes of action of polysaccharide hydrolases have been studied by using their oligosaccharide substrates, and information on subsite structure has been obtained for many of these enzymes [1,2]. More detailed information on the mode of action is available by using a modified oligosaccharide substrate, in which the reducing end residue is linked to a chromophore facilitating the use of spectrophotometry in the evaluation of enzyme activity [3–5]. Moreover, modification of the reducing-end residue of a homo-oligosaccharide enables a more precise determination of the cleavage position by chromatographic analysis of the reaction products. Oligosaccharides reduced with sodium borohydride, which converts the reducing-end residue into an alditol unit, are also useful for investigating the cleavage patterns of carbohydrolases. In fact, the alditol derivatives of chito-oligosaccharides have been used for analysis of the splitting mode of chitinolytic enzymes [6,7].

However, a modified oligosaccharide may not retain its native conformation, hence the data obtained by using such oligosaccharides may not reflect the behavior of the native substrate. This suggests the necessity of characterizing the binding of a modified substrate to its corresponding hydrolase. In this paper, we report a study of the mode of binding of the alditol derivative of chitotetraose to hen egg white lysozyme (HEWL) by analysis of the fluorescence and ^{1}H NMR spectra of the enzyme in the presence of the saccharide.

2. Experimental

Materials.—Six times recrystallized hen egg white lysozyme was purchased from Seikagaku Kogyo Co. *N,N′,N″*-Triacetylchitotriose [$(GlcNAc)_3$] was prepared by acid hydrolysis of chitin followed by charcoal chromatography [8]. *N,N′,N″,N‴*-Tetra-acetylchitotetraitol [$(GlcNAc)_4$-ol] [1] was from Yaizu Suisan Kagaku Industry Co., Ltd. D_2O (99.96 atom%) was purchased from MSD Isotopes. Deuterated acetic acid (acetic-d_3 acid-*d*, 99.5 atom%) and its sodium salt (sodium acetate-d_3, 99 atom%) were from Aldrich Chemical Co., Inc.

Fluorescence.—Lysozyme was dissolved in 0.1 M sodium acetate buffer, pH 5.0, to give a 4.0×10^{-6} M solution. The lysozyme solution (2 mL) was mixed with each of the different concentrations of saccharide solutions (2 mL). Fluorescence measurements were performed with a Hitachi 650-60 spectrofluorometer at 30°C using a thermostated cell. Excitation was at 290 nm. Fluorescence intensity at 360 nm is enhanced by addition of the saccharide, and the enhancement (ΔF) was used for the determination of binding parameters. Association constants (K_{assoc}) were obtained from Scatchard plots of the

[1] Abbreviation: $(GlcNAc)_n$-ol represents the reduction product of $(GlcNAc)_n$, having *N*-acetyl-D-glucosaminitol as its reducing-end unit.

experimental data (Fig. 1), and were converted to binding free energy changes (ΔG^0) according to the equation,

$$\Delta G^0 = -RT \ln K_{assoc} - \Delta G_{mix} \quad (1)$$

where ΔG_{mix} is the free energy change of mixing, calculated to be 2.4 kcal/mol [2].

NMR measurement.—The lysozyme (6 mg) was dissolved in 1 mL of 99.9 atom% D_2O, and the solution was incubated at 80°C for several minutes to replace the exchangeable hydrogens with deuterium. After lyophilization three times from D_2O, the lysozyme was dissolved in 0.5 mL of 0.01 M sodium deuterioacetate buffer made with 99.9 atom% D_2O (pD 4.7). The saccharide was also dissolved in the sodium deuterioacetate buffer to give a 0.03 M solution. A portion of the oligosaccharide solution was added to the lysozyme solution, and the ^1H NMR spectrum was recorded at 270 MHz with a spectral width of 3501 Hz using a Jeol EX-270 spectrometer. The HDO resonance was saturated by means of selective gated irradiation. All measurements were performed at 35°C. In order to investigate the spectral change concomitant with increase in oligosaccharide concentration, the oligosaccharide solution was added stepwise, and the spectra of the lysozyme–oligosaccharide mixtures in various molar ratios were obtained.

For investigation of the tryptophan indole N^1H signals, the lysozyme (30 mg) was directly dissolved in 0.01 M deuterioacetate buffer made with 10% D_2O–90% H_2O (pH 5.1). Then, the ^1H NMR spectrum was recorded under the same conditions as described above, except that the spectral width was 4000 Hz.

Since the rate of conformational change concomitant with saccharide binding is not fast on the NMR timescale, the changes in the chemical shifts of the affected protons are discontinuous. With increase in the saccharide concentration the intensities of signals of free lysozyme were gradually reduced without change in the chemical shifts, and the signals of bound lysozyme appeared gradually at different positions (Figs. 2 and 3). Thus, the chemical shift changes resulting from saccharide binding were directly determined from the chemical shift differences of the individual signals between free and bound states.

3. Results

Fluorescence measurements.—Fig. 1 gives the Scatchard plots of the fluorescence changes at 360 nm measured for the binding of $(GlcNAc)_3$ and $(GlcNAc)_4$-ol at pH 5.0 and 30°C. The experimental points show good linearity in each case. From the slopes of the fitted lines, association constants were found to be 1.07×10^5 M^{-1} for $(GlcNAc)_3$ and 0.70×10^5 M^{-1} for $(GlcNAc)_4$-ol, and the free energy changes of the binding were calculated to be −9.4 and −9.1 kcal/mol, respectively. The value for $(GlcNAc)_3$ is in accord with the values reported previously [1,9].

Changes in ^1H NMR spectra in 99.9 atom% D_2O.—The NMR signals of many hydrogens in HEWL are perturbed by addition of chito-oligosaccharides [11–13]. Thus,

[2] By the equation $\Delta G_{mix} = RT \ln 55.5$, the value of 55.5 being the molarity of water.

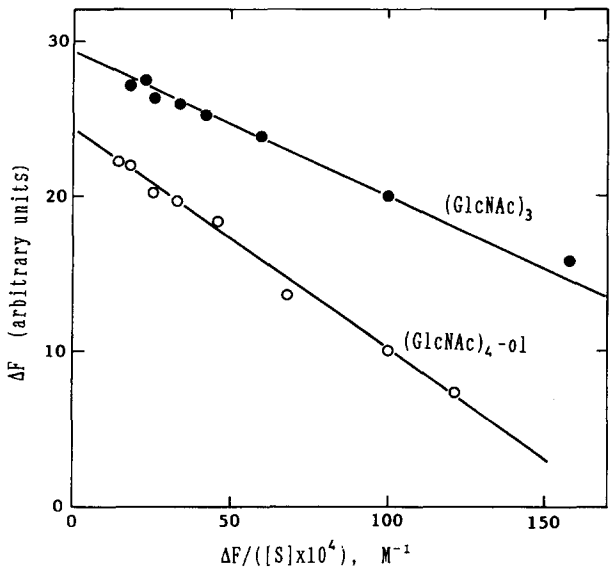

Fig. 1. Scatchard plots of fluorescence changes induced by the binding of $(GlcNAc)_3$ and $(GlcNAc)_4$-ol to lysozyme (2.0×10^{-6} M) in 0.1 M acetate buffer, pH 5.0, at 30°C. Excitation was at 290 nm and emission was measured at 360 nm.

the binding properties of these oligosaccharides can be evaluated by analyzing the chemical shift changes induced by complex formation [11]. Fig. 2 shows the changes resulting from saccharide binding. The peaks were assigned according to Redfield and Dobson [10]. The signals of Trp 63 H^2 and Trp 28 H^5 were strongly perturbed by the addition of $(GlcNAc)_4$-ol or $(GlcNAc)_3$, and in most cases the chemical shift changes induced by $(GlcNAc)_4$-ol were almost the same as those induced by $(GlcNAc)_3$. However, the effect of $(GlcNAc)_4$-ol on the Asn 59 H^α signal was quite different from that of $(GlcNAc)_3$. When the molar ratio of $(GlcNAc)_4$-ol to lysozyme was 2.0, the H^α signals of Asn 59, Asn 65, and Asn 39 were overlapped, while the signals were still separated in the lysozyme-$(GlcNAc)_3$ mixture at a molar ratio of 2.4. For the Asn 59 H^α signal, the chemical shift change induced by $(GlcNAc)_4$-ol was larger than that induced by $(GlcNAc)_3$. From the high field region of the ^1H NMR spectra of the lysozyme–oligosaccharide mixture (data not shown), the signals of Ile 98 $H^{\gamma 1}$ and $H^{\gamma 2}$ were found to shift upfield upon addition of $(GlcNAc)_3$. When $(GlcNAc)_4$-ol was added to the lysozyme, a similar effect was observed on Ile 98 signals. The chemical shift changes induced by $(GlcNAc)_3$ and $(GlcNAc)_4$-ol are listed in Table 1. Although many signals were perturbed by the addition of the saccharides, only those which exhibited differences of more than ± 0.04 ppm are listed in Table 1.

Effect on tryptophan indole N^1H signals.—As reported by Cassels et al. [14], the tryptophan indole N^1H signals of lysozyme are well separated from other signals and have been assigned to the individual N^1H protons of the tryptophan residues. When $(GlcNAc)_3$ was added to the lysozyme solution, the N^1H signals of Trp 62, Trp 63, Trp 108, and Trp 111 were affected as shown in Fig. 3a. These results are consistent with the

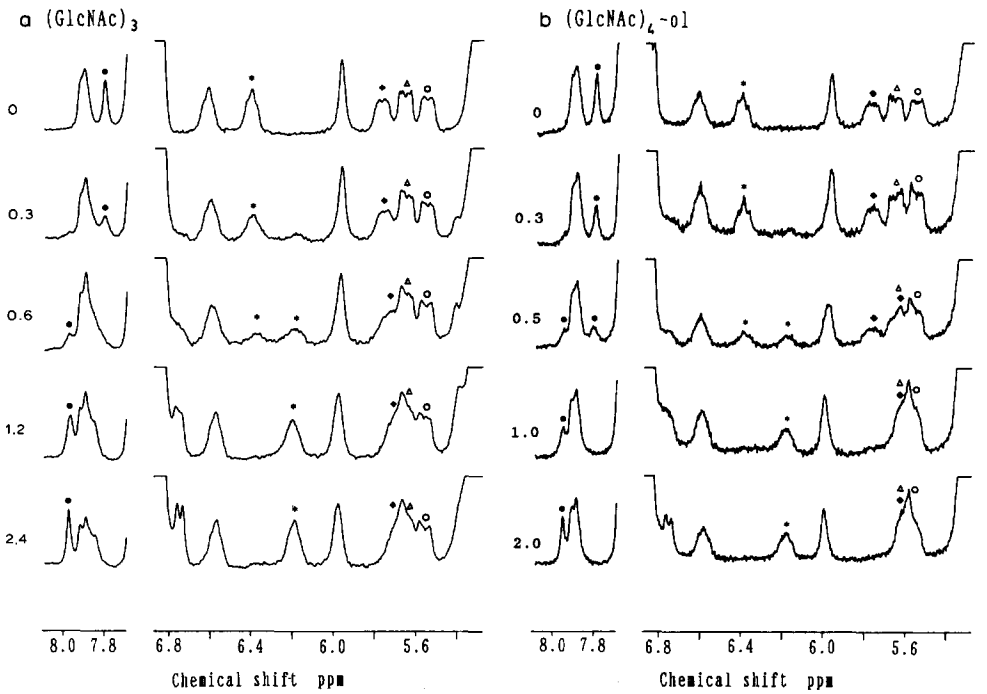

Fig. 2. Changes in the aromatic and H^{α} NMR signals of lysozyme induced by saccharide binding. (a) Effect of $(GlcNAc)_3$; lysozyme concentration, 5.5×10^{-4} M; pD 4.7. (b) Effect of $(GlcNAc)_4$-ol; lysozyme concentration, 6.5×10^{-4} M; pD 4.8. Molar ratios of saccharide to the lysozyme are indicated at the left of the individual spectra. The signals of Trp 63 H^2 (●), Trp 28 H^5 (∗), Asn 59 H^{α} (◆), Asn 65 H^{α} (△), and Asn 39 H^{α} (○) are labelled.

Table 1
Chemical shift changes of signals of individual lysozyme protons induced by saccharide binding [a]

Proton(s)	$\Delta\delta$ (ppm)	
	$(GlcNAc)_3$	$(GlcNAc)_4$-ol
Ile 98 $H^{\gamma1}$	−0.24	−0.28
Met 105 H^{β}	−0.04	−0.04
Ile 98 $H^{\gamma2}$	−0.10	−0.10
Asn 39 H^{α}	0.04	0.03
Asn 65 H^{α}	−0.01	−0.08
Asn 59 H^{α}	−0.10	−0.19
Trp 28 H^5	−0.21	−0.22
Trp 108 H^5	−0.04	−0.03
Trp 63 H^2	0.18	0.16

[a] Only signals that exhibited differences of more than ±0.04 ppm are listed. The positive and negative signs indicate shifts downfield and upfield, respectively.

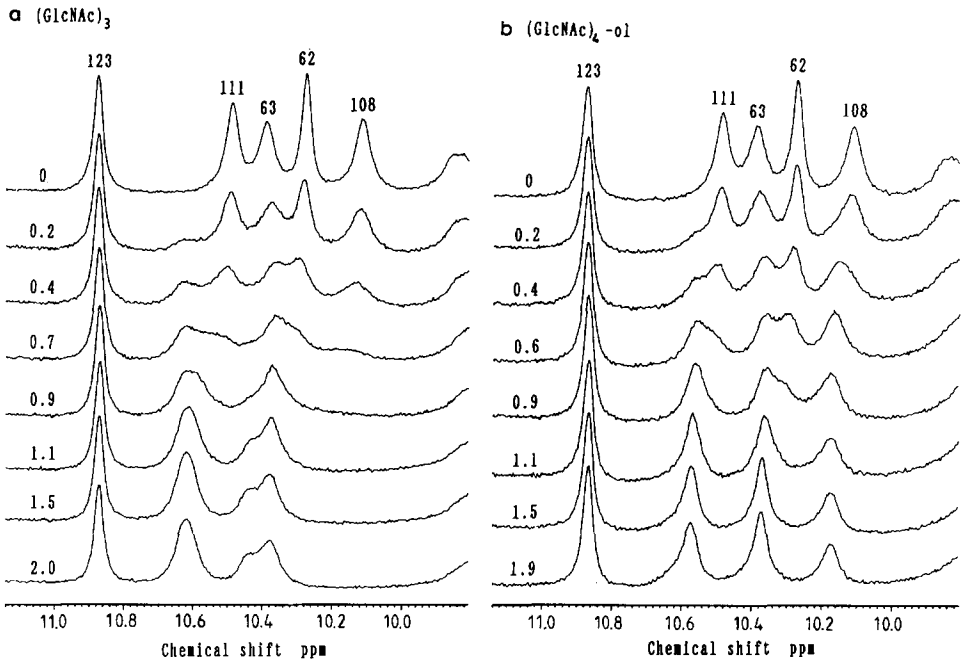

Fig. 3. Changes in the tryptophan indole N^1H NMR signals of lysozyme induced by saccharide binding. (a) Effect of $(GlcNAc)_3$; lysozyme concentration, 2.8×10^{-3} M; pH 5.1. (b) Effect of $(GlcNAc)_4$-ol; lysozyme concentration, 2.9×10^{-3} M; pH 5.1. Molar ratios of saccharide to the lysozyme are indicated at the left of the individual spectra. Numerals above the peaks of the uppermost spectra indicate the residue numbers of the tryptophans.

data reported by Blake et al. [11]. Upon the addition of $(GlcNAc)_4$-ol, the chemical shift change of the N^1H signal of Trp 111 was somewhat smaller than that induced by $(GlcNAc)_3$ (Fig. 3b). However, a drastic difference between the effects of $(GlcNAc)_3$ and $(GlcNAc)_4$-ol was seen on the Trp 108 N^1H signal. The strong perturbation of this signal which was observed for $(GlcNAc)_3$ did not occur upon the addition of $(GlcNAc)_4$-ol. The difference in the effect on the spectrum must result from presence of the additional *N*-acetylglucosaminitol residue in $(GlcNAc)_4$-ol.

4. Discussion

From the fluorescence measurements, the free energy changes of the binding were calculated to be -9.4 kcal/mol for $(GlcNAc)_3$ and -9.1 kcal/mol for $(GlcNAc)_4$-ol. There is no substantial difference between these values, suggesting that the binding modes of the two saccharides are quite similar to each other. From this it can be inferred that the $(GlcNAc)_3$ moiety of $(GlcNAc)_4$-ol binds to subsites A, B, and C, and *N*-acetyl-D-glucosaminitol residue located near subsite D does not interact significantly with the enzyme (Fig. 4).

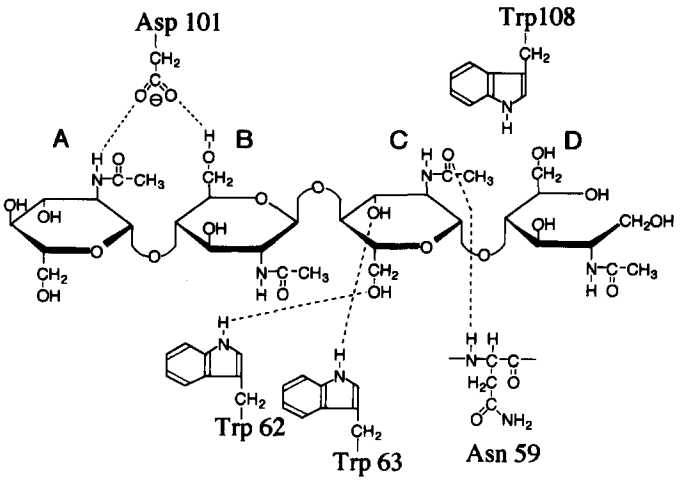

Fig. 4. Binding mode of $(GlcNAc)_4$-ol to lysozyme. The broken lines indicate hydrogen bonds.

On the other hand, analysis of the ^1H NMR spectra of the lysozyme in the presence of the two saccharides indicated a minor difference in their binding modes. For most of the lysozyme signals responding to the saccharide binding, the chemical shift changes induced by $(GlcNAc)_4$-ol were similar to those induced by $(GlcNAc)_3$ (Table 1). On the signal of Asn 59 H^{α}, however, the effect of the tetraitol was considerably greater than that of the trisaccharide, even though the alditol residue of $(GlcNAc)_4$-ol does not seem to bond to the enzyme. Since the main chain NH of Asn 59 forms a hydrogen bond with the acetamido group of the GlcNAc residue at subsite C, the effect on the Asn 59 H^{α} signal is most probably related to the binding mode of the sugar residue at subsite C. On the other hand, the binding of $(GlcNAc)_4$-ol to lysozyme hardly affects the Trp 108 indole N^1H signal, whereas the binding of $(GlcNAc)_3$ strongly affects that signal. As shown in Fig. 4, the side chain of Trp 108 is located on the opposite side of the binding cleft from Asn 59. Thus, it can be inferred that at subsite C the sugar residue of $(GlcNAc)_4$-ol binds to the enzyme in such a way that it is close to Asn 59 and away from Trp 108, in contrast to the corresponding residue of $(GlcNAc)_3$. In lysozyme complexed with $(GlcNAc)_3$ the reducing end residue located at subsite C takes either the α- or β-anomeric form, whereas in lysozyme complexed with $(GlcNAc)_4$-ol the residue at subsite C is restricted to the β configuration. This situation may be responsible for the difference in the binding modes of the two sugar residues, hence for the trivial difference between the binding free energy values of the two saccharides.

In stable complexes between lysozyme and chito-oligosaccharides the saccharide binds to the upper region of the binding cleft (subsites A, B, C, and D). Therefore, information on the interaction of ligands with the lower region of the binding cleft (subsites E and F) is not obtained from analyses of the stable complex. Instead, knowledge of subsites E and F is available from data on the time course of product formation [6,15]. We have already analyzed the time course of the lysozyme-catalyzed reaction of $(GlcNAc)_6$-ol with the aid of a modified kinetic model based on the model

used for $(GlcNAc)_n$ [16], and this analysis yielded information on the interaction of the *N*-acetylglucosaminitol residue with subsites E and F. The data will be reported in the subsequent paper.

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