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Note

Competitive binding of highly de-*N*-acetylated chitosans and *N*,*N*′-diacetylchitobiose to lysozyme from chicken egg white studied by ^{1}H NMR spectroscopy

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Chitosan is a linear polysaccharide which contains 2-acetamido-2-deoxy-β-D-glucose (GlcNAc; **A**-unit) and 2-amino-2-deoxy-β-D-glucose (GlcN; **D**-unit) residues linked through (1 → 4) glycosidic linkages. It has previously been shown that **A**-units and **D**-units are randomly distributed along the polymer chain in water-soluble, partially *N*-acetylated chitosans prepared by homogeneous or heterogeneous deacetylation of chitin [1,2]. Thus, the neighbourhood of units (e.g. the fractions of diads and triads) in the polymer is determined only by the fraction of acetylated units, F_A ($F_{AA} = F_A^2$; $F_{AD} \approx F_{DA} = F_A * F_D$; $F_{DD} = F_D^2$; $F_{AAA} = F_A^3$ etc.).

Lysozyme hydrolyses β-(1 → 4) linkages in chitin. Berger and Weiser [3] showed that fully de-*N*-acetylated chitosan ($F_A = 0$) is not degraded by lysozyme. Nordtveit et al. [4] reported that the initial degradation rate (by lysozyme from chicken egg white) of partially *N*-acetylated chitosans increases in proportion to F_A to the power of 3.6, and that the degradation rate is negligible at low fractions of acetylated units ($F_A < 0.04$). Lysozyme contains six binding subsites normally designated by the letters A–F, with cleavage occurring between sugar residues bound to subsites D and E. It has been suggested that subsites C, D, and E preferentially are occupied by **A**-units in productive enzyme-substrate complexes [5–7]. Accordingly, the chemical composition of the

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polymer (e.g. the F_A) is important for understanding the interactions between chitosans and lysozyme. Whether the observed differences in lysozyme degradation rates between chitosans are due to variation in catalytic rate constants (k_{cat}) or binding affinities (or a combination) is not known. ^{1}H NMR Spectroscopy has been extensively used to study binding between lysozyme and GlcNAc-oligosaccharides [9–15], but no experimental data on the binding of lysozyme to different hexamer sequences, from the fully acetylated sequence **A–A–A–A–A–A** to the fully deacetylated one **D–D–D–D–D–D**, have been reported, except for the fully acetylated sequence [8]. However, Fukamizo et al. [9] have reported on the binding of some partially acetylated trisaccharides to lysozyme. Their data suggested that also the **D**-units may interact favourably with lysozyme. However, no binding was detected to lysozyme of the fully deacetylated trisaccharide. The objective of this work is to study quantitatively the binding of low molecular weight chitosans ($dp_n \approx 17–25$) with a negligible or low fraction of acetylated groups ($F_A \leq 0.022$), using ^{1}H NMR spectroscopy.

The acetyl-region of the ^{1}H NMR spectrum of $(GlcNAc)_2$ is shown in Fig. 1A. Assignments with respect to reducing and non-reducing ends are according to Dahlquist and Raftery [12], while the assignments with respect to α/β-anomers are deduced from comparison with ^{1}H NMR data on GlcNAc [16] and the observed intensity ratios compared to the anomeric protons. The ligand $(GlcNAc)_2$ binds primarily to subsites B and C of lysozyme [8,15], and the NMR signal of the reducing end acetyl-group is shifted towards higher field upon binding due to chemical exchange between the free state and the enzyme bound state in subsite C [12,14].

The acetyl region of the ^{1}H NMR spectrum of $(GlcNAc)_2$, in the absence and presence of lysozyme, is shown in Fig. 1(A–G). The reducing end signal of $(GlcNAc)_2$ broadens when lysozyme is added, implying that fast exchange conditions are not completely fulfilled, in accordance with previously published data [12]. However, the coalescence temperature of this resonance was determined to ~ 40 °C (data not shown), and the change in the chemical shifts with increasing ligand concentration therefore represents the weighted average of the chemical shifts of free and enzyme-bound states.

Taking L_0 and E_0 as the total concentration of ligand and enzyme, respectively, the dissociation constant is defined as $K_D = (E_0 - [EL])(L_0 - [EL])/[EL]$. Assuming that the shifts δ (the upfield shift relative to the NMR signal with no enzyme present) observed in Fig. 1 are proportional to the bound fraction of ligand (i.e. $\delta/\Delta = [EL]/L_0$, where Δ is the shift when all ligand molecules are bound), K_D and Δ can be extracted by plotting L_0 against $1/\delta$. In cases where $L_0 - [EL] \approx L_0$, these data can be fitted to the equation $L_0 = \Delta * E_0 * (1/\delta) - (K_D + E_0)$ [11]. Using this procedure, we obtained a value for the dissociation constant of $(GlcNAc)_2$ to lysozyme of $K_D \approx 1.5$ mM at ionic strengths of 0.005 and 0.15 M at 55 °C. Note that due to denaturation of the lysozyme above approximately 65 °C [17], limiting fast exchange is unattainable for the native state of this system.

Our measured K_D for the binding of $(GlcNAc)_2$ to lysozyme is in agreement with other data on the dissociation constant for this complex, considering previous reports on the thermodynamic parameters of saccharide binding [8]. The value of $K_D \approx 1.5$ mM obtained in this work at $T = 55$ °C is approximately equivalent to a value of $K_D = 0.25$ mM at $T = 25$ °C, mainly due to a negative entropy of binding of approximately 25

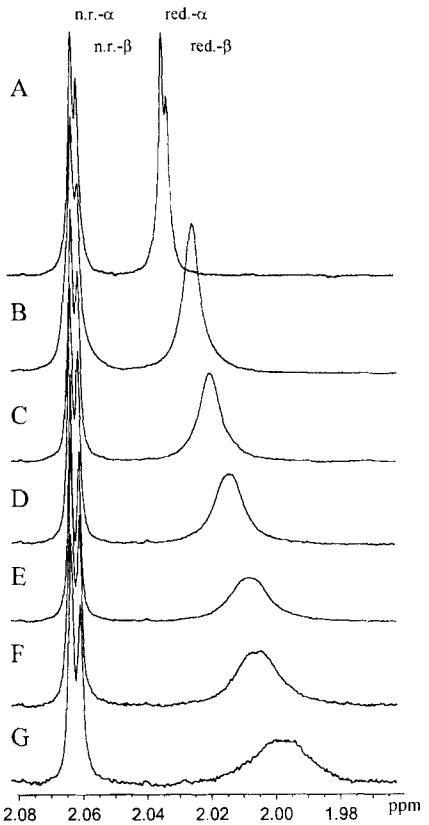

Fig. 1. (A) The acetyl region of the ^{1}H NMR spectrum of $(GlcNAc)_2$ (L_0) in the absence of lysozyme (A) and in the presence of lysozyme (B–G). n.r. = non-reducing end, red. = reducing end. (B) $L_0 = 9.16$ mM, (C) $L_0 = 2.81$ mM, (D) $L_0 = 1.95$ mM, (E) $L_0 = 0.99$ mM, (F) $L_0 = 0.50$ mM, (G) $L_0 = 0.20$ mM. The lysozyme concentration was 0.11 mM, and the ionic strength was 0.15 M (NaCl). $T = 55$ °C, pH 6.5.

cal/(mol K). Furthermore, there is good internal agreement of the values of Δ obtained for $(GlcNAc)_2$ in the different experiments described in this work ($\Delta = 0.82$–0.87 ppm at ionic strength 0.15 M, pH 5.7).

For the competitive binding studies between $(GlcNAc)_2$ (ligand) and chitosans (inhibitor), a reference solution was prepared in which the enzyme was approximately 85% saturated with the ligand before titration with the inhibitor. When a chitosan ($dp_n \approx 17$) with no detectable acetyl groups as determined by ^{1}H NMR [1] (i.e. $F_A < 0.001$) was added to the reference solution, no effect on the $(GlcNAc)_2$ acetyl resonance lines was observed with increasing concentrations of chitosan (up to 75 mM of dimer units, which is equivalent to approximately equimolar concentrations of $(GlcNAc)_2$ and chitosan molecules). This result was obtained at both ionic strengths of 0.005 and 0.15 M. Thus, the fully deacetylated chitosan does not compete with $(GlcNAc)_2$ for binding to subsites B and C in lysozyme.

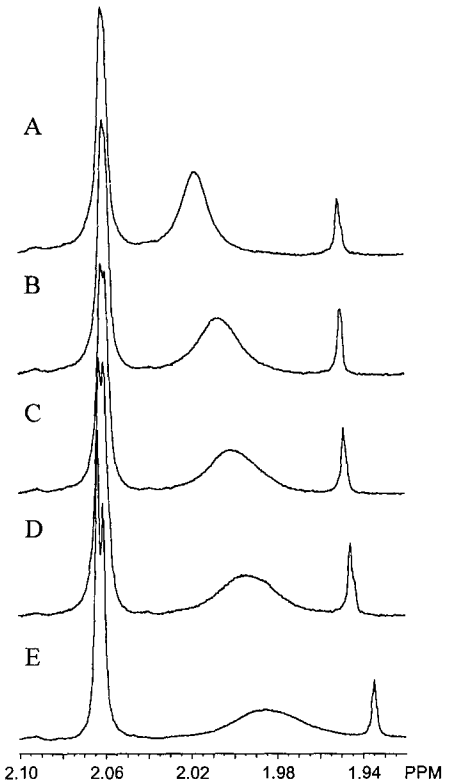

Fig. 2. The effect on the acetyl-region of the ^{1}H NMR spectrum of $(GlcNAc)_2$ (10.0 mM) with increasing chitosan ($F_A = 0.022$, $dp_n \approx 20$) concentration, $E_0 = 0.75$ mM, [NaCl] = 0.15 M. I_0 is the molar concentration of acetyl-groups from chitosan. (A) $I_0 = 2.20$ mM, (B) $I_0 = 1.12$ mM, (C) $I_0 = 0.79$ mM, (D) $I_0 = 0.54$ mM, (E) $I_0 = 0$ mM. $T = 55$ °C, pH 5.7. The resonance line at 1.94–1.95 ppm is acetate.

As a fully de-*N*-acetylated chitosan does not bind to lysozyme, an attempt was made to measure the dissociation constant for binding of a low-acetylated chitosan ($F_A = 0.022$) to lysozyme, by studying the NMR signal from the acetyl-group of the chitosan at different fractions of enzyme-bound chitosan. However, strong binding and slow exchange rates on the NMR time scale prevented the determination of K_D in a similar way as for $(GlcNAc)_2$ (data not shown).

The NMR signals in the acetyl region of $(GlcNAc)_2$, with increasing concentrations of a low-acetylated chitosan ($F_A = 0.022$, $dp_n \approx 20$), are shown in Fig. 2. The chemical shift of the reducing end acetyl line is shifted towards the resonance position of the free ligand, equivalent to the observation of a smaller fraction of $(GlcNAc)_2$ bound to the enzyme. Thus, this chitosan competes efficiently with $(GlcNAc)_2$ for the binding to subsites B and C. The observed effect is more pronounced at the higher ionic strength, as expected since both chitosan and lysozyme are positively charged at the chosen pH-value. No enzymatic cleavage was expected nor could be detected for neither $(GlcNAc)_2$ nor the chitosans in this system.

As the fully de-N-acetylated chitosan does not bind to subsites B and C, the effect observed here must be due to the small fraction of **A**-units ($F_A = 0.022$) in the chitosan. Thus, we can relate the effect observed to the concentration of **A**-units added to the reference solution. Assuming a random distribution of **A**- and **D**-units in the chitosan molecule [1,2], all **A**-units can be considered to be 'single' (–**D**–**A**–**D**–). This was also confirmed by ^{1}H NMR spectra of the chitosans, showing only the resonance of the –**A**–**D**– diad (no –**A**–**A**– diads were observed). To analyze the competitive binding data, we assumed that the effect of the chitosan with $F_A = 0.022$ observed on the NMR-spectra of $(GlcNAc)_2$ with lysozyme is mainly due to the effect of one single acetyl group in a hexameric sequence (containing 1 **A**-unit and 5 **D**-units), i.e. a monoacetylated sequence.

When I_0 is the total molar concentration of acetyl groups, and K_I is the dissociation constant for the monoacetylated sequence, we get $K_I = [E](I_0 - [EI])/[EI]$, where $[E] = E_0 - [EL] - [EI]$. Combining this with the expression for the dissociation constant K_D, we obtain $K_D(1 + [I]/K_I) = (E_0 - [EL])(L_0 - [EL])/[EL]$, showing that the expression for the dissociation constant of the ligand with only a small modification can be used to analyze the competitive system. Introducing $\delta/\Delta = [EL]/L_0$, as previously, and rearranging gives $[I] = (K_I/K_D) * \Delta * E_0 * (1/\delta) - (K_I/K_D)(L_0 + E_0 + K_D)$. Unfortunately, as I_0 in our experiments are of the same order as E_0, the approximation $[I] \approx I_0$ is not valid for the experiments performed, and a linear fit to the measured data will fail. By introducing $[I] = I_0 - [EI] \approx I_0 - (E_0 - [EL])$ (it was shown by the exact solution to the equations that [E] is in the range of $[0.04–0.14] * E_0$ during the competitive experiment performed), we obtain eq (1).

$$I_0 = E_0 - \frac{K_I}{K_D}(L_0 + E_0 + K_D) + \frac{K_I}{K_D}\Delta E_0 \frac{1}{\delta} - L_0 \frac{\delta}{\Delta} \qquad (1)$$

Although eq (1) is an approximate solution (valid when $L_0 - [EL] \approx L_0$ and $[E] \ll E_0$), the fitted values of K_I and Δ were insignificantly different from a fit to the exact solution of the basic equations (data not shown). Note that if $L_0 \gg K_D$, this fit gives a direct estimate of K_I/K_D, without prior knowledge of the value of K_D. The experimental data from Fig. 2 is shown in Fig. 3 together with the fit to eq (1). A competitive experiment with a chitosan with $F_A = 0.018$ was also performed and analyzed in the same manner as described above, and the values of K_I/K_D for all experiments are summarized in Table 1.

Table 1 shows that the fully deacetylated chitosan ($F_A < 0.001$) has no detectable binding to subsites B and C. In contrast, the monoacetylated sequence binds approximately 20 times stronger than $(GlcNAc)_2$ at pH 5.7 and an ionic strength of 0.15 M. It has previously been found that the monomer GlcNAc binds to lysozyme (in subsite C) with considerable less strength than the dimer $(GlcNAc)_2$ (in subsites B and C) [8]. We conclude that the **D**-units in the monoacetylated sequence contribute significantly to the binding to lysozyme, and that at least one **A**-unit is needed to initiate the binding. Similar conclusions have been obtained by Fukamizo et al. [9] for some partially *N*-acetylated trisaccharides. A similar highly de-*N*-acetylated chitosan ($F_A = 0.010$) has previously been tested in a kinetic assay [4], and has been shown to competitively inhibit

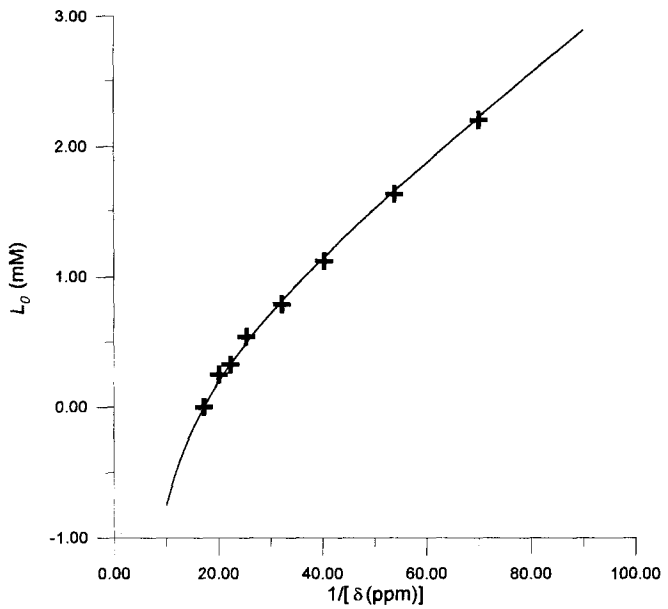

Fig. 3. Plot of L_0 versus $1/\delta$ for the data in Fig. 2, showing the fit to eq (1). The fit gives the value of $K_I/K_D \approx 1/19$.

lysozyme degradation of partially *N*-acetylated chitosans with F_As ranging from 0.17 to 0.60.

The fully acetylated hexamer, $(GlcNAc)_6$, bound in subsites A–F in lysozyme has a dissociation constant that is approximately 1/30 to that of $(GlcNAc)_2$ [8], i.e. $(GlcNAc)_6$ binds approximately 30 times stronger to lysozyme than $(GlcNAc)_2$. The present results indicate that the monoacetylated sequence binds 2–3 times weaker to lysozyme than $(GlcNAc)_6$ at pH 5.7–4.5 (ionic strength 0.15 M), with no subsequent enzymatic cleavage. The striking difference in K_D between two chitosans of relatively similar chemical composition ($F_A < 0.001$ and $F_A = 0.018/0.022$) demonstrates clearly the importance of a precise method for their characterization. These results are of importance not only with respect to biomedical and pharmaceutical applications of chitosan,

Table 1
Values of K_I/K_D as determined in competitive binding experiments between $(GlcNAc)_2$ and chitosans to lysozyme. Values are determined from fitting data to eq (1)

	[NaCl] = 0.005 M pH 5.5	[NaCl] = 0.15 M pH 5.7	[NaCl] = 0.15 M pH 4.5
$F_A < 0.001$	∞ [a]	∞ [a]	– [b]
$F_A = 0.018$	– [b]	1/21	– [b]
$F_A = 0.022$	1/2	1/19	1/9

[a] No competition with $(GlcNAc)_2$ for the binding to subsites B and C in lysozyme could be detected.
[b] Not measured.

but also for the interpretation of the kinetics of lysozyme degradation of chitosans [4]. Furthermore, our results suggest that purified monoacetylated oligosaccharides can be useful as substrate-analogues for lysozyme that binds with high affinity without subsequent hydrolysis.

The decrease in K_I/K_D for the chitosan with $F_A = 0.022$ with increasing ionic strength (Table 1) is probably due to long range electrostatic interactions between the positively charged chitosan and the lysozyme (which contain 12 positive charges at pH 5 [8]), as no difference in the K_D for $(GlcNAc)_2$ with increasing ionic strength was found. Such an effect does not necessarily change the value of the dissociation constant, but rather modulates the effective concentrations of chitosan near the lysozymes. The increase in K_I/K_D with decreasing pH might also be related to long range electrostatic interactions, or to short range interactions in the binding site (i.e. protonation of acidic amino acids [Glu 35, Asp 101] in the active site cleft of lysozyme [18]). The binding site for the monoacetylated sequence is unknown (that is, to which subsite(s) in lysozyme the **A**-unit is bound), but based on earlier reports on the binding of oligosaccharides to lysozyme [8,9], one might suggest subsite C. Further work on the nature of the interactions described in this work and probable binding patterns is in progress.

1. Experimental

$(GlcNAc)_2$ was purchased from Sigma (USA), D 1523. Lysozyme from chicken egg white was used (Sigma L-6876, Grade 1) without further purification. A chitosan with a fraction of acetylated groups (F_A) of 0.15 was kindly provided by Pronova Biopolymer. This chitosan was further deacetylated by heterogeneous deacetylation to $F_A < 0.001$ (no detectable acetyl groups by ^{1}H NMR), $F_A = 0.018$, and $F_A = 0.022$. These samples were depolymerized with nitrous acid and subsequently reduced with $NaBH_4$ as previously described [19] to dp_n values of 17 ($F_A < 0.001$), 25 ($F_A = 0.018$), and 20 ($F_A = 0.022$). The F_A were determined by ^{1}H NMR spectroscopy [1]. The dp_n was determined by estimating the fractions of non-reducing ends based on ^{13}C NMR assignments reported by Domard et al. [20]. ^{13}C NMR Spectroscopy was performed with proton decoupling during delays and acquisition (repetition time 3 s), and for one chitosan also with inverse gated decoupling of protons to eliminate the effect of different nOe's. No differences in the measured dp_n could be found with these 2 techniques.

^{1}H NMR Spectroscopy in binding studies and competitive studies were performed in D_2O at 400 MHz, $T = 55$ °C and at pH 4.5 or 5.5–5.7 (reading on the pH-meter, no corrections made). 32–400 Scans were recorded, depending on the concentrations of oligosaccharides. Solutions with salt were prepared with NaCl. For the competitive binding studies a reference solution was prepared, containing concentrations of enzyme and $(GlcNAc)_2$ of $E_0 = 0.75$ mM and $L_0 = 10.0$ mM, respectively. The reference solution was titrated with increasing amounts of chitosans up to a maximum concentration of GlcN-residues of approximately 150 mM. ^{1}H Chemical shifts were measured relative to internal TSP at -0.01 ppm (pH 5.5), according to the recommendations of Wishart et al. [21].

References

[1] K.M. Vårum, M.W. Anthonsen, H. Grasdalen, and O. Smidsrød, *Carbohydr. Res.*, 211 (1991) 17–23.
[2] K.M. Vårum, M.W. Anthonsen, H. Grasdalen, and O. Smidsrød, *Carbohydr. Res.*, 217 (1991) 19–27.
[3] L.R. Berger and R.S. Weiser, *Biochim. Biophys. Acta*, 26 (1957) 517–521.
[4] R.J. Nordtveit, K.M. Vårum, and O. Smidsrød, *Carbohydr. Polymers*, 23 (1994) 253–260.
[5] K. Amano and E. Ito, *Eur. J. Biochem.*, 85 (1977) 97–104.
[6] K. Ishiguro, N. Yoshie, M. Sakurai, and Y. Inoue, *Carbohydr. Res.*, 237 (1992) 333–338.
[7] B.T. Stokke, K.M. Vårum, H.K. Holme, R.J.N. Hjerde, and O. Smidsrød, *Can. J. Chem.*, 73 (1996) 1972–1981.
[8] T. Imoto, L.N. Johnson, A.T.C. North, D.C. Phillips, and J.A. Rupley, *Vertebrate Lysozymes*, in P.D. Boyer (Ed.), *The Enzymes*, Vol. 7, Academic Press, New York, 1972, pp 665–868.
[9] T. Fukamizo, Y. Ikeda, T. Ohkawa, and S. Goto, *Eur. J. Biochem.*, 210 (1992) 351–357.
[10] F.W. Dahlquist and M.A. Raftery, *Biochemistry*, 7(9) (1968) 3269–3276.
[11] M.A. Raftery, F.W. Dahlquist, S.I. Chan, and S.M. Parsons, *J. Biol. Chem.*, 243(16) (1968) 4175–4180.
[12] F.W. Dahlquist and M.A. Raftery, *Biochemistry*, 8(2) (1969) 713–725.
[13] B.D. Sykes and C. Parravano, *J. Biol. Chem.*, 244(14) (1969) 3900–3904.
[14] S.J. Perkins, L.N. Johnson, D.C. Phillips, and R.A. Dwek, *Biochem. J.*, 193 (1981) 553–572.
[15] K.J. Lumb, J.C. Cheetham, and C.M. Dobson, *J. Mol. Biol.*, 235 (1994) 1072–1087.
[16] S.J. Perkins, L.N. Johnson, D.C. Phillips, and R.A. Dwek, *Carbohydr. Res.*, 59 (1977) 19–34.
[17] C.C. MacDonald, W.D. Phillips, and J.D. Glickson, *J. Am. Chem. Soc.*, 93(1) (1971) 235–246.
[18] K. Bartik, C. Redfield, and C.M. Dobson, *Biophys. J.*, 66 (1994) 1180–1184.
[19] M.W. Anthonsen, K.M. Vårum, and O. Smidsrød, *Carbohydr. Polymers*, 22 (1993) 193–201.
[20] A. Domard and N. Cartier, *Int. J. Biol. Macromol.*, 11 (1989) 297–302.
[21] D.S. Wishart, C.G. Bigam, J. Yao, F. Abildgaard, H.J. Dyson, E. Oldfield, J.L. Markley, and B.D. Sykes, *J. Biomol. NMR*, 6 (1995) 135–140.